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Unique patterns of mitochondrial genes, transcripts and proteins in different male-sterile cytoplasms of *Daucus carota*

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Summary. Restriction fragment analysis of mitochondrial and chloroplast DNAs from a brown anther and a petaloid cytoplasmic male-sterile (cms) line revealed unique patterns for each cms line distinct from those of normal fertile cytoplasms, but identical restriction fragments for all chloroplast DNAs. The restauration of fertility through the introduction of nuclear restorer genes had no effect on the overall mitochondrial DNA (mtDNA) structure. The genomic environment and transcription patterns of several mitochondrial genes differ between cms and normal cytoplasms, while no difference has so far been detected between cms and the corresponding fertility-restored lines in mitochondrial DNAs and mRNAs. Mitochondrial translation products analysed by in-organello synthesized proteins revealed a number of polypeptides unique to each cytoplasm. Most prominent is a 17-kDa polypeptide that is present in the brown anther cms line but not in fertile mitochondria. Synthesis of this protein was not visibly affected by fertility restauration. The different cms phenotypes in carrot are thus associated with extensive and unique mtDNA rearrangements and distinct alterations in transcription and translation patterns.

Key words: Carrot cms – Mitochondrial DNA – Mitochondrial transcription – Mitochondrial proteins

Introduction

The maternally inherited trait of cytoplasmic male sterility (cms) disturbs normal pollen development and prevents the maturation of viable pollen. This extrachromosomally transmitted phenotype has been observed in many different plant species and has an important role in commercial hybrid seed production. The disturbances in pollen development are unique to each species and also to different types of cms within a species. Investigations of the underlying molecular events in several species and cms plasmtypes have confirmed that a diversity of mitochondrial mutations is most likely involved in this phenotype.

Mutations in the mitochondrial genomes of the T-cytoplasm in maize (Forde et al. 1978; Forde and Leaver 1980; Dewey et al. 1986, 1987), of the 9E plasmtype in sorghum (Bailey-Serres et al. 1986, 1987) and of a cms cytoplasm of *Petunia* (Young and Hanson 1987) are responsible for altered open reading frames and the resulting synthesis of aberrant polypeptides in these organelles. In most cases of cytoplasmic male sterility specific nuclear genes (or sets of genes) can restore a normal fertile phenotype. The interaction of these nuclear genes, called restorer genes, with mitochondrial gene expression is still unknown.

We have initiated molecular analysis of two cms cytoplasms in carrot and in the present article report genetic analysis of the brown anther (Sa) and petaloid (Sp) types of cms together with comparative investigations of mitochondrial and chloroplast restriction fragment patterns, mitochondrial transcripts of several genes and protein synthesis in sterile, restored and normal cytoplasms of *Daucus carota*.

Materials and methods

Plant material

Analyses of restriction fragments from mitochondrial and chloroplast DNAs and of mitochondrial transcription and

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translation products were done with organelles isolated from young seedling leaves. All three types of cytoplasms known in the genetic stock of cultivated carrots were represented, including normal fertile, male-sterile and male-fertility restored lines.

One line has the common fertile N-cytoplasm (F-54157), which leads to male fertiliy regardless of the nuclear genetic condition. One of the two lines with Sp-cytoplasm (56105), the prerequisite for petaloid male sterility, exhibited exclusively male-sterile flowers (Mehring-Lemper 1987), whereas in the other line (46174) male fertility was restored. The Sa-cytoplasm of the brown anther male sterility was present in three lines. One of them was male sterile (46017); in the other two lines (56953 and 56982) male fertility was restored. Earlier studies had shown that all lines were homogeneous in the expression of male sterility and fertility, respectively (Mehring-Lemper 1987).

Male sterility of the brown anther type based on the Sa-cytoplasm is characterized by shrivelled anthers in otherwise normal looking white flowers. Brown anther sterile plants occur in many cultivars at a low frequency (Banga et al. 1964; Timin 1979).

Plants with Sp-cytoplasm show five additional petals, the shape and structure of which are more or less like those of normal petals. In this cytoplasm, which originates from a North American wild carrot species (Thompson 1961), green or greenish flower colours occur. This phenotype is expressed almost exclusively in male-sterile genotypes (Mehring-Lemper 1987). Inheritance of both types of male sterility is controlled by the cytoplasm and three to four nuclear genes (Mehring-Lemper 1987).

