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Comparison of the organization and expression of mtDNA of fertile and male-sterile sugar beet varieties (*Beta vulgaris* L.)

M. Duchenne [1,2,*,], B. Lejeune [1], P. Fouillard [2] and F. Quetier [1]**

[1] Laboratoire de Biologie Moléculaire Végétale, UA CNRS 1128, Bât. 430, Université Paris Sud, F-91405 Orsay Cedex, France
[2] Institut de Recherche Mennesson, B.P. 19, F-02320 Anizy Le Chateau, France

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Summary. Analysis of minicircle occurrence in different samples of sugar beet mitochondrial (mt) DNA invalidates the postulated relationship between cytoplasmic male sterility (CMS) phenotype and the absence of minicircle **c** and **d.** In high molecular weight mt DNA, two types of restriction patterns are found for fertile genomes and only one type for the CMS; in spite of the multiplicity of crosses carried out by plant breeders, all the CMS varieties analyzed seem to have derived from the original cytoplasm discovered by Owen in 1945. Southern hybridizations with mitochondrial genes coding for cytochrome oxidase subunits II and III, ATPase subunits α, 6 and 9 and 26S ribosomal RNA indicate that gene organization is different between fertile and sterile genomes but similar in all fertile genomes. Transcription analysis with the same genes indicate several differences between fertile and sterile varieties but also within some fertile varieties. These results suggest that the mt genome found in male-sterile sugar beet may originate not from modifications of the fertile mitochondrial genome but from a particular source of cytoplasm, of which a possible origin is discussed.

Key words: Sugar beet – Mitochondrial DNA – CMS – Southern hybridization analysis – Transcription

Introduction

Cytoplasmic male sterility (CMS) in sugar beet (*Beta vulgaris* L.) is recognized as an important tool for the production of hybrid varieties. Owen's (1945) source of CMS is the only one commericially used so far by plant breeders. According to Owen, CMS is governed by two recessive nuclear genes interacting with a sterile cytoplasm. But other authors (Theurer and Ryser 1969) have suggested that more than two genes are involved.

Small DNA molecules have been found in mtDNAs from maize (Kemble and Bedbrook 1980), *Oenothera herteriana* (Brennicke and Blanz 1982), *Brassica* (Palmer et al. 1983), *Vicia faba* (Goblet et al. 1985) and *Helianthus annuus* (Leroy et al. 1985); these molecules can be linear or circular and seem to replicate independently from the main mtDNA.

Sugar beet mtDNAs were first studied by Powling (1981). In addition to the high molecular weight main DNA, he reported the existence of four differently sized DNA minicircles named **a** (1.62 kb), **b** (1.5 kb), **c** (1.4 kb) and **d** (1.31 kb). Minicircle **c** has been cloned and sequences by Hansen and Marcker (1984), and minicircles **a** and **d** by Thomas (1986). Minicircle **b** is supposed to be a derivative of minicircle **a** but has not been extensively studied. The function and origin of these sugar beet minicircles are still unclear.

According to Powling (1981), who examined the occurrence of these minicircles in several different fertile and cytoplasmic male-sterile (CMS) varieties, minicircle **a** (replaced by **b** in some fertile varieties) was present in all cases, whereas only fertile varieties possess in addition minicircles **c** and/or **d.**

In the same work, Powling (1981) examined the main mtDNAs and found differences in restriction patterns between fertile and CMS mtDNAs, which he classified into two types: type 1, for fertile mtDNA and type 2, for CMS mtDNA pattern. The physical map of Owen's mtDNA and the location of several mitochondrial genes have been recently published (Brears and Lonsdale

* Present address: Laboratoire de Biologie Moléculaire et Cellulaire Végétale, BIOSEM (LIMAGRAIN), Campus des Cézeaux 24, Avenue des Landais, F-63170 Aubiere, France
** To whom offprint requests should be addressed

1988); this genome is 386 kb in size and contains repeated sequences, allowing homologous recombination generating its multimolecular organization.

In the present study our aim was to see whether cytoplasms differing from those studied by Powling exist and what differences exist between fertile and CMS mitochondrial genomes. Therefore, we compared the mitochondrial genomes of twelve isogenic pairs of fertile and CMS sugar beet varieties, by characterizing the occurrence of these minicircles and the location and expression of some mitochondrial genes.

