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Molecular characterization of mitochondrial DNA of different subtypes of male-sterile cytoplasms of the sugar beet *Beta vulgaris* L.

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Summary. Mitochondrial (mt) DNA from eight cytoplasmic male-sterile (cms) lines of sugar beet from different breeding stations was investigated by restriction fragment analysis and Southern hybridization. All cms lines showed similar but not identical restriction and hybridization signal patterns, readily distinguishable from those of fertile (N) cytoplasm. Digestion of the mtDNA with BamHI, EcoRI, SalI, and XhoI revealed distinct differences between the sterile lines, and six subtypes of the S cytoplasm could be distinguished. Differences between the sterile lines were confirmed by hybridization with a *COXII* gene probe revealing minor, line-specific hybridization signals. The data presented provide evidence for the existence of considerable variation within the only commercially used source of cms in the sugar beet, the Owen's type of cytoplasm.

Key words: *Beta vulgaris* – Cytoplasmic male sterility – mtDNA – Restriction fragment patterns – Filter hybridization

Introduction

Cytoplasmic male sterility (cms) is a common trait in many higher plants and is widely used for hybrid seed production. There is considerable evidence suggesting that cms is encoded by the mitochondrial genome and controlled by nuclear dominant restorer genes (*Rf*), which repress cms, and recessive maintainer genes (*rf*), which maintain the trait (reviewed by Lonsdale et al. 1988; Levings and Brown 1989). cms in the sugar beet relies on one type of cytoplasm called S or Owen's type (Owen 1942, 1945). It is generally assumed that all commercially available hybrid seed contains this type of cytoplasm. Sugar beet mtDNA was first studied by Powling (Powling 1981, 1982; Powling and Ellis 1983), who found striking differences in the restriction patterns between fertile and male-sterile mtDNAs. All published restriction fragment patterns of mtDNA isolated from sugar beet cms plants seem to be identical (Powling 1982; Mikami et al. 1985; Weihe et al. 1985; Brears and Lonsdale 1988; Duchenne et al. 1989).

A physical map of the S-(Owen's) type cytoplasm mtDNA has been published, and several mitochondrial genes have been localized on it (Brears and Lonsdale 1988). The mitochondrial genome is 386 kb in size and contains five repeats which allow homologous recombination, resulting in a multipartite structure of the genome. Thus, the sugar beet mitochondrial genome exhibits a more complex structure than any other dicotyledonous plant so far studied. In addition to the high molecular weight DNA, sugar beet mitochondria contain four minicircular DNAs: mc a (1.62 kb), mc b (1.5 kb), mc c (1.45 kb), and mc d (1.31 kb) (Powling 1981; Hansen and Marcker 1984; Thomas 1986). The data regarding a correlation between the presence or absence of these minicircular DNAs and the expression of male sterility are controversial. In most cases, the fertile cytoplasms of *Beta vulgaris* contained mc c and/or mc d, which were lacking in all cms lines thus far examined (Powling 1981; Hansen and Marcker 1984; Budahn et al. 1989), except for three varieties described recently by Duchenne et al. (1989). A loss of mc c was observed during spontaneous reversion to male sterility in fertile sublines of *Beta vulgaris* (Dudareva et al. 1990). The function and origin of the minicircular mtDNAs of the sugar beet still remain to be elucidated.

In our search for divergent or new types of cytoplasms, we investigated a number of cms lines that originated from different breeding stations. In this paper, we

present restriction analysis and hybridization data that demonstrate the existence of diverging subtypes of the Owen's type cytoplasm.

Materials and methods

Plant material

Eight cytoplasmic male-sterile and one fertile sugar beet (*Beta vulgaris* L.) lines were used in this study. Their origins are listed in Table 1. Lines KW-S/89, KW-N/89, "Regina," and JAP were from the collection of the Institute for Beet Research, Klein Wanzleben and were maintained by O-type plants, except JAP, which was a hybrid obtained on the basis of the sterile form. Lines P1492, P1517, 8577, 83115, and C94 were from the Laboratory of Population Genetics of the Institute of Cytology and Genetics, Novosibirsk, and represented the male steriles of isogenic pairs with four to six generations of backcrosses.

