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Diversity of *Vicia faba* circular mtDNA in whole plants and suspension cultures

V. I. Negruk [1], G. I. Eisner [2], T. D. Redichkina [1], N. N. Dumanskaya [1], D. I. Cherny [3], A. A. Alexandrov [3], M. F. Shemyakin [2] and R. G. Butenko [1]

[1] Institute of Plant Physiology, USSR Academy of Science, Botanicheskaya 35, 127273, Moscow, USSR
[2] All-Union Institute of Applied Molecular Biology and Genetics, Academy of Agriculture, Vuchetich 23, 125206 Moscow, USSR
[3] Institute of Molecular Genetics, USSR Academy of Science, Kurchatov Square, 123182, Moscow, USSR

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Summary. A comparative analysis of the *Vicia faba* mitochondrial genome in whole plants and in long-term suspension culture has been conducted. Restriction fragment patterns of the mtDNA isolated from these two sources were notably different. Electron-microscopic analysis also revealed significant differences. Large circular mtDNA patterns shifted from a 37–80 kb subpopulation, which was predominant in whole plants, to 18–34 kb subpopulations although in both classes notable quantities of circular molecules of 80 to 120 kb and more were also found. Both in whole plant and suspension culture cells very large circular DNAs were observed. Some of them had lengths nearly 290 kb and could be considered as evidence of the existence of master chromosomes. The minicircular DNA population was also altered. In the suspension culture we observed a notable increase of percentage of minicircles with sizes near 1 kb. Simultaneously, the percentage of minicircles with sizes near 3.5–10 kb significantly increased in suspension culture cells. In addition, a new peak (10–12 kb) of minicircles appeared. Copy number alterations for some sequences homologous to CCC1A, CCC1B and CCC2 (Negruk et al. 1982, 1985) were shown. Southern hybridization revealed the existence of a family of minicircles having sizes 1.4–2 kb with predominance of CCC1A, CCC1B and CCC2. The copy numbers of CCC1B and some minor minicircles was changed in the suspension culture when compared with the whole plants.

Key words: Mitochondrial DNA of plants – Electron microscopy – Suspension culture – *Vicia faba*

Introduction

Electron microscopic analysis of the circular fraction of higher plant mitochondrial (mt) DNA preparations reveals an apparently heterogeneous population of circles. This has been observed in both whole plants and suspension culture cells (Synenky et al. 1978; Levings et al. 1979; Fontarnau and Hernandes-Jago 1982; Sparks and Dale 1980; Quetier and Vedel 1977; Dale 1981; Brennicke and Blanz 1982; Eisner et al. 1984). The sizes of these circular molecules ranges from 0.5 to 30–40 μm and they are distributed in different ways in each plant species. MtDNA preparations from tissue culture and those from plant material have been compared by restriction analysis. Some differences in the stoichiometry of some restriction fragments have been revealed (Quetier and Vedel 1977; Dale et al. 1981; McNay et al. 1984; Lonsdale 1984). It was suggested (McNay et al. 1984) that such differences reflect differences in the subgenomic circular mtDNA population which represent the mitochondrial (mt) genome. However, Dale et al. 1981 have observed notable alterations in the subgenomic circular mtDNA molecules from two different lines of tobacco suspension culture and at the same time they did not find significant differences in the restriction patterns of the mtDNA from these two lines. Comparing these data one could suggest that the intensity of rearrangement events in mt genomes from the whole plant and correspondent suspension cultures depends on the plant species, type of suspension culture line, and its age. It is possible that in more prolonged suspension cultures alterations could be more significant (McNay et al. 1984).

To examine this suggestion we compared mtDNA preparations isolated from whole plants and long-term suspension culture of *Vicia faba* using restriction

analysis, electron microscopy and molecular hybridization.

