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Structure of the mitochondrial genome of *Beta vulgaris L.*

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Summary. The structure of mitochondrial DNA (mtDNA) from sugarbeet (*Beta vulgaris L.*) has been studied by biochemical methods and electron microscopy. It was found to be complex multipartite consisting of two main classes of molecules: high molecules weight (HMW) mtDNA and low molecular weight (LMW) mtDNA. The HMW mtDNA consists of rosette-like structures and globules resembling chromomeres (150–200 nm). A typical rosette has a protein core and radially stemming closed DNA loops (from 0.6–1.5 μm). The number of loops in a rosette varies from 16–30. The bulk of HMW mtDNAs are represented by interconnected rosettes (total contour length about 130–160 μm, 403–496 kbp). Such large circular DNAs may be evidence of the master chromosome arrangement of the sugarbeet genome. Globules and rosettes are interconnected by thick and thin DNA fibrils, along which nucleosome- and nucleomere-like structures are distributed. The LWM mtDNA is composed of two groups of supercoiled circular molecules, 0,2–1.5 μm and 0.02–0.05 μm in size. Electrophoretic analysis demonstrated that LWM mtDNA is represented by minicircle plasmid-like DNA molecules of 1.3, 1.4 and 1.6 kbp.

Key words: Plant mitochondrial genome – Minicircle DNA – Electron microscopy – *Beta vulgaris L.*

Introduction

The mitochondrial genome of higher plants differs from that of animals in size and structural organization (Levings 1983; Sederoff 1984). Thus, plant mitochondrial DNA (mtDNA) attains 2,500 kbp in marked contrast to that of animals, which does not exceed 18 kbp. The genome of plant mitochondria contains, besides the high molecular forms of DNA, multiple subgenomic circular DNA, generated by recombination between directly repeated sequences, and occasionally plasmid-like DNA (Leaver and Gray 1982; Lonsdale 1984; Mulligan and Walbot 1986).

Recently, the mitochondrial genome has attracted attention because of its recognition as a structure not only coding for the enzymes of energy conversion, but also for conferring plants with important traits such as resistance to pathotoxins and cytoplasmic male sterility (cms) (Leaver and Gray 1982; Leaver et al. 1982; Levings 1983). For this reason, a further study of the mitochondrial genome in terms of the structural and spatial organization of mtDNA appeared worthwhile.

In this paper we present the results of biochemical and electron microscopic analyses of the multipartite composition of the mitochondrial genome of sugarbeet (*Beta vulgaris L.*) plants.

Materials and methods

Isolation of mtDNA

MtDNA was isolated from plants of a fertile sugarbeet line SOAN-102 (collection of the Laboratory of Polyploidy of this Institute).

Mitochondria were isolated by method of Synenki et al. (1978) from 5-day-old etiolated seedlings, treated with DNase (50 μg/ml 1 h at 2 °C), and the crude mitochondrial fraction was centrifuged for 1 h at 20,000 rpm (Beckman SW27 rotor) in discontinuous (1.2–1.6 *M*) sucrose gradient. To isolate DNA, the purified mitochondria were incubated with 1% sarkosyl to which proteinase K (100 μg/ml) was added to lyse the membranes and proteins. MtDNA was then prepared by phenol deproteinizations.

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Electrophoretic analysis of mtDNA

About 30 μg of native mtDNA was subjected to electrophoresis in 1.5% agarose gel. The products of Pst I restriction of phage λ DNA were used as markers. The conditions for electrophoresis and staining with ethidium bromide were standard (Maniatis et al. 1982).

Enzyme treatment of native mtDNA

The mtDNA samples were incubated for 10 min at 20 °C with S_1-nuclease (Sigma, 5 units activity/μg DNA) to transform the supercoiled DNA molecules into the open circular form, and 10 min at 37 °C for their transformation into the linear form.

