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Isolation, characterization and restriction endonuclease mapping of the Petunia hybrida chloroplast DNA

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ABSTRACT

A procedure is developed for the isolation of intact chloroplast DNA (ctDNA) from *Petunia hybrida*. The molecular weight, calculated from contour length measurements, is 96.0 $\pm$ 4.5 x 10^6 daltons. This value is in good agreement with the value of 101.2 x 10^6 daltons that was estimated from the electrophoretic mobilities of restriction endonuclease fragments of ctDNA. Analysis of petunia ctDNA in neutral CsCl gradients revealed the presence of only one type of DNA at a buoyant density of 1.6987 $\pm$ 0.0005 gcm^{-3}. This corresponds with a GC-content of 39.3 $\pm$ 0.5%. A physical map of petunia ctDNA was constructed by using the restriction endonucleases Sal I, Bgl I, Hpa I and Kpn I. The map indicates that petunia ctDNA contains two copies of a sequence in an inverted orientation. The inverted repeat regions have a minimum length of 10 x 10^6 daltons. Hybridization data indicate that part of the inverted repeat regions contain the genes for chloroplast ribosomal RNAs.

INTRODUCTION

For the study on the genetic organization and regulation of expression of ctDNA genes, involved in chloroplast biogenesis, we use tissue cultures of *Petunia hybrida*. Such plant cell cultures are valuable tools for the study of chloroplast biogenesis because cytokinin can be used to induce developmental processes that lead to the transition of undifferentiated plastids into chloroplasts (1, 2). In addition, chemical mutagenesis can be employed to isolate various mutants with an altered pattern of chloroplast biogenesis (3). For the study of the expression of at least part of the chloroplast genome, we can use isolated petunia chloroplasts. Such chloroplasts are able to perform light-driven and ATP-driven protein synthesis (4). However, for a full understanding of the coding properties and genetic organization of the petunia ctDNA, the physical mapping of genes and the study of the expression of cloned ctDNA in various *in vitro* systems will be essential. A prerequisite for this research is the availability of a restriction endonuclease map of the ctDNA. Restriction

endonuclease maps have been described for the ctDNAs from a number of higher plants, e.g. *Zea mays* (5), *Spinacia oleracea* (6), *Nicotiana tabacum* (7), *Vicia faba* (8) and also for the algae *Chlamydomonas reinhardii* (9) and *Euglena gracilis* (10). Electronmicroscopic studies and hybridization of chloroplast ribosomal RNA (rRNA) to ctDNA restriction fragments have indicated that ctDNA of *Spinacia oleracea* (11), *Zea mays* (12) and *Chlamydomonas reinhardii* (13) contain two copies of rRNA genes, located in inverted repeat regions. In *Pisum sativum* (11) two copies and in *Euglena gracilis* (14) three copies of these genes are present in a tandem array. *Vicia faba* (8) contains only one set of rRNA genes.

In this paper we present a procedure for the isolation of intact petunia chloroplast DNA, because methods described for the isolation of ctDNA from other plant species were not suitable for petunia. A restriction endonuclease map of the ctDNA was constructed by using the enzymes Sal I, Bgl I, Hpa I and Kpn I.

MATERIALS AND METHODS

Preparation of chloroplast DNA

About 400g of leaves from 4-5 months old petunia plants were washed in water and homogenized in 2 l of ice-cold buffer A (0.05 M Tris-HCl pH 8.0, 0.35 M sucrose, 7 mM EDTA and 5 mM 2-mercaptoethanol), by using a Braun blendor (four 10 seconds bursts at medium speed). The homogenate was filtered through four layers of nylon netting of 200 µm and again through four layers of 50 µm. The filtrate was centrifuged at 1,000 x g for 8 min at 4°C. The crude chloroplast pellets were resuspended in buffer A and washed twice by centrifugation at 1,000 x g for 8 min. The final chloroplast pellet was gently resuspended in buffer B (0.05 M Tris-HCl pH 8.0, 0.02 M EDTA) in a total volume of 51.2 ml. In order to lyse the chloroplasts, 12.8 ml of buffer B containing 10% sodium sarkosyl was added and the mixture was incubated for 1 h at 20°C. (When the chloroplasts contained a lot of starch the lysate was centrifuged at 2,700 x g for 5 min to remove most of the starch grains). The lysate was transfered to polyallomer tubes (8 ml per tube) and 8.4 g of CsCl was added to each tube and dissolved by gently rolling the tubes on a flat surface. Ethidium bromide was added (120 µg/ml) and the tubes were centrifuged in a Beckmann 65H roter at 35,000 rpm for 60 h at 15°C. Under ultraviolet light two fluorescent DNA bands were visible in each gradient. The DNA bands were collected seperatäly and extracted three times with an equal volume of isopropanol that had been