Materials and methods

Plant material

Six-day old etiolated seedlings of *Vicia faba* L. var. 'Russian Blacks' were grown in a dark room at 20 °C. A suspension culture of *Vicia faba* var. 'Russian Blacks' derived initially from callus has been available in our laboratory since 1979. Callus was originally initiated in our laboratory in 1969 using 1-day old embryos. Suspension culture cells were grown in SH liquid media without amino acids and with NH_4NO_3 instead of KNO_3 (Schenk and Hildebrandt 1972; Mitchel and Gildow 1975). Cultures were grown in the dark at 25 °C on a gyratory shaker at 120 rpm. Cultures were routinely transferred every 3 weeks and diluted 1 to 20. There were nearly 85% live cells with modal chromosome number 2n and growth index near 20 in these cultures.

Mitochondrial DNA isolation

Seedlings were homogenized in Waring blendor and suspension culture cells were disrupted in a Potter homogenizer. In all cases 500 µg/ml of ethidium bromide (EtBr) was added to the homogenizing buffer A (0.05 M Tris-HCl, pH 8.0; 0.3 M mannitol; 0.1% bovine serum albumine; 0.003 M ethylenediaminetetraacetic acid (EDTA); 0.004 M 2-mercaptoethanol) (Synenky et al. 1978; Kislev and Rubinstein 1980). The homogenate was centrifuged at 100×g for 15 min, the supernatant at 100×g for 15 min, and the second supernatant at 24,000×g for 15 min. The pellet was suspended in buffer 1 (0.1 M EDTA, pH 8.0; 0.15 M NaCl) containing 500 µg/ml EtBr (Synenky et al. 1978; Kislev and Rubinstein 1980), repelleted, re-suspended in buffer 3 (0.1 M EDTA pH 8.0; 0.1 M Tris-HCl, pH 8.0) and subsequently lysed 10 min at 37 °C. The lysate was treated with 2 volumes of 0.2 N NaOH according to Birnboim and Doly (1979), neutralized with a 1.5 volume of 3 M sodium acetate, pH 4.8 for 1 h at 0 °C, and centrifuged 15 min at 10,000×g. The resulting supernatant was treated with 2 volumes of ethanol. After 1 h at −20 °C, the mixture was centrifuged at 10,000×g for 15 min and the pellet was dissolved in buffer 3. The solution was treated with pancreatic RNAse (100 µg/ml for 1 h at room temperature) and deproteinized with phenol saturated with 0.1 M Tris, pH 12.0 until a clear interphase appeared. The water phase was treated with 2 volumes of ethanol and washed twice with 70% ethanol. The pellet was vacuum-dried and was dissolved in a small volume of double distilled water. The resulting preparations were used for the electron microscopic analysis.

MtDNA prepared by direct phenol deproteinization or by CsCl – ethidium bromide equilibrium centrifugation (Synenki et al. 1978) was used for restriction analysis and hybridization.

Electron microscopic analysis was done according to Davis et al. (1971). Southern blotting, nick translation and molecular hybridization were carried out according to Maniatis et al. (1982). Plasmid-like DNA sequences of *Vicia faba* mitochondria cut out from the recombinant plasmids (Negruk et al. 1985) were used for nick translation.

Results

Comparative restriction analyses of mtDNA preparations isolated from whole plants and from suspension culture revealed notable quantitive differences (Fig. 1). The most evident alterations are shown by white dots, however a more detailed analysis of the restriction fragment patterns shows a higher number of such alterations. The rearrangement events in the suspension culture mt genome could be caused by recombination or by alterations in the copy number of the subgenomic circular mtDNA population. The latter hypothesis was verified by comparative electron microscopic analysis of circular mtDNA isolated from both whole plants and suspension culture cells.