Isolation of chloroplast DNA

Freshly harvested green leaves (approximately 30 g) were disrupted in three-fold excess (w/v) of cp-buffer (0.3 *M* mannitol, 50 m*M* TRIS-HCl pH 8.0, 3 m*M* EDTA, 0.1% BSA) with a Waring blender (2 × 5 s at high speed) and subsequently ground with quartz sand in a mortar. The suspension was filtered through four layers of cheese cloth and one layer of miracloth. Large cell debris was sedimented by centrifugation at 100 *g* for 10 min. Chloroplasts were pelleted at 1,800 *g* for 15 min, redissolved in 6 ml cp-buffer and loaded on a three-step sucrose gradient (20%, 45%, 60%). After centrifugation at 72,000 *g* for 20 min the interface between 45% and 60% was removed and slowly diluted five-fold with cp-buffer. Chloroplasts were pelleted at 1,800 *g* for 15 min and digested in 3 ml cp-buffer with 2% triton and 3 mg/ml proteinase K for 1 h at 40 °C. All steps prior to proteinase K digestion were done in the cold at 4 °C. The chloroplast lysate was extracted with phenol and chloroform/octanol (24/1; v/v). Nucleic acids were precipitated with ethanol, and DNA was purified through Qiagen columns as described by the manufacturer. DNA yields were estimated in sample digests of aliquots by intensity of fluorescence after staining with ethidium bromide.

Isolation of mitochondrial DNA

Mitochondrial DNA was routinely isolated from 50 g green leaves. Cells were disrupted in ten-fold (w/v) mt-buffer (0.4 *M* mannitol, 1 m*M* EGTA, 25 m*M* MOPS adjusted to pH 7.8 with 5 *N* KOH; before use BSA was added to 0.1%, cysteine to 8 m*M* and PVP to 0.1%). The suspension was filtered through four layers each of cheesecloth and miracloth, and large debris was removed by centrifugation at 2,800 *g* for 15 min. Mitochondria were pelleted from the supernatant by centrifugation at 16,000 *g* for 20 min and resuspended in 3 ml sterile wash buffer (0.4 *M* mannitol, 1 m*M* EGTA, 25 m*M* MOPS adjusted to pH 7.5 with KOH, 0.1% BSA). Mitochondria were digested with DNase I adjusted to 200 µg/ml and $MgCl_2$ adjusted to 25 m*M* at 4 °C for 30 min. After addition of a further 100 µg/ml of DNase I and raising the $MgCl_2$ concentration to 37 m*M*, mitochondria were re-incubated for another 30 min at 4 °C. DNase was inhibited by the addition of 100 µl 0.5 *M* EGTA/ml suspension. Mitochondria were washed twice by the addition of 50 ml resuspension buffer (0.4 *M* mannitol, 10 m*M* tricine pH 7.2, 5 m*M* EGTA) and pelleting at 12,000 *g* for 20 min. The further steps of DNA purification are the same as those described for the isolation of chloroplast DNA.

Isolation of total cellular RNA

Total cellular RNA was isolated from green leaves as described in Gelvin and Schilperoort (1988).

Nucleic acid analysis

Gel and hybridization techniques in nucleic acid analysis followed standard procedures as described, for example, in Sambrook et al. (1989).

In organello protein synthesis

Mitochondria for in organello protein synthesis were isolated from young green leaves and mature roots. The analysis of proteins synthesized in isolated carrot mitochondria essentially followed the protocol devised by Forde et al. (1978) and Leaver et al. (1983). Labelled proteins were identified after gel electrophoresis by fluorography (Leaver et al. 1983).

Results

Analysis of chloroplast DNAs

Chloroplast DNAs from sterile and restored lines of the two cms types as well as from a normal line were compared by restriction analyses to investigate the stability of this genome and any potential connection with the cms phenotype. Several different restriction enzymes gave identical fragment patterns for the lines investigated; a sample gel is shown in Fig. 1. The restriction fragments generated from these lines, however, differ from the chloroplast DNAs of other members of the *Daucus* family (Matthews and DeBonte 1985). No differences were evident between fertile (N) and sterile (Sa) or restored chloroplast DNA in fragments of normal stoichiometry. Some variation was consistently observed in substoichiometric fragments, but that might have been due to inefficient digestion of the DNA samples.

These results make the direct involvement of the plastid genome in the cms phenytope unlikely. The observed connection between greening of petals in the petaloid cytoplasms and the cms phenotype might be a secondary effect of a mitochondrial mutation and needs to be investigated thoroughly in this respect.