Electron microscopic analysis

To obtain mtDNA spreads we utilized Kleinschmidt protein monolayer technique with formamide (Kleinschmidt 1968). For this purpose, we used the spreading standard solution. A 100 μl of the solution contained 30 μl formamide, 20 μl 0.02 *M* Tris-HCl pH 8.0, 20 μl 0.02 *M* EDTA, 30 μl double distilled water, 5 μl cytochrome c (5 mg/ml) and 5 μl mtDNA. MtDNA was spread on a hypophase that consisted of double distilled water. Structural studies of HMW mtDNA were based on analysis of mtDNA preparations subjected to one-, three- or eight-fold phenol deproteinizations. After eight-fold successive phenol deproteinizations, the high molecular fractions of mtDNA were treated with proteinase K (100 μl/ml for 30 min at 37 °C). The three small DNA bands from the 1.5% agarose gel were isolated by the method of Chen and Thomas (1980). The preparations were shadowed with a platinum-palladium alloy at an angle of 7°. The purity of mitochondria was checked by examination of ultrathin mitochondrial sections under the electron microscope. The material was fixed with 1% OsO_4 and embedded in araldyte. The preparations were examined with a JEM-100 electron microscope (Japan). The contour lenghts of the DNA molecules were measured with a curvimeter, Phage M13 DNA 7,200 bp long served as a length marker.

Results and discussion

Electron microscopic analysis of the isolated mitochondrial fractions provided assurance that the mitochondria were sufficiently pure and that the integrity of the mitochondrial membranes and cristae was spared (Fig. 1 a).

Electron microscopic analysis of the mtDNA spreads demonstrated that they consisted of heterogenous populations of circular molecules differing in degree of packing and occasionally occurring linear fragments (Figs. 1 b–f and 2). The results allowed us to distinguish two main molecular groups of mtDNA: (1) molecules with high molecular weights from 20–160 μm (62–496 kbp) and more; and (2) molecules with low molecular weights represented by plasmid-like DNA with contours accepted as long (1–10 μm or 3.1–31 kbp) and short (0.2–0.8 μm or 0.6–2.4 kbp).

After three-fold phenol deproteinizations the proteins remained attached to HMW mtDNA molecules, and they were isolated in a relaxed and supercoiled state. The relaxed HMW mtDNA are visualized electronmicroscopically as discrete (Fig. 1 b and f) or associated (Fig. 1 c) rosette-like structures containing a central dense globule (40–60 nm) and closed DNA loops (0.6–1.5 μm) radially stemming from the globule. The number of loops in a rosette varies from 16–30, or 22 ± 3 on the average. A contour length of DNA in a single rosette ranges from 20–37 μm.

Similar structures were observed for the animal mtDNA after mild osmotic shock of mitochondria (van Bruggen et al. 1968) or after separation of DNA through sucrose gradient (van Tiule and Pherson 1979). Similar structures were also found in *Crithidie fasciculata* kinetoplast DNA (Renger and Wolstenholme 1972) and chloroplast DNA of marine chromophyte *Olisthodiscus* (Aldrich and Cattolico 1981). As for the packing of plant mtDNA, an accumulation of loops devoid of the central globule, yet similar to rosettes, were observed on the electron micrographs of *Pisum sativum* mtDNA (Mikulska et al. 1970) and on the giant supercoiled DNA from the mitochondria of *Vicia faba* seedlings (Negruk et al. 1986). These observations suggest that a rosette-like organization of DNA underlies the packing of large supercoiled molecules of the mitochondrial genomes and that this organization is common to animal and plants.

The DNA-bound proteins, which are not completely removed even after eight phenol deproteinizations, are presumably involved in the maintenance of the rosette-like organization of HMW mtDNAs. Only additional treatment of mtDNA with proteinase K results in the appearance of protein free coiled molecules with lost rosette-like organization (Fig. 2 b). The results obtained with mtDNA isolated from animals cells also suggest the involvement of the tightly DNA-bound proteins in the stabilization of the mtDNA structure (van Tiule and Pherson 1979).

The HMW mtDNA were found to contain structures other than the rosette-like ones. There were formations of interconnected or scattered (150–200 nm) globules (Fig. 1 d) and their intermediates at different stages of decompactization associated with the appearance of DNA loops (Figs. 1 e and 2 a). These formations were observed only after a single phenol deproteinization of mtDNA. These large globules may be densely packed rosettes with supercoiled loops bound to the histone-like proteins whose presence in the mitochondria has been demonstrated for yeast (Caron et al. 1979; Kuroiwa 1982) and protozoa only (Suzuki et al. 1982). These compact globules are similar in size and other morphological features to the chromomere regularly detected in the chromatin and metaphase chromosomes of plant (Gornung et al. 1986) and animals (Zatsepina et al. 1983). We recently observed similar chromomere- and rosette-like structures in prokaryotes (Kiseleva et al. 1986).