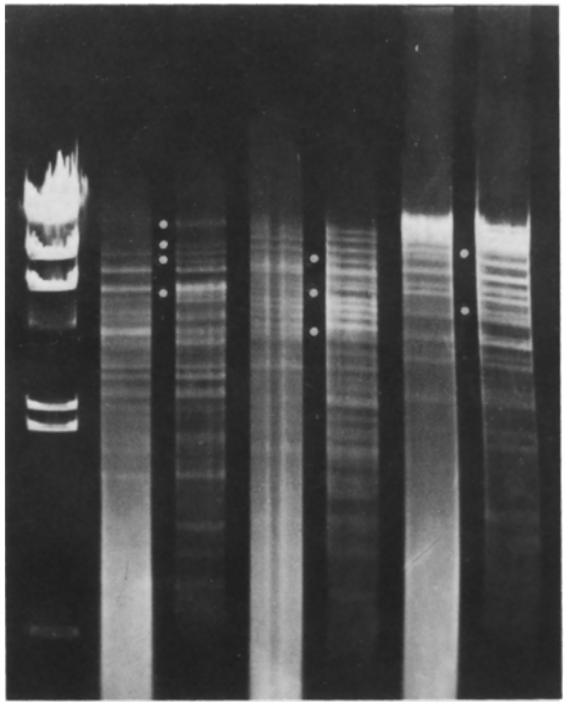

Fig. 1. Comparative restriction analysis of *Vicia faba* mtDNA isolated from whole plants and from suspension culture. *A* DNA digested with HindIII; *B* HindIII digest of suspension culture mtDNA; *C* HindIII digest of whole plant mtDNA; *D* EcoRI digest of suspension culture mtDNA; *E* EcoRI digest of whole plant mtDNA; *F* SalGI digest of suspension culture mtDNA; *G* SalGI digest of whole plant mtDNA

An analysis of the distribution of the circular mtDNAs population as the cells move from a log growth phase to a stationary and senescing culture showed no detectable alterations, a result also observed by Dale et al. (1981) in tobacco suspension culture. However, we observed significant differences in patterns of circular molecules between those isolated from the mitochondria of whole plants and those from suspension culture cells. The histograms of large (more than 16 kb) circular mtDNAs from both 6-day old

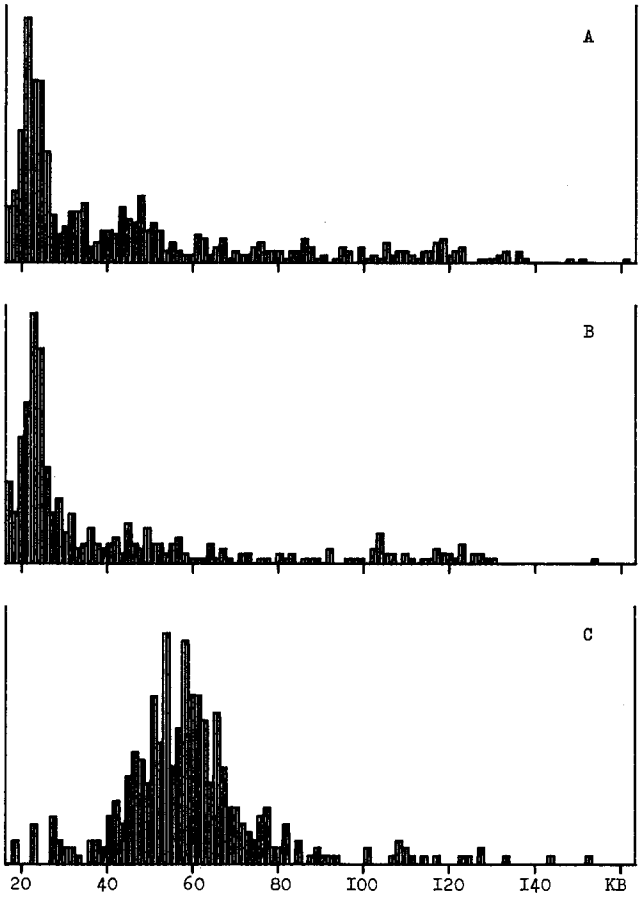

Fig. 2 A–C. Histogram of large circular DNA lengths. **A** suspension culture (8 days old) mtDNA preparations (585 molecules from 2 preparations were measured); **B** suspension culture (22 days old) mtDNA preparations (378 molecules from 2 preparations were measured); **C** etiolated seedling (6 days old) mtDNA preparations (395 molecules from 3 preparations were measured)

etiolated seedlings and suspension culture cells harvested after 8 days (the end of log phase) and 22 days (the late stationary phase) are shown in Fig. 2.