Restriction fragment comparisons of mitochondrial DNAs

Mitochondrial DNA restriction fragment patterns are unique for each of the lines of the three different cytoplasms (Fig. 2). Fragment profiles differ extensively be-

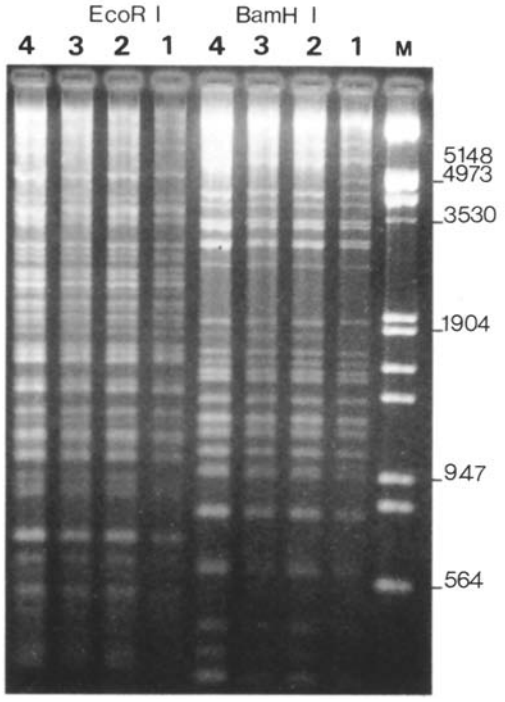


Fig. 1. Agarose gel electrophoresis of chloroplast DNA restriction fragments from the normal fertile cytoplasm and the Sa cytoplasm. Digests with the two enzymes *Eco*RI and *Bam*HI of the following lines are compared in the different tracks: *1* F54157 (N-cytoplasm, fertile), *2* 46017 (Sa-cytoplasm, sterile), *3* 56953 (Sa-cytoplasm, restored), *4* 56982 (Sa-cytoplasm, restored). Some fragment variation is detectable in the 0.9- to 1-kb region in both digests between the normal and Sa lines, all other fragments are identical. The sizes of lambda DNA *Hin*dIII/*Eco*RI standards (*M*) are given alongside in nucleotides

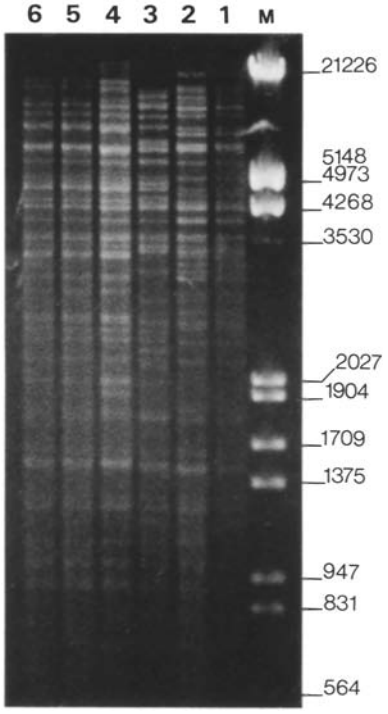


Fig. 2. Agarose gel electrophoresis of mitochondrial DNA *Bam*HI restriction fragments from the normal fertile cytoplasm, the petaloid (Sp) cytoplasm and the brown anther (Sa) cytoplasm. Digests of the following lines are compared in the individual tracks: *1* 46174 (Sp-cytoplasm, restored), *2* 56105 (Sp-cytoplasm, sterile), *3* F54157 (N-cytoplasm, fertile), *4* 46017 (Sa-cytoplasm, sterile), *5* 56953 (Sa-cytoplasm, restored), *6* 56982 (Sa-cytoplasm, restored). The fragment variation between the three cytoplasm types suggests that extensive sequence rearrangements have occurred in the mitochondrial genomes. The sizes of lambda DNA *Hin*dIII/*Eco*RI standards (*M*) are given alongside in nucleotides

tween the two sterile (Sa and Sp) and normal (N) lines and also between other *Daucus* sterile and fertile lines (Pingitore et al. 1989). The three types of cytoplasms investigated here can thus be identified and classified by their individual restriction patterns. Classification into one of the three types of cultivars based on mitochondrial DNA restriction fragment analyses (Ichikawa et al. 1989) was unfortunately not possible in our investigations due to the different enzymes used. Restriction fragment patterns of the sterile cytoplasms were not altered by different nuclear backgrounds and nuclear restoration to fertility. The carrot mitochondrial genome once established thus appears to be stable in any one of the three forms investigated here. Carrot mitochondrial DNA has previously been reported to be also stably maintained in various carrot cultivars during tissue culture (Matthews and DeBonte 1985).

