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Isolation and physicochemical characterization of mitochondrial DNA from cultured cells of *Petunia hybrida*

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Summary. Mitochondrial DNA of *Petunia hybrida* was purified from cell suspension cultures. Up to 50% of the DNA could be isolated as supercoiled DNA molecules by CsCl-ethidium bromide density gradient centrifugation. The DNA purified from DNase-treated mitochondria bands at a single buoyant density of 1.760 gcm^{-3} in neutral density gradients and runs on agarose gels as a single band with an apparent molecular weight exceeding 30 megadaltons (Md). Summing of the restriction endonuclease fragment lengths indicates a mitochondrial genome size of at least 190 Md. Electron microscopic analysis reveals the presence of a heterogeneous population of circular DNA molecules, up to 60 Md in size. Small circular DNA molecules, ranging in size from 2–30 Md are present, but unlike in cultured cells of other plant species they do not form discrete size classes and furthermore, they constitute less than 5% of the total DNA content of the mitochondria. The restriction endonuclease patterns of mitochondrial DNA do not qualitatively alter upon prolonged culture periods (up to at least two years).

Key words: *P. hybrida* – mtDNA – Cell cultures

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait in plants which is of great economic importance for the commercial production of hybrid seed because it prevents normal pollen development and thus eliminates self fertilization of the seed parent plant (Marrewijk 1979). The aim of our research is to broaden the applicability of this property which is as yet limited because the trait is only found in a small number of plant species, including *Petunia hybrida.* For this purpose we study the molecular basis of CMS in *Petunia hybrida,* which has been shown to be a good model system for plant molecular biology studies (Hanson 1980).

In maize, CMS is apparently caused by a mutation in the mitochondrial genome and may result in the synthesis of variant polypeptides in mitochondria (Forde and Leaver 1980). In other plant species, such as tobacco, field beans and sorghum, mitochondrial modifications associated with CMS have also been observed (Belliard et al. 1979; Boutry and Briquet 1982; Dixon and Leaver 1982). We therefore will focus our attention on the genetic organization and expression of the mitochondrial genome of CMS and normal fertile plants. A prerequisite for this research is the availability of a good procedure for the isolation of mitochondrial DNA that is sufficiently pure and representative of the total population.

Methods described for the isolation of mitochondrial DNA from higher plants such as pea and maize usually involve the use of etiolated seedlings because of their relative high content of mitochondria (Kolodner and Tewari 1972; Kemble et al. 1982; Ward et al. 1981). Etiolated plant tissue of *Petunia hybrida* can not be obtained in large quantities while light grown, green tissue contains only few mitochondria. Dale (1981) has shown that mitochondrial DNA can also be isolated from cultured cells of maize and bean. We therefore have turned to suspension cultures of *Petunia hybrida* and developed a procedure for the isolation of mitochondrial DNA from such cultured cells.

In this paper we describe the purification and characterization of mitochondrial DNA from *Petunia hybrida* with respect to buoyant density, contour length and restriction fragments. We show that restriction endonuclease patterns of mitochondrial DNA isolated from cultured cells do not differ with prolonged periods of culture and we conclude that cultured cells of *Petunia hybrida* are the material of choice when the objective is to isolate mitochondrial DNA for studies on genetic organization and expression.

Materials and methods

Plant material and cell suspension cultures

The origin of the *Petunia hybrida* cultivars 'Rosy Morn', PZ5050 D1 and C7158 has been described previously (Marrewijk 1969; Colijn et al. 1979; Colijn et al. 1981). Callus cultures were initiated from these *P. hybrida* plants by incubating surface sterilized stem sections on modified Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 1 mg/l) and 0.6% agar as described previously (Colijn et al. 1982). Suspension cultures were obtained after transfer of the calli to liquid MS medium supplemented with naphthaleneacetic acid (NAA, 1 mg/l) and 0.3% sucrose. The liquid suspension cultures were grown on rotary shakers as described by Colijn et al. (1981). Unless otherwise indicated cell line AK 5000 obtained from *P. hybrida* C7158 was used throughout this study.

Isolation of mitochondrial DNA of Petunia hybrida

For the isolation of mitochondrial DNA (mt DNA) from suspension cells of *P. hybrida*, the method described by Sparks and Dale (Sparks and Dale 1980) was modified as follows: cells from liquid suspension cultures were harvested on a scintered glass filter, washed twice with ice-cold 20 mM KCl/5 mM EDTA and twice with 20 mM KCl. The cells were weighed (a typical preparation starts with 250–300 g of cells) and suspended in twice their weight of ice-cold buffer I (50 mM Tris-HCl pH 8.0, 0.3 M mannitol, 3 mM EDTA, 1% w/v polyvinylpyrrolidine, 1 mM 2-mercaptoethanol, 0.1% bovine serum albumine (BSA), and 5 mM sodium D-isoascorbic acid). Breakage of the cells was performed by three 10 s bursts with a Polytron homogenizer at maximum speed, and completed by their passing through a prechilled French pressure cell at 300 p.s.i. The homogenate was filtered through two layers of 200 µm nylon netting and subsequently through two layers of 30 µm netting. The filtered homogenate was then centrifuged at 200×g for 2 min at 4 °C to remove nuclei and starch. The pellet was discarded and the supernatant was recentrifuged at 1,500×g for 6 min at 4 °C. The resulting chloroplast pellet was discarded and the supernatant was centrifuged at 15,000×g for 15 min at 4 °C. The resulting mitochondrial pellets were resuspended (with a cotton wool stick) in 20 ml of buffer I, adjusted to 10 mM $MgCl_2$ and incubated with DNase (50 µg/ml) for 45 min at 22 °C. The samples were adjusted to 20 mM EDTA and centrifuged for 15 min at 15,000×g at 4 °C. The pellets were carefully resuspended with a glass homogenizer in a total volume of 24 ml of buffer II (50 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 0.02 M EDTA, 0.1% (w/v) BSA) and layered on top of four discontinuous sucrose gradients, composed of 12 ml of 1.6 M sucrose and 18 ml of 0.9 M sucrose in buffer II. The gradients were centrifuged in a Beckmann SW 27 rotor at 25,000 rpm at 4 °C for 1 h. The mitochondria which banded at the 1.6/0.9 M interphase were removed, diluted slowly 1 in 3 with buffer II and centrifuged at 15,000×g for 20 min at 4 °C. The purified mitochondrial pellets were resuspended in a total volume of 8 ml of buffer III (0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA) and lysed by the addition of 8 ml of buffer III containing 1% sodium sarkosyl. Solid CsCl was added immediately to the lysate (1.14 g of CsCl/ml of lysate) and dissolved by gently rolling the tubes on a flat surface. The lysates were transferred to polyallomer tubes, ethidium bromide was added (30 µg/ml) and the tubes were centrifuged for at least 65 h at 41,000 rpm at 15 °C in a Beckmann 65 H angle rotor. The gradients were examined under longwave ultraviolet illumination and the fluorescent DNA was removed. Ethidium bromide was extracted three to five times with an equal volume of isopropanol, saturated with CsCl in buffer IV (0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA). The DNA was dialyzed against four 1 l changes of buffer IV for 40 h at 4 °C. The dialyzed DNA solution was then transferred to collodium dialyzing tubes SM 1320 (Sartorius, Göttingen) and concentrated by several cycles of dialysis against 80% (w/v) sucrose in buffer IV, alternated by dialysis against buffer IV without sucrose.