Materials and methods

Sugar beet varieties

Sugar beet varieties were used as isogenic pairs, i.e. the male-fertile variety (maintainer "O" type) associated with its corresponding CMS. All the material has been obtained from the Institut de Recherche Mennesson (Anizy Le Chateau, France) and is listed in Table 1.

Mitochondrial gene probes

Mitochondrial genes used in hybridization studies were: *18S* and *5S* from wheat (Falconet et al. 1984), *26S* from wheat (Falconet et al. 1985), *cox2* from wheat (Bonen et al. 1984), *cox1* and *cox3* from *Œnothera* (Hiesel et al. 1987), *atpA* from *Œnothera* (Schuster and Brennicke 1986), *atp6* from maize (Dewey et al. 1985a), *atp9* from maize (Dewey et al. 1985b), *cob* from maize (Dawson et al. 1984), minicircles **a** (our own cloning), **b** and **c** (Thomas 1986) from sugar beet.

mtDNA extraction and hybridization

Mitochondria were isolated by Thomas's (1986) method from sugar beet taproots grown in the fields and stored at 4°–6°C.

Table 1. Presence of the minicircles **a, b, c** and **d** in pairs of isogenic fertile and CMS varieties. The plants were obtained from the Institut de Recherche Mennesson (Anizy le Chateau, France), where the phenotypes were checked. Although they were thought to be characteristic of fertile cytoplasms, minicircles **c** and **d** are present in three CMS varieties: 55CMS, 61 CMS and 84 CMS (bold type)

	Minicircles					Minicircles			
Variety	**a**	**b**	**c**	**d**	Variety	**a**	**b**	**c**	**d**
55		+	+	+	61	+			+
55 CMS	+		+	+	**61 CMS**	+		+	+
56	+		+	+	71	+		+	+
56 CMS	+				72 CMS	+			
57		+	+	+	81	+		+	+
57 CMS	+				82 CMS	+			
58		+	+	+	83	+		+	+
58 CMS	+				**84 CMS**	+		+	+
59		+	+	+	522	+		+	+
59 CMS	+				524 CMS	+			
60	+				607	+		+	+
60 CMS	+				607 CMS	+			

Mitochondrial DNA was purified by using CsCl-ethidium bromide gradients. Digestion by restriction enzymes, agarose gel electrophoresis and transfer on Hybond N (Amersham) membrane followed standard procedures (Maniatis et al. 1982).

For hybridization studies, probes were prepared from cloned mitochondrial genes by digestion and electrophoresis; the inserts were isolated by electroelution and labelled by nick-translation (Maniatis et al. 1982).

Prehybridization of blots was performed for 2 h at 42°C in 0.1 ml/cm^2 of buffer containing 50% formamide, 1% SDS, 6 × SSC [1 × SSC: citrate 3 Na 0.015 *M*, NaCl 0.15 *M*, 5 × Denhardt's solution (Denhardt's solution: 2% ficoll, 2% polyvinyl-pyrrolidone, 2% bovine serum albumin)]. Hybridization was carried out at 42°C for 16–24 h in the prehybridization buffer to which denatured nick-translated probes (10^8 cpm/μg) had been added. Non-specifically bound DNA was then removed by washing twice for 15 min in 2 × SSC, 1% SDS at room temperature, twice for 15 min in 1 × SSC, 1% SDS at 42°C and twice for 15 min in 0.1 × SSC, 1% SDS at 65°C.

In some cases a non-isotopic method (Chemiprobe, Orgenics Ltd) based on probe sulfonation was used (Lebacq et al. 1988); sulfonated probe concentration in hybridization was 10 μg/ml.

mtRNA extraction and Northern hybridization

Mitochondria were isolated from 200 g of 4-week-old plantlets by the slightly modified standard method (Thomas 1986): β-mercaptoethanol concentration was raised to 0.1% and DNase (50 μg/ml) treatment was for 1 h at 4°C.

Mitochondria were lysed in 50 m*M* TRIS-HCl (pH8), 20 m*M* EDTA, 2% lauroyl sodium sarkosinate and one drop of DEPC (diethylpyrocarbonate) for 1 h at room temperature. The lysate was first deproteinized with an equal volume of phenol saturated with 50 m*M* TRIS-HCl (pH8) containing 0.1% hydroxyquinoline, then successively with a phenol-chloroform (1:1) mixture and chloroform. Nucleic acids were precipitated with ethanol and stored at −80°C.

