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Studies on the organelle genomes of sugarbeet with male-fertile and male-sterile cytoplasms

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Summary. Chloroplast DNA (ctDNA) from male-fertile and cytoplasmically male-sterile (cms) sugarbeet has been investigated by restriction endonuclease digestion. Three enzymes (SalGI, BamHI and PstI) did not distinguish between the ctDNA from the two different types of plant. Another enzyme (EcoRI) revealed one variant fragment in one of the male-fertile lines examined, but this variant is not thought to be associated with the cms trait. This situation contrasts with that for mitochondrial DNA (mtDNA) since it has previously been shown that mtDNA of cms sugarbeet differs considerably from that of male-fertile sugarbeet. The presently used form of cms in sugarbeet was discovered in the variety 'US1'. Individual plants of this variety were examined to determine which types of mtDNA they contained. Some plants contained mtDNA typical of male-fertile plants and some mtDNA typical of cms plants. These results suggest that the cms trait in sugarbeet is encoded by mtDNA and not ctDNA. The small, circular DNA species previously found in mitochondria were cloned and used in hybridization experiments to determine their homologies to the other mtDNA molecules. The small DNA species showed no homology to the main band of high molecular weight DNA, but they did hybridize to molecules which were apparently their own dimers, tetramers, octamers, etc. If a small DNA species was absent from a particular sugarbeet line then no hybridization to any mtDNA of that line was detected.

Key words: Sugarbeet – Cytoplasmic male sterility – Mitochondrial DNA – Chloroplast DNA

Introduction

Cytoplasmic male sterility (cms) was first discovered in sugarbeet (*Beta vulgaris* L.) by F. V. Owen (1945). He found that approximately 2% of individual plants of the variety 'US1' were cms and used these plants to develop cms inbred lines which could be used in breeding programmes. This material has given rise to all the cms lines presently used by breeders in both America and Europe (Theurer and Mumford 1976; Bosemark 1979).

It has been shown previously that the mitochondrial genomes of sugarbeet with male-fertile and male-sterile cytoplasms differ in both the DNA fragments they yield after restriction endonuclease digestion and the number and type of small, supercoiled DNA molecules they possess (Powling 1981, 1982). This suggests that mitochondrial DNA (mtDNA) encodes the defect causing the cms trait. However the cytoplasm contains another group of organelles with their own genetic system potentially capable of encoding the cms trait – the plastids, which include the chloroplasts. This paper describes the isolation of chloroplast DNA (ctDNA) from male-fertile and cms sugarbeet and the comparison of the two types by restriction endonuclease digestion.

The relationships of the small, supercoiled mtDNA molecules (minicircles) to the main, high molecular weight (HMW) mtDNA are also described. The minicircles have been cloned in *Escherichia coli* by recombinant DNA techniques and the multiplied DNA hybridized to mtDNA on nitrocellulose filters. This allowed detection of other mtDNA molecules with homologies to the various minicircles.

Some individual plants of the variety 'US1' have been examined to determine which types of mtDNA and which combinations of minicircles were present in the source population for all cms lines presently used. This was done to test the possibility that all 'US1' plants had mtDNA of the type now always associated

with the cms trait. If this were found to be the case then we could conclude that the cause of the cms trait is not simply the presence of this particular form of mtDNA.

Materials and methods

Sugarbeet lines and varieties

The inbred lines I 13M4 and 01 I 13M4 were obtained from Bush-Johnson Ltd., Maldon, England, SA3, SA8, RA18 and RD1 from the Plant Breeding Institute, Cambridge, England, and SRA5 from Anglo-Maribo, Lincoln, England. A sample of seed of the variety 'US1' was obtained from Dr. J. C. Theurer, Utah State University, Logan, USA. 'Vytomo' is a commercial variety produced by Hilleshög Ltd.

Isolation of chloroplast DNA

Leaves from 4 to 8 week old greenhouse grown plants were used to isolate cpDNA from the inbred lines investigated. The individual plants of variety 'US1' were greenhouse grown for about 8 months. Chloroplasts were isolated by the method of Herrmann et al. (1975), except that the phosphodiesterase digestion step was omitted. The chloroplasts were lysed with Sarkosyl (2%) and autodigested Pronase (0.1 mg/ml) at 37 °C for 2 h. The ctDNA was purified by three phenol extractions, ethanol precipitation and ethanol washes, then resuspended in 10 mM tris-HCl, pH 8.0, 1 mM EDTA.

Isolation of mitochondrial DNA

MtDNA was isolated as described previously (Powling 1981).