The intermediate forms of DNA (Fig. 2 a) were found to contain 7–8 nm thin fibrils representing uncoiled DNA strands and also to have thick fibrils (12–14 nm

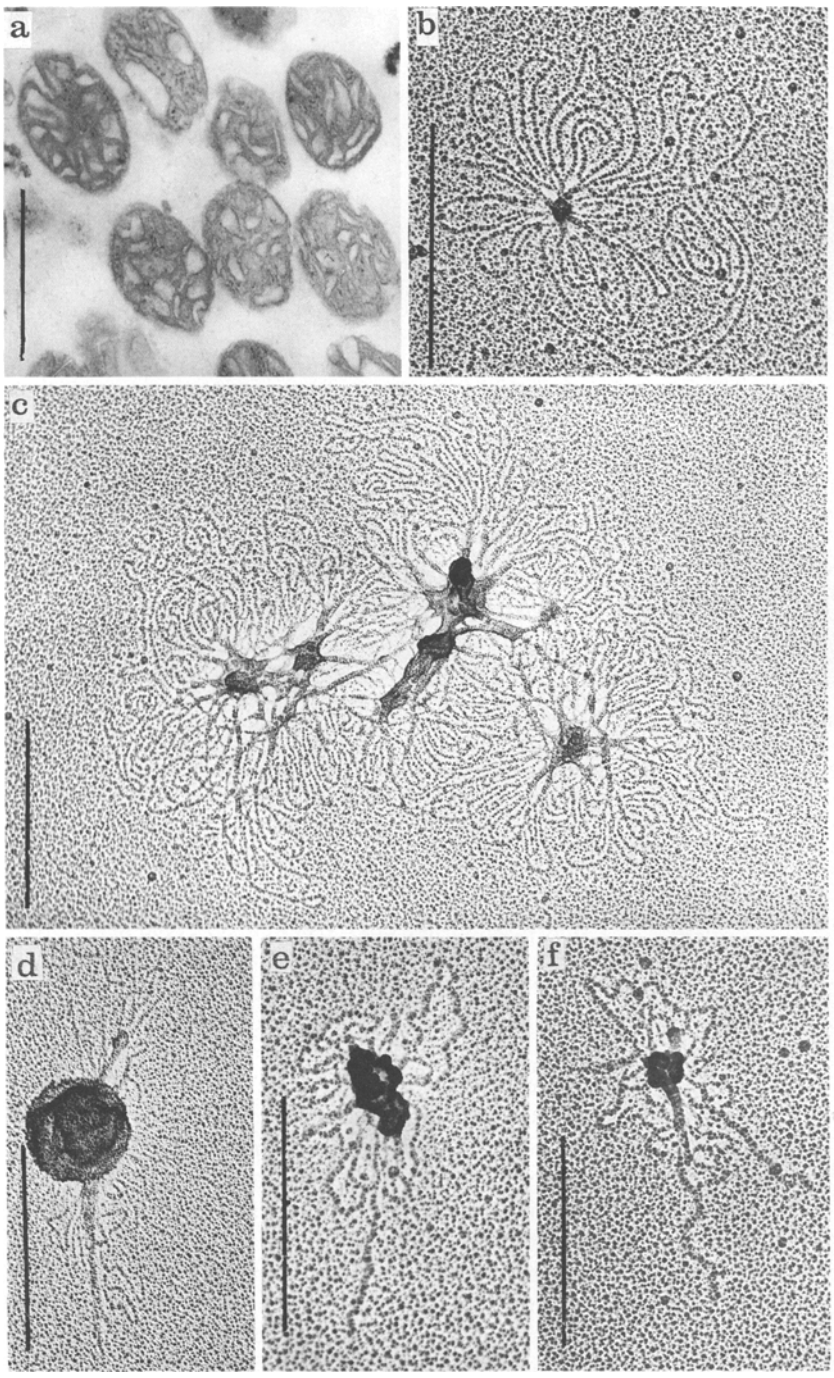

Fig. 1 a–f. Electron micrographs of **a** representative isolated mitochondria of sugarbeet in a thin section and **b–f** spreadings of sugarbeet mtDNA molecules: **b** a circular molecule of mtDNA forming a rosette; **c** a high molecular weight mtDNA consisting of interconnected rosettes; **d–f** successive steps of decondensation of a separate compact globule into a rosette. *Bar* represents 0.5 μm