As reported earlier (Eisner et al. 1984) and confirmed herein large molecules ranged in size from 27 to 120 kb and more, and the majority of large circular DNA was represented by molecules ranging from 37 to 80 kb (Fig. 2C). The patterns of large circular mtDNA from the suspension culture were completely different (Fig. 2A, B): the majority of molecules ranged in size from 18 to 34 kb.

These differences cannot be considered a consequence of unspecific endonuclease activity in the mitochondria of suspension culture cells because a large number of circular DNA with sizes not only more than 80 kb, but more than 120 kb, were also observed. In experiments analyzing suspension culture mtDNA 950 molecules had sizes of more than 16 kb; 123 molecules more than 100 kb and 19 molecules ranged from 132 to 290 kb.

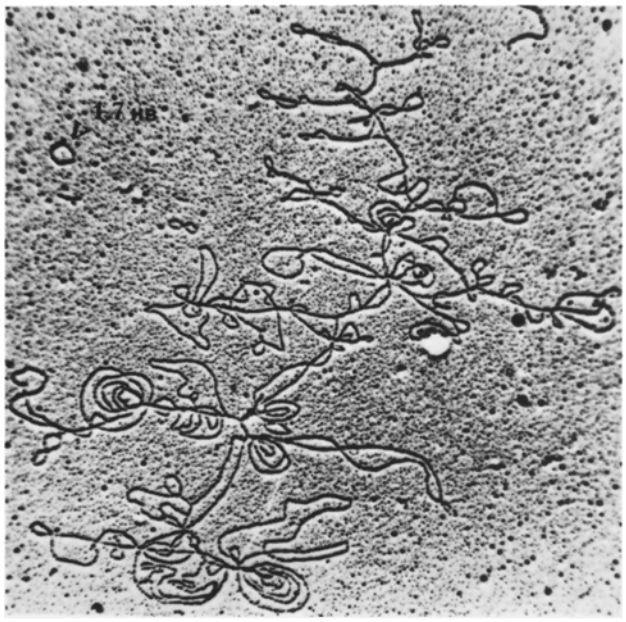

Fig. 3. Electron micrograph of giant supercoiled DNA from mitochondria of (6 days old) *Vicia faba* etiolated seedlings

The largest circular mtDNAs were distributed in the following way: 10 molecules had sizes near 135 kb, three near 154 kb, two near 167 kb, two near 184 kb and two near 290 kb. Without doubt the real proportion of very large circular molecules is higher, but it is impossible to estimate the losses of such molecules during the isolation and spreading procedures.

Mitochondria of 6-day old etiolated seedlings also contain very large circular molecules: one of these supercoiled DNAs is shown in Fig. 3. This molecule was found to have a length close to 290 kb but its real length is more than 300 kb.

From Fig. 4 we see that the population of minicircular mtDNA found in the suspension culture was more heterogeneous than that found in the whole plant. In addition to the main peak of the circles which consisted of 3 types of molecules: CCC1A, CCC1B and CCC2 (Negruk et al. 1982; Goblet et al. 1983; Eisner et al. 1984; Goblet et al. 1985; Negruk et al. 1985) there was a notable increase in molecules near 3.5 kb. Minicircular mtDNA population from 6-day old etiolated seedlings contained virtually no molecules with sizes between 3.5 and 15 kb. In the suspension culture we found many such molecules and especially those with sizes near 10–12 kb (Fig. 4).