Restriction endonucleases

These enzymes were isolated by Mr. P. Dickerson, John Innes Institute.

Agarose gel electrophoresis

Vertical agarose gels were made in 40 mM Tris-HCl pH 7.9, 5 mM Na acetate, 1 mM EDTA. Details of the individual gels are given in the figure legends. Staining and visualization were as described elsewhere (Powling 1981). The marker fragments were bacteriophage λ DNA digested with HindIII and double digested with HindIII and EcoRI. Transfer of DNA from agarose gels to nitrocellulose filters (Schleicher and Schull BA8S) was performed as described by Southern (1979).

Hybridization

Hybridization probes were prepared by nick translation (Maniatis et al. 1975) of DNA to a specific activity of approximately 2 μCi/μg with ^{32}P α dCTP (from Amersham). Filters for hybridization were prewashed in 25 mls of 3×SSC, 4× Denhardt's solution at 65 °C for 4 h. [SSC is 0.15 M NaCl, 0.015 M trisodium citrate. Denhardt's solution is 0.02% w/v Bovine Serum Albumin, Polyvinyl pyrrolidone, and Ficoll (Denhardt 1966)]. Hybridization was performed overnight (16 h) in 25 mls of fresh 3×SSC, 4×Denhardt's solution also at 65 °C; the nick translated DNA was added to this solution after denaturation by the addition of 100 μl 1 M NaOH and subsequent neutralization with 100 μl 1 M HCl.

After hybridization the filters were washed extensively in 0.1×SSC, 0.1% w/v SDS at 50 °C for 4 h. Filters were then dried in air at 65 °C and autoradiographed at −70 °C with film (Fuji Rx) prefogged to an enhanced $OD_{650\,nm}$ of 0.1 (Laskey and Mills 1975).

Preparation of plasmid DNA

Recombinant plasmids and vectors were prepared from broth cultures under appropriate drug selection, according to the cleared lysate procedure of Clewell and Helinski (1970) and using Ethidium Bromide-containing Caesium Chloride equilibrium density gradient centrifugation.

Cloning of minicircle DNA

Minicircle DNA was isolated from gels of low melting point agarose (BRL) by melting the agarose, then phenol extraction and ethanol precipitation to purify the DNA. Minicircles a and d were digested with HindIII and ligated into the HindIII site in the plasmid vector pAT153 (Twigg and Sherratt 1980). Minicircle c was cut with EcoRI and ligated into the EcoRI site in the plasmid vector pBR328 (Soberon et al. 1980).

Results

Chloroplast DNA

Chloroplast were isolated and their DNA purified from I 13M4 (male-fertile), 01 I 13M4 (cms), SA3 (male-fertile) and RA18 (cms). These DNA samples were subjected to digestion with restriction endonucleases SalGI, BamHI, PstI and EcoRI. The first three of these enzymes did not distinguish the ctDNA from male-fertile and cms lines, although they all clearly distinguished mtDNA from the same lines. Figure 1 shows ctDNA from a male-fertile (lane 4) and a cms line (lane 5) after digestion with SalGI, both samples showing identical digestion products. Also shown for comparison are digestion products of mtDNA from a male-fertile and a cms line. Mitochondrial DNA has been investigated previously (Powling 1982) when it was shown that all male-fertile lines contain mtDNA of a particular type (type 1 – Fig. 1, lane 1) and all cms lines contain mtDNA of a different type (type 2 – Fig. 1, lane 2).

During the course of the present work it was found that the method used to isolate mtDNA usually resulted in the presence of a small amount of contaminating plastid DNA. In an earlier publication (Powling 1982) the bands, described as "noticeably faint", formed by this contaminating DNA were assumed to be mtDNA and were taken into account in calculating the size of the mitochondrial genome. If these bands are ignored when estimating the size of the mitochondrial genome the value obtained is reduced but remains within the confidence limits of two estimates given previously i.e. 375±24 kilobase pairs (kbp) and 379±39 kpb (Powling 1982).