Denaturation of nucleic acids and conditions for gel electrophoresis (size marker: EcoRI/HindIII digest of λ DNA) followed the procedures of Stern and Newton (1986). Gels were transferred onto Hybond N (Amersham) with 2 × SSC buffer (Maniatis et al. 1982). Northern prehybridization and hybridization were performed at 42°C in 50% formamide, 1% SDS, 10 × Denhardt's solution and 5 × SSPE (1 × SSPE: NaCl 0.18 *M*; NaH_2PO_4 0.01 *M*, EDTA 0.1 m*M*). Washing conditions were the same as above.

Results

Occurrence of minicircles

The occurrence of minicircles in native mitochondrial DNA has been investigated in 12 isogenic pairs of fertile and CMS sugar beet varieties. Figure 1 A shows the results obtained for four couples.

Since minicircles are detectable only as relatively faint bands whose stoichiometry can vary, their presence in mtDNA was further investigated by hybridization (Fig. 1 B) with minicircles **a** (cloned by us and labelled by a non-isotopic method, Lebacq et al. 1988), **c** and **d** (gifts of C. Thomas, labelled radioactively).

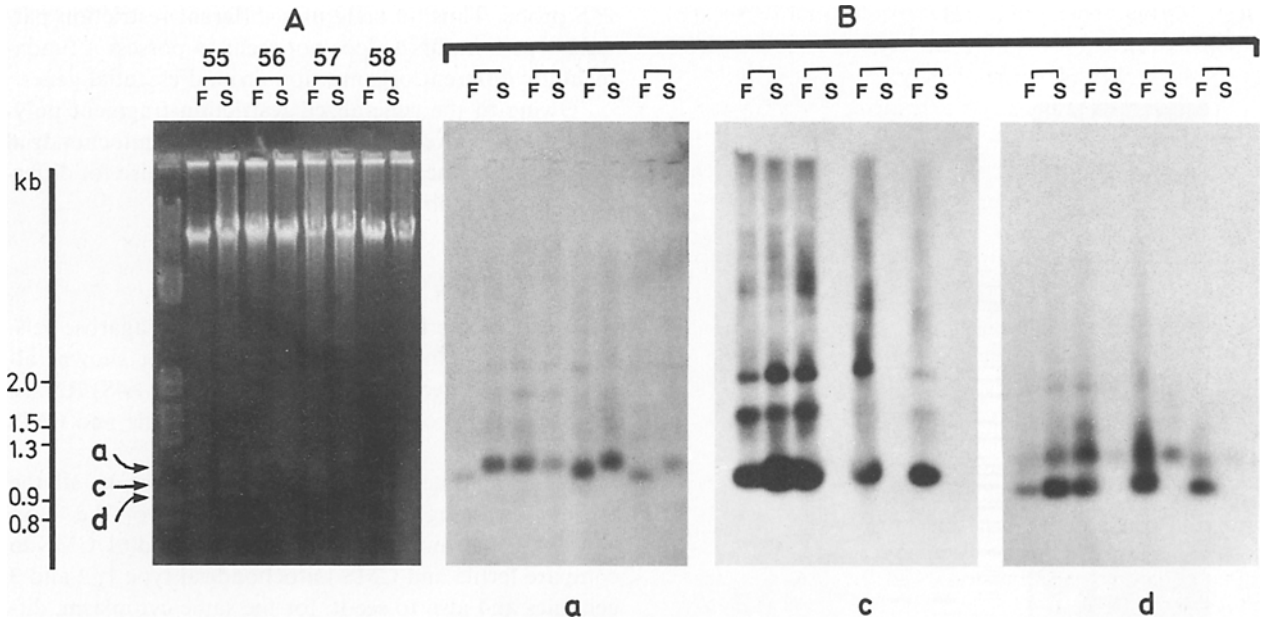

Fig. 1 A and B. Hybridization of cloned minicircle DNAs to native mtDNAs from some sugar beet varieties (nos. 55, 56, 57 and 58; F: fertile. S: male sterile). **A:** ethidium bromide-stained gel from which the blot was made. **B:** hybridizations with minicircles **a, c,** and **d.** Molecules **a** (or **b**) are present in all samples; **c** and **d** are present in all fertile varieties and in variety 55CMS, phenotypically male sterile. In this experiment, probes **c** and **d** were radioactively labelled; **a** was labelled by the non-radioactive method, Chemiprobe (Lebacq et al. 1988), based on the sulfonation of cytidyl residues in DNA and their subsequent immunoenzymological detection with monoclonal antibodies