Preparation of mtDNA

Mitochondria were isolated by differential centrifugation and DNase I treatment from 400 g taproot tissue or 200 g 5-day-old etiolated seedlings, as described by Chase and Pring (1985). The mitochondria were lysed with cetyltrimethylammoniumbromide (CTAB) and the mtDNA was isolated according to Rogers and Bendich (1985). RNA was removed by ammonium acetate precipitation (Dörfel et al. 1989).

Restriction analysis and gel electrophoresis of mtDNA

Samples of mtDNA (3–5 μg) were digested with restriction endonucleases (Boehringer, Mannheim, and Genofit S.A., Geneva) for 5 h under the conditions recommended by the enzyme suppliers. Digested mtDNA was loaded onto horizontal 0.8% agarose gels and electrophoresed at 50 V for 16 h in TRIS-borate buffer (Maniatis et al. 1982). Gels were stained with ethidium bromide and photographed on a UV transilluminator (312 nm).

Filter hybridization

DNA was transferred from the gels onto nylon membranes (Compas, Genofit S.A., Geneva) using vacuum blotting and an alkaline transfer protocol, as recommended by the manufacturer of the membrane. Mitochondrial gene probe *COXII* (cytochrome c oxidase subunit II) from *Zea diploperennis*, a 2.5-kb EcoRI clone (kindly provided by Dr. C. S. Levings III, Raleigh/NC), was labelled with ^{32}P-dATP (Amersham) by random priming, according to Feinberg and Vogelstein (1984). Filter hybridization was performed in 1 *M* NaCl, 1% SDS, 50 m*M* TRIS-HCl (pH 7.5), 50% formamide, and 0.5% nonfat dry milk at 42 °C overnight. Filters were washed twice at 65 °C in 2 × SSC, 0.5% SDS and twice in 0.5 × SSC, 0.5% SDS for 30 min, followed by an additional wash in 0.1 × SSC, 0.1% SDS at room temperature. Filters were exposed to ORWO X-ray film HS11 at −70 °C for 1–2 days using intensifying screens.

Results

Restriction analysis of mtDNAs

mtDNA from one fertile and eight cms sugar beet lines was digested with restriction endonucleases BamHI, BglII, EcoRI, HindIII, PstI, SalI, SmaI, and XhoI. In all cases, the mtDNA from the male-sterile lines differed

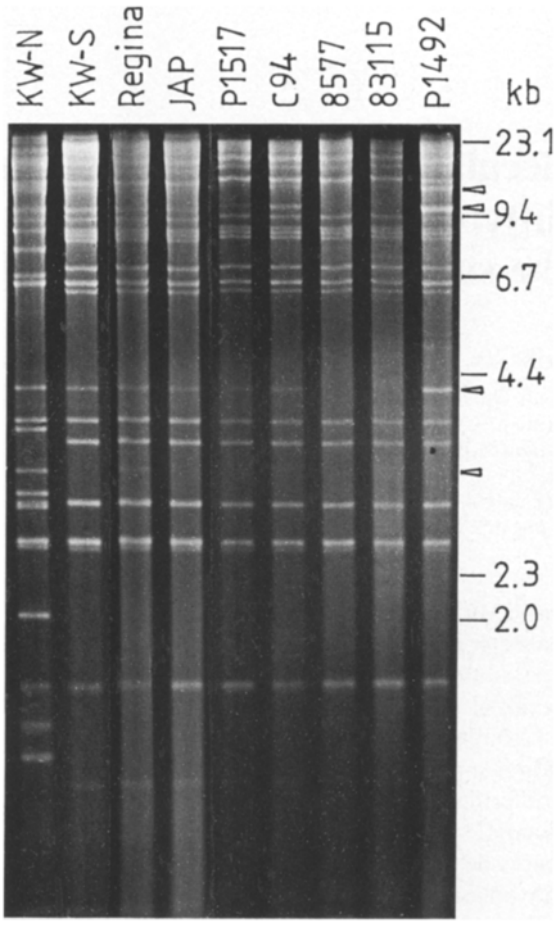

Fig. 1. SalI restriction patterns of mtDNA from one fertile (KW-N/89) and seven male-sterile lines of *Beta vulgaris*. Lambda DNA-Hind III restriction fragments were used as molecular weight markers (kb). The *arrows* indicate the positions of the line-specific fragments (12.3, 10.5, 4.0, 3.0 kb)

