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Two new types of cytoplasmic male sterility found in wild *Beta* beets

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Summary. Mitochondrial (mt) and chloroplast (ct) DNAs from sugar beet carrying normal fertile and different cytoplasmic male sterile (cms) cytoplasms were compared by restriction analysis and for the occurrence of minicircles. One of the cms materials had the Owen cms cytoplasm currently used for hybrid production in sugar beet; the other three cms materials were derived from wild *Beta* beets. The mtDNAs from two of the latter cms types (C 7051, C 8640) differed from both the Owen and the fertile cytoplasms in fragment patterns seen after restriction enzyme analysis and in minicircle composition. The third cms type (C 8684) differed from the Owen cytoplasm in minicircle composition, but restriction enzyme analysis revealed no differences. The presence of the different minicircles was confirmed by Southern hybridization using minicircle-specific clones. All bands hybridized as predicted by gel electrophoresis except a band in the cms type C 8640, which migrated in a similar manner as the c.c.c. form of the *a* minicircle. This band hybridized only faintly to a minicircle *a*-specific probe and could be removed by treatment with nuclease S1. In contrast to the large mtDNA variation, restriction analysis of ctDNA detected little variation between cytoplasms. The molecular characterization of the new sources of cms supports the results of previous crossings. Two of the cytoplasms are not only of independent origin, but are also most likely functionally different and thus may be of value in future production of hybrid sugar beet varieties.

Key words: Cytoplasmic male sterility – Mitochondrial DNA – Minicircle – Sugar beet

Introduction

Cytoplasmic male sterility usually implies pollen abortion in the anthers, resulting from an interaction of organelle genes and a certain nuclear genotype referred to as a maintainer. Similarly, a genotype that counteracts the effect of the organelle sterility genes is called a restorer. Studies of organellar DNA from several plant species, e.g. maize (Pring and Levings 1978), sorghum (Pring et al. 1982 a) and rapeseed (Erickson et al. 1986) have provided strong evidence of a mitochondrial origin of cytoplasmic male sterility (cms) in these species. Correlations between cms and mitochondrial (mt) protein synthesis have also been found in several species (Boutry et al. 1984).

In sugar beet a reliable source of cms is a prerequisite for commercial production of hybrid sugar beet seed. So far the only male sterile sugar beet cytoplasm available is the one discovered by Owen (1945). Consequently all current hybrid sugar beet cultivars are based on this type of cytoplasm, referred to in this paper as the Owens cms. However, this cytoplasmic uniformity may render the crop vulnerable to certain diseases and a broadening of the cytoplasmic genetic variation is therefore desirable (Bosemark 1979). With this in mind, one of us has worked for several years with three new male sterile cytoplasms discovered in wild beets collected in the mediterranean countries in the 1950s and belonging to the section *Beta* (formerly *Vulgares*) of the genus *Beta*.

All cultivated beets and a number of highly polymorphic and widely distributed wild forms belong to the section *Beta*. Since all *Beta* beets cross easily and produce fully fertile hybrids, most taxonomists consider *B. vulgaris* L. the only authentic species and group the wild forms into a varying number of subspecies in-

cluding *B. maritima* and *B. cicla.* In this paper we refer to all truly wild *Beta* beets as *B. maritima* and the primitive leaf beets as *B. cicla.* Since their discovery, the above mentioned cytoplasmic male sterile wild beets have been converted to sugar beets by repeated backcrossing with male sugar beet parents. Concurrently maintainers have also been isolated. Because the wild cms plants all came from the original seed samples and were collected well before the introduction of hybrid sugar beet varieties in the countries in question, there can be no doubt that they constitute new and independent origins of cms. Two of the new cms types also require maintainers very different from that of Owen cms.

So far molecular studies of cms in beets have focused mainly on the fertile and the Owen cytoplasms. These studies have shown that the Owen cytoplasm differs from that of a normal pollen fertile plant with respect to the number and kind of minicircles present in the mitochondria (Powling 1981; Powling and Ellis 1983). Differences have also been found in mtDNA restriction patterns (Powling 1982) and in mt in organello translation products (Boutry et al. 1984). Recently, however, different sources of male sterile cytoplasms originating from wild *Beta* beets have been compared with the two previously known types. Restriction analysis (Mikami et al. 1985) revealed at least one distinctly different cms type and analysis of the minicircle content (Mikami et al. 1986) showed additional low molecular DNA bands in all male sterile lines when compared with the Owen cms.