On the other hand, in the main minicircular DNA peak the relative number of smaller molecules increased (Fig. 4). It is necessary to emphasize the appearance of molecules having sizes near 1 kb in the sus-

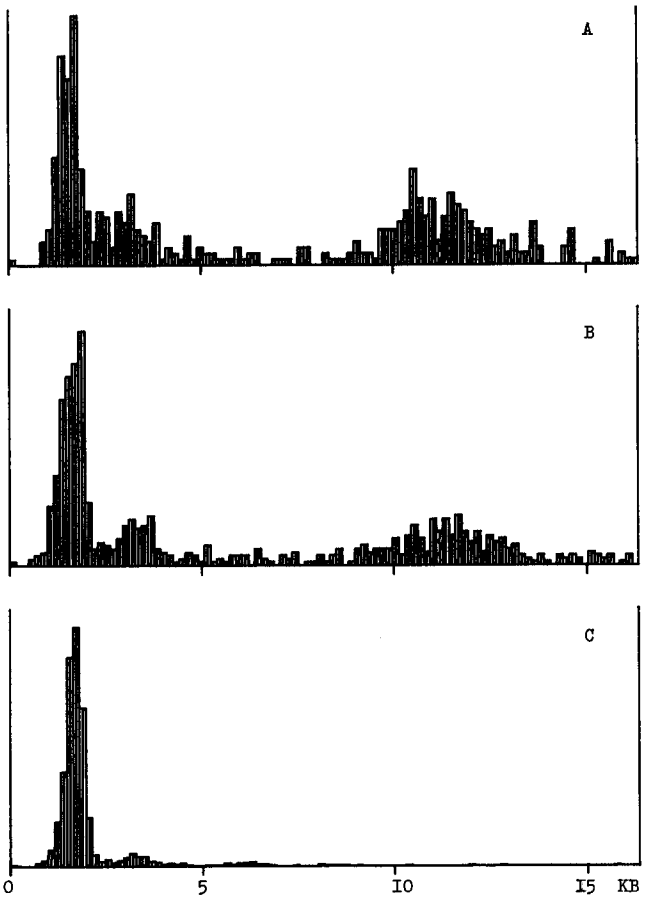

Fig. 4 A–C. Histogram of minicircular DNA lengths. **A** suspension culture (8 days old) mtDNA preparations (487 molecules from 2 preparations were measured); **B** suspension culture (22 days old) mt DNA preparations (720 molecules from 2 preparations were measured); **C** etiolated seedlings (6 days old) mtDNA preparations (1347 molecules from 3 preparations were measured). Circular molecules were measured in relation to circular pBR322 DNA on the same grid square

pension culture; in whole plants these were found in negligible quantities.

Alterations in the minicircular mtDNA population in suspension culture in comparison with those in the whole plants were accompanied by alterations in the patterns of sequences homologous to CCC1A, CCC1B and CCC2 (Fig. 5). As was shown earlier, cloned minicircular DNA sequences hybridized in total mtDNA preparations from 6-day old etiolated seedlings, not only with minicircles and their derivatives, but also with several discrete classes of DNA having larger sizes (Negruk et al. 1985) (Fig. 5 C, E, G). CCC1A and CCC1B sequences hybridized with slow migrating small DNA species of mtDNA from the suspension culture and from the whole plant with almost the same intensity (Fig. 5 D, F). However, relatively more intensive hybridization with small mtDNA species, probably correspondent to dimers of CCC1A and CCC1B (arrows) occurred. On the contrary, CCC2 sequences in the suspension culture contain significantly larger quantities of homologous small DNA species migrating as linear fragments with sizes near 2 kb (probably dimers of CCC2) and between 3.2 and 12 kb (probably oligomeres of CCC2) (Fig. 5 G, H). These results are in good agreement with the electron microscopic data and reflect some functional differences between CCC1A and CCC1B on one hand and, CCC2 on the other.