saturated with CsCl in buffer C (0.01 M Tris-HCl pH 8.0, 1 mM EDTA), in order to remove ethidium bromide. The DNA fractions were dialyzed against four 1 l changes of buffer C for 40 h at 4°C, followed by ethanol precipitation for 16 h at -20°C. The precipitated DNA was pelleted at 2,700 x g for 20 min at 4°C and dissolved by soaking the pellets in 300 µl of buffer D (5 mM Tris-HCl pH 8.0, 0.5 mM EDTA) for 72 h at 4°C. The same method as described above was used for the isolation of ctDNA from exponentially growing *Petunia hybrida* cell suspension cultures. Petunia cell suspension cultures were grown as described previously (3).

Preparation of mitochondrial DNA

Homogenates from leaves were prepared and filtered as described above. The filtrate was centrifuged twice at 1,000 x g for 10 min in order to remove most of the chloroplasts. The remaining chloroplasts (small and disrupted chloroplasts) were removed by centrifugation at 3,000 x g for 15 min. The resulting supernatant was centrifuged at 20,000 x g for 15 min. Mitochondrial pellets were washed twice in buffer A and resuspended in buffer B. Mitochondria were lysed in 2% sarkosyl and mitochondrial DNA (mtDNA) was purified by dye-buoyant density centrifugation as described for ctDNA.

Analytical ultracentrifugation

Equilibrium centrifugation of 1-2 µg of DNA in neutral CsCl solutions (ρ = 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.* (15).

Electron microscopy

Chloroplast DNA, purified by CsCl density gradient centrifugation, was prepared for electron microscopy as described by Ferguson and Davis (16). Fifteen microliter of a solution containing 0.1 M Tris-HCl pH 8.5, 10 mM EDTA, cytochrome c (100 µg/ml), 30% formamide, 0.1 µg ctDNA and 1.4 µg Clo DF13 DNA (added as marker) was spread on a hypophase that consisted of 10 mM Tris-HCl pH 8.5 and 10% formamide. The protein-nucleic acid layer was picked up on parlodion-coated grids (Pellco, mesh 400), stained with uranyl acetate, rotary shadowed with platinum-palladium (80:20) wire and covered with carbon. The grids were examined at 60 kV in a Zeiss electron microscope EM10.

Restriction endonucleases

Sal I was prepared by the method of Greene *et al.* (17). Bgl I and Bam HI were purchased from Boehringer, Mannheim; Hpa I and Kpn I were purchased from New England Biolabs. Chloroplast DNA was digested with restriction endonucleases as described by the suppliers. 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% bromphenol blue.

Agarose gel electrophoresis

Digests of ctDNA and mtDNA were analysed by electrophoresis on horizontal slab gels (25 x 20 x 0.5 cm), containing 0.5 - 1.5% agarose (Sigma, low EEO) depending on the size of the DNA fragments to be seperated. Electrophoresis was performed in 40 mM Tris-acetate pH 7.5, 20 mM sodium acetate, 2 mM EDTA and ethidium bromide (0.5 µg/ml). Gels were run for 16-20 h at 2 mA/cm, examined under UV-light and photographed.

Molecular weights of ctDNA restriction fragments were estimated relative to molecular weight markers: phage λ DNA and HindIII and Eco RI digestion fragments of λ DNA (Boehringer, Mannheim).