In the present investigation the three new sources of cytoplasmic male sterility, none of which have the same origin as any of the lines investigated by Mikami et al. (1985, 1986), have been characterized with respect to the variation in their mitochondrial and chloroplast (ct) DNAs. The object was to study how differences in origin and apparent maintainer requirement is reflected at the molecular level and to provide conclusive evidence of truly qualitative differences between the new cms sources and the Owen cms.

Materials and methods

Beet material

The following sugar beet materials, obtained from Hilleshög AB, Sweden, were studied: a) Primahill – a commercial diploid, monogerm hybrid variety, carrying the Owen male sterile cytoplasm; b) LNSZ 1 – a normal male fertile, slightly inbred, multigerm population; and c) C 7051, C 8640, C 8684, three breeding populations with male sterile cytoplasms originating from wild or primitive *Beta* species and developed through repeated back crossing to a broad-based monogerm sugar beet population.

Previous crosses with restored male steriles as pollen parents have shown that in all three populations the sterility is cytoplasmic in nature (NO Bosemark, unpublished). The origin of the new sources of cms is as follows: (1) C 7051 – an annual *B. maritima* collected in Morocco in 1953, location unknown; (2) C 8640 – a semi-annual *B. cicla* found in a garden near Omnis, 25 km from Split in Yugoslavia in 1956; and (3) C 8684 – an annual *B. maritima* collected near Aliaga north of Izmir in Turkey in 1956.

The C 8640 and C 8684 samples were collected during a plant collecting expedition to the mediterranean countries in 1956 (Olsson and Ellerström 1957; Ellerström 1963).

Isolation of mitochondria

Mitochondria were isolated at 4 °C from taproot tissue of one or more greenhouse grown plants. Fifty to five-hundred grams were chopped and homogenized in 3 vol. of ice-cold homogenization buffer (0.25 *M* sucrose, 0.005 *M* EDTA, 0.2% BSA, 0.05% cysteine, 0.02 *M* MOPS pH 7.4) for 30 s using a Waring blender. The slurry was filtered through 4 layers of cheesecloth and 2 layers of a 30 μm net before centrifugation at 2,000 g for 5 min. The supernatant was centrifuged at 12,000 g for 15 min and the resulting pellet was re-suspended with a P-E homogenizer in 25 ml homogenization buffer. The homogenate was centrifuged at 1,000 g for 5 min; $MgCl_2$ was then added for a final concentration of 0.01 *M* and DNase I was added (10 μg/g fresh weight). The mitochondria were incubated at 4 °C for 1 h and then centrifuged through 150 ml of 0.6 *M* sucrose, 0.02 *M* EDTA, 0.01 *M* MOPS pH 7.2 at 12,000 g for 15 min. The pellet was lysed in 2% SDS, 0.01 *M* EDTA, 0.05 *M* Tris pH 8.0 at 20 °C for 10 min before the extraction of DNA.

Isolation of chloroplasts

Chloroplasts were isolated at 4 °C by a modification of the method of Palmer (1982). Leaf tissue (100–200 g) from the same plants used for taproot mitochondria isolation was washed with 70% ethanol and distilled water. The leaves were homogenized in 4 vol of homogenization buffer (0.33 *M* sorbitol, 0.005 *M* EDTA, 0.1% BSA, 0.05 *M* Tris pH 8.0) in a Waring blender for 20 s. The homogenate was filtered through 4 layers of cheesecloth and 2 layers of a 30 μm net. The filtrate was centrifuged at 50 g for 5 min and the resulting supernatant re-centrifuged at 2,000 g for 3 min. The chloroplast pellet was re-suspended in 25 ml homogenization buffer containing 0.01 *M* $MgCl_2$, DNase I was added (10 μg/g fresh weight) and the mixture was incubated at 4 °C for 1 h. The chloroplasts were washed by 3 centrifugations through 150 ml of 0.3 *M* sucrose, 0.05 *M* EDTA, 0.05 *M* Tris pH 8.0 at 2,000 g for 3 min. Finally, the chloroplasts were lysed with the same method as the mitochondria.

Isolation of DNA

To the lysed mitochondria or chloroplasts, NH_4Ac was added for a final concentation of 0.2 *M* and the DNA was purified by three extractions with aq-saturated phenol and ethanol precipitation. The pellet was re-suspended in 0.5% SDS, 0.005 *M* EDTA, 0.01 M Tris pH 7.8 and incubated with 500 μg/ml proteinase K at 37 °C for 1 h. Finally, the DNA was purified as described above and dissolved in TE-buffer.