Hybridization of labelled CCC1A, CCC1B and CCC2 sequences with mtDNA preparations digested with different restriction endonucleases revealed a number of features. If mtDNA from the whole plants was treated with the mixture of HindIII and EcoRV, labelled CCC1A hybridized primarily with a 1.13 and 0.5 kb HindIII fragment of itself and only partly with a 1.47 kb HindIII fragment of CCC1B and 1.45 kb EcoRV fragment of CCC2. Labelled CCC1B hybridized mainly with the 1.47 kb HindIII fragment of itself and partly with the 1.13 and 0.5 HindIII fragments of CCC1A. Labelled CCC2 hybridized with the 1.45 kb EcoRV fragment of itself and partly with the 1.13 kb HindIII fragment of CCC1A (Fig. 6). Existence of sequence homology between CCC1A and CCC1B and between CCC2 and 1.13 kb HindIII fragment of CCC1A (Negruk et al. 1985; Goblet et al. 1985) explains the cross hybridization of labelled minicircular mtDNA sequences (Fig. 6).

However, in all cases minor bands of hybridization having other mobilities could also be recognized (shown by arrows). These bands probably contain mtDNA fragments of low sequence homologies or highly homologous fragments with low copy numbers. Such homologous sequences were also found in mtDNA from the suspension culture digested with the mixture of HindIII and EcoRV and in mtDNA of whole plants and suspension culture digested with the mixture of HindIII, EcoRV and PstI (Fig. 6). The use of PstI, which does not cut neither CCC1A and CCC1B nor CCC2, allowed us to identify in the total mtDNA of whole plants the low quantities of minicircles of similar sizes and high sequence homology but with additional PstI sites (Fig. 6, shown by double arrows).

The comparison of copy numbers of minicircular mtDNA in whole plants and in suspension culture revealed strong differences for CCC1B (Fig. 6). Preliminary analysis of minicircular mtDNA copy numbers in the mt genome showed for 300–350 kb genome size 2–3 copies of CCC1A, 4–6 copies for CCC1B and 2–3 copies for CCC2. Fig. 6 B, 3 shows that in suspension culture the copy number of CCC1B is at least ten times less than that of CCC1A. It follows from Fig. 6 that the copy number alterations in suspension cultures could be found not only for CCC1B but also for some minor