Preparation of chloroplast DNA

Chloroplast DNA was prepared essentially as described by Kumar et al. (1982) with some modifications: approximately 40 g of young, fully expanded leaves were washed in ice-cold 20 mM KCl and homogenized in buffer A (50 mM Tris-HCl, pH 8.0, 0.35 M sucrose, 7 mM EDTA and 5 mM 2-mercaptoethanol) by using a Braun blendor. The homogenate was filtered through nylon netting (30 µm). Chloroplasts were isolated from the filtrate by centrifugation at 1,500×g. Chloroplasts were lysed in 2% sarkosyl and the cpDNA was purified from the lysate by two phenol and two phenol-chloroform (1 : 1) extractions. Nucleic acid was ethanol precipitated from the resulting water layer and the pellet was dissolved in a small volume of TE buffer (5 mM Tris-HCl pH 8.0 and 0.5 mM EDTA).

Analytical ultracentrifugation

Equilibrium centrifugation of 2 µg of DNA in neutral CsCl solutions ($=1.710\ gcm^{-3}$) was performed in an AN-F Ti rotor in a Beckmann model E analytical ultracentrifuge equipped with a photoelectric scanner. Centrifugation was at 40,000 rpm for 24 h at 22 °C. *Micrococcus lysodeikticus* DNA of density $1.731\ gcm^{-3}$ was included with the sample as a density standard. Buoyant densities and base compositions were calculated according to the method of Schildkraut et al. (1962).

Restriction endonuclease analysis and agarose gel electrophoresis of DNA

Sal I was prepared by the method of Green et al. (1978). Bgl I and Bam HI were purchased from Boehringer, Mannheim. Samples of chloroplast DNA (2 µg) were digested with two units of enzyme as described by the suppliers. Samples of mitochondrial DNA (4–5 µg) were digested with 10 units of restriction endonucleases for 1.5 h at 37 °C. Reactions were terminated by adding 0.25 vol. of a solution containing 50 mM Tris-HCl pH 7.8, 125 mM EDTA and 0.06% bromophenol blue. Digests of DNA were analysed by electrophoresis on horizontal slab gels (25×20×0.5 cm), containing 0.7–1.0% agarose (Sigma, low EEO), depending on the size of the DNA fragments to be separated. Electrophoresis was performed as described previously (Bovenberg et al. 1981). Molecular weights of DNA restriction fragments were estimated relative to molecular weight markers: Hind III and Eco RI digestion fragments of λ DNA (Boehringer, Mannheim).

Electron microscopy

Purified mitochondrial DNA was prepared for electron microscopy as described by Kleinschmidt (1968). Six ml of hypophase containing 2 µg of mitochondrial DNA and 0.5 µg of Clo DF13 DNA (added as an internal standard: 9,600 bp (Elzen et al. 1980) in a solution of 0.2 M ammonium acetate was transferred to a plastic petri dish (∅ 4 cm). A hyperphase containing 0.024% (w/v) cytochrome c in 0.96 M ammoniumacetate was spread on the surface of the hypophase. The DNA was allowed to diffuse and adsorb to the cytochrome c film for 30 min at room temperature. The cytochrome-nucleic acid

layer was picked up on parlodion-coated grids (Pellco, 400 mesh), stained with uranyl acetate, dehydrated with isopentane and rotary shadowed with platinum. The grids were examined in a Zeiss EM10 Electron microscope. Contour length measurements relative to plasmid Clo DF13 DNA, were performed using a Beckmann 2D apparatus.

Blot hybridization analysis of mitochondrial DNA

Mitochondrial DNA, separated on agarose slab gels, was transferred to nitrocellulose according to Southern (1975) and hybridized with ^{32}P-labelled mitochondrial DNA (Wahl et al. 1979). The in vitro labeling of the mitochondrial DNA with 5′-α-^{32}P-dATP was performed as described by Rigby et al. (1977). By this method mitochondrial DNA was labeled to a specific activity of about 10×10^6 dpm/μg.

Results

Isolation and buoyant density analysis of mitochondrial DNA from cultured Petunia hybrida cells

The procedure for the isolation of mitochondrial DNA from cell suspension cultures of *P. hybrida* that is described in detail in the section "Materials and methods" involves a DNase treatment of the mitochondria to remove contaminating DNA present outside the mitochondria. If this DNase treatment is omitted, two fluorescent DNA bands are visible in CsCl-ethidium bromide gradients when examined under ultraviolet light. As much as 50% of the total mitochondrial DNA could be isolated on the gradient in the lower DNA band which contains supercoiled circular DNA molecules (see section on electron microscopy). Analysis on neutral CsCl equilibrium density gradients indicates that the DNA from the upper band, and to a lesser extent also the DNA from the lower band, contains a considerable amount of contaminating nuclear DNA that bands at a buoyant density of 1.6961 gcm^{-3} (Fig. 1 A, B).