Each probe reveals a prominent band corresponding to the supercoiled form of the minicircle, and a complex pattern of linear, open circular and multimeric molecular forms. Minicircle **a** hybridizes strongly either with a band having the expected mobility of **a** or with a faster band corresponding to the mobility of minicircle **b** (Powling 1981; Powling and Ellis 1983) (see, e.g. fertile mtDNA nos. 55, 57 and 58). Minicircles **c** and **d** are present in the four fertile varieties and also in CMS variety 55CMS.

We also detected the cross-hybridization to other minicircles shown by Thomas (1986), who reported partial sequence homology between **a** and **d** and also between **d** and **c.** Only hybridization between **a** and **d** could be detected here, owing to the stringent hybridization conditions used.

No hybridization of the different minicircles with the main mtDNA could be detected, in agreement with Powling's data (1981).

The occurrence of the different minicircles in the 12 pairs (fertile and isogenic male sterile) tested is summarized in Table 1; **a** and **b** are mutually exclusive and **b** (or presumably a shortened form of **a,** as shown by our hybridizations) is found only in fertile varieties. Minicircles **c** and **d** are not always necessarily present together, since, in fertile variety no. 61, **c** is missing. Finally, **c** and **d** have been unexpectedly found in male-sterile varieties 55CMS, 61CMS and 84CMS.

Restriction endonuclease analysis of mitochondrial DNA

Samples of total mitochondrial DNA from the isogenic pairs of sugar beet varieties were digested with different restriction enzymes. The resulting fragments were separated by gel electrophoresis. For all CMS plants, the restriction patterns are identical to Powling's (1982) type 2 pattern (Fig. 2). Most of the fertile varieties display the mtDNA pattern of type 1, but we revealed in the fertile variety no. 61 a new restriction pattern (type 3), which differs slightly from type 1 (Fig. 2A). We checked that this was not due to an incomplete digestion.

Microdensitometer scans of negatives of the gels have allowed the establishment of schemes of the three types (Fig. 2B). The microdensitometer analysis of all the restriction patterns of all the varieties tested (data not shown) displays for each type identical bands, with only fluctuations of the intensity depending on the samples.

Southern hybridization analysis

In order to see whether the differences in restriction patterns between mtDNAs affect the gene location or not, probes representing different mitochondrial genes were hybridized to membrane blots containing EcoRI and BamHI digests from all sugar beet mtDNA samples. An instance of a complete set of hybridization patterns is

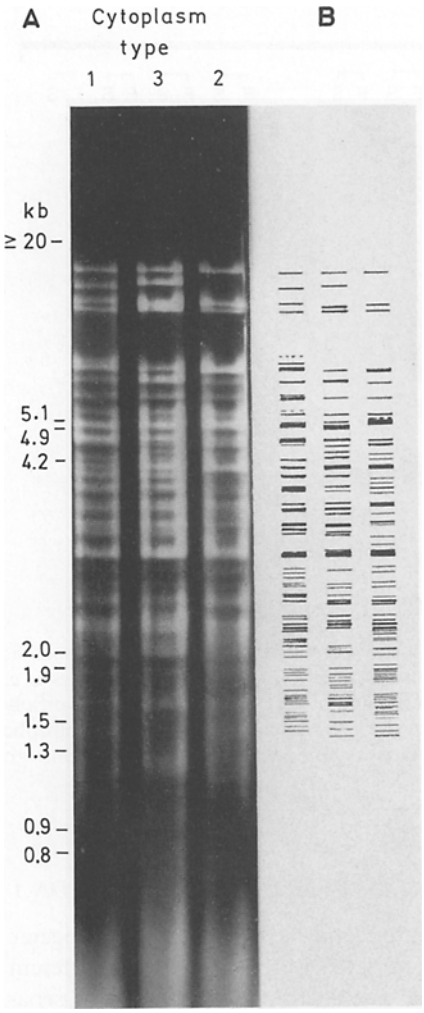

Fig. 2. **A.** EcoRI restriction patterns of types 1, 2 and 3 sugar beet cytoplasms. **B.** Corresponding schemas obtained from microdensitometer tracings

given for pair no. 56/56CMS in Fig. 3. All the results are compiled in Table 2.