and 30–40 nm) appearing as regularly arranged granules of almost the same size. The latter resembled the nucleosomes (10–12 nm in diameter) and nucleomeres (25–30 nm in diameter) that are usually observed in the nuclear genomes of eukaryotes. To our knowledge, chromomere-, nucleomere- and nucleosome-like structures in plant mtDNAs have not been previously described. Our data concerning plant mtDNA suggest a universal mode of DNA packing not only in the nuclear, but also in the mitochondrial genomes of various organisms.

The bulk of the HMW mtDNAs consists of interconnected rosettes (146 molecules were measured) with an approximate total contour length of 130–160 μm (403–496 kbp) (Figs. 1c and 3). These values are close to those obtained for the sugarbeet HMW mtDNAs (375 ± 24 kbp) by restriction analysis (Powling 1982). It

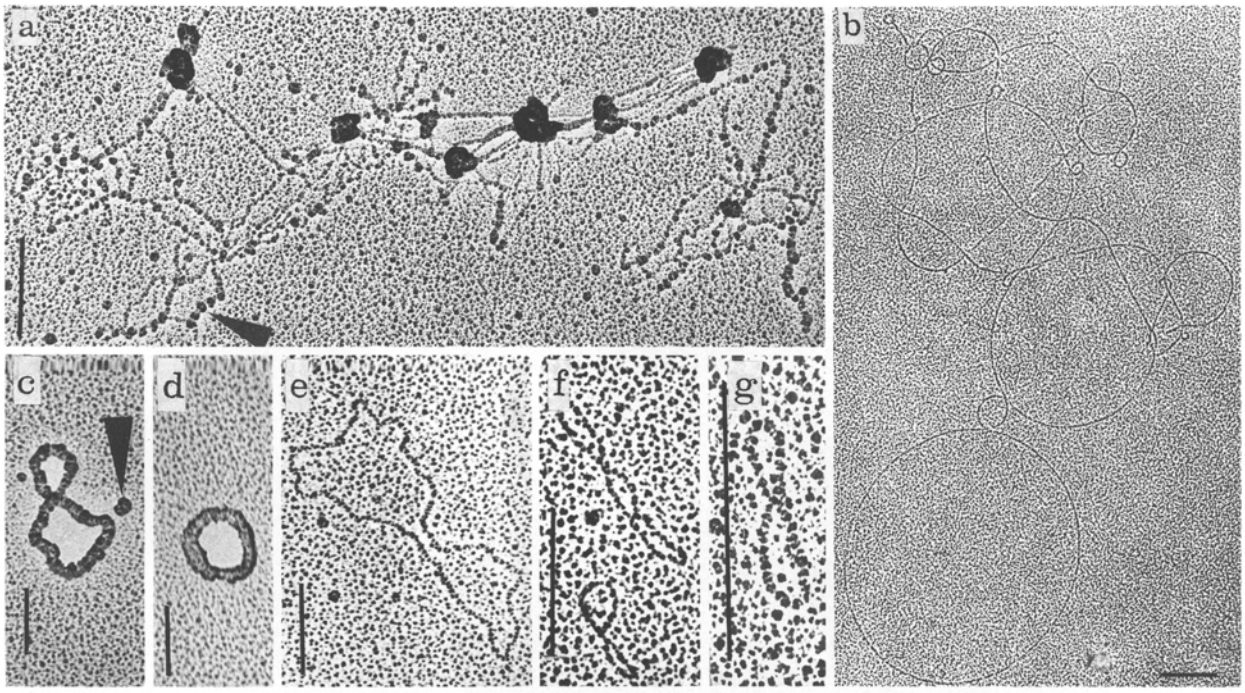

Fig. 2a–g. Electron micrographs showing different forms of molecules of sugarbeet mtDNA in spreadings; **a** a high molecular mtDNA form seen as interconnected compact globules and rosettes containing fibrils and regularly distributed granules (*arrows*) of different sizes; **b** a large circular DNA molecule after incubation of the material with proteinase K; **c–d** supercoiled circular mtDNA forms (*arrow* indicates compact particle of supercoiled minicircular DNA); **e–g** relaxed forms of minicircular DNA. *Bar* represents 0.25 μm