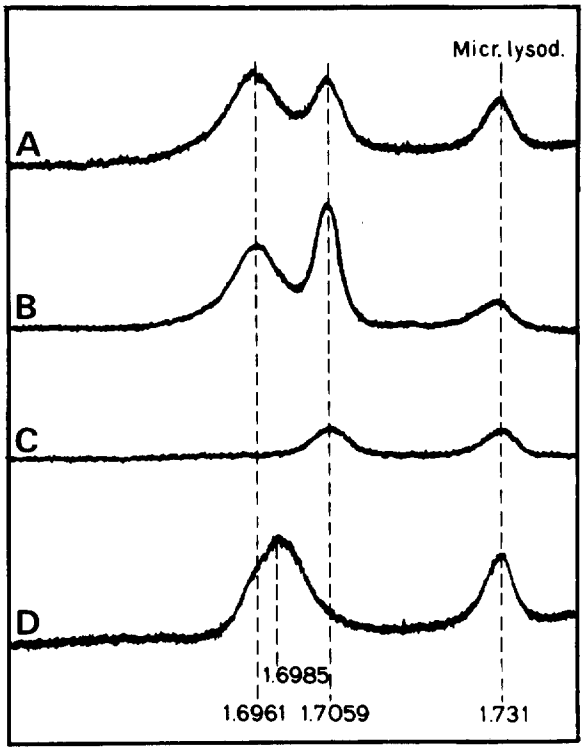

Fig. 1. Photoelectric scans of mitochondrial DNA banded in neutral CsCl equilibrium density gradients. *Micrococcus lysodeikticus* DNA was added as density standard. (*A*) Upper band and (*B*) lower band mitochondrial DNA; (*C*) mitochondrial DNA from DNase treated mitochondria; (*D*) chloroplast and nuclear DNA from *P. hybrida*

To avoid the presence of contaminating nuclear DNA, the mitochondria are incubated with DNase prior to final purification through the discontinuous sucrose gradients. CsCl-ethidium bromide gradient centrifugations of lysates of mitochondria thus incubated results in only one band that contains open circular and linear DNA molecules (see section on electron microscopy). This indicates that incubation of the mitochondria with DNase results in nicking of the supercoiled DNA molecules present in the mitochondria. Centrifugation of this DNA from DNase treated mitochondria on neutral CsCl gradients results in only one peak that bands at a buoyant density of 1.7059 ± 0.005 gcm^{-3} (Fig. 1 C). This value corresponds with a GC-content of $46.5 \pm 0.5\%$. The buoyant density of *P. hybrida* mitochondrial DNA agrees with the value of 1.706 gcm^{-3} that has been found for the buoyant density of mitochondrial DNA from most higher plants thus far analyzed (Quetier and Vedel 1977).

Chloroplast DNA and nuclear DNA of *P. hybrida* band at buoyant densities of respectively 1.6985 and 1.6961 ± 0.0005 gcm^{-3} (Fig. 1 D). These densities differ sufficiently from the density of mitochondrial DNA to distinguish the DNA species on analytical CsCl gradients. Therefore it can be concluded from the banding patterns in Fig. 1 C that mitochondrial DNA preparations that are isolated from DNase treated mitochondria do not contain detectable amounts of chloroplast or nuclear DNA.

Restriction endonuclease analysis of mitochondrial DNA

The presence of contaminating nuclear DNA in the various mitochondrial DNA preparations, as visualized on neutral CsCl gradients, results in a background smear when these DNA's are electrophoresed on agarose gels (Fig. 2 lane 1–3). The amount of background decreases in the order upper band DNA, lower band DNA and DNA from DNase treated mitochondria, which is consistent with the results of the neutral CsCl gradients. The quality of the mitochondrial DNA was further analyzed by digestion with

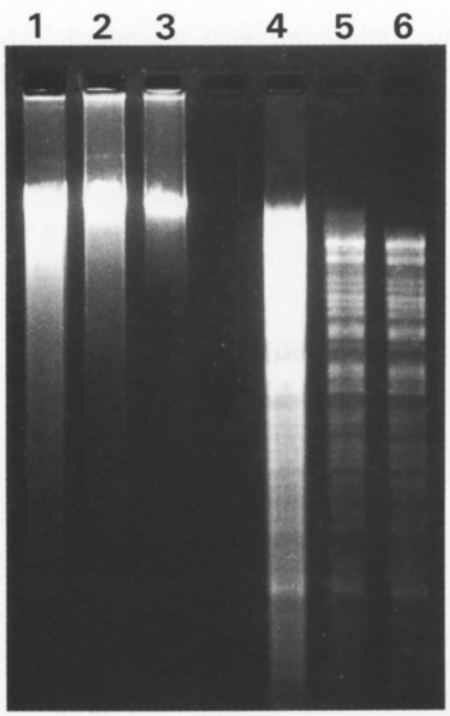

Fig. 2. Agarose gel electrophorese of undigested (*lane 1–3*) and Bam HI digested (*lane 4–6*) mitochondrial DNA. Upper band (*Lane 1 and 4*) and lower band (*lane 2 and 5*) mitochondrial DNA from CsCl-ethidiumbromide gradient centrifugation of non-DNase treatment mitochondria; *lane 3 and 6* contain mitochondrial DNA purified from DNase treated mitochondria

restriction endonucleases. The results of digestion with the enzyme Bam HI can be seen in Fig. 2, lane 4–6. The digestion patterns of all three mitochondrial DNA preparations look identical as far as the bands can be distinguished. However, the presence of nuclear DNA in the upper and lower band mitochondrial DNA preparations again causes a background smear, especially in the upper band DNA. DNA from the lower band appears to be quite suitable for restriction fragment analysis. The mitochondrial DNA that is isolated from the DNase treated mitochondria can be digested with various restriction endonucleases and produces the cleanest pattern of DNA fragments (Fig. 2, lane 6 and Fig. 3). This DNA has been used for all further restriction endonuclease analysis studies.