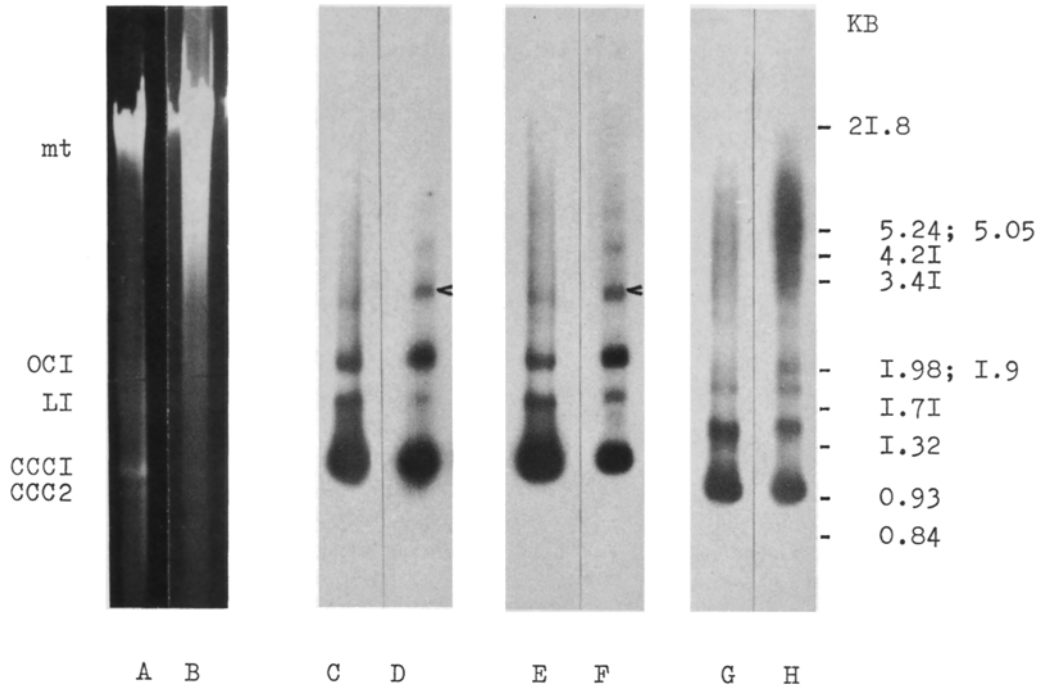

Fig. 5. One percent agarose gel electrophoresis of total mtDNA preparations from 6 days old etiolated seedlings (*A, C, E, G*) and a 22 days old suspension culture (*B, D, F, H*). *A, B* electropherograms of mtDNA stained with ethidium bromide. The autoradiographs of the hybridization of ^{32}P-labeled (1.13 kb + 0.5 kb) HindIII-fragments of CCC1A (*C, D*), 1.47 kb HindIII fragment of CCC1B (*E, F*) and 1.45 kb EcoRV fragment of CCC2 (*G, H*) to mtDNA from whole plants and suspension culture blotted onto nitrocellulose filters. The numbers to the right of the electropherogram (*H*) show the fragment sizes in kb of (HindIII + EcoRI) digest of λ DNA. CCC1 and CCC2 are minicircular supercoiled DNAs; L1 and OC1 are linear and open circular derivatives of CCC1; Mt is the main mtDNA

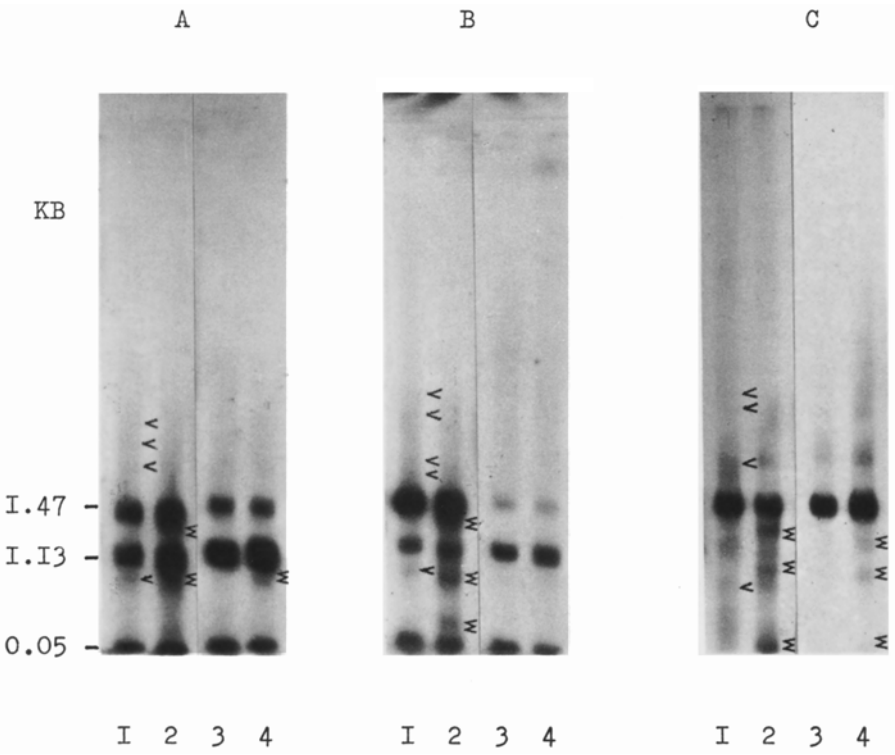

Fig. 6A–C. One percent agarose gel electrophoresis of total mtDNA preparation from 6 days old etiolated seedlings and from suspension culture digested with restriction endonucleases. The autoradiographs of the hybridization of ^{32}P-labeled (1.13 kb + 0.5 kb) HindIII-fragments of CCC1A (**A**), 1.47 kb HindIII-fragments of CCC1B (**B**) and 1.45 kb EcoRV-fragment of CCC2 (**C**) to mtDNA from whole plant digested with HindIII + EcoRV (*1*) and HindIII + EcoRV + PstI (*2*), and to mtDNA from suspension culture digested with HindIII + EcoRV (*3*) and HindIII + EcoRV + PstI (*4*). The numbers to the left of the electropherogram (**A**) show the sizes of the CCC1A and CCC1B HindIII fragments in kb