The fourth enzyme used to digest the ctDNA samples, EcoRI, showed that I 13M4, one of the male-fertile lines tested, was slightly different from the others (Fig. 2). Since this variation was not present in the

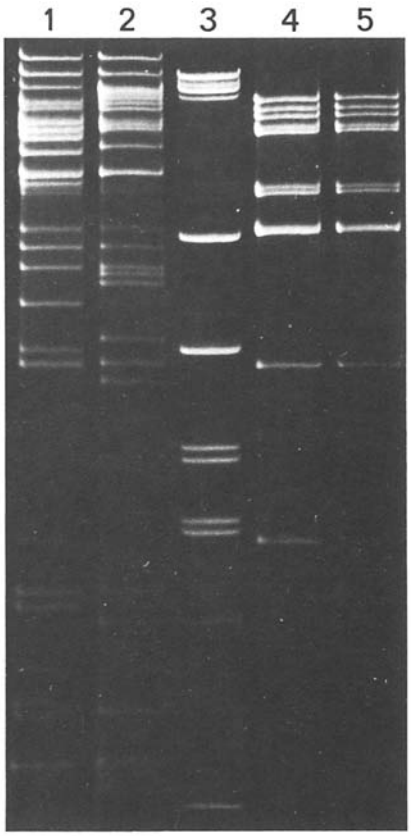

Fig. 1. SalGI digestion products of mitochondrial DNA and chloroplast DNA from sugarbeet. Digestion with 8 units was overnight at 30 °C. The gel was 0.8% agarose and was run for 34 h at 20 mA at 4 °C. Lanes – *1* mtDNA from SA8 (male-fertile), *2* mtDNA from Vytomo (cms), *3* marker fragments, *4* ctDNA from I 13M4 (male-fertile), *5* ctDNA from 01 I 13M4 (cms)

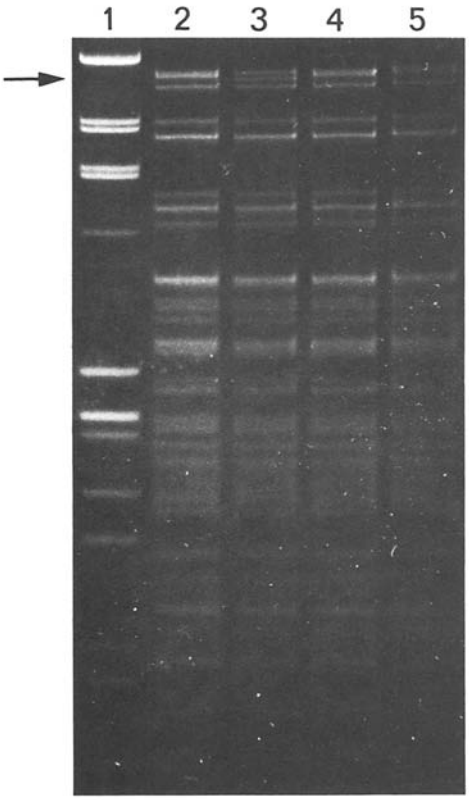

Fig. 2. EcoRI digestion products of chloroplast DNA from sugarbeet. Digestion with 4 units was for 4 h at 37 °C; RNase (2.5 μg/ml) was included in the reaction mixes. The gel was 1% agarose and was run for 19 h at 30 mA at room temperature. Lanes – *1* marker fragments, *2* 01 I 13M4 (cms), *3* I 13M4 (male-fertile) *4* RA18 (cms), *5* SA3 (male-fertile). The arrow marks the variant band in lane *3*

other male-fertile line (SA3) it is probably not associated with the cms trait.

The molecular weight of the sugarbeet chloroplast genome was estimated by summing the molecular weights of the fragments generated by SalGI digestion, and doubling the value for the third smallest band which densitometer traces showed to be a double band. The molecular weights of the fragments were obtained by comparison with the marker fragments of known molecular weight run on the same gel according to the method of Southern (1979), using the non-linear regression analysis of Duggleby (1981). The resulting figure of 99.9 ± 2.0 megadaltons (SD, $N = 4$) compares with the value of 97 ± 3.7 Md given by Herrmann et al. (1975) and corrected to 99.6 Md by Bedbrook and Kolodner (1979). These latter values were obtained by measurement of the length of open circular molecules in the electron microscope.

Relationship of minicircles to other mtDNA molecules

Sugarbeet mitochondria contain small, supercoiled DNA molecules in addition to the HMW mtDNA (Powling 1981). Four different minicircles were distinguished on the basis of size. Their approximate sizes are: minicircle a: 1.5 kbp, b: 1.45 kbp, c: 1.4 kbp and d: 1.3 kbp. The mitochondria of all genotypes of male-fertile sugarbeet that have been examined contain either a, c and d or b, c and d or a and c, depending on the particular line, whilst cms sugarbeet mitochondria contain only minicircle a.