Digestion of *P. hybrida* mitochondrial DNA with restriction endonucleases such as Sal I, Bam HI and Bgl I results in a complex pattern of over 30 bands (Fig. 3). Very characteristic for higher plant mitochondrial DNA is the presence of a great number of bands in non-stoichiometric amounts. We can rule out the possibility that this is the result of contaminating nuclear DNA because of the purity of the mitochondrial DNA preparation from DNase treated organelles as was ascertained by analysis on analytical density gradients (Fig. 1 C) and furthermore because the most prominent bands in the chloroplast DNA digests that were run adjacent to each mitochondrial DNA digest

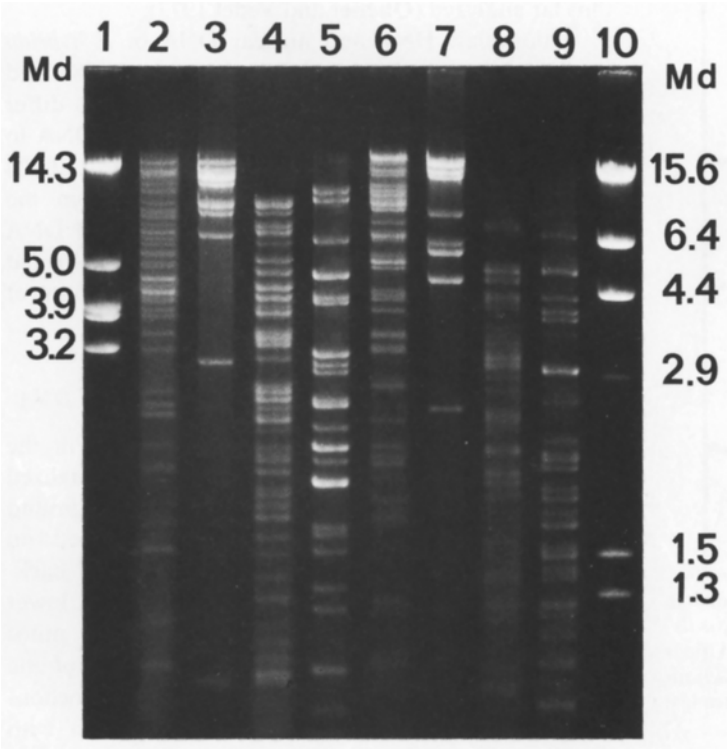

Fig. 3. Agarose gel electrophoretic patterns of restriction endonuclease digested mitochondrial DNA and chloroplast DNA. Mitochondrial DNA from DNase treated mitochondria and chloroplast DNA was digested with Sal I (*lane 2 and 3*, respectively), Bam HI (*lane 4 and 5*, resp.), Bgl I (*lane 6 and 7*, resp.) and Eco RI (*lane 8 and 9*, resp.). *Lane 1 and 10* contain molecular weight markers of, respectively, Eco RI digested and Hind III digested λ DNA

do not line up with the non-stoichiometric bands in the mitochondrial DNA digests (Fig. 3).

The molecular weight of *P. hybrida* mitochondrial DNA obtained by summation of the restriction endonuclease fragments ranges from 190 to 219 Md when differences in the fluorescence intensity of the bands are not taken into account (Table 1). Densitometer tracings of photonegatives of the gels revealed that some bands contain up to six times the amount of DNA of neighbouring bands (Fig. 4). Because these non-stoichiometric bands do not represent contaminating chloroplast or nuclear DNA, these bands must either contain several different sequences of about similar length and/or multiple copies of the same sequence. Ward et al. (1981) found for watermelon mitochondrial DNA a good correlation between genetic complexity values obtained from reassociation kinetics and by summing restriction endonuclease fragments taking the non-stoichiometry of the bands into account. This indicates that at least most non-stoichiometric bands contain different sequences. Assuming that the non-stoichiometric bands in the mitochondrial DNA restriction patterns of petunia mitochondrial DNA also contain several different sequences or multiple copies of the same sequence then the estimated molecular weight of mitochondrial DNA should be adjusted to a value of about 321–324 Md (Table 1).

Genomic stability of mitochondrial DNA in Petunia cell cultures

The restriction endonuclease patterns described above are derived from mitochondrial DNA isolated from cell cultures. Recently it has been reported for tobacco that the tissue culture stage may cause substantial differences in the range and frequency of size classes of the mitochondrial DNA molecules, although in that case this did not lead to qualitative differences in the mitochondrial DNA restriction pattern (Dale 1981). As we intend to use the mitochondrial DNA from petunia cell cultures for physical mapping and studies on the genetic organization and expression of mitochondrial DNA, it is essential that the nucleotide sequences in *P. hybrida* mitochondrial DNA are similarly conserved under our cell culture conditions. We therefore have analyzed the mitochondrial DNA restriction patterns of three independently initiated cell lines of different cultivars that have been continuously grown as suspension culture for 1 month, 6 months and 2 years, respectively. Figure 5 A shows that mitochondrial DNA isolated from all three cell lines has the same Bam HI restriction fragment pattern. These results indicate that in *P. hybrida* cell cultures major alterations in the nucleotide sequence of the mitochondrial DNA do not occur upon prolonged growth of the cell cultures.