Enzyme treatments and gel electrophoresis

Digestion with nuclease S1 (Boehringer-Mannheim) transformed covalently closed minicircles into their open circular and linear forms. Digestions were performed in 0.3 *M* NaCl, 0.03 *M* sodium acetate pH 4.6, and 1 m*M* zinc sulphate. After treatment the mtDNA was separated on 1.5% horizontal agarose

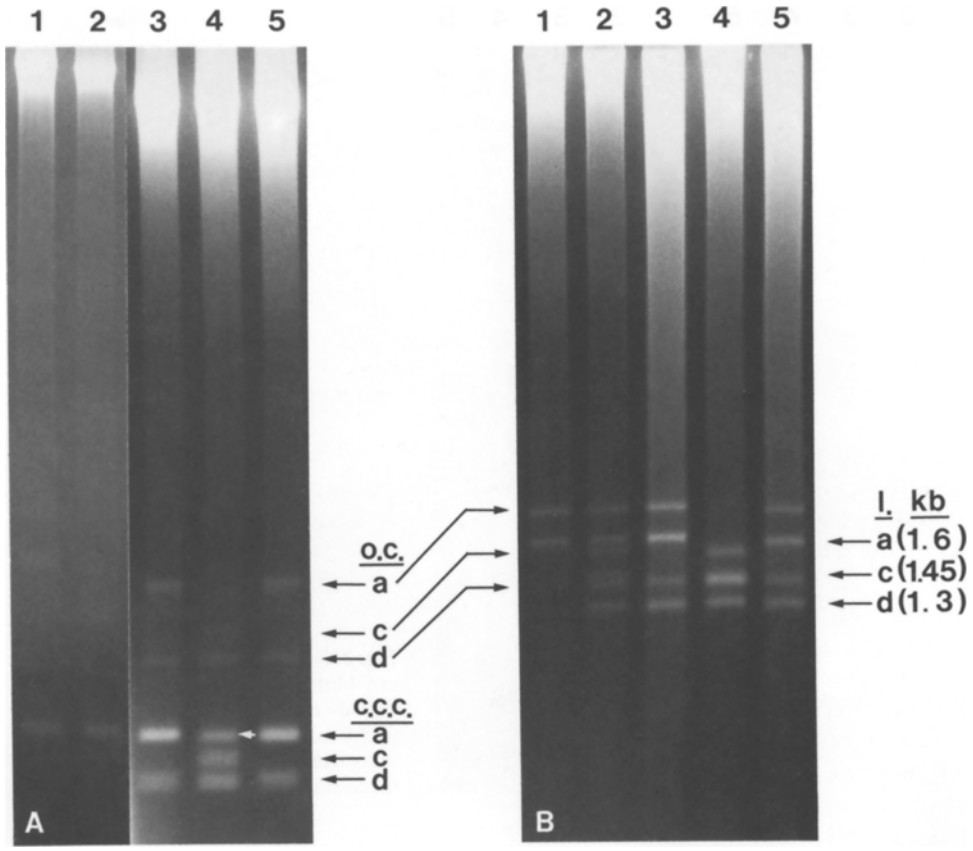

Fig. 1 A, B. Electrophoresis in 1.5% agarose gels of mtDNA from fertile and cms sugar beet materials. *Lanes 1* Primahill (cms), *2* LNSZ 1 (fertile), *3* C 7051 (cms), *4* C 8640 (cms), *5* C 8684 (cms). Minicircle species are in their linear, (l) open circular, (*oc*) and covalently closed circular (*c.c.c.*) forms, respectively. **A** Native mtDNA **B** mtDNA digested with 100 units of nuclease S1 for 30 min at 37 °C

gels at 1.3 V/cm for 16 h at room temperature. Restriction endonucleases Sal I, Hpa II, Xba II, Pvu II, Bam HI, Bcl I and Hind III (Boehringer-Mannheim) were used as recommended by the manufacturer. The DNA fragments were separated on 0.7% horizontal agarose gels at 2.0 V/cm for 16 h at room temperature. The electrophoresis buffer used was 0.04 *M* Tris-acetate, pH 7,8, 0.002 *M* EDTA. The gels were stained with ethidium bromide (3 μg/ml) for 30 min, de-stained for 30 min and analysed under UV-light. Molecular size markers were bacteriophage lambda DNA digested with Hind III and pBR 322 digested with Hinf I or Bgl I.