Three of the minicircles (a, c and d) were digested with restriction endonucleases and the fragments ligated into vectors derived from pBR322, as described in the Methods section. The resulting molecules were transformed into *E. coli*. The multiplied DNA was made radioactive by nick translation and hybridized to

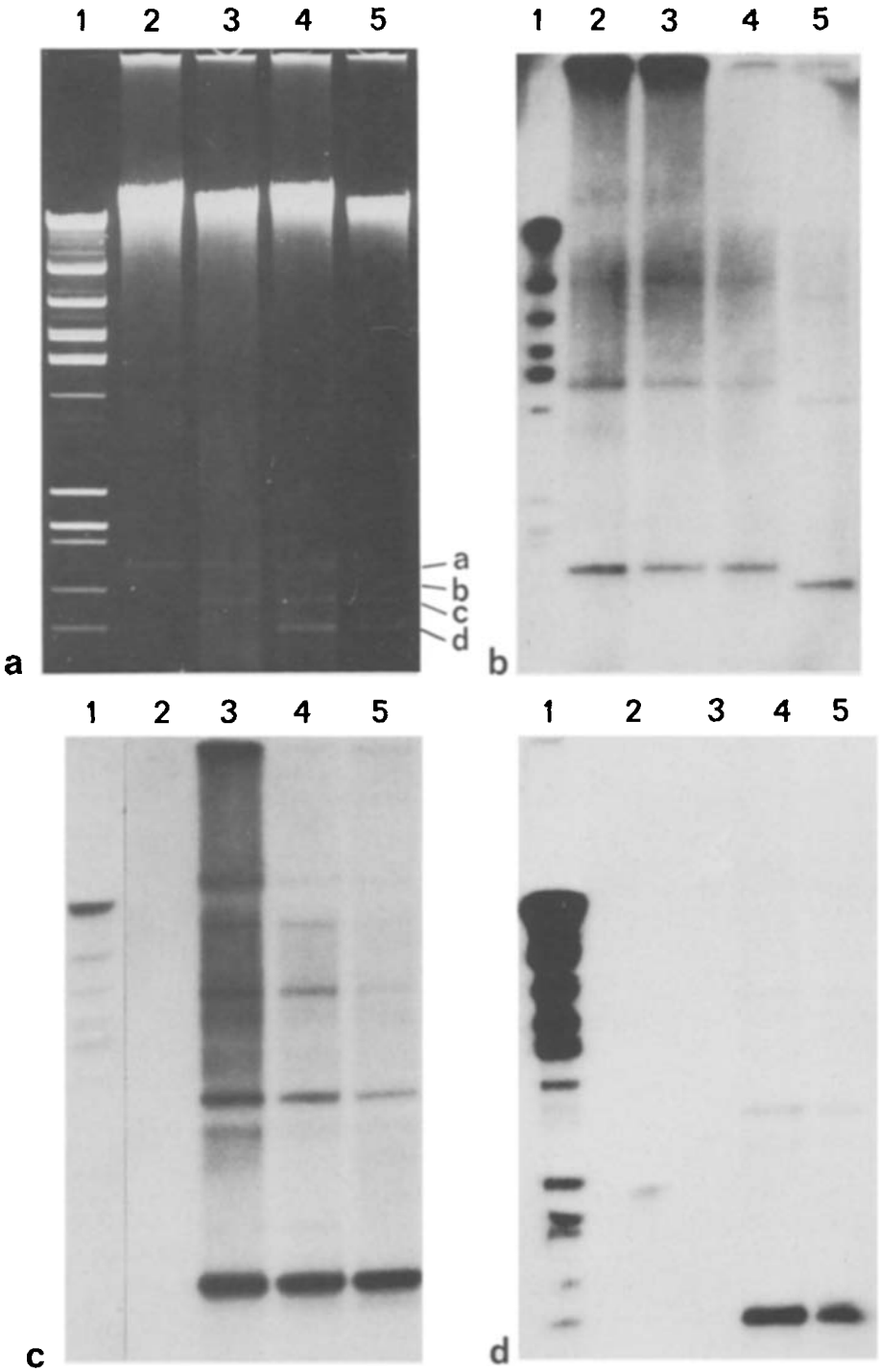

Fig. 3a–d. Hybridization of cloned minicircle DNA to total mtDNA. **a** Example of gel from which Southern blots were taken. The 1.2% agarose gel was run for 5 h at 60 mA at room temperature. The mtDNA samples were treated with nuclease S1 (0.5 units) for 5 to 10 min prior to electrophoresis. Lanes – *1* marker fragments, *2* RD1 (cms), *3* SRA5 (male-fertile), *4* SA8 (male-fertile), *5* SA3 (male-fertile). Minicircles *a*, *b*, *c* and *d* are indicated. **b** Autoradiograph of Southern blot after hybridization to ^{32}P-labelled, cloned minicircle *a* sequence. Lanes are as in Fig. 3a. **c** Autoradiograph of Southern blot after hybridization to ^{32}P-labelled, cloned minicircle *c* sequence. Lanes are as in Fig. 3a. **d** Autoradiograph of Southern blot after hybridization to ^{32}P-labelled, cloned minicircle *d* sequence. Lanes are as in Fig. 3a. Bacteriophage lambda DNA, labelled with ^{32}P by nick translation, was included in all hybridization mixes to indicate the marker fragments