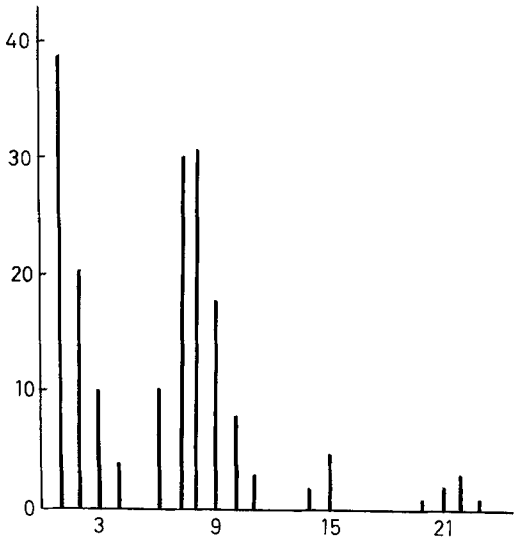

Fig. 3. A histogram of the distribution of HMW mtDNAs according to the number of rosettes. Abscissa = number of rosettes (estimated as the number of central compact globules); ordinate = number of molecules

should be noted that accumulations of DNA larger than the ones described above were found in the preparations. These consisted of a greater number of rosettes. The presence of such structures may be the result of the replication and amplification of the mitochondrial genome (Jamet-Vierney et al. 1980). However, chance aggregation of rosettes during the isolation procedure cannot be excluded.

The plasmid-like mtDNAs were observed in the preparations as supercoiled 0.2–1.5 μm circular molecules (Fig. 2c and d) or as compact 0.02–0.05 μm particles (Fig. 2c). When supercoiled, the molecules most frequently had sizes of 0.4, 0.5, 0.6 and 0.8 μm (Fig. 4). After treatment with S_1-nuclease (10 min, 20 °C), these molecules were transformed into the relaxed circular form with a DNA contour length of 1–10 μm (Fig. 2e). Subgenomic circular molecules of this kind have been found in the mtDNAs of some other higher plants (Levings and Pring 1978; Handa et al. 1984). There are data indicating that the circular forms resulted from recombination between the direct repeats of the HMW mtDNA (Lonsdale 1984; Mulligan and Walbot 1986). Analysis of the primary structure of these circular DNAs demonstrated the presence of unique sequences (Quetier and Vedel 1977) with a function as yet unknown.

A second subgroup of plasmid-like mtDNAs was observed in a supercoiled state as small compact particles (Fig. 2c). After treatment with S_1-nuclease (10 min, 20 °C), they assumed the appearance of minicircular DNA with a contour length of 0.2–0.6 μm (Fig. 2f and g), most frequently of 0.4–0.5 μm (1.3–1.5 kbp).

Upon 1.5% agarose gel electrophoresis, the mtDNA separated into a broad band representing HMW DNA and additionally into three discrete bands of low appar-

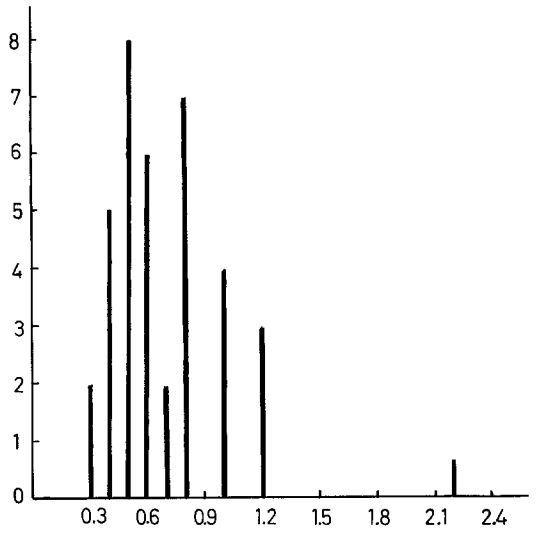

Fig. 4. A histogram of the distribution of circular supercoiled mtDNA molecules according to their lengths. Abscissa = sizes of superspiralised molecules, μm; ordinate = number of molecules