Hybridization

The hybridization probes were plasmid pBR 322 with minicircles *a, c* and *d* inserted in the Nru I, Eco RI and Hind III sites,, respectively (Hansen and Marcker 1984). The plasmids were a gift from M. Lund, Department of Molecular Biology and Plant Physiology, University of Aarhus, Denmark. They were labelled to a specific activity of $1-5\times10^7$ cpm/μg by nick translation using α^{32}P dCTP (Maniatis et al. 1982).

MtDNA samples were separated on 1.5% agarose gels and partially depurinated in 0.2 *M* HCl for 15 min. The gels were then denatured, neutralized and transferred to Gene Screen membranes by electroblotting at 0.4 A for 16 h. Membranes were baked in vacuo at 80 °C for 2 h and pre-hybridized at 65 °C for 5 h in 5×SSC, 1% SDS and 10× Denhardt's solution with sheared salmon DNA (0.1 mg/ml). The denatured probe was added and the blots were hybridized for 16 h at 65 °C, washed with two changes of 1×SSC, 1% SDS at room temperature and then washed in the same buffer for 2 h at 65 °C. Autoradiography was carried out at −70 °C using Kodak X-omat G film.

Results

Mitochondrial DNA

Analysis of low molecular weight DNA molecules. Mitochondrial DNA preparations from fertile and cms sugar beets were fractionated by electrophoresis in 1.5% agarose gels (Fig. 1 A). The mtDNA was separated into high molecular weight (HMW) mtDNA at the top of the gel and discrete bands of low molecular weight (LMW) DNA molecules at the bottom. Most of these LMW molecules were shown by nuclease S1 digestion to be minicircles of three different molecular weights in the form of covalently closed and open circular DNA molecules.

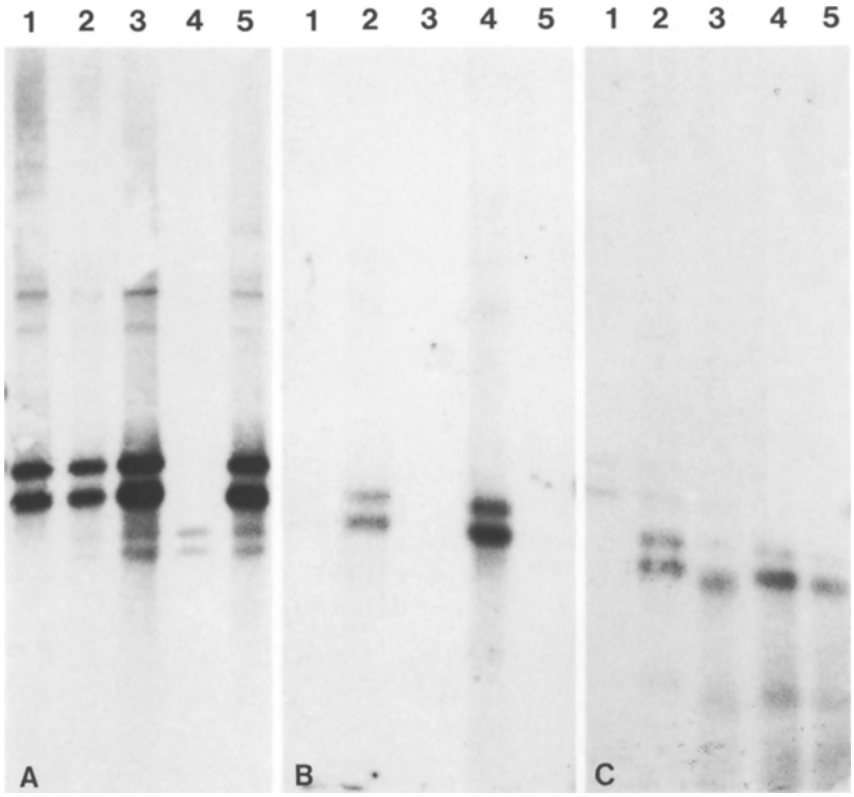

Fig. 2 A–C. Autoradiographs of Southern blots after hybridization to ^{32}P-labelled recombinant plasmids specific for: **A** minicircle *a*, **B** minicircle *c*, and **C** minicircle *d*. The Southern blots were taken from gels as in Fig. 1 B