Table 1. Origin of sugar beet lines used in the experiments

Material	Cytoplasm	Origin
KW-N/89	N (fertile)	Institute for Beet Research, Klein Wanzleben
KW-S/89	cms	Institute for Beet Research, Klein Wanzleben
"Regina"	cms	Comercial cultivar, Sweden
JAP	cms	Hybrid, Japan
P1492	cms	SLC-91 cytoplasm, USA
P1517	cms	Station for Selection and Genetics, Uman, USSR
8577	cms	All-Russian Sugar Beet and Sugar Institute, USSR
83115	cms	Experimental Breeding Station Uladovskaya, USSR
C94	cms	Sterile cytoplasm obtained as a result of spontaneous reversion from fertile line SOAN-31-19, Laboratory of Population Genetics, Novosibirsk, USSR

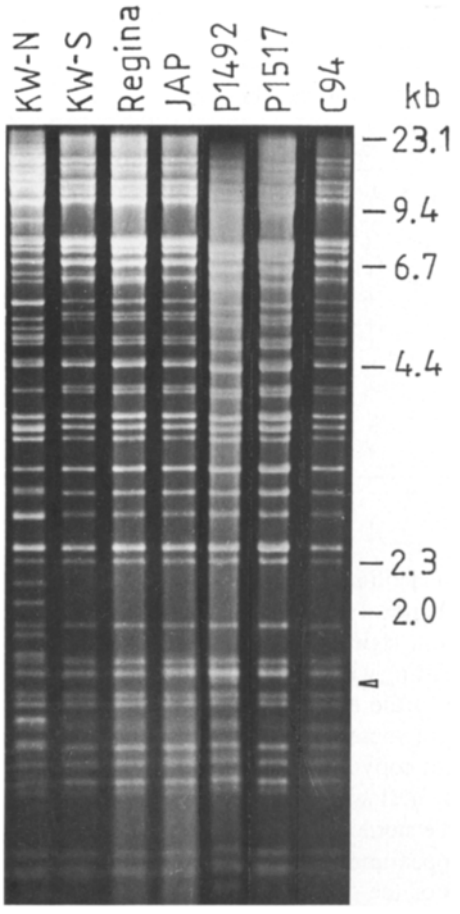

Fig. 2. XhoI restriction patterns of mtDNA from one fertile (KW-N/89) and seven male-sterile lines of *Beta vulgaris*. Lambda DNA-Hind III restriction fragments were used as molecular weight markers (kb). The *arrow* indicates the position of the line-specific fragment (1.7 kb)