Mitochondrial genome reorganizations involving coding regions

The genomic surroundings of most of the genes investigated in this study differ among the three types of cytoplasm. Southern hybridizations of *Oenothera* gene probes with coding regions of cytochrome oxidase subunits II (*coxII*) and III (*coxIII*), NADH-dehydrogenase subunits 2 and 3 (*nad2* and *nad3*) and ATPase subunits alpha and 6 (*atpA* and *atp6*) revealed that different restriction fragments carry the respective open reading frames for each of the three plasmtypes (Fig. 3 and data not shown).

The *atpA* probe hybridizes with four *Bam*HI restriction fragments in the mtDNA of the normal, fertile cytoplasm. Two of these fragments are also present in the Sa-cytoplasm, while the other two fragments are specifi-

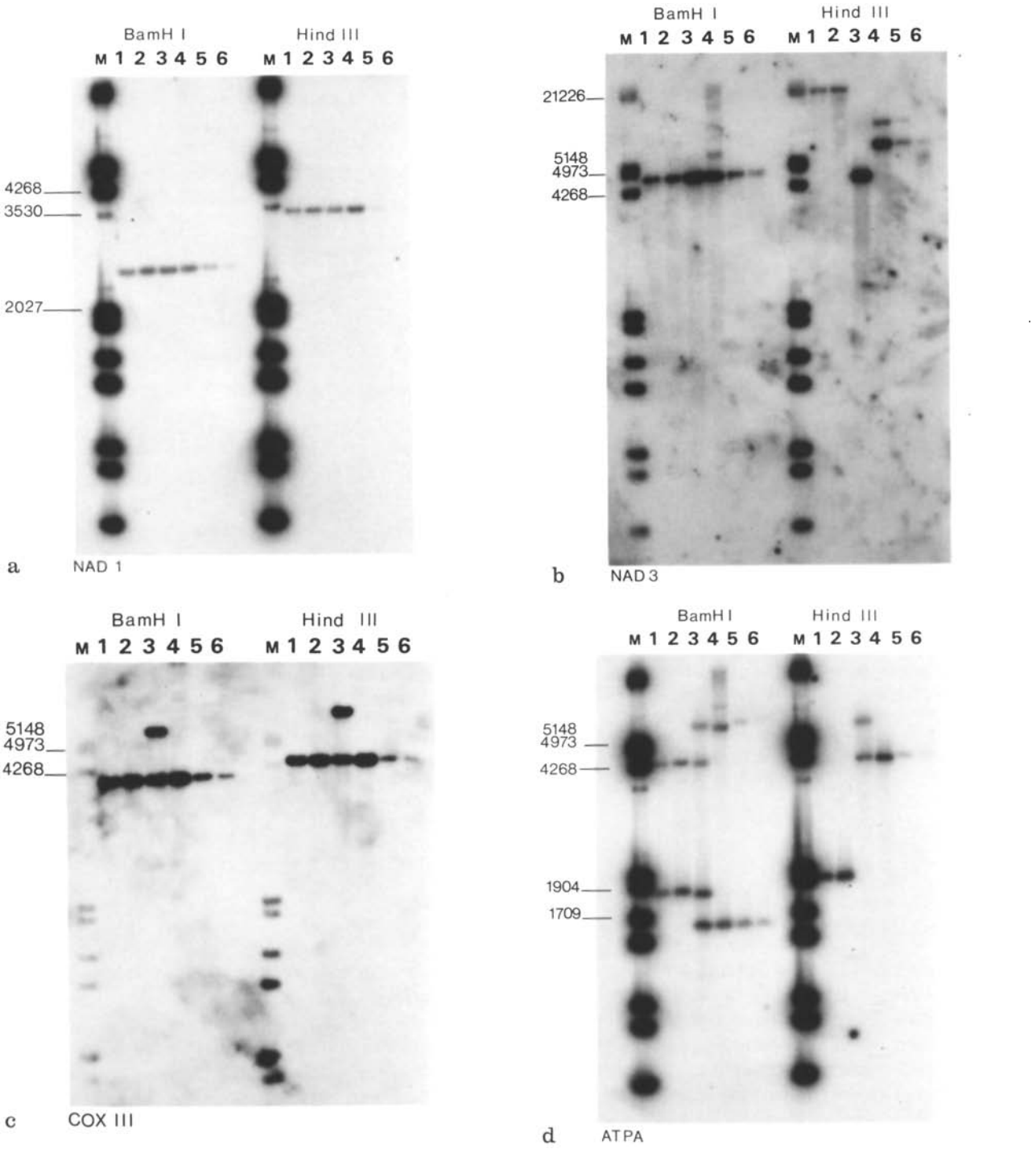
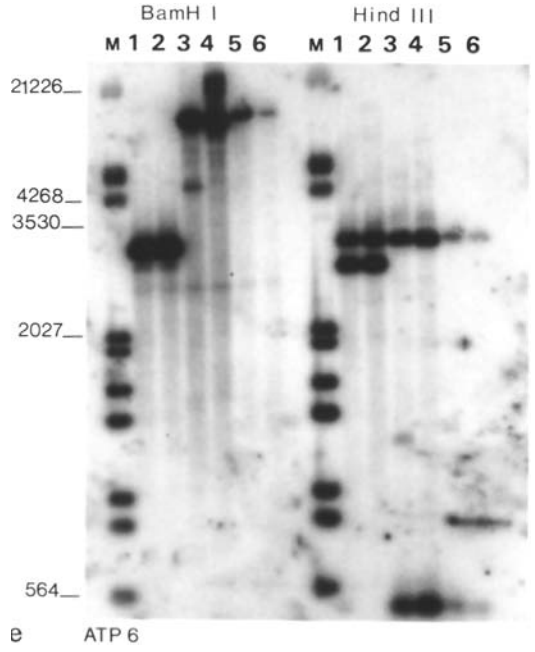