Table 1. Minicircle composition of mtDNA from different sugar beet materials

Materials	Origin of cytoplasm	Fertile/ sterile	Minicircles		
			1.6 *a*	1.45 *c*	1.3 kb *d*
Primahill	Hybrid sugar beet variety, Hilleshög AB	S	+		
LNSZ 1	Multigerm sugar beet population, Hilleshög AB	F	+	+	+
C 7051	*B. maritima* Morocco	S	+		+
C 8640	*B. cicla* Yugoslavia	S	*	+	+
C 8684	*B. maritima* Turkey	S	+		+

* a molecule migrating as the c.c.c. form of the *a* minicircle is present in agarose gels but does not hybridize to the cloned *a* minicircle

The sizes of the LMW molecules were determined by digestion of the mtDNA samples with nuclease S1. The resulting linear forms were analyzed by electrophoresis and compared with a set of marker fragments (Fig. 1 B). Table 1 summarizes the minicircle patterns observed in the five cytoplasms. The male fertile LNSZ 1 contained minicircles of approximate molecular weight 1.6 kb, 1.45 kb and 1.3 kb. Primahill, which carries the cms source originally discovered by Owen (1945), contained only DNA molecules of 1.6 kb molecular weight. The other three cms cytoplasms possessed different combinations of low molecular weight DNA molecules. All bands were resistant to digestion with RNase but disappeared after treatment with DNase (data not shown).

Powling (1981) and Thomas (1986) detected minicircles with 1.6 kb, 1.45 kb and 1.3 kb sizes in their beet material and named them minicircles *a*, *c* and *d*, respectively. The presence of *a*, *c* and *d* minicircles in our different cytoplasms was confirmed by Southern blot hybridization to recombinant plasmids specific for the different minicircles. As can be seen in Fig. 2 A-C, the hybridization was, in all cases except one, as predicted by size comparisons using gel electrophoresis. The cms cytoplasms show three different sets of minicircles: *a* (Primahill); *a*, *d* (C 7051, C 8684) and *c*, *d* (C 8640). In agreement with previous results there was no hybridization between the minicircle-specific probes and HMW mtDNA in any of the materials tested (Powling and Ellis 1983; Hansen and Marcker 1984; Thomas 1986). The recombinant plasmid specific for the *a* minicircle cross hybridized faintly with the *d* minicircle and vice versa (Fig. 2 A, C). For all three

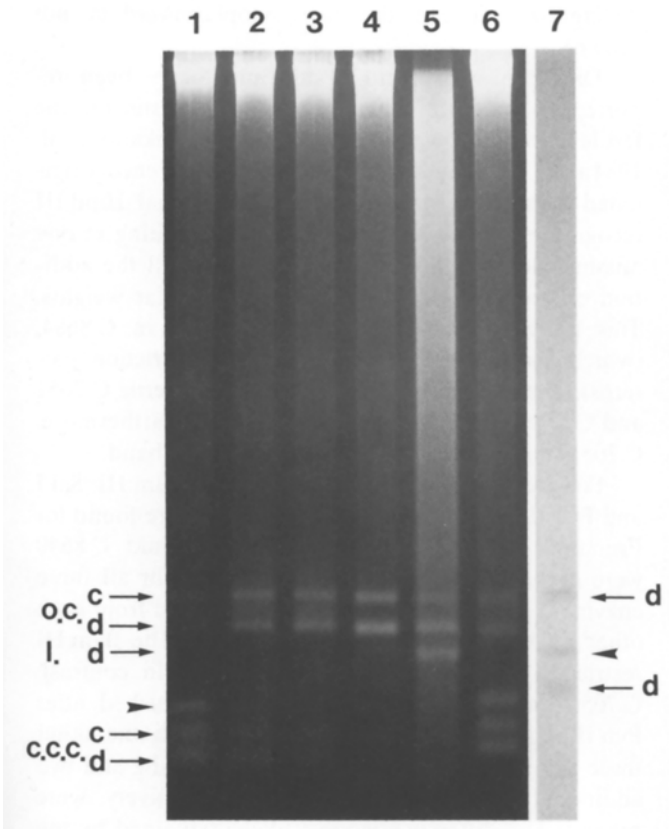

Fig. 3. MtDNA from C 8640 analysed on a 1.5% agarose gel. *Lanes 1* untreated mtDNA, *2* mtDNA digested with 2.5 units nuclease S1 for 5 min at room temperature, *3* mtDNA digested with 5 units of nuclease S1 for 5 min at room temperature, *4* mtDNA digested with 5 units of nuclease S1 for 30 min at 37 °C, *5* mtDNA digested with 50 units of nuclease S1 for 2 h at 37 °C, *6* mtDNA incubated with ethidium bromide (10 µg/ml) for 30 min under UV-light, *7* autoradiography of Southern blot of native C 8640 mtDNA after hybridization to ^{32}P-labelled recombinant plasmid specific for minicircle *a*