Table 1. Molecular weights of restriction endonuclease fragments of mitochondrial DNA from *Petunia hybrida*. Molecular weights are given in megadaltons. The number given in brackets refers to the molar ratio of that band

Sal I – digest				Bgl I – digest			
Fragment	Mol. wt.	Fragment	Mol. wt.	Fragment	Mol. wt.	Fragment	Mol. wt.
1	18 (2×)	21	4.0	1 a, b	26, 23	21	3.4
2	13.2 (2×)	22	3.9	2	14	22	3.3
3	12.8	23	3.8	3	12.2	23	3.2
4	11	24	3.3	4	10.7	24	2.7
5	9.6	25	3.2	5	10.2 (2×)	25	2.4
6	8.5	26	2.7	6	9.4 (2×)	26	2.2
7	7.9 (2×)	27	2.5 (2×)	7	9.0	27	2.1
8	7.4 (2×)	28	2.4 (3×)	8	8.6 (2×)	28	2.1
9	7.0	29	2.2 (2×)	9	8.5 (2×)	29	2.0
10	6.6 (3×)	30	2.0	10	7.0	30	1.8
11	6.4	31	1.7 (3×)	11	6.2 (4×)	31	1.7
12	6.0	32	1.5 (6×)	12	6.0	32	1.3 (2×)
13	5.5	33	1.2 (2×)	13	5.3	33	1.2 (2×)
14	5.4	34	1.0 (5×)	14	5.1 (5×)	34	1.1 (2×)
15	5.0	35	0.8	15	4.9 (2×)	35	1.0 (2×)
16	5.6	36	0.7 (2×)	16	4.1	36	0.9 (2×)
17	4.6 (5×)	37	0.6	17	4.0	37	0.8 (2×)
18	4.4 (2×)			18	3.9	38	0.7
19	4.3 (6×)			19	3.7	39	0.7
20	4.0			20	4.5		
Sum of Mol. wts.			190.1 (320.7)				219.8 (323.9)

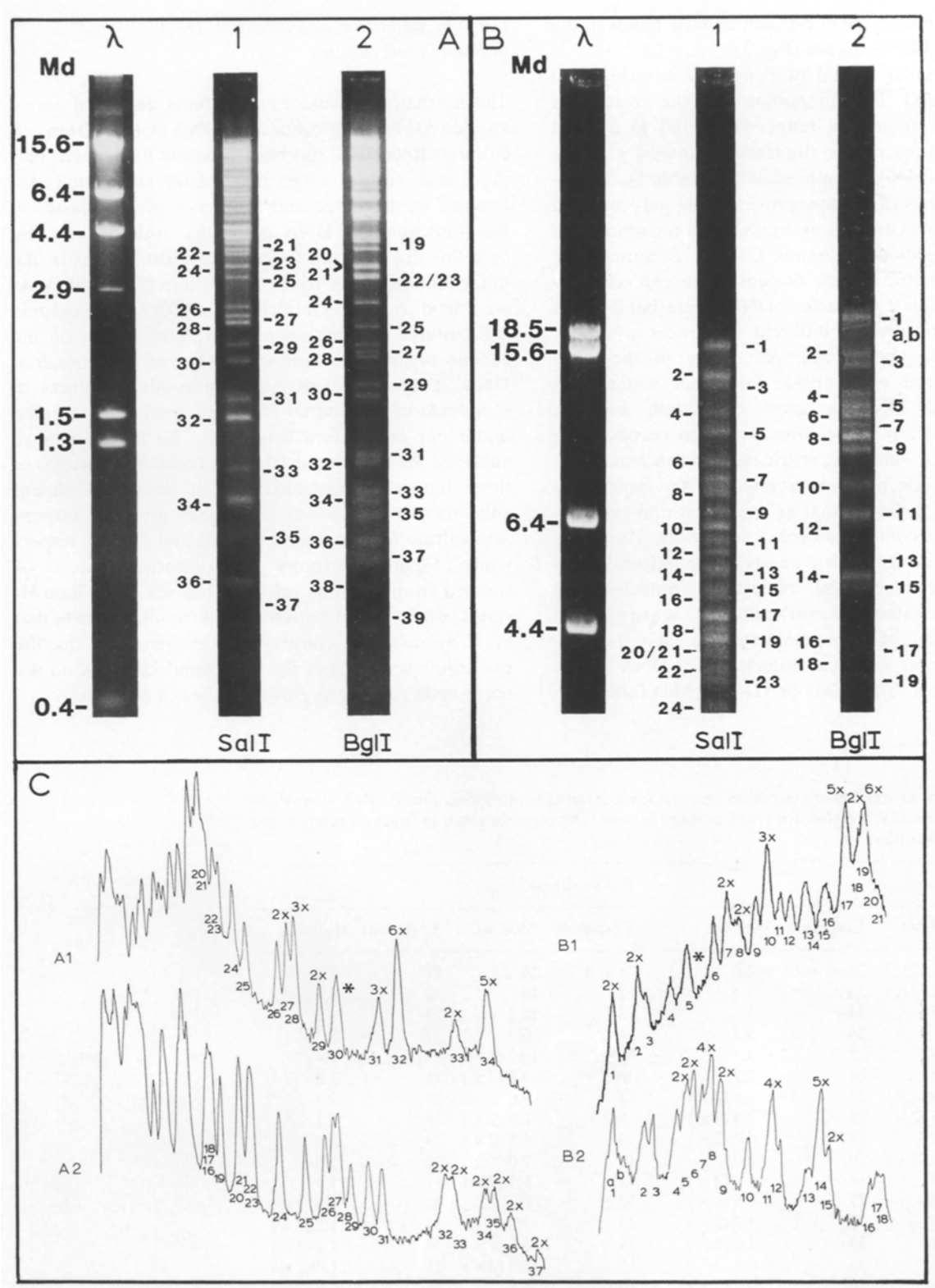

Fig. 4A–C. Sal I and Bgl I digest patterns of *P. hybrida* mitochondrial DNA. **Panel A and B** represent the agarose gels, **panel C** represents the densitometric tracings of the photographic negatives of these gels. Sal I digests were separated on 0.7% agarose gels for 25 h (*lane A1*) and 48 h (*lane B1*), Bgl I digests ran for 25 h (*lane A2*) and 48 h (*lane B2*). The λ marker lane contains λ DNA digested with Hind III. The relative increase in DNA content of the bands that are present in non-stoichiometric amounts is indicated above the peaks. (The *asterisk* indicates the position of a scratch in negative *A1* and *B1*.) On other gels Bgl I band 1 separates into two DNA fragments: 1a and 1b

