Circular DNAs in mitochondria of *Vicia faba*

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Electron microscopic analysis of *Vicia faba* mitochondrial DNA revealed two different types of circular DNA molecules. The class of small DNA molecules consisted mainly of circles near 1.6 kb in size, although a number of molecules were 2–3-times longer. Large molecules ranged from 27 to 120 kb, and the majority of large circular DNA was represented by the molecules ranging from 37 to 67 kb.

Circular DNA *mtDNA* *Electron microscopy* Vicia faba

1. INTRODUCTION

Electron microscopic analysis has revealed the existence of a heterogeneous population of circular and linear DNA molecules in higher plant mitochondria [1–4]. Different ratios of circular and linear DNA molecules have been demonstrated in a number of plant species. It is not yet clear whether the existence of linear molecules is a natural distribution or a result of endonuclease activity. Nevertheless, it was shown that linear plasmid-like DNAs existed in the mitochondrial genomes of sorgum and maize [1,5]. Analysis of the length distribution of mitochondrial circular DNAs revealed that the patterns of circular DNA molecules in different plant species differed. After we discovered small minicircular DNAs in *V. faba* mitochondria [6] it was necessary to clarify the size of other molecules found. We knew [7] that *V. faba* bean mitochondria contained circular DNA so it was interesting to determine its length distribution. We present here the results of electrophoretic and electron microscopic analysis of *V. faba* mitochondrial DNA (mtDNA). mtDNA was isolated by alkaline extraction and consequently was significantly enriched with circular molecules. Two general classes of heterogeneous circular molecules with sizes ranging from 25 to 120 kb and about 1.6 kb were found.

2. MATERIALS AND METHODS

V. faba L. var. Russian Blacks were used. Mitochondria were isolated from 6-day-old etiolated seedlings as in [3]. mtDNA was extracted according to [8] and by an alkaline procedure [9] with some modifications. mtDNA preparations were analysed on 1–1.5% agarose gels as in [10].

The restriction enzyme preparations were kindly supplied by Dr C.A. Puntegis and Dr V.I. Tanyashin. The restriction analysis was performed according to the supplier's instructions. Minicircular DNA was eluted as in [6].

Spreading of DNA for electron microscopy was performed as in [11]. The hyperphase contained 50 mM Tris–HCl, 5 mM EDTA, 50% formamide and 50 μg/ml of cytochrome *c* (pH 8.5). Deionized water was used as hypophase. The grids were shadowed with platinum at an angle of 7° and analysed with a Philips 400 electron microscope at a magnification of ×6000. Length measurements of the molecules were done on an HP 9825A digi-

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tizer (Hewlett Packard, USA) with a programme kindly provided by Dr E.I. Golovanov. DNA of pBR322 and ColE1 were used as length standards.

3. RESULTS AND DISCUSSION

We used a modified alkaline extraction procedure [9] for the isolation of *V. faba* circular mtDNA. In fig.1 the electropherogram of an mtDNA preparation extracted by this method (A) is compared with an mtDNA preparation (B) isolated by the usual procedure [8]. Comparing the slots A and B it is clear that the preparations isolated by the alkaline procedure were enriched with minicircular mtDNA. Electron microscopic analysis also showed the increased proportion of circular molecules in the slow-moving fraction of *V. faba* mtDNA. As preferential losses of large DNA molecules could occur it was important to compare alkaline-prepared mtDNA with the mtDNA isolated by the usual CsCl procedure. As shown in fig.1C,D the content of small DNA fragments was much higher in alkaline-prepared DNA. These species were found to consist of linear and open circular derivatives of minicircular DNAs CCC1 and CCC2 (fig.1). In addition, we cannot exclude some other differences in small DNA fragments although in general the patterns of *Eco*RI restriction fragments of these two DNA preparations were very close.