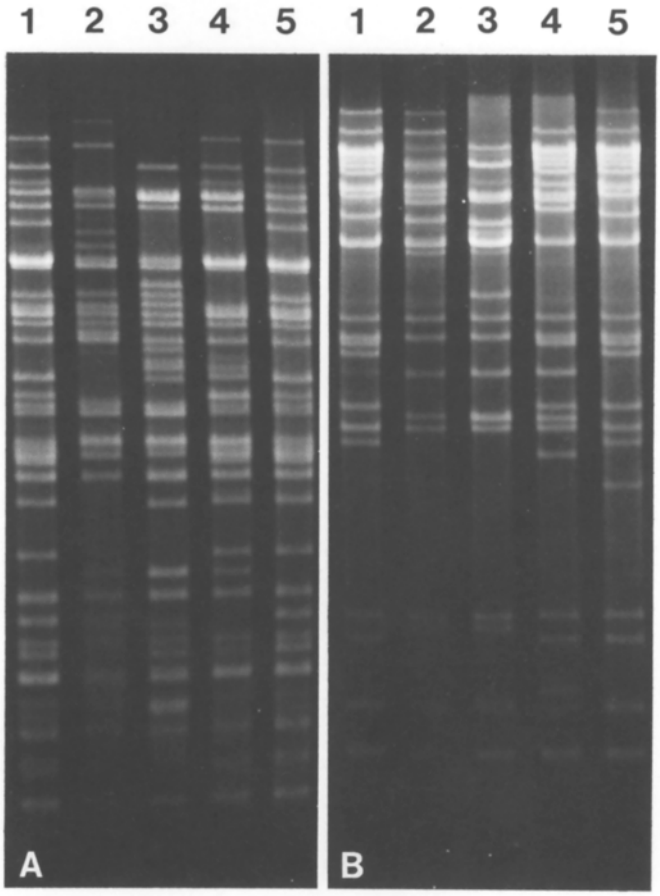

Fig. 4 A, B. Electrophoresis in a 0.7% agarose gel of **A** Bam H1 and **B** Sal 1 digested mtDNA from fertile and cms sugar beet materials. *Lanes 1* Primahill (cms), *2* LNSZ 1 (fertile), *3* C 7051 (cms), *4* C 8640 (cms), *5* C 8684 (cms)

minicircles, in all cytoplasms, the predominant forms in native mtDNA were c.c.c.-forms but o.c.-forms were also detected, possibly due to mechanical shearing when isolating the DNA. Multimeric forms of the *a* minicircle were detected but were present in much lower numbers than were the monomeric forms (Fig. 2 A). In all cytoplasms where comparisons were possible, the minicircle *a* was present in higher copy numbers than minicircles *c* and *d* (Fig. 1 A, lanes 2, 3 and 5).

A band was observed in C 8640 which migrated as the c.c.c. form of the *a* minicircle (Fig. 1 A, *lane 4*) but very little hybridization occurred with the *a* minicircle-specific probe (Fig. 3, *lane 7*). The level of hybridization was approximately that of the cross-hybridizing *d* minicircle. The band immediately disappeared when treated with low concentrations of nuclease S1 (2.5 units for 5 min at room temperature; Fig. 3, *lane 2*). No transformation to open circular or linear molecules could be detected for this particular molecule (Fig. 3, *lanes 1–6),* either upon nuclease S1 digestion or after addition of Ethidium bromide and radiation with UV-light. The origin and nature of the band is still unknown.

Restriction endonuclease analysis. Mitochondrial DNA from the five cytoplasms was digested with the restriction endonucleases Bam HI and Sal I, which previously have been shown to distinguish normal fertile from Owen cms cytoplasms (Powling 1982). Fig. 4 A shows the distribution of fragments obtained after Bam HI digestion. The restriction patterns of the fertile (*lane 2*) and of the Primahill cms cytoplasm (*lane 1*) correspond to the type 1 and type 2 patterns described by Powling (1982), although a different beet material was used. The Bam HI restriction analysis revealed apparently identical restriction fragment patterns for the Primahill and C 8684 cms cytoplasms. The two remaining cms sources and the fertile type differed from each other and from