Fig. 3a–e. Southern blot hybridizations of the different carrot lines with gene-specific probes from *Oenothera berteriana* as indicated for subunits 1 and 3 of the NADH-dehydrogenase (**a** *NAD1* and **b** *NAD3*, respectively), subunit III of the cytochrome oxidase (**c** *COXIII*) and the alpha-subunit and subunit 6 of the ATPase (**d** *ATPA* and **e** *ATP6*, respectively). Numbering of the lanes is as in Fig. 2. The genomic surroundings of the genes shown here differ in at least one of the cytoplasms, sometimes also in the number and nature of alleles. Only the gene-internal fragment probed here for the *NAD1* gene appears to be unaltered in all lines



cally maintained in the Sp-cytoplasm only (Fig. 3). The single hybridizing *Hin*dIII fragment in Sa-mtDNA is identical in size with one of the two fragments with homology in the N-cytoplasm, while the Sp-mtDNA contains atpA sequences on a unique *Hin*dIII fragment. The two hybridizing *Bam*HI fragments probably result from a gene internal *Bam*HI recognition site similar to that which has been found in the *Oenothera atpA* gene (Schuster and Brennicke 1986) and is expected to be maintained in carrot also due to nucleotide sequence conservation within the open reading frame. Nucleotide sequences surrounding the *atpA* coding regions thus appear to have been maintained between the N and the two cms mtDNAs as far as the *Bam*HI sites, but to diverge before the next *Hin*dIII site, at least on one side of the gene. Only one of the presumably two copies of the gene in the N-genome is kept in each cms mtDNA.

The vicinity of the *coxII* coding regions appears to have been rearranged in the sterile and fertile lines of carrot analysed. Two *Bam*HI fragments are found in all three genomes; this may be explained by the size of this gene, which contains intervening sequences in carrot mitochondria (Turano et al. 1987; Lippok et al. 1992) and contains two gene-internal *Bam*HI sites that presumably have to be maintained in a functionally intact gene. The Sp-mtDNA appears to have duplicated parts or all of the *coxII* coding sequences since three additional restriction fragments are found in a *Bam*HI digest with homology to *coxII* (data not shown).

Of all the genes tested only *nad1* and *nad5* do not show any alteration of the homologous *Bam*HI and *Hin*dIII restriction fragments. Both these genes contain several introns in carrot and internal recognition sites for both enzymes within the examined coding region (Wissinger et al. 1989; Wissinger et al. 1991; B. Wissinger, unpublished results). Functional constraints on sequence conservation presumably maintain these gene internal fragments, which therefore do not permit deduction of recombination events outside the coding regions.

The frequently rearranged vicinities of the mitochondrial genes investigated in this respect thus confirm, at the level of individual genes, the global genome rearrangements deduced from the altered restriction fragment patterns in the two cms cytoplasms and in the normal, fertile mitochondrial genome.

Transcript comparisons of mitochondrial genes

The effect of genome rearrangement in the cms cytoplasms on the transcription of individual genes was investigated in Northern blot experiments. Transcript sizes and abundances of most of the genes investigated were found to differ between normal and cms plasmatypes (Fig. 4 and data not shown). The example of the *atpA* transcription patterns (Fig. 4) demonstrates the consequences of genome arrangement on transcription of this gene. A transcript of 1.8 kb is present in all cytoplasms of normal, fertile and restored idiotypes that is comparable with the single transcript of 2.1 kb found for the *atpA*-gene in *Oenothera* mitochondria (Schuster and Brennicke 1986). The Sp-cytoplasm of sterile and restored plants contains an additional mRNA species of 2.4 kb. More detailed investigations will be required to decide whether this additional transcript is caused by transcription initiation from new promoter regions or differences in mRNA maturation.