^{32}P-labeling of ctDNA

To improve detection of low molecular weight DNA restriction fragments on agarose gels, the ctDNA was labeled *in vitro* with 5'- α-^{32}P dATP. The use of radioactive DNA has the additional advantage that high and low molecular weight DNA fragments can be made visible on the same gel, simply by varying the exposure time for autoradiography. The nick-translation method of Rigby *et al.* (18) was modified as follows: Chloroplast DNA was digested with the appropriate restriction endonuclease, precipitated in ethanol and dissolved in TE buffer (5 mM Tris-HCl pH 8.0, 0.5 mM EDTA). The digested DNA (about 2 µg) was added to an incubation mixture containing: 50 mM NaCl, 6 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, BSA (100 µg/ml), 1 µl DNA polymerase I (Kornberg polymerase, Boehringer, specific activity 7635 U/mg), 0.01 mM dCTP, dTTP, dGTP and 0.001 mM 5'- α-^{32}P dATP (specific activity 400 Ci/mmol) in a total volume of 50 µl. DNAse I was added to the incubation mixture in a final concentration of 5 pg/50 µl. After incubation for 2 h at 15°C, 5 µl of a 250 mM EDTA solution was added and the mixture was heated for 10 min at 65°C. Ammonium acetate was added to a final concentration of 2M and the labeled ctDNA fragments were precipitated in ethanol for 15 min at -70°C. The precipitate was centrifuged for 15 min at 12,000 x g, dissolved in 250 µl 0.3 M Na acetate (pH 5.6) and again precipitated. The latter step was repeated three times.

Finally the pellet was vacuum dried and dissolved in 50 µl TE buffer. By this method the ctDNA was labeled to a specific activity of about 6 x 10^6 dpm/µg. The nick-translation procedure causes some degradation of the large fragments, by which these fragments are present in less than equimolar amounts.

Secondary digestion of DNA fragments isolated from LGT agarose gels

For the physical mapping of DNA fragments, generated by digestion with restriction endonucleases, the procedure described by Herrmann *et al.* (19) was modified as follows: About 500,000 dpm of ^{32}P-labeled primary ctDNA restriction endonuclease digests were mixed with 3 µg of unlabeled primary digests and subjected to electrophoresis on horizontal slab gels, containing 0.5% low-gelling-temperature (LGT) agarose (Seaplaque, Marine Colloids Inc.). Gels were run at 80 mA for 16-24 h, examined under UV-light, and DNA fragments were cut out. Gel slices containing individual DNA bands were liquified by heating for 2 min at 68°C. The salt concentration was adjusted and secondary digestion was performed with 5-10 units of restriction endonuclease for 1.5 h at 37°C. Reactions were terminated as described above and the samples were subjected to electrophoresis on horizontal agarose slab gels, containing 0.8 - 2.0% agarose. Gels were dried under vacuum and exposed to Kodak XR-1 films.

Hybridization of ^{32}P-labeled rRNA to ctDNA restriction fragments

The ^{32}P-labeled rRNA was prepared from *E. coli* P678-54(20) by *in vivo* labeling with ^{32}P-orthophosphate as described by Vereijken (21). The isolated ^{32}P-labeled rRNA had a specific activity of about 6.10^7 dpm/µg.

Preparation of diazobenzyloxymethyl-paper (DBM-paper), transfer of ctDNA restriction endonuclease fragments to DBM-paper, and hybridization of ^{32}P-rRNA to DBM-paper strips, containing the transferred ctDNA fragments was performed according to Wahl *et al.* (22).

RESULTS AND DISCUSSION

Isolation of petunia ctDNA

Methods described for the isolation of ctDNA from higher plants (23, 24) were not suitable for the isolation of DNA from *Petunia hybrida* chloroplasts, probably because these chloroplasts are much more fragile. The DNAse treatment, that is used in these methods to remove contaminating nuclear DNA (nDNA) from the chloroplast preparation, resulted in degradation of petunia ctDNA. Therefore we developed an isolation procedure that lacks the DNAse treatment. Furthermore, centrifugation steps used for the

isolation and washing of the chloroplasts may not exeed 1,200 x g to prevent disruption of the chloroplasts.

When petunia chloroplast lysates are subjected to CsCl-ethidium bromide centrifugation, two fluorescent DNA bands are visible in the gradients under ultraviolet light. Electron microscopic analysis of the lower band indicates that this band represents covalently closed circular ctDNA. Digestion of this DNA with restriction endonuclease Bam HI did not show any contamination with mitochondrial DNA which has a quite different Bam HI restriction profile (results not shown). The ctDNA from the upper band in the CsCl gradient is also suitable for restriction endonuclease mapping work, although some contaminating nuclear DNA may be present, resulting in a slight smear when the DNA is analyzed by electrophoresis on agarose gels. Starting from 400 g of leaves, the method described in Materials and Methods yields about 250 µg of lower and upper band ctDNA.

The same method is also suitable for the isolation of ctDNA from *Petunia hybrida* cell suspension cultures that have been grown in our laboratory for four years. The fragments obtained upon digestion of this DNA with several restriction endonucleases did not differ from the fragments obtained upon digestion of ctDNA isolated from leaf chloroplasts (results not shown). This implies that major genetic alterations in the ctDNA did not occur during this period of *in vitro* culture of petunia cells.