The pattern displayed by CMS cytoplasm is in agreement with the data of Brears and Lonsdale (1988). According to the probes and the enzymes used, differences in the hybridization patterns can be detected between the three types (fertile-1 or -3 and sterile-2) of cytoplasms. On EcoRI digest, they can be distinguished by their *cox2, atp6, atp9* and *26S* patterns; on BamHI digests, by their *cob, cox2, cox3, atp6, atp9, atpA* and *26S* patterns.

Although the restriction profiles look identical in all fertile type 1 varieties, some differences appear in the EcoRI organization of the *26S* gene; two patterns are found depending on the size (1.7 or 1.5 kb) of the smallest hybridizing band.

The only specific difference between type 3 and type 1 fertile cytoplasms concerns the EcoRI pattern of the *26S* probe. Thus, in spite of a different restriction pattern, type 3 mtDNA does not seem to possess a fundamentally different organization around essential genes.

Owing to the generalized restriction fragment polymorphism between fertile and CMS mitochondrial genomes, it seemed to us advisable to look also for differences in transcription pattern.

Northern hybridization analysis

After extraction, RNAs were separated on agarose gels. Staining with ethidium bromide (data not shown) allowed visual detection of *26S, 18S* and (5S+4S) RNAs. No differences could be seen between fertile and CMS varieties.

We have tested by Northern hybridization all the protein encoding genes mentioned above. We have chosen the pair nos. 57/CMS, 607/CMS and 61/CMS to compare fertile and CMS mitochondrial type 1, 2 and 3 genomes and also to see if, for the same cytoplasm, differences in nuclear backgrounds had an influence. Significant profiles are represented in Fig. 4.

atp9 ist the only gene for which an identical transcript can be seen throughout all the tested varieties, with a size (0.8 kb) close to that of the tobacco transcript of the *atp9* (Bland et al. 1986). *cox3* transcript sizes are also very much alike in fertile and CMS mitochondria with one major and minor transcript. For all the other probes used, differences exist between fertile and CMS varieties. This happens for *cox1, cox2* and *atp6,* where simple patterns make the distinction easy between both types, and also for *cob,* where gene transcription profiles are more complex and show numerous variations between fertile and CMS plants.

In fertile genome, the approximate size of the major transcript for *cox2* is similar to that found in wheat (Bonen et al. 1984) and *Oenothera* (Hiesel and Brennicke 1983). In the CMS genome, the *cox2* main transcript is larger and is associated with two smaller minor transcripts.

In the case of *atpA,* differences exist not only between fertile and CMS mitochondria (three transcripts are present in CMS varieties but only two in fertile varieties) but also between fertile types; transcription in the fertile genome no. 607 (one major transcript) differs from that on other varieties.

Discussion

Previous work on sugar beet (Powling 1981, 1982; Powling and Ellis 1983) has shown that CMS, when compared with male fertility, is specifically associated with one form of mtDNA, whereas very few changes can be detected in ctDNA (Mikami et al. 1984, 1985). The exis-