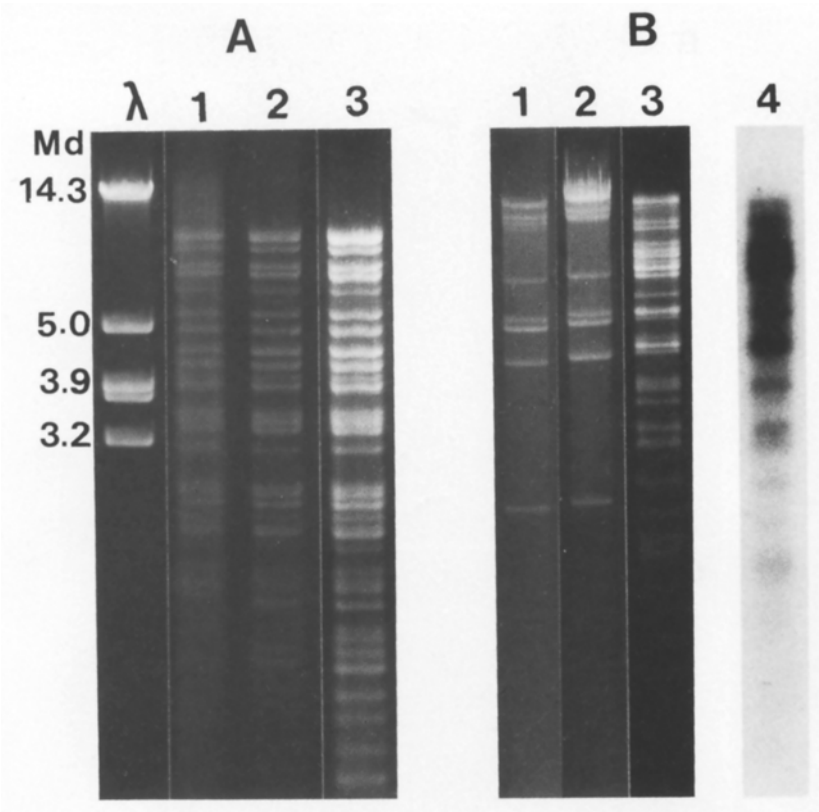

Fig. 5A, B. Gel electrophoresis of restriction endonuclease digests of mitochondrial DNA from leaves and from cell cultures of various cultivars at various culture age. **Panel A**: Bam HI digest of mitochondrial DNA from a cell culture of *P. hybrida* cv. 'Rosy Morn', 1 month in culture (*lane 1*); *P. hybrida* C7158 cell culture AK5000, 2 years in culture (*lane 2*) and after 6 months in culture (*lane 3*). The mol wt marker lane contains λ DNA digested with Eco RI. **Panel B**: Bgl I digests of mitochondrial DNA (*lane 1*) and chloroplast DNA (*lane 2*) from leaves of *P. hybrida* E5050 (Colijn et al. 1983) mitochondrial DNA from cultured cells of *P. hybrida* cv. 'Rosy Morn' (*lane 3*); *lane 4* represents a Southern blot of lane 1 hybridized with ^{32}P-labeled mitochondrial DNA from cultured of *P. hybrida* cv. 'Rosy Morn'

The results of these experiments do not rule out the possibility that alterations have already occurred immediately after initiating the tissue cultures. To test this we have isolated mitochondrial DNA from *P. hybrida* leaves. Upon digestion of this DNA with restriction endonucleases one does not observe a typical mitochondrial DNA fragment pattern but rather a chloroplast DNA digestion pattern because of the large amount of contaminating chloroplast DNA present in mitochondrial DNA preparations from leaves (Fig. 5 B, lane 1). The visualize the mitochondrial DNA fragment pattern we have hybridized a Southern blot of this lane with ^{32}P-labeled mitochondrial DNA from cultured cells. Autoradiographs of this hybridized blot show a pattern of bands that line up with the bands in Bgl I digests of mitochondrial DNA from cell suspensions (Fig. 5 B, lane 3 and 4). None of these bands co-migrate with one of the Bgl I fragments of chloroplast DNA (lane 2). This indicates that the pattern of radioactive bands represents the restriction endonuclease digest of the small amount of mitochondrial DNA present in this DNA preparation from leaves. All the bands that show up on the autoradiograph are also present in the digestion pattern of mitochondrial DNA from cultured cells. This implies that at least for all the DNA fragments of leaf mitochondrial DNA that can be distinguished on the autoradiographs no extensive rearrangements of the DNA have occurred by growing the cells in tissue culture. Therefore, these results suggest that most probably there are no major differences between the genetic organization of the mitochondrial DNA in plants and in cultured cells of *P. hybrida.*

Electron microscopy of Petunia hybrida mitochondrial DNA

In all plant species examined so far, mitochondrial DNA populations have been shown to contain a heterogeneous population of circular DNA molecules ranging in size from 0.5 μm to more than 30 μm (Dale 1981; Sparks and Dale 1980; Quetier and Vedel 1977; Ferguson and Davis 1978). CsCl-ethidium bromide gradients of petunia mitochondrial DNA reveal the presence of two bands. This suggests that *P. hybrida* mitochondria contain supercoiled circular DNA molecules. To verify this more directly we have examined mitochondrial DNA preparations in the electron microscope.