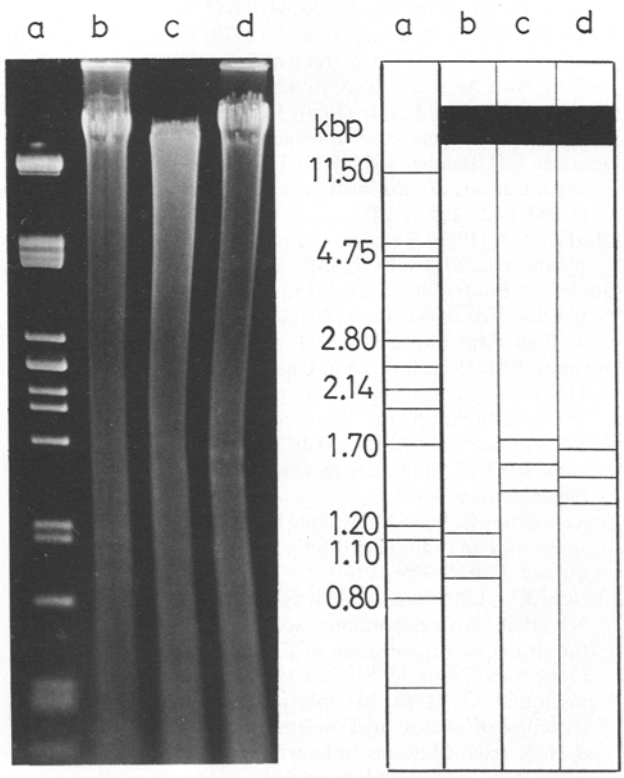

Fig. 5. Electrophoresis on 1.5% agarose gel of mtDNA preparations from a fertile line of sugarbeet. Products of digestion of phage λDNA with PstI were taken as markers (*a*); *b* intact mtDNA; *c* mtDNA digested with S_1-nuclease for 10 min at 20 °C (relaxed circular forms); *d* mtDNA digested with S_1-nuclease for 10 min at 37 °C (linear DNA forms)

ent molecular weight (Fig. 5b). Judging from the changes in their electrophoretic mobilities after treatment of mtDNA with S_1-nuclease under different conditions, the LMW mtDNAs are supercoiled circular molecules (Fig. 5c and d). It should be noted that limited treatment with nuclease-S_1 produced a transformation of supercoiled DNA molecules into open circular ones; at higher concentrations of the enzyme or elevated temperature the supercoiled forms were transformed into linear molecules (Wiegand et al. 1975; Goblet et al. 1983). Thus, the transformations we observed conform with those described in the literature.

The lengths of the LMW DNAs were estimated as 1.3, 1.4 and 1.6 kbp, when determined by comparison of the electrophoretic mobilities of the linear forms (Fig. 5d) with those of the marker fragments (Fig. 5a).

The three small DNA bands were eluted and examined under the electron microscope. The electron microscopic observations assured us that the bands represented minicircular molecules (Fig. 6a–f). The contour lengths of these molecules were measured. Figure 7 is a histogram showing the distribution of the mtDNA species according to their sizes. There is good agreement between the electron microscopic and electrophoretic data. These data also agree with Thomas (1986), who distinguished minicircles of similar sizes (1.3, 1.4 and 1.6 kbp).

Small size mtDNA may be relate to the expression of cytoplasmic male sterility. This appears to be possible because of the correlation found between this trait and the absence of some minicircular molecules in the mitochondria of sugarbeet plants (Powling and Ellis 1983).