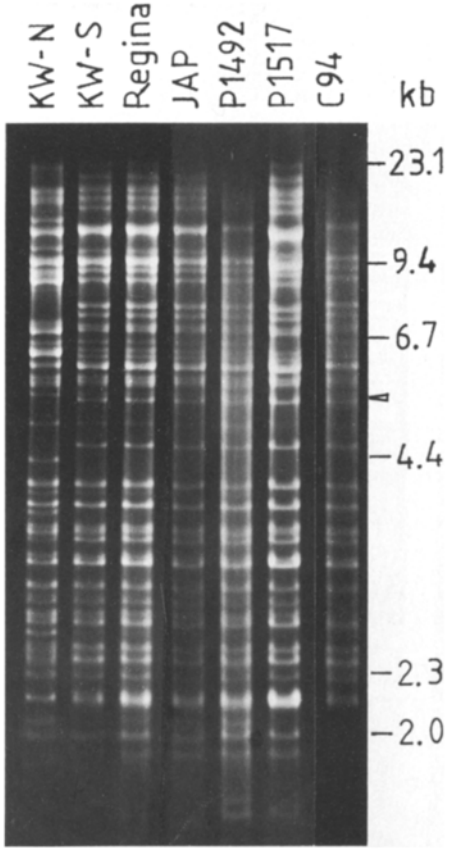

Fig. 3. BamHI restriction patterns of mtDNA from one fertile (KW-N/89) and seven male-sterile lines of *Beta vulgaris*. Lambda DNA-Hind III restriction fragments were used as molecular weight markers (kb). The *arrows* indicate the position of the line-specific fragment (5.2 kb)

markedly from the fertile line pattern (see Figs. 1–4). Restriction digests obtained with BglII, HindIII, PstI, and SmaI showed no differences between the cms lines investigated (not shown). Restriction analyses with the enzymes BamHI, EcoRI, SalI, and XhoI, however, revealed distinct differences between the cms lines (Figs. 1–4). The most remarkable variations were found with SalI: a fragment of 12.3 kb present in lines KW-S/89, "Regina," P1492, C94, and also in the fertile KW-N/89 was missing in lines JAP, 8577, 83115, and P1517 (see Fig. 1). A 10.5-kb fragment was not detected in two of the sterile cytoplasms, whereas a 4-kb fragment was lacking in three of them. Line "Regina" was the only one to contain a 3-kb fragment (see Fig. 1). All the specific fragments occurring in one or more of the cms lines were also found in the fertile pattern, although we cannot rule out that the bands at the same position in the fertile and the sterile cytoplasms represent different sequences. When the mtDNA from the different cms cytoplasms was digested with BamHI, EcoRI, and XhoI, different patterns were also obtained, but the variations were less remarkable than in the case of SalI in regard to one (BamHI, XhoI) or three (EcoRI) bands, respectively. mtDNA of a cms line originating from the USA (P1492) exhibited one additional fragment of 1.7 kb in the XhoI pattern (Fig. 2) that did not occur in any of the other sterile lines or in the fertile cytoplasm. Similarly, one additional minor band of 5.2 kb was found in the BamHI pattern of four sterile cytoplasms (see Fig. 3). Digestion with EcoRI revealed the absence of three minor fragments in three of the sterile cytoplasms (see Fig. 4). Table 2 summarizes the occurrence or loss of specific fragments obtained by analysis with four enzymes. On the basis of these results, seven different subtypes of sterile cytoplasms can be distinguished.

Hybridization analysis of mtDNA

To investigate a possible correlation between the different restriction patterns in the cms lines and the location of mitochondrial genes, we hybridized mtDNA digested

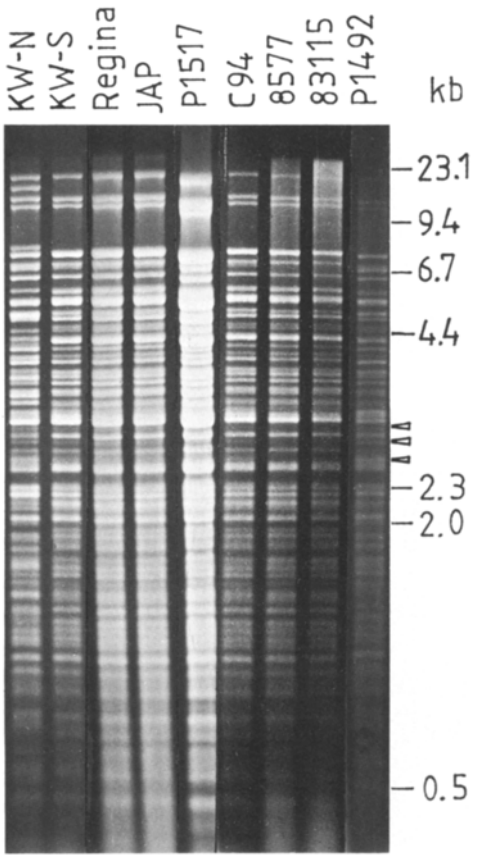

Fig. 4. EcoRI restriction patterns of mtDNA from one fertile (KW-N/89) and seven male-sterile lines of *Beta vulgaris*. Lambda DNA-Hind III restriction fragments were used as molecular weight markers (kb). The *arrows* indicate the positions of the line-specific fragments (2.8, 2.7, and 2.5 kb)