In our initial experiments we examined mitochondrial DNA that was spread using the standard formamide technique described by Ferguson and Davis (1978). We observed mainly long linear DNA and only occasionally a small, circular DNA molecule, even when lower band DNA was spread. This might be attributed

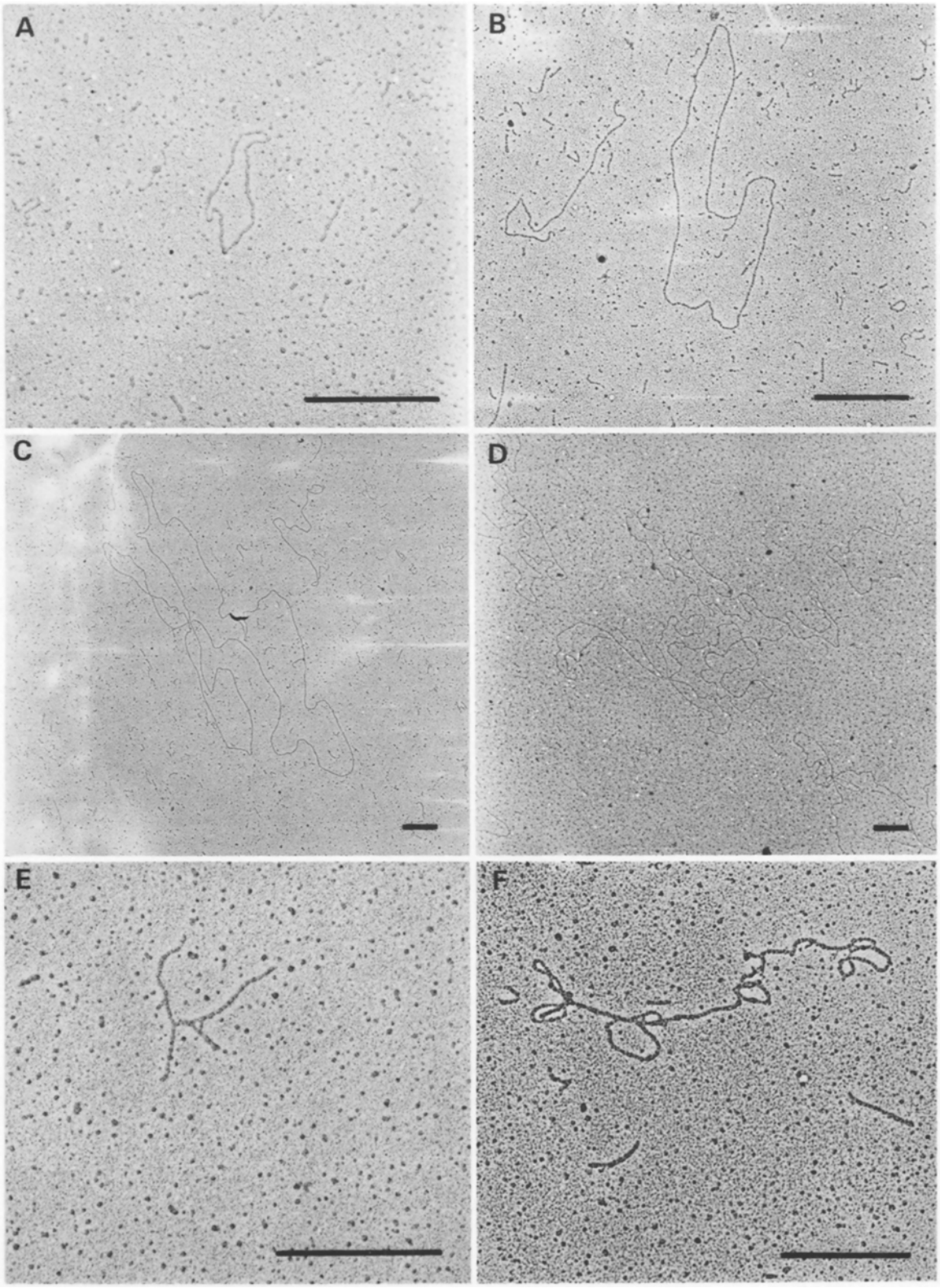

Fig. 6 A–F. Electron micrographs of *P. hybrida* mitochondrial DNA. **Panels A–D** represent open circular DNA molecules from DNase treated mitochondria. **Panel E** represents a typical supercoiled circular mitochondrial DNA molecule and **panel F** represents a supercoiled circular DNA molecule converting to the open configuration, present in the lower band of the CsCl-ethidium bromide gradient. The bars indicate 0.5 μm

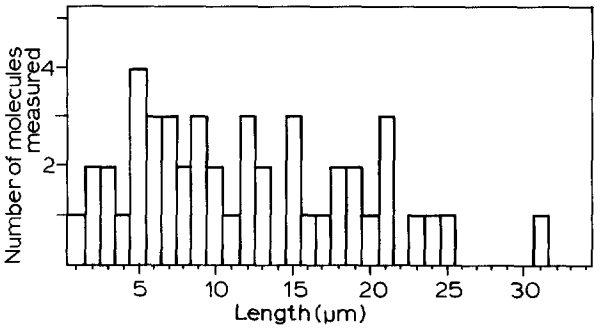

Fig. 7. Frequency distribution of open circular mitochondrial DNA molecules purified from DNAse-treated mitochondria by CsCl-ethidium bromide gradient centrifugation

to extensive shearing of circular DNA during the spreading procedure. We therefore turned to the 'classical' diffusion spreading technique (Kleinschmidt 1968) in which shearing forces are minimized. If this diffusion method is applied to lower band DNA only 20% of the molecules observed have a linear or open circular configuration. The remainder is either fully supercoiled (Fig. 6 E) or supercoiled and converting to the open circular form (Fig. 6 F), as can be expected for DNA molecules present in the lower band of CsCl-ethidium bromide gradients. DNA in this supertwisted configuration is not suitable for contour length measurements and large circles may have been lost from the lower band because after nicking or breakage such molecules will be found in the upper band position in the gradient. Therefore we performed contour length measurements on DNA molecules purified from DNase-treated mitochondria that band in only one position in the gradient and thus represent the total population of mitochondrial DNA.

We have measured about forty randomly selected open circular molecules (Fig. 6 A–D). A histogram summarizing the data is presented in Fig. 7. In contrast to data reported for other plant mitochondrial DNA's (Sparks and Dale 1980; Levings et al. 1979; Pring and Hannah 1983) no predominant size classes are observed but rather a continuous range of mitochondrial DNA circles which vary in size from 2–60 Md. We also observed large linear mitochondrial DNA molecules up to 90 Md indicating that larger circular DNA molecules may exist in mitochondria but get broken during isolation and preparation for electron microscopy.