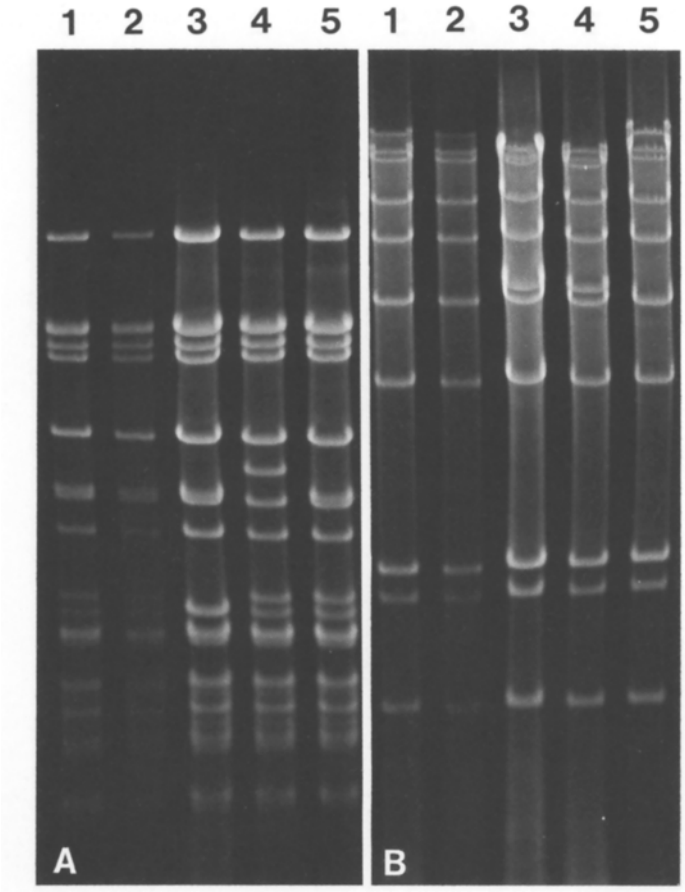

Fig. 5 A, B. Electrophoresis in a 0.7% agarose gel of **A** Bam HI and **B** Pvu II digested chloroplast DNA from fertile and cms sugar beet materials. *Lanes* are as in Fig. 4

Primahill and C 8684 in all combinations by at least 6 out of approximately 30 bands.

Similar results were obtained when Sal I was used for analysis (Fig. 4 B). Again, the two cms cytoplasms Primahill and C 8684 had identical restriction patterns and the four cytoplasmic types could be distinguished from each other by a difference of 9 or more bands. Together, these results indicate that C 7051 and C 8640 represent two new forms of male sterile cytoplasms in the sugar beet which can be distinguished from the presently used Owen cms cytoplasm by simple restriction analysis.

Chloroplast DNA

Chloroplasts from the fertile and the four cms cytoplasms were isolated and their DNA purified for restriction analysis. The DNA samples were digested with Hpa II, Bcl I, Xba I, Hind III, Bam HI, Sal I and Pvu II and the restriction fragments were separated on 0.7% agarose gels. Hpa II, Bcl I and Xba I could not distinguish between the five cytoplasms (data not shown).

Digestion with Hind III has previously been reported to generate different ctDNA patterns for the fertile and the Owen cms cytoplasms (Mikami et al. 1984 a, b; Kishima et al. 1987). The difference originated from the generation of one additional Hind III recognition site in the Owen cms type resulting in one missing band in this cytoplasm (5.3 kb) and the addition of two new bands with lower molecular weights. This pattern was seen in Primahill and in C 8684, (which also showed identical mtDNA restriction patterns). The male fertile LNSZ 1 and male sterile C 7051 and C 8640 all contained the 5.3 kb band. Furthermore, C 7051 showed one extra cytoplasm specific band.

For each of the other three enzymes – Bam HI, Sal I and Pvu II – identical restriction profiles were found for Primahill, LNSZ 1 and C 8684. C 7051 and C 8640 were different from the other cytoplasms for all three enzymes. The two groups were distinguished from each other by 3 bands with Bam HI and Sal I. The Bam HI restriction analysis is shown in Fig. 5 A. In contrast, C 7051 and C 8640 could not be distinguished after Pvu II digestion (Fig. 5 B). Compared with the other three cytoplasms, one 32 kb band was missing and two additional bands, 21 kb and 11 kb, respectively, were present. The difference is most likely explained by the creation of a Pvu II recognition site within the 32 kb fragment.