Thus, the mitochondrial genome of sugarbeet (like that of higher plants) consists of a heterogenous population of molecules among which high molecular forms with complex organization and subgenomic plasmid-like DNAs occur. The salient findings of this study were the different levels of DNA organization of the mitochondrial genome. The HMW DNAs presumably consist of very large circular molecules attaining about 500 kbp. This may be a situation where the genome can be arranged into a single master chromosome. Similar situations have been envisaged for maize (Lonsdale 1984) and bean (Negruk et al. 1986) mitochondrial genomes. The packing of these molecules is maintained by: (1) the formation of nucleosome- and nucleomere-like globular structures (Fig. 2a); and (2) DNA loops giving rise to rosette-like structures (Fig. 1b, c and f). The core of these rosettes is composed of proteins with a possible affinity for particular nucleotide sequences in the mtDNA.

This principle of nuclear DNAs packing into loops has been studied in detail in nuclear genomes (Georgiev and Bakaev 1978; Glazkov 1986). It was assumed that such loops are transcriptional units. The rosette- and

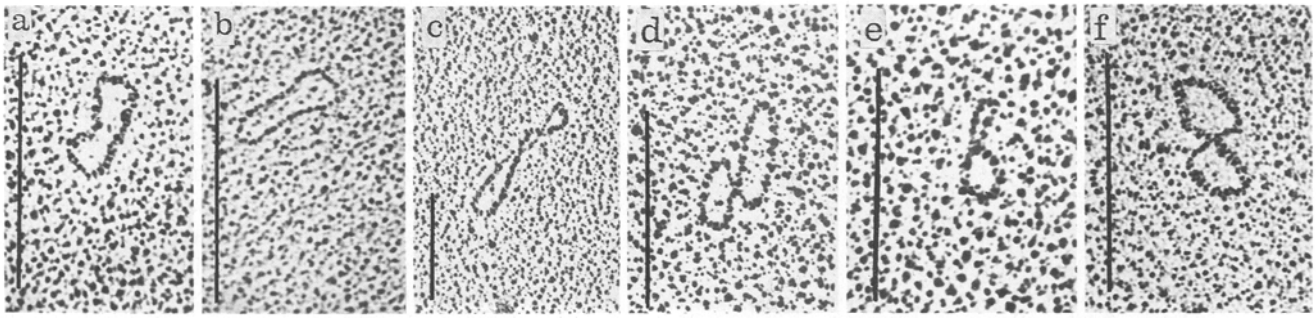

Fig. 6a–f. Electron micrographs of relaxed minicircular mtDNA species isolated from agarose gel. *Bar* represents 0.25 μm

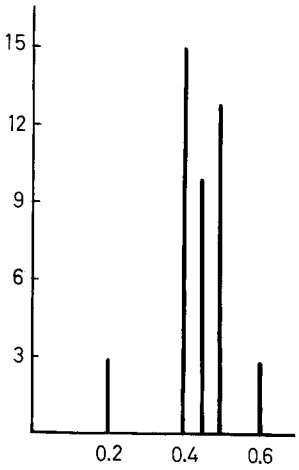

Fig. 7. A histogram of the distribution of sugarbeet minicircular mtDNA molecules isolated from agarose gel according to their sizes. Abscissa = size of molecules, μm; ordinate = number of molecules

chromomere-like structures, which we identified in the mtDNA, have been observed in the metaphase chromosomes and interphase chromatin of plant and animal cells (Zatsepina et al. 1983; Gornung et al. 1986) and also in the bacterial nucleoid (Kiseleva et al. 1986).

Based on the results of electrophoretic analyses and electron microscopic observations, it was concluded that three DNA species of sizes 1.3, 1.4 and 1.6 kbp mainly represent the plasmid-like minicircular DNA in the mitochondrial genome of sugarbeet. Even if they are a result of recombination between directly repeated HMW mtDNA sequences (Thomas 1986), these DNA species may perhaps accomplish some important functions. This appears plausible because they have been retained by the mitochondrial genome of sugarbeet and other higher plants, i.e., they have been spared by evolution. Additionally, complete or partial absence of these plasmid-like DNA in maize, sorghum and sugarbeet is associated with cms (Leaver and Gray 1982; Powling and Ellis 1983; Thomas 1986).

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