Physical characterization of chloroplast DNA

Buoyant density and base composition. Samples containing 1 µg of upper band or lower band ctDNA, mtDNA and nDNA were subjected to equilibrium CsCl density gradient centrifugation. Both upper band and lower band ctDNA bands at a buoyant density of 1.6987 $\pm$ 0.0005 gcm^{-3} (Fig. 1a and b). This value corresponds with a GC-content of 39.3 $\pm$ 0.5%. The buoyant density of petunia ctDNA agrees with the value of 1.698 $\pm$ 0.001 that has been found for the buoyant density of ctDNA from several higher plants (24). Mitochondrial DNA and nuclear DNA were found to band at buoyant densities of respectively 1.7066 and 1.6960 $\pm$ 0.0005 gcm^{-3} (Fig. 1c and d). The buoyant density of ctDNA and mtDNA differ sufficiently to distinguish both DNA species from each other on analytical CsCl gradients. Therefore it can be concluded from the banding patterns in Fig. 1a and b that the ctDNA preparations do not contain detectable amounts of mtDNA. Analytical ultracentrifugation could not exclude the presence of nDNA in the upper and lower band ctDNA preparation because nDNA and ctDNA band very closely in the gradient. However the fact that the lower ctDNA band represents

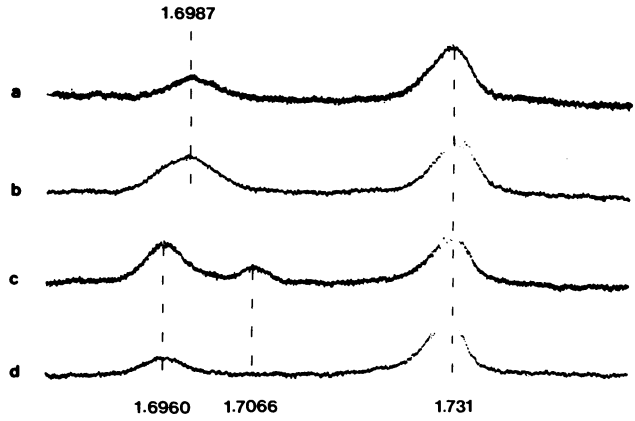

Fig. 1. Photoelectric scans of DNA, banded in neutral CsCl equilibrium density gradients. *Micrococcus lysodeikticus* DNA of buoyant density 1.731 gcm^{-3} was added as a density standard. (a) lower band ctDNA, (b) upper band ctDNA, (c) mtDNA and nDNA, (d) nDNA.

covalently closed circular DNA molecules excludes the possibility that contaminating nDNA is present in the ctDNA from this band.

Electron microscopy. Electron microscopic analysis revealed that petunia ctDNA molecules are circular and have a homogeneous contour length. The length of relaxed circular ctDNA molecules was measured relative to the length of the bacterial plasmid Clo DF13, which was used as an internal standard in all experiments (Fig. 2). The size of this plasmid was previously determined at 9,600 bp. (25). The ratio of the length of ctDNA to Clo DF13 DNA was 15.3 $\pm$ 0.7, which corresponds to a molecular weight of 96.0 $\pm$ 4.5 Md for ctDNA. This value is in good agreement with the estimated value of 101.2 Md, that was obtained from restriction endonuclease analysis of ctDNA (see Table 1). The molecular weight found for petunia ctDNA falls within the molecular weight range of 85.2 - 103,2 Md, established for ctDNA from several higher plants (26).

Cleavage of ctDNA by restriction endonucleases. CtDNA was digested with several restriction endonucleases in order to find out which endonucleases cut the DNA infrequently and produce fragments that can be easily seperated on agarose slab gels. For these reasons the restriction endonucleases Sal I, Bgl I, Hpa I and Kpn I were selected for the construction of a restriction map of petunia ctDNA. The fragment patterns of petunia ctDNA obtained upon digestion with the selected endonucleases