Only transcripts of *coxII* do not show any detectable size difference among the three cytoplasms (Fig. 4). The single mRNA species of 1.4 kb is presumably the mature, spliced transcript, suggesting that in carrot mitochondria intron excision from the *coxII* precursor occurs rapidly as in other species like maize or wheat (Fox and Leaver 1981; Covello and Gray 1989).

Since the transcription patterns of almost all of the genes investigated were different between fertile and sterile lines, the potential causal cascade from genome rearrangements to altered transcription to altered gene product and to the cms phenotype may reside in any of these genes. No gross transcript alterations between sterile and restored lines could be detected, however, suggesting that either one of the as yet unidentified mitochondrial genes is responsible for these cms phenotypes or that the restorer effects act more subtly or at other levels than genome organization and transcript sizes. To investigate possible effects of the cms cytoplasms and restoration of fertility

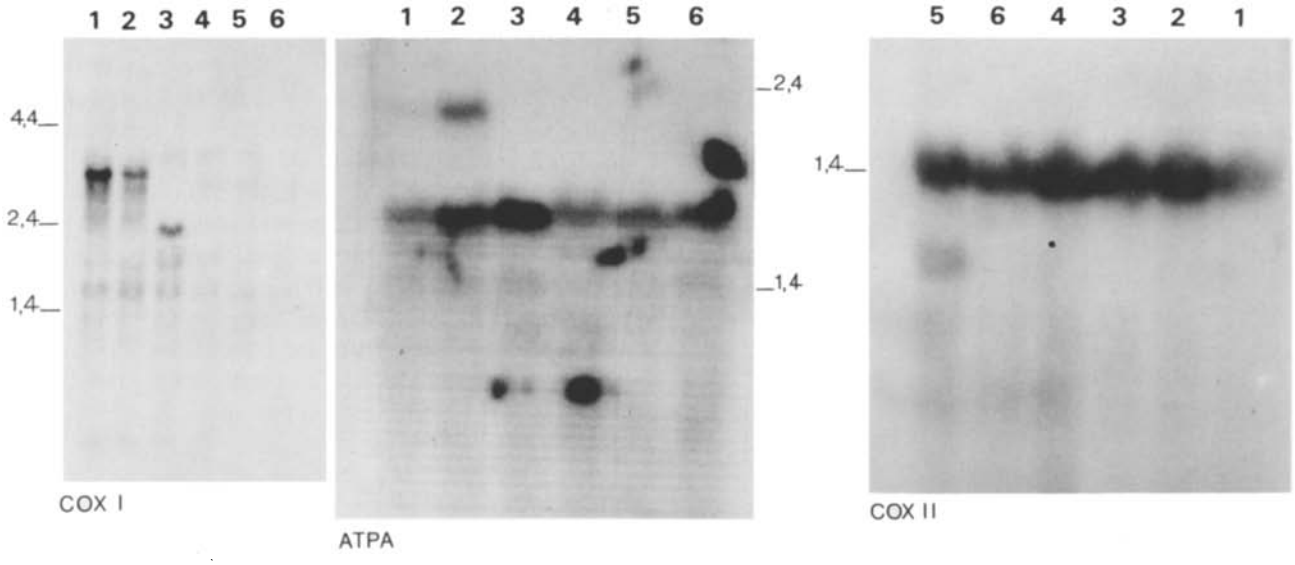


Fig. 4. Northern blot hybridizations of three gene probes specific for subunits I and II of the cytochrome oxidase (*COXI* and *COXII*, respectively) and the alpha-subunit of the ATPase (*ATPA*) to total cellular RNA of the different carrot lines investigated. Identification of the lines by numbering follows Fig. 2. The transcript patterns of *COXI* give an example of mRNA differences correlated with rearrangements in the mitochondrial DNA (not shown). Transcription of *COXII* on the other hand is apparently unaltered with the strongest signal being derived from the mature, processed mRNA although the genomic environment of this gene also differs between lines. Transcripts of the *ATPA* gene differ between the Sp-cytoplasm and the normal line, although the normal line apparantly also contains the genomic arrangement of the Sp-cytoplasm *ATPA* locus. No influence on transcription of the nuclear restorer genes is detected in these analyses (further data not shown)

at the level of the protein products encoded by the mitochondrial genomes we analysed the in-organello synthesized polypeptides.