Southern blots of unrestricted sugarbeet mtDNA run out on agarose gels. The mtDNA had been treated with nuclease S1 which relaxes supercoiled minicircles to open circular forms (Powling 1981) and so enhances their transfer to nitrocellulose. Figure 3a shows the mtDNA samples on the gel, with a representative cms line in lane 2, then three different male-fertile lines in lanes 3–5, each with a different combination of minicircles. Figure 3b, c and d show the autoradiographs following the hybridizations. It can be seen that the cloned minicircle DNA hybridizes to the minicircle from which it originated but not to the other minicircles (with the exception of a which hybridized to b, indicating that the two have sequences in common). It is clear that if a particular minicircle is absent from a sugarbeet line there is no hybridization of the cloned

minicircle sequence to any of the mtDNA of that line. There was also very little, if any, hybridization of the cloned sequences to the main band of HMW mtDNA, suggesting that no sequences homologous to the minicircles are present in the HMW mtDNA. In all three cases the cloned DNA hybridized to a series of molecules of increasing molecular weight. By comparison of their mobilities with the mobilities of the marker fragments it appears that these molecules are the dimers, tetramers, octamers, etc. of the original minicircle. We cannot be certain about this since the hybridizing molecules are probably open circles, whilst the marker fragments are linear. Electron microscope studies would be necessary to confirm the size and conformation of these molecules.

The variety 'US1'

Mitochondrial DNA samples from eight individual plants of this variety were tested by SalGI digestion to see whether they were characteristic of male-fertile sugarbeet (type 1), or of cms sugarbeet (type 2) (Powling 1982; and Fig. 1). Six plants contained type 1 mtDNA and two contained type 2 mtDNA. The plants with type 1 mtDNA also contained minicircles a, c and d, whilst the plants with type 2 mtDNA contained only minicircle a. During the period of the experiments two of the plants flowered. Both were male-fertile and both contained type 1 mtDNA typical of male-fertile plants previously examined. Chloroplast DNA was isolated from two plants, one of which had type 1 mtDNA and the other type 2 mtDNA. The ctDNA from both these plants gave SalGI digestion patterns identical to those shown for ctDNA in Fig. 1. The enzymes BamHI and PstI also failed to distinguish the two ctDNA samples.

Discussion

There have been previous reports that cms in crop plants is associated with distinctly different forms of mtDNA, but with unchanged or little changed forms of ctDNA, when compared with male-fertile forms. Examples are *Zea mays* (Pring and Levings 1978), *Sorghum bicolor* (Pring et al. 1982; Conde et al. 1982) and *Petunia hybrida* (Kool et al. 1982). The present paper, together with previous work (Powling 1982), shows that sugarbeet (*Beta vulgaris*) can be added to the list. These results do not in themselves prove that mtDNA encodes the cms trait, but a considerable amount of work indicates that, at least in *Zea mays*, this is the case (review, Leaver and Gray 1982).

The minicircles and the HMW mtDNA do not hybridize, at least under the conditions used in these experiments. Similar observations have been made in *Zea mays* (Kemble and Bedbrook 1980) and *Neurospora crassa* (Collins et al. 1981) where small circular mtDNA species exist which also do not hybridize to the HMW mtDNA. The function of the minicircles, if any, is unknown. Since minicircle c is present in all male-fertile lines investigated, but is absent from the cms lines, it might encode information necessary for pollen production, although this is perhaps unlikely due to its small size. However the cloned sequence of minicircle c does provide a probe which may allow quick identification of male-fertile and sterile cytoplasms in the laboratory.

The existence of the two different types of mtDNA in the US1 population is consistent with the assumption that mtDNA encodes the cms trait, and with the US1 population being the source of all cms lines presently used. Had all the individual plants of the US1 population contained mtDNA of type 2 it would have indicated that the cause of the cms trait is not simply the presence of this type of mtDNA. However, type 1 mtDNA as well as type 2 mtDNA was found in the US1 population, which suggests, but does not prove, that type 2 mtDNA is indeed the cause of the cms trait.

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