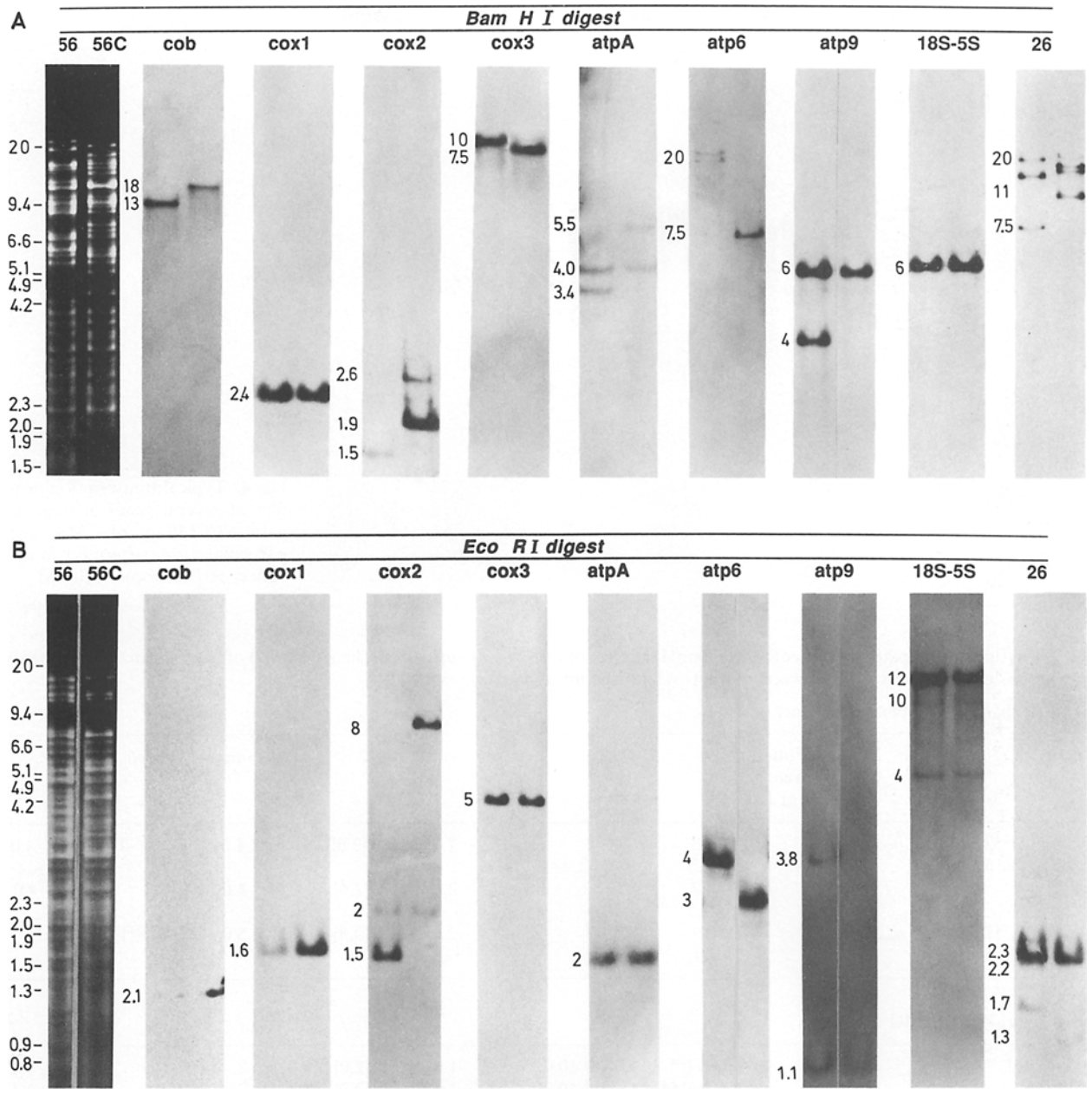

Fig. 3A and B. Hybridization patterns of mtDNAs from isogenic pair 56/56CMS restricted by **A** BamHI and **B** EcoRI

tence of two different types of mtDNA is consistent with the assumption that mtDNA encodes the CMS trait. In this work, we have investigated: (i) whether some CMS varieties differ from Owen's, and (ii) what differences (including those already reported by Powling and Mikami) could be found between fertile and CMS mitochondrial genomes concerning both their structures and their expression.

No CMS variety tested displays restriction patterns different from those reported by Powling (1982) and Mikami et al. (1984); all the CMS varieties used so far thus originate from the same cytoplasm discovered by Owen (1945). Nevertheless, the analysis of minicircle distribution shows some variations: since minicircles **c** and **d** are found in some CMS varieties, their absence in all other CMS lines has no causal relationship with male sterility. This links up with similar conclusions about the plasmid in *Brassica* mtDNA described by Kemble et al. (1986) and about those of *Helianthus annuus* (Leroy et al. 1985), which are not exclusively associated with CMS.