with eight restriction endonucleases with mitochondrial gene probes. It had been previously shown that the rearrangement of the mitochondrial genome in cms lines of the sugar beet, compared to the fertile cytoplasm, affects the localization of genes: apocytochrome b (*cob*), α-subunit of the F_1-ATPase (*atpA*), and cytochrome c oxidase subunit II ($\overline{COXII}$) (Weihe et al. 1988; Duchenne et al. 1989; Dudareva et al. 1989). We used the $\overline{COXII}$ gene in the present study to reveal differences between cms lines of different origin. With all eight restriction enzymes used in this study, the hybridization pattern of the fertile line differed from that of the sterile lines when $\overline{COXII}$ was used as a hybridization probe (shown only for SalI, BamHI, EcoRI; see Fig. 5). Differences between sterile lines were obtained only when mtDNA was digested with SalI, BamHI, and EcoRI. In the case of SalI (Fig. 5a), one additional minor hybridization signal was observed in lines "Regina" and JAP. This signal coincides with the line-specific band of 3 kb in the restriction pattern (see Fig. 1, Table 2). Interestingly, a fragment of the same size present in the fertile line does not hybridize with the $\overline{COXII}$ gene, indicating that the sterile line-specific fragment contains nucleotide sequences different from those in the mtDNA band of the fertile line. In line JAP, the 3-kb hybridization signal is much weaker than in line "Regina," correlating with the observation that the additional fragment in the restriction pattern of this line is very faint. The fragment should be present in these two lines in a different copy number. When blots of mtDNA digested with BamHI were probed with $\overline{COXII}$, differences between the sterile lines were also observed, again, regarding the appearance of additional, minor signals in some lines: two of the sterile lines, "Regina" and JAP, exhibited two additional hybridization signals of 2.7 kb and 2.4 kb, the latter being present in a lesser amount also in line KW-S/89. Again, in the case of EcoRI, line "Regina" exhibited an additional minor hybridization signal (3.5 kb). Differences in minor hybridization signals between the eight sterile cytoplasms could also be obtained when atp9 and atpA were used as probes (not shown).

Table 2. Characteristics of male-sterile cytoplasms by line-specific restriction fragments of mtDNA

Line	Restriction fragments (kb)								
	SalI				EcoRI			BamHI	XhoI
	12.3	10.5	4	3	2.8	2.7	2.5	5.2	1.7
KW-N/89	+	+	+	+	+	+	+	+	−
KW-S/89	+	+	+	−	+	+	+	+	−
"Regina"	+	+	+	+	+	+	+	+	−
JAP	−	+	+	−	+	+	+	−	−
P1517	−	+	−	−	+	+	+	−	−
8577	−	−	−	−	−	−	−	N.E.	−
83115	−	−	−	−	−	−	−	N.E.	−
P1492	+	+	+	−	+	+	+	+	+
C94	+	+	+	−	−	−	−	+	−

N.E. – Not examined

Discussion

mtDNA restriction patterns of different sugar beet cms lines have been published by several authors (Powling 1982; Weihe et al. 1985; Mikami et al. 1986; Hallden et al. 1988; Duchenne et al. 1989; Dudareva et al. 1989). Powling (1982) analyzed 13 lines of *Beta vulgaris* by restriction of the mtDNA employing enzymes BamHI, PstI, and SalI. He was able to distinguish two types of patterns: type 1 for fertile cytoplasms, and type 2 for cms lines. Also, comparison of 12 isogenic paris of fertile and cms sugar beet varieties by restriction analysis recently confirmed the existence of only one type of cms mtDNA pattern identical to Powlings type 2 (Duchenne et al. 1989). All variant cms mtDNA patterns thus far reported were due to cytoplasms of wild *Beta* beets (Boutin et al.