Discussion

Analysis of organelle DNA has demonstrated a considerable variation in cultivated and wild *Beta* beets carrying the cms trait. In general, the more conservative nature of the ct-genome when compared with the high rate of change found in the mt-genome has made restriction analysis of ctDNA useful in the study of taxonomic relationships between species. Kishima et al. (1987) detected little ctDNA variation between five different species in the genus *Beta,* section *Beta.* In this investigation we have demonstrated that ctDNA shows much less variation than the correponding mtDNA. Thus, only one difference was found in ctDNA patterns between the male fertile LNSZ 1 when compared with the Owen cms (both sugar beets), which strongly suggests that mtDNA is the carrier of the cms trait. The ctDNA differences found in this study are probably a reflection of evolutionary changes rather than being associated with differences in fertility. As both the main mt-genome and the different minicircles have been implicated as potential carriers of the cms trait in several plant species, both were investigated to try to resolve this question.

Analysis of minicircular mtDNA

A number of LMW linear or circular molecules have been shown to exist in the mitochondria of many higher plants. The presence of such LMW-molecules has been correlated with cms: in maize (Levings et al. 1980), faba been (Goblet et al. 1985), sunflower (Leroy 1985), *Brassica* (Palmer et al. 1983), *Sorghum* (Pring et al. 1982b) and sugar beet (Powling 1981; Mikami et al. 1986). In sugar beet, the *c* and *d* minicircles were postulated to be involved in the maintenance of fertility, since they were absent in all cms lines and present in all fertile lines investigated by Powling (1981). However, results presented by Mikami et al. (1986) show the *c* minicircle to be present not only in wild sources of cms beets but also in an American variety carrying the Owen type of cytoplasm. We detected only the *a* minicircle in the Primahill variety carrying the Owen cytoplasm, but in C 8684, which was shown to generate a mt-restriction pattern identical to that of Primahill, both the *a* and *d* minicircles were present. The same minicircle pattern was also found in C 7051. Furthermore, we detected both the *c* and *d* minicircles in C 8640, where the minicircle *a* was missing. Minicircle *a* was previously found in the majority of the cytoplasms tested (both fertile and cms), with the exception of the few that contained *b* instead. Thus, a correlation between cms and presence or absence of specific minicircles does not exist in the *Beta* beets. The correlation reported by Powling and Ellis (1983) was due to the fact that all cms-lines tested had a common origin.

Restriction analysis of mtDNA

Restriction analysis of mtDNA shows that there are at least three distinctly different cytoplasmic types of cms *Beta* beets. All three clearly differed from the male fertile sugar beet studied. However, identical restriction patterns were found in the mtDNA (and ctDNA) of C 8684 and Primahill cytoplasms. Maintainers for the Owen type also sterilise the C 8684 type (NO Bosemark, unpublished), which further confirms their resemblance. The other sources of cms, C 7051 and C 8640, which had different restriction patterns, are not maintained by maintainers for the Owen type, further implying their difference from C 8684 and Primahill. Since C 8684 was collected in Turkey in 1956 (Ellerström 1963), we assume that the Owen type of cytoplasm may have its origin in populations of *B. maritima* from Turkey. This hypothesis is supported by the investigation of Mikami et al. (1985) on different sources of cms from wild beets. Three lines gave identical restriction fragment patterns for both mtDNA and ctDNA when compared with the Owen cytoplasm. All three lines had a cytoplasmic origin in wild beets from Turkey.

cms in sugar beet

While "alloplasmic cms" can be interpreted as poor cooperation between the nuclear genes of one species and the organelle genes of another, it is interesting to note that the new sources of cms investigated in this study do not have their origin in the interspecific cross, but were also cms in their original nuclear background. As a result it can be assumed that part of the variation detected is due to evolutionary changes and part of it may be due to the difference in fertility. Since cytoplasmic male fertile cytoplasms from different wild sources were not compared with the corresponding cms cytoplasms, we cannot be certain that the organellar DNA variation (or any part of it) observed among the male sterile cytoplasms is closely associated with the sterility trait. However, Boutin et al. (1987), in a study of a natural population of *Beta maritima*, compared normal fertile lines of *B. vulgaris* and *B. maritima* with restriction analysis and found them to differ only by three fragments of mtDNA. The cms *B. maritima* found in that study was very different to both the Owen cms type and the normal fertile types, indicating variation resulting from differences between fertile and cms types of mtDNA to be larger than the corresponding interspecific differences.

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