In the cases reported here, correlation between type 1 cytoplasm and fertility or type 2 and male sterility has

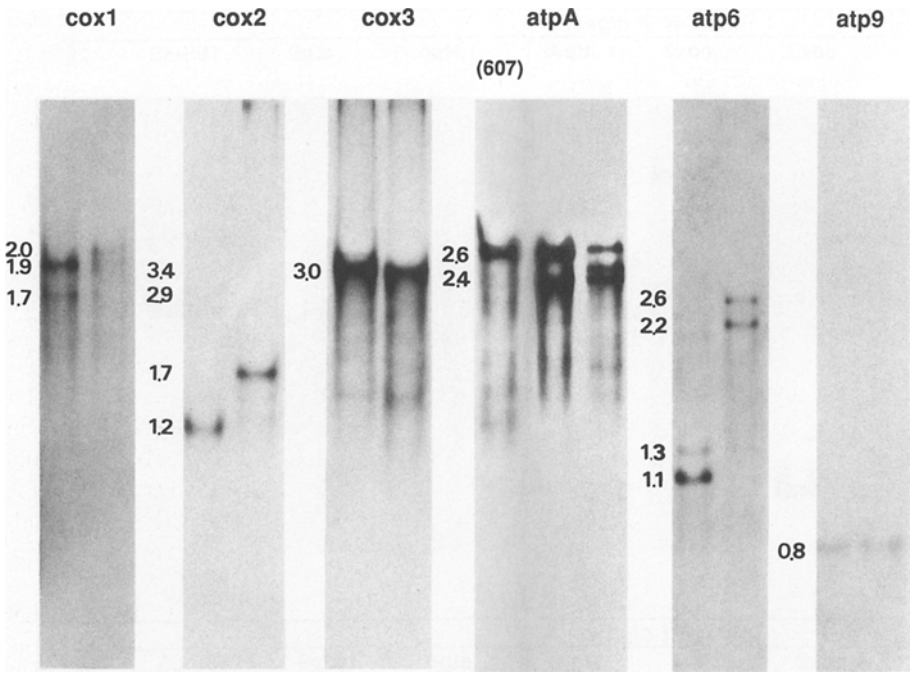

Fig. 4. Typical transcription profiles of several genes in isogenic pairs 57/CMS or 61/CMS. The particular *atpA* pattern for fertile variety 607 has been included

Table 2. Hybridization patterns of EcoRI and BamHI digests of mtDNA of sugarbeet. The sizes in kb of the hybridizing fragments are given for each probe; values between brackets relate to faint hybrization signals

Size of the hybridizing BamHI fragments

Varieties	Phenotype	DNA type	Minicircle **a** or **b**	*26 S*	*18/5 S*	*cob*	*cox1*	*cox2*	*cox3*	*atpA*	*atp6*	*atp9*
55, 56, 607, 522 71, 57, 58, 59	F	1		22–20–15–7.5	6.0	13.0	2.4	1.5 [2.6]	10.0	4.0–3.4	20–22	6.0–4.0
61	F	3		22–20–15–7.5	6.0	13.0	2.4	1.5 [2.6]	10.0	4.0–3.4	20	6.0–4.0
55, 56, 607, 524 72, 57, 58, 59	S	2		21–19–18–11	6.0	18.0	2.4	1.9 [2.5]	7.5	5.0–4.0	7.5 [1.2]	6.0

Size of hybridizing EcoRI fragments

56, 607, 522, 71	F	1	**a**	2.3 [1.7]*	12 [4.0/10.0]	2.1	1.6	1.5 [2.0]	5	2	4	3.8+1.1
55, 57, 58, 59	F	1	**b**	2.3 [1.5]**	12 [4.0/10.0]	2.1	1.6	1.5 [2.0]	5	2	4	3.8+1.1
61	F	3	**a**	2.3	12 [4.0/10.0]	2.1	1.6	1.5 [2.0]	5	2	4	3.8+1.1
55, 56, 607, 524 72, 57, 58, 59	S	2	**a**	2.3 [1.3]	12 [4.0/10.0]	2.1	1.6	8.0 [2.0]	5	2	3	1.1

* this band is found in all the lines having the minicircle a
** this band is found in all the lines having the minicircle b

been verified as in Powling's work. Differences between fertile mitochondrial genomes can exist, as evidenced by type 3.