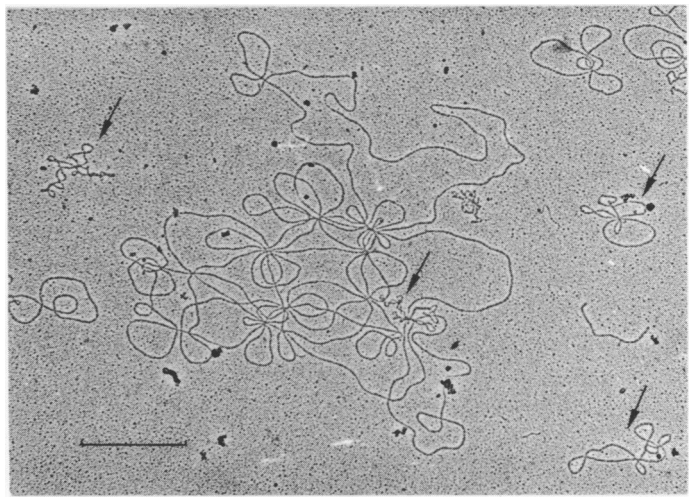

Fig. 2. Electron micrograph of *Petunia hybrida* chloroplast DNA. The arrow indicates Clo DF13 plasmid DNA (9,600 bp), added as marker. The bar represents 1 µm.

are shown in Fig. 3. The molecular weights of the ctDNA restriction endonuclease fragments were estimated from the electrophoretic mobilities of DNA molecular weight markers and are summarized in Table 1.

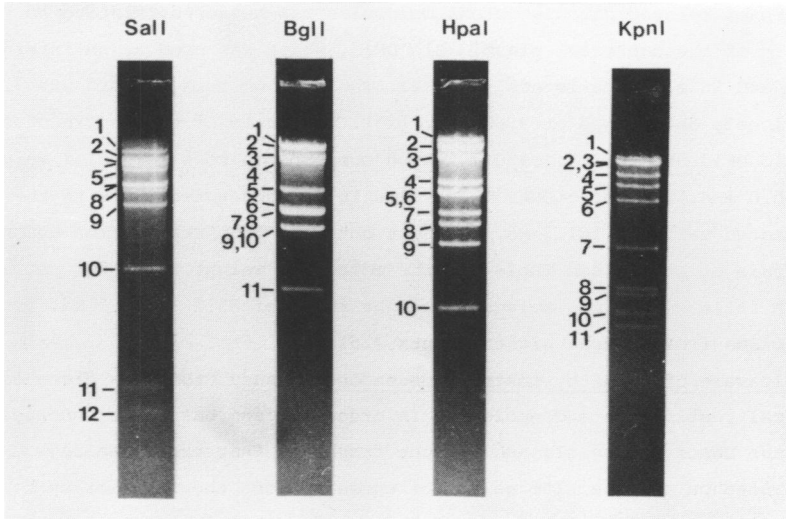

Fig. 3. Agarose gel electrophoresis of petunia ctDNA digested with restriction endonuclease Sal I, Bgl I, Hpa I and Kpn I. The fragments S13, K12, 13 and 14 are not visible on this photograph.

Table 1. Chloroplast DNA fragments resulting from digestions with various restriction endonucleases.

Fragment no.	Sal I	Bgl I	Hpa I	Kpn I	Sal I/Bgl I	Sal I/Hpa I	Sal I/Kpn I
1	17.6	20.1	29.3	21.6	12.4	14.3	14.3
2	14.3	18.7	19.5	17.7	9.9	8.2	11.3
3	12.4	14.4	14.0	17.3	9.8	8.0	8.0
4	11.9	13.2	8.2	12.1	8.2	7.3	6.0
5	10.7	7.3	7.2*	9.6	7.3	7.2 (2x)	5.95 (5x)
6	8.2	5.5	7.2*	7.4	6.1	6.7	4.0
7	8.0	5.3*	5.3	4.0	5.8	5.3	3.7
8	7.3	5.3*	4.65	2.8	5.3 (2x)	4.45	3.3
9	5.7	4.4*	3.7	2.75	4.8	4.3	2.85
10	2.85	4.4*	2.2	2.3	4.6 (2x)	4.05	2.8
11	0.95	2.55		2.1	2.85	3.9	2.75
12	0.85			0.5*	2.5 (2x)	3.7	2.1 (2x)
13	0.45			0.5*	1.9 (2x)	3.3	1.5
14				0.45	1.45	2.85	1.45
15					0.95	2.45	1.3
16					0.9	2.2	0.95
17					0.8	1.35	0.8
18					0.65	1.15 (2x)	0.5 (2x)
19					0.45	0.95	0.45 (2x)
20					0.2	0.8	0.2
21						0.45	
Sum of Mol. wts.	101.2	101.15	101.25	101.1	101.15	101.25	101.05

Molecular weights of the fragments are given in megadaltons. The number given in brackets refers to the molar ratio of that band.