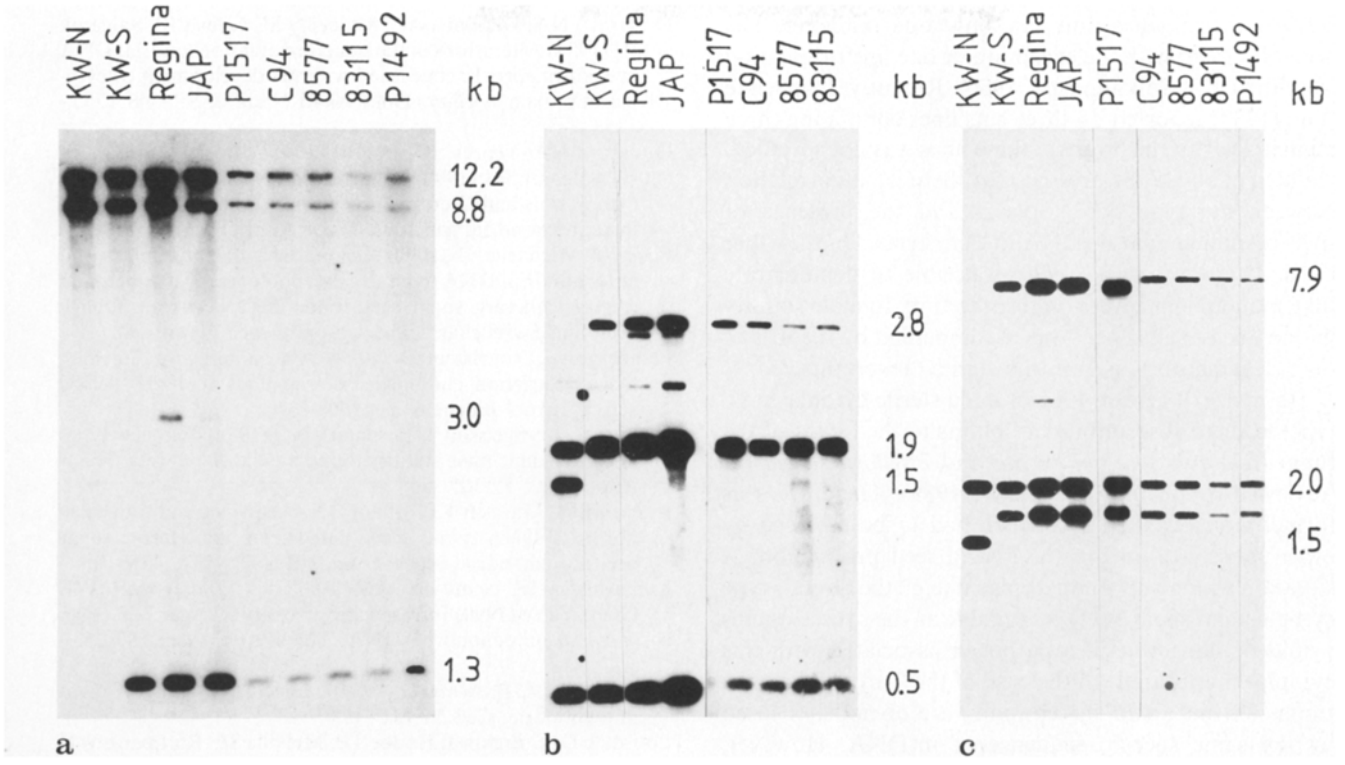

Fig. 5a–c. Hybridization of $\overline{\overline{COXII}}$ with mtDNA from one fertile (KW-N/89) and seven male-sterile lines of sugar beet. DNA was restricted with SalI (*a*), BamHI (**b**), and EcoRI (**c**)

1987; Hallden et al. 1988), except one cms line selected from O-type fertilizer populations, which showed one additional BamHI fragment as compared to the standard Owen cytoplasm mtDNA (Komarnitsky et al. 1990). Also, investigation of five morphologically distinct *Beta vulgaris* cultivars revealed the same restriction profile of mtDNA (type 1, N cytoplasm) (Ecke and Michaelis 1990). Based on these data, it is generally agreed that all cms cytoplasms involved in commercial hybrid seed production of *Beta vulgaris* are of the same type, originating from the cytoplasm introduced by Owen (1942, 1945).