components of the minicircular mtDNA family. Thus, both electron microscopy and hybridization data show that the mt genome in prolonged suspension culture of *Vicia faba* undergoes alterations in circular mtDNA copy numbers.

Discussion

We observed significant molecular diversity in circular mtDNA in suspension culture in comparison with the whole plant. Such diversity was found both for large circular molecules and for minicircular DNAs. We believe that these data reflect the observed differences in the stoichiometry of the same restriction fragments in mtDNA from whole plant and suspension culture (Quetier and Vedel 1977; Dale et al. 1981; McNay et al. 1984). We also found such differences for *Vicia faba* mtDNA. Our observations are therefore in a good agreement with the suggestion about higher plant tissue culture as a factor increasing the molecular diversity of the mitochondrial genome (Kemble and Shepard 1984).

The lability of higher plant mitochondrial genomes in a suspension culture was shown earlier by Dale et al. (1981). These authors found notable differences between supercoiled mtDNA populations of old (several years of cultivation) and recently initiated (about a year) *N. tabacum* W38 culture lines. Such differences probably could be considered a result of subcloning the tobacco cells in vitro because authors did not observe any alterations in the suspension mtDNA population from the older culture of tobacco cells over the last 18 months. However, we cannot exclude the existence of such alterations during the initial period of in vitro cultivation.

Comparing and analysing the data reported here and the data from the physical mapping of *Brassica campestris* and *Zea mays* mitochondrial genomes (Palmer et al. 1984; Lonsdale et al. 1984), we suggest that the circular DNAs with sizes near 300 kb observed by electron microscopy could be considered master chromosomes or subgenomic molecules with sizes very close to the size of the master chromosomes (Bendich 1982; Lonsdale et al. 1983; Lonsdale 1984). Giant DNA molecules were also observed in mitochondria of citrus by Fontarnau and Hernandes-Jago (1982). Therefore, it is possible that master chromosomes do exist in the mitochondria of higher plants and that their visualization is only a technical problem.

The number of circular mtDNA classes may correspond to the number of repeats in the master chromosome, or may be more because of duplications (Palmer et al. 1984; Lonsdale et al. 1984; Lonsdale 1984). We observed in *Vicia faba* suspension culture mitochondria having no less than 20 classes of circular DNAs. At least half of them could be mono- and dimeric forms of the same sequences. Comparing circular DNA populations in mitochondria of whole plant and suspension culture one can suggest that the sizes of circular molecules predominant in suspension cultures (10–12 kb and 18–34 kb) correspond to minimal distances between short repeats (Lonsdale 1984). In agreement with this suggestion, 10–12 kb and 18–34 kb circles could appear as a result of intramolecular recombination of 37–80 kb circular DNAs predominant in 6-day old etiolated seedlings. Such lability can be explained by the necessity to change the efficiency of expression of different genes (Levings et al. 1979; Spruill et al. 1980; Leaver and Gray 1982).

MtDNA rearrangements of *Vicia faba* suspension cultures resemble structural alterations of the *Neurospora crassa* mitochondrial chromosome in stopper mutants (Gross et al. 1984), where 21 kb circular molecules, one third the length of the normal chromosome, became the predominant form during the stopped phase.

The appearance of mtDNA with other patterns of circular molecules in a new physiological situation possibly could reflect a more intensive replication of other subgenomic circular DNAs or the higher rate of recombination at a given point on a master chromosome.

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