*Bgl I fragments B7 and B8, B9 and B10, Hpa I fragments H5 and H6, and Kpn I fragments K12 and K13 are identical.

A number of restriction fragments, obtained after primary digestion of ctDNA, were present in bimolar amounts as was concluded from the relative intensity of ethidium bromide fluorescence of some DNA bands. Redigestion of such bands with a second enzyme resulted in fragments with molecular weights that added up to the molecular weight of the original DNA band and not twice that molecular weight. From these results it was concluded that the fragments B7 and B8 are identical, and also B9 and B10, H5 and H6 and K12 and K13.

Physical mapping of restriction endonuclease fragments

Ordering of the Sal I, Bgl I, Hpa I and Kpn I restriction endonuclease fragments of ctDNA on a cleavage site physical map was obtained by determining the cleavage sites of the enzymes Bgl I, Hpa I and Kpn I relative to those of Sal I. For this purpose the individual Sal I fragments were isolated and redigested with Bgl I, Hpa I or Kpn I, and *vice versa*. In order to improve the detection of small DNA fragments (smaller than 1.0 Md), produced upon redigestion of individual fragments, we used ^{32}P-labeled primary digests of ctDNA. The isolation of DNA fragments from gels was facilitated by the use of low-gelling-temperature (LGT) agarose (19). In a typical experiment ^{32}P-labeled Sal I fragments of ctDNA were separated on 0.5% LGT agarose gels. The DNA bands were cut out, melted, and incubated with Hpa I. Fig. 4 shows the agarose electrophoresis of the resulting DNA fragments. Sal I/Hpa I double digest and Sal I and Hpa I single digests were coelectrophoresed as markers. By comparing the redigestion products of the individual Sal I fragments with the Sal I/Hpa I double digest

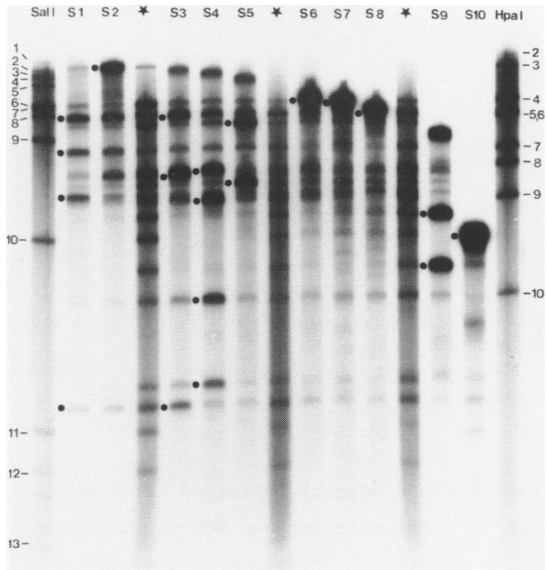

Fig. 4. Autoradiograph of agarose gel electrophoresis of individual ^{32}P-labeled Sal I restriction fragments of petunia ctDNA redigested with restriction endonuclease Hpa I. Lanes indicated by an asterix contain a ^{32}P-labeled Sal I/Hpa I double digest, added as marker. The cleavage products resulting from redigestion of the individual Sal I fragments are marked by a dot.

pattern, it could be established from which primary Sal I fragments a certain Sal I/Hpa I double digest fragment originates. The fragment patterns are sometimes slightly complicated by the presence of incompletely digested fragments or by cross-contamination of the fragments, due to close migration on LGT agarose gels. However, interpretation of the patterns presents little difficulty when differences in the intensities of redigestion products are taken into consideration and also because the molecular weights of the fragments are known. The results obtained from this gel and those obtained from the gels of the Sal I/Bgl I and Sal I/Kpn I digestions are summarized in Table 2. From the common double digest fragments, the overlap between the primary Sal I, Bgl I, HpaI and Kpn I fragments could be assigned. This allowed the determination of the order of most of the DNA

Table 2. Relationship between primary restriction endonuclease fragments and double digest fragments of petunia ctDNA.