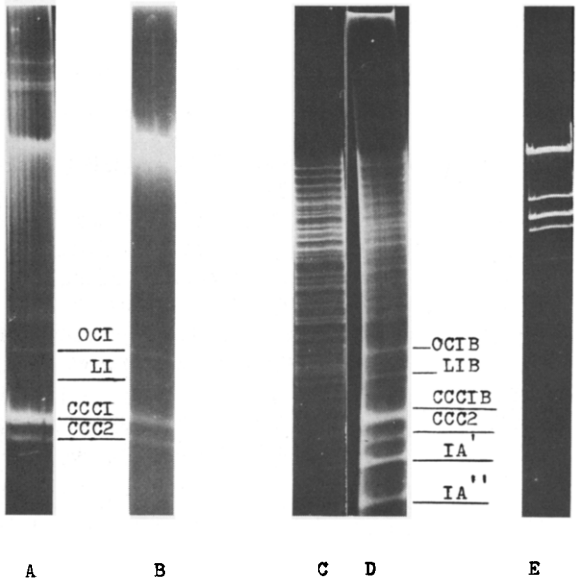


Fig.1. Agarose gel electropherogram of *V. faba* mtDNA. (A) mtDNA isolated with the alkaline procedure; (B) mtDNA isolated with the CsCl procedure; (C) *Eco*RI digest of CsCl-isolated DNA; (D) *Eco*RI digest of alkaline-isolated DNA; (E) *Eco*RI digest of λ DNA. Electrophoresis was done in 1% agarose gel. CCC1 and CCC2: supercoiled minicircular DNAs; OC1 and L1: relaxed CCC1 and linear CCC1, respectively. As shown earlier, CCC1 consisted of CCC1A and CC1B circles [6]. CCC1B was not cut by *Eco*RI. CCC1A as found was cut by *Eco*RI into IA′ and IA″ fragments with sizes of about 0.9 and 0.7 kb, respectively (not shown).

Electron microscopic analysis of *V. faba* mtDNA isolated by the alkaline procedure revealed two different classes of circular molecules with lengths less than 2 μm and more than 8 μm. The class of minicircular DNA was represented mainly by 0.5 μm molecules (fig.2A). Restriction analysis of the circles revealed the existence of 3 types of molecules with similar sizes [6]. In addition to 0.5 μm (1.6 kb) circles a small number of molecules with lengths 1–2 μm (3.1–6.2 kb) were also found. Possibly these molecules were oligomers of 0.5 μm circles as found in other plants [12].

More detailed electrophoretic analysis of alkaline-isolated mtDNA also revealed zones that possibly corresponded to oligomeric forms of minicircular DNA (fig.3). Two of the zones migrating slower than covalently closed circular DNA 1 (CCC1) were relaxed and linear forms of CCC1. This was confirmed by elution of bands CCC1 and

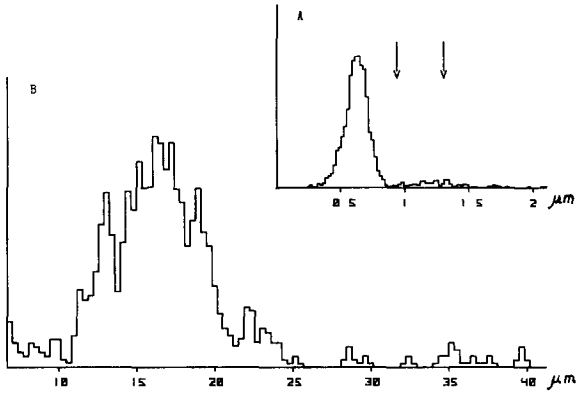


Fig.2. Histogram of the circular mtDNA lengths (expressed in μm). (A) Histogram of minicircular DNA lengths; 1009 molecules were measured. (B) Histogram of the large circular DNA lengths; 273 molecules were measured.