Protein synthesis in mitochondria of sterile and fertile cytoplasms

Mitochondrial proteins synthesized in isolated organelles and resolved in SDS-PAGE gels (Laemmli 1970) show the overall characteristic pattern of mitochondrially encoded proteins of higher plants (Forde et al. 1978; Forde and Leaver 1980; Leaver et al. 1983; Bailey-Serres et al. 1986, 1987). Comparison of the polypeptides synthesized in mitochondria of the normal, fertile and each of the two sterile lines on the other hand revealed specific differences in in-organello synthesised proteins (Fig. 5). Most prominent is the abundant presence of a 17-kDa polypeptide uniquely synthesized in Sa-mitochondria that is absent in the normal, fertile line. Synthesis of this protein does not appear to be affected by restauration of the Sa-cytoplasm to fertility, since it is present in comparable amounts in the restored line. The 17-kDa polypeptide, which was only observed in Sa-mitochondria, is synthesised in both leaves (Fig. 5A) and roots of carrot (Fig. 5B), suggesting constitutive expression at least in these two tissues.

Other less abundant proteins show quantitative differences between the different cytoplasms in these analyses. These observations, however, need to be investigated further to clearly determine potential correlations between the type of cytoplasms and these proteins.

Similar observations can be made for the Sp-cytoplasm, where several differences in the mitochondrially synthesized proteins can be detected but still need to be defined in detail (Fig. 5). The presence or absence of polypeptides unique to these plasmatypes and their possible connection with the cms phenotype remain unclear as long as no differences in the in-organello synthesized proteins can be detected between sterile and restored lines.

Discussion

In this investigation two types of cytoplasmic male sterility in carrot were characterized by genetic and molecular analyses.

The genetic experiments revealed the existence of several types of nuclear backgrounds that restore fertility with both cms cytoplasms. The two cms cytoplasms have been characterized as petaloid (Sp) and brown anther (Sa) types, originating from an American wild carrot species and from cultivars, respectively.

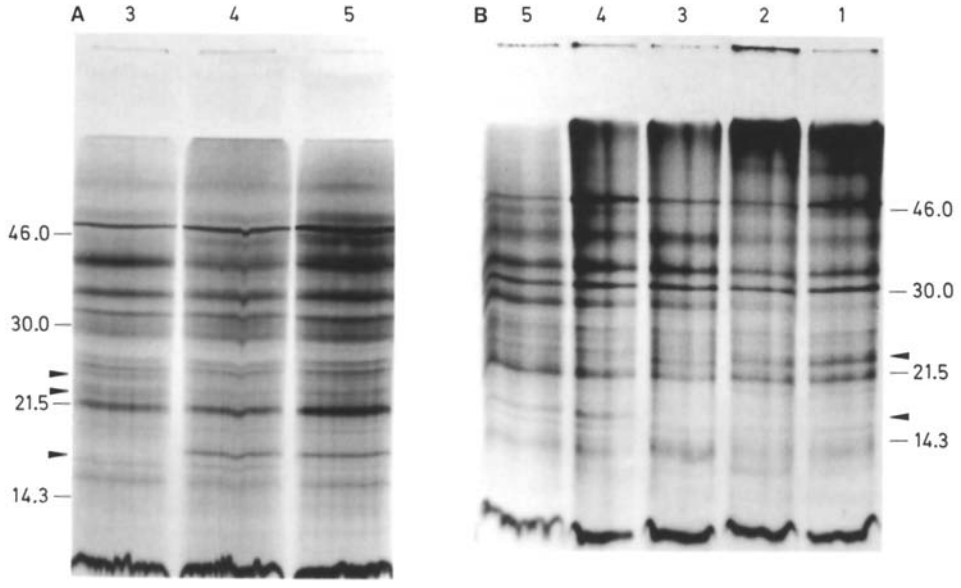


Fig. 5 A, B. Mitochondrially encoded polypeptides were labelled in organello, separated on a SDS-PAGE gel, enhanced and autoradiographed. Each carrot cytoplasm synthesizes several unique proteins not detected in the other lines. Especially striking is the example of the 17-kDa polypeptide synthesized in the Sa-cytoplasm, but not present in the normal and the Sp-lines (indicated by arrows). Synthesis of these proteins, however, appears to be unaffected by the restorer functions and may thus not be causally related to the cms phenotype. **Figure A** shows the in-organello synthesized protein patterns of mitochondria isolated from young green leaves; **Figure B** the labelled products of mitochondria isolated from carrot roots. The lanes contain proteins of the following lines: *1* 46174 (Sp-cytoplasm, restored), *2* 56105 (Sp-cytoplasm, sterile) *3* F54157 (N-cytoplasm, fertile), *4* 46107 (Sa-cytoplasm, sterile), *5* 56953 (Sa-cytoplasm, restored)