Sal I/Bgl I double digest fragments	Primary Sal I and Bgl I fragments from which derived		Sal I/Hpa I double digest fragments	Primary Sal I and Hpa I fragments from which derived		Sal I/Kpn I double digest fragments	Primary Sal I and Kpn I fragments from which derived	
SB1	S2	B4	SH1	S2	H2	SK1	S2	K1
SB2	S5	B1	SH2	S6	H1	SK2	S1	K3
SB3	S1	B3	SH3	S7	H1	SK3	S7	K2
SB4	S6	B2	SH4	S8	H1	SK4	S3	K1
SB5	S4	B1	SH5 (2x)	S1, S3	H5, H6	SK5 (5x)	S1, S3	K2, K3
SB6	S7	B5	SH6	S5	H3		S4(2x), S6	K4, K5, K6
SB7	S8	B2	SH7	S1	H7	SK6	S9	K7
SB8 (2x)	S1, S3	B7, B8	SH8	S4	H3	SK7	S8	K5
SB9	S9	B2	SH9	S3	H4	SK8	S5	K4
SB10 (2x)	S3, S4	B3, B6	SH10	S5	H2	SK9	S10	K4
SB11	S10	B1	SH11	S1	H4	SK10	S5	K8
SB12 (2x)	S1, S3	B9, B10	SH12	S4	H9	SK11	S5	K9
SB13 (2x)	S2, S7	B9, B10	SH13	S9	H8	SK12 (2x)	S6, S8	K10, K11
SB14	S8	B11	SH14	S10	H3	SK13	S8	K2
SB15	S11	B5	SH15	S9	H1	SK14	S9	K6
SB16	S9	B6	SH16	S4	H10	SK15	S5	K1
SB17	S5	B4	SH17	S4	H8	SK16	S11	K2
SB18	S12	B11	SH18 (2x)	S1, S3	H1, H2	SK17	S12	K2
SB19	S13	B11	SH19	S11	H1	SK18 (2x)	S1, S3	K12, K13
SB20	S12	B5	SH20	S12	H1	SK19 (2x)	S5, S13	K2, K14
			SH21	S13	H1	SK20	S9	K10

The number given in brackets refers to multiple copies of a fragment. Molecular weights of the fragments are given in Table 1.

fragments. The relative order of Hpa I fragments H9 and H10 was established by redigestion of these fragments with Bgl I. The relative order of fragments K8 and K9 was determined by redigestion with Hpa I (results not shown). As yet the relative order of K8 and K14 could not be determined unambiguously. The position of several cleavage sites was confirmed by reciprocal digestion of various Bgl I, Hpa I and Kpn I restriction fragments (results not shown). The resulting restriction fragment map of petunia ctDNA is shown in Fig. 5.

The physical map shows that petunia ctDNA contains two copies of a region in a inverted orientation. The inverted repeat regions have a minimum size of 10 Md but may very well extend into the adjacent part of the ctDNA molecule. A similar arrangement of inverted repeat regions on restriction endonuclease fragment maps has been found for ctDNA from a number of other higher plants, e.g. *Zea mays*, *Spinacia oleracea* (see also Introduction). The sizes of the inverted repeat regions observed in the ctDNA from these and other plants range from about 13-17 Md (27).

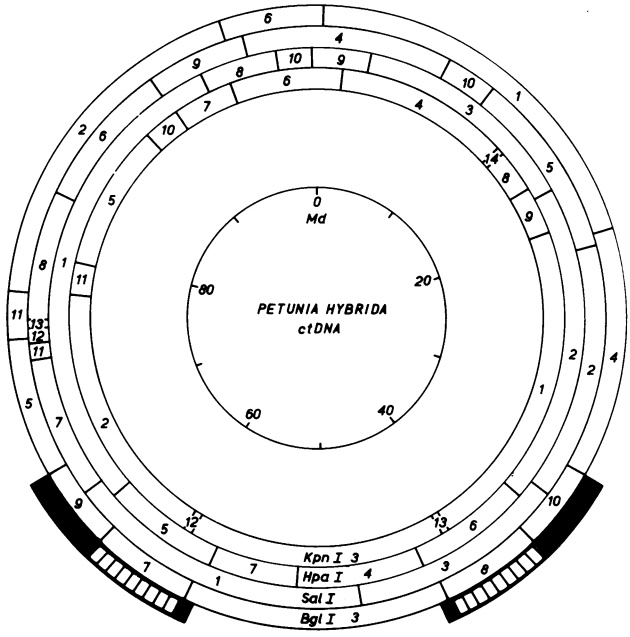