In order to determine if the structural differences between fertile and CMS mtDNAs involve mitochondrial genes, we have realized hybridization experiments with plant mitochondrial probes.

Both fertile cytoplasms 1 and 3 display exactly the same pattern of hybridization with all probes except for *26S* gene (EcoRI digest). Moreover, for this gene, the

type 1 pattern can be divided into two subtypes: the first one displays a small 1.5-kb band accompanying the main hybridizing 2-kb band and possesses minicircle **a**; in the second subtype, this 1.5-kb band is replaced by a 1.7-kb band and minicircle **a** by **b**. The occurrence of type 3 and also of minicircle **b** in the fertile mtDNAs indicates that changes are possible in the fertile varieties but are presumably not very important. It is interesting, nevertheless, to note that this structural variation involves the *26S* gene which, in the CMS cytoplasm (Brears and Lonsdale 1988), is part of a repeated element and thus found associated with structural rearrangements. The same situation occurs in wheat (Falconet 1987), maize (Dewey et al. 1986), petunia (Young and Hanson 1987), *Oenothera* (Manna and Brennicke 1985) and *Brassica* (Vedel et al. 1987).

All hybridization patterns of CMS mtDNA are identical, but differ from the corresponding patterns in fertile varieties for all the probes tested but *cox1* and *18S* genes. The organization of this CMS genome is thus quite different, as already shown by the comparison of restriction patterns.

Our results indicate in some cases the presence of several copies of the same gene in the mt genome of sugar beet. As an instance of this, we have compared the results obtained for *cox2* with the location of this gene on the physical map published for CMS sugar beet (Brears and Lonsdale 1988) and also with several other plants (Bonen et al. 1984; Fox and Leaver 1981; Kao et al. 1984; Hiesel and Brennicke 1983; Moon et al. 1985; Turano et al. 1987). In all cases, restriction site positions emphasize the high degree of conservation of the two exons and of some parts of the intron. Our probe, originating from wheat, allows only the detection of the 5′ half of the *cox2*gene, which is present in a 2-kb EcoRI fragment as well as in a 1.5-kb (fertile varieties) or in an 8-kb (CMS varieties) fragment. Two copies of the *cox2* gene are present in both fertile and sterile genome of sugar beet, as suggested by the map of Brears and Lonsdale (1988), in which this gene is adjacent to a repeated sequence.

Further analysis is in progress to precisely determine the copy number for other genes, the existence of pseudocopies and fine mapping.

The study of the transcription profiles of the same genes confirms the real differences between fertile and CMS sugar beet mitochondrial genome; the lengths of the transcripts for each gene vary, except those of *atp9*, *18S* and *5S* and *26S*. In the absence of gene maps in both genomes, one cannot relate these differences to changes in either initiation or termination of transcription.

Furthermore, we have revealed in 1-month-old plantlets that expression variations can occur between a priori identical fertile mitochondrial genomes. The instance of *atpA* transcription is shown here: variety 607 displays only one major transcript instead of two for other fertile varieties having the same cytoplasm. This can, therefore, possibly be attributed to the nuclear background. This observation emphasizes the importance of the relationship between nuclear and mitochondrial genomes.

What might the genetical origin be of the numerous changes observed in mtDNA organization between CMS and fertile varieties? Two explanations are possible: either Owen's (1945) cytoplasm is a derivative of the actual fertile mitochondrial genome or it originates from an early interspecific cross generating a CMS alloplasmic variety (A. Bervillé personal communication). In the absence of data on this point in Owen's paper, both explanations are a priori valid. The mitochondrial genome in higher plants is very recombinogenic (Leaver and Gray 1982; Quetier et al. 1985; Newton 1988) and two specific instances of recombination associated with CMS are already known in maize (Dewey et al. 1986) and in petunia (Young and Hanson 1987).

Nevertheless, the hypothesis that the CMS type is an alloplasmic variety is more tempting to explain all the noticeable differences reported here between fertile and CMS mitochondrial genomes. It should be interesting, via a thorough screening of existing *Beta* species, to check whether one of them harbours at mtDNA identical – or very close – to that found in the sugar beet CMS varieties.

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