Discussion

Mitochondrial DNA of *P. hybrida* isolated from green plants is not suitable for further analyses because of the heavy contamination with chloroplast DNA and the low yields. Therefore, we turned to cultured cells of *P. hybrida* and developed a procedure for the isolation of mitochondrial DNA from such cultured cells. The mitochondrial DNA isolated from these cultured cells has a buoyant density of 1.7059 gcm^{-3} which is similar to that described for most other higher plant mitochondrial DNAs. As much as 50% of the mitochondrial DNA from cultured petunia cells can be isolated as lower band supertwisted DNA in a CsCl-ethidium bromide gradient. This configuration of the DNA molecules has been confirmed by electron microscopy. The lower band mitochondrial DNA has the same density in neutral CsCl gradients as total mitochondrial DNA (Fig. 1 B). This observation, together with the fact that the restriction endonuclease fragment pattern of this DNA is identical to that of total DNA from DNase treated mitochondria, indicates that these supertwisted DNA molecules contain all the nucleotide sequences present in the total population of mitochondrial DNA molecules. They therefore do not represent a special class of DNA molecules with specific nucleotide sequences. The observed percentage most probably does not reflect the actual part of the DNA molecules that is present in a circular configuration in vivo because a number of the DNA molecules, and especially the larger DNA circles, may have been nicked during the extraction procedure and will then be present in the upper band of the CsCl ethidium bromide gradient. Therefore, it is very well possible that in vivo all mitochondrial DNA molecules are circular.

Electron microscopic analysis of mitochondrial DNA purified from DNase-treated mitochondria shows a series of open circular DNA molecules that range in size from 2–60 MD. The molecular weight of mitochondrial DNA as estimated by summing restriction fragments yields a value of at least 190 Md which is far larger than the longest open circular molecule measured (60 Md). Larger DNA molecules, up to 90 Md, can be isolated. However, these molecules are in a linear configuration indicating that larger circular DNA molecules may exist in mitochondria but get broken during isolation and preparation for electron microscopy. In fact, only 20% of the DNA molecules on the grids are intact circular DNA molecules. Therefore, it can not be concluded from our results whether the mitochondrial DNA genome of *P. hybrida* consists of one large circular molecule of about 100 μm or of several somewhat smaller circular molecules. If the latter is the case then the non-stoichiometry of certain bands in the restriction fragment patterns could also reflect the difference in relative amounts of the various subgenomes.

At first sight, the presence of many small DNA circles, as seen under the electron microscope, is in disagreement with the results presented in Fig. 2 where

the electrophoretic mobility of mitochondrial DNA molecules in agarose gels is analyzed. In agarose gels lower and upper band mitochondrial DNA, as well as DNA isolated from DNase-treated mitochondria migrate as a single band with an apparent molecular weight exceeding 30 Md. There is no trace of small circular DNA species as observed under the electron microscope. We think that the major DNA band found in the gel represents the actual large mitochondrial DNA genomes (and large degradation products thereof) of which the majority gets broken during isolation and preparation for electron microscopy. This explains why only 20% of the DNA molecules on the grids are intact circular DNA molecules. The smaller DNA circles (size less than 15 μm) represent therefore only a minor fraction of the total mitochondrial DNA, and due to their size heterogeneity remain undetected in the agarose gels.

The presence of small circular DNA molecules in mitochondrial DNA preparations is not specific for cultured cells of *P. hybrida.* They have also been observed in mitochondria from a number of other plant species (Kemble et al. 1982; Dale 1981; Sparks and Dale 1980). The possibility that these small circular molecules in *P. hybrida* originate from contaminating bacteria or chloroplasts can be rejected for a number of reasons. Bacterial contamination is excluded because the mitochondria were isolated from sterile cell cultures. The chloroplast origin of these circles can be excluded on the basis of the buoyant density (Fig. 1) and furthermore because we have shown previously that chloroplast DNA of *P. hybrida* exists exclusively in circular molecules of 101 Md (Bovenberg et al. 1981).

In *P. hybrida* cultured cells, these small mitochondrial DNA circles make up just a small percentage of the total population of mitochondrial DNA molecules. In tobacco, field bean and maize cell cultures small mitochondrial DNA circles (14 Md and less) can make up a large part of the mitochondrial DNA population (up to at least 50% of the isolated supercoiled DNA fraction) and, depending on the culture conditions used, substantial differences in the range and frequency of size classes occur (Sparks and Dale 1980). However, in all these situations the mitochondrial DNA restriction patterns do not qualitatively differ, suggesting that these small DNA circles may simply be amplified genes or defective mitochondrial DNA (Dale 1981; Pringe and Hannah 1983). Similarly, in *P. hybrida* we do not observe any qualitative differences between restriction endonuclease patterns of mitochondrial DNA isolated from cells that have been in culture for various periods of time. Therefore, despite the presence of a low amount of small circular DNA molecules that perhaps does not entirely reflect the in vivo plant situation, we conclude that cultured cells of *P. hybrida* are the material of choice when the objective is to isolate mitochondrial DNA for studies on physical mapping, expression and genetic organization.

We have tried to obtain more support for this conclusion by showing that restriction endonuclease patterns of mitochondrial DNA isolated from leaves and cultured cells are identical. For this purpose the leaf mitochondrial DNA pattern had to be visualized by hybridizing a southern blot of this digested DNA with a pure DNA probe, that is mitochondrial DNA from cultured cells. However, by doing so we could in theory fail to visualize those nucleotide sequences present in the mitochondrial DNA of the plant that get deleted when cells are cultured in vitro. This possibility appeared to be not very likely because all the bands that showed up on the autoradiograph of the leaf mitochondrial DNA digest comigrated with bands in the digest of mitochondrial DNA from cultured cells which implicates that at least in those parts of the mitochondrial genome that are represented by these DNA fragments such major deletions or other major rearrangements do not occur when the cells are cultured in vitro.

Our current research is focussed on the elucidation of the molecular basis of cytoplasmic male sterility in petunia. For this study we have successfully used cell cultures of cytoplasmic male sterile and fertile *P. hybrida* lines to demonstrate that mitochondrial modifications in DNA and translation products are associated with cytoplasmic male sterility.

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