We have analyzed eight sugar beet cms lines from different breeding stations to investigate whether or not variants of the common cms type exist. From the eight restriction endonucleases employed in our study, four enzymes revealed considerable differences in the restriction patterns of the mtDNA, whereas the other enzymes yielded identical patterns for all the sterile lines. Relying on these findings, we would like to point out that conclusions regarding the identity of different cytoplasms that are based on only two or three enzymes are not sufficiently reliable. Using BamHI, EcoRI, SalI, and XhoI we were able to distinguish seven different mtDNA subtypes among the eight cms lines investigated (and one fertile pattern). We consider these variant mtDNA patterns as evidence for the existence of different subtypes of the one common, Owen's type of cms cytoplasm. To our knowledge, this is the first demonstration of the existence of such subtypes.

In our experiments, hybridization analyses with mitochondrial gene probes also revealed differences between the different cms lines. On the basis of hybridization with $\overline{\overline{COXII}}$, remarkable differences were obtained between the fertile line and all sterile lines, whereas differences between sterile lines involved only the occurrences of additional minor hybridization signals in some of them. Similar results were obtained using *atp9* and *atpA* as probes (not shown). In the variety "Regina," one minor hybridization signal obtained with $\overline{\overline{COXII}}$ coincided with one "extra" band of 3 kb in the SalI pattern. Line "Regina" is the only one to contain this 3-kb SalI fragment. The occurrence of minor hybridization signals in some of the sterile lines that cannot be correlated to differences in the restriction patterns probably reflect the existence of substoichiometric fragments, referred to in the literature as "sublimons" (Lonsdale et al. 1988).

All restriction and hybridization analysis data suggest that considerable rearrangements occurred in the mitochondrial genomes of the cms lines, which are a result of intra- and intermolecular recombinations via the direct and inverted repeats of the sugar beet mitochondrial genome (Brears and Lonsdale 1988). Analogous processes have been reported for a number of other higher plants (Levings and Brown 1989).

The present study did not involve investigation of the minicircular mtDNA of sugar beet, but preliminary results suggest that all eight cms lines contain only one minicircular mtDNA (mc a). In all sterile lines of *Beta*

vulgaris investigated thus far, only one minicircle has been observed, with the exception of one line originating from the USA (Mikami et al. 1986). Recently, Duchenne et al. (1989) reported on three cms lines containing three minicircles, but the origin of these lines was not specified. Hallden et al. (1988) stressed that there is no correlation between the type of cytoplasm and the presence of specific minicircular mtDNA in *Beta* beets. On the other hand, Dudareva et al. (1990) was able to demonstrate that spontaneous reversion from fertility to male sterility in one line of sugar beet was accompanied by the loss of mc c, leaving only mc a in the sterile "revertants."

In maize, a certain type of male-sterile cytoplasm (T type) leads to susceptibility of plants to the toxins of the fungi *Helminthosporium maydis* and *Phyllosticta maydis* (reviewed by Levings and Brown 1989). Therefore, this formerly widely used cytoplasm had to be replaced by other sources of cms in the hybrid seed production. A situation where only one cytoplasm (e.g., the Owen's type cytoplasm of sugar beet) is available as the source of cms would be dangerous if a pathogen associated with this cytoplasm appeared. In the case of the T cytoplasm, the traits cms and toxin susceptibility are obviously caused by the same specific sequence of mtDNA. However, there is no reason to assume that a mitochondrial DNA-related pathogen susceptibility is in general absolutely linked to cms. In this respect our data are of interest. Although we could not find striking differences between the sterile cytoplasms studied, the results clearly demonstrate a certain degree of variability within the Owen's type cytoplasm, i.e., there exists a group of subtypes within this source of cms.

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