Molecular analysis of chloroplast and mitochondrial DNAs shows that the chloroplast genomes are not altered in the cytoplasm of normal, sterile and restored lines. Mitochondrial genomes on the other hand are highly and uniquely rearranged in each sterile cytoplasm in comparison to the normal fertile line. The two types of cms cytoplasms thus appear to have arisen by different recombination and amplification events that have restructured the architecture of the mitochondrial genome to two distinct and stable confirmations. The *atpA* gene, for example, is one result of these recombinations that lead to different genomic surroundings of the coding regions. In addition to such direct sequence recombinations only one of the presumably two copies of the *atpA* gene in the normal cytoplasm is detectable in the mitochondrial genome of each sterile line. Two intact copies of the *atpA* gene are also present in the maize mitochondrial genome and have been used as markers in the determination of relative mitochondrial DNA-molecule abundances (Isaac et al. 1985; Small et al. 1987, 1989). The substoichiometric presence of the respective other allele in both sterile cytoplasms of carrot, however, cannot be excluded from the hybridizations reported here. Further investigation is required to decide between actual deletion of one copy and selective amplification of one genomic allele. The unique dominance of different alleles in the two sterile cytoplasms nevertheless shows the independent derivation and unique genomic constitution of these mitochondrial genomes. The two male-sterile genomes are thus likely to be defect in different functions that may possible be causally involved in creating the specific types of phenotypic defects preventing correct pollen maturation. The numerous altered gene arrangements observed in the mitochondrial genomes of these cms lines, however, make a direct correlation of the gene involved in the phenotype extremely difficult, since all of these genes are potential candidates.

A correlation of altered transcription concurrent with genomic reorganization of a mitochondrial gene in these carrot lines might allow a reduction in the number of candidate genes. The investigations of transcript patterns, however, revealed divergent transcript patterns, a result of the different genomic environments of the respective open reading frames, for most of the genes analysed. This transcript analysis therefore only permits the further focusing and narrowing of the investigation of the molecular basis of the cms phenotype in these carrot lines to a limited degree.

The responsible altered genes and transcripts should also result in detectably different polypeptide product(s), being synthesized by the rearranged genomes. The number of affected variant polypeptides would be expected to

be considerably smaller than the number of rearranged genes and altered mRNAs. The identification of new protein products might thus offer the best chance to identify those proteins and the corresponding mRNAs and genes related to the cms phenotypes.

This approach has been very successful in maize and sorghum male-sterile cytoplasms (Forde et al. 1978; Forde and Leaver 1980; Dewey et al. 1986; Bailey-Serres et al. 1986, 1987). The variant 13-kDa polypeptide in maize cms T mitochondria, for example, is drastically reduced by fertility-restoring nuclear backgrounds (Forde et al. 1978; Forde and Leaver 1980; Dewey et al. 1986). These results thus revealed the consistent pattern of a primary involvement of this protein and the responsible chimeric gene in the sterile phenotype.

Several altered mitochondrially encoded polypeptides, however, are detected in the sterile Sa- and Sp-cytoplasms, and none of them appears to be influenced by the nuclear restorer functions. The unaffected stability of the abundant 17-kDa variant polypeptide specific for the Sa-cytoplasm in restored nuclear backgrounds (Fig. 5) suggests that this protein may not be causally involved in the sterile phenotype, but could be among the chance results of genomic reorganization without major effect on the phenotype.

The overall number of mitochondrial polypeptides altered in the different cms cytoplasm lines appears to be smaller than the many different reorganizations of the genome and the numerous divergent transcription patterns and will thus narrow the range of altered genes and their products that have to be investigated. Future work still requires further analysis of a clear correlation of altered protein(s) with the cms phenotype and will have to focus on the identification of protein(s) possibly affected by the restorer functions.

The identification of the mitochondrial genes involved in the cms phenotype of these carrot lines can only proceed with a thorough protein-phenotype correlation since the high percentage of altered genes and transcripts described in this study suggests that the genomic environment and transcription of most of the mitochondrial genes will be affected, precluding a direct identification of the responsible gene(s). The observation of numerous rearrangements in the vicinity of mitochondrial genes, but not within coding regions, shows that genomic recombinations can be stabilized and maintained in a functional mitochondrial genome as long as no vital information is disrupted.

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