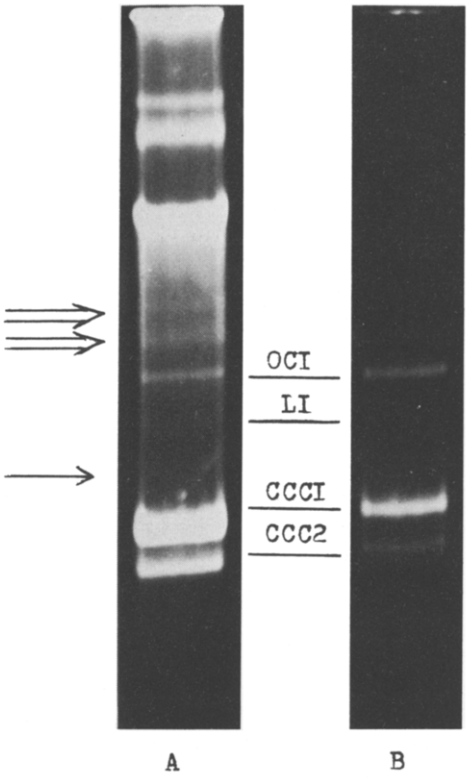


Fig.3. Comparative electrophoretic analysis in 1% agarose gel. (A) mtDNA isolated with the alkaline procedure; (B) minicircular DNAs eluted from an agarose gel by the freeze–squeeze procedure. One of the CCC2 derivatives is shown by an arrow. Minicircular DNA oligomers are indicated by double arrows.

CCC2 (covalently closed circular DNA 2) from an agarose gel by the freeze–squeeze procedure (fig.3B). Part of the supercoiled DNA was broken during elution which led to zones of relaxed and linear molecules on a gel. A weak zone between linear forms of CCC1 and CCC1 is a CCC2 derivative.

Large molecules ranged from 8.5 μm (27 kb) to 38 μm (120 kb). The majority of large circular DNA was represented by molecules ranging from 12 μm (37 kb) to 21 μm (67 kb) as seen in fig.2B. It is worth mentioning that the standard deviation from the real length of these molecules is proportional to the square root of their length which prevents full separation of single peaks. In independent experiments with T7 DNA the standard deviation was 5–10%. Based on these results we assume that the region of 37–67 kb consists of at least 5–7 species of molecules that belong to different size classes.

Thus, electron microscopic examination of *V. faba* mtDNA isolated with the alkaline procedure reveals that the mitochondrial genome of *V. faba* beans also comprises a heterogeneous population of circular DNAs that can be subdivided into several classes.

However, it is not excluded that the absence of discrete size classes on the histogram of the large circular DNA (fig.2B) reflects a real situation and could be a result of intensive rearrangements in the *V. faba* mitochondria genome.

REFERENCES

[1] Pring, D.R., Levings, C.S. iii, Hu, W.L. and Thimoty, D.H. (1977) Proc. Natl. Acad. Sci. USA 74, 2904–2908.
[2] Sparks, R.B. Jr and Dale, R.M.K. (1980) Mol. Gen. Genet. 180, 351–355.
[3] Synenky, R.M., Levings, C.S. iii and Shah, D.M. (1978) Plant Physiol. 61, 460–464; 351–355.
[4] Vedel, F. and Quetier, F. (1974) Biochim. Biophys. Acta 340, 374–387.
[5] Pring, D.R., Conde, M.F., Schertz, K.F. and Levings, C.S. iii (1982) Mol. Gen. Genet. 186, 180–184.
[6] Negruk, V.I., Cherny, D.I., Nikiforova, I.D., Alexandrov, A.A. and Butenko, R.G. (1982) FEBS Lett. 142, 115–117.
[7] Kolodner, R. and Tewari, K.K. (1972) Proc. Natl. Acad. Sci. USA 69, 1830–1834.
[8] Boutry, M. and Briquet, M. (1982) Eur. J. Biochem. 127, 129–135.
[9] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523.
[10] Nikiforova, I.D. and Negruk, V.I. (1983) Planta 157, 81–84.
[11] Davis, R.W., Simon, M. and Davidson, N. (1971) Methods Enzymol. 21, 413–428.
[12] Dale, R.M.K. (1981) Proc. Natl. Acad. Sci. USA 78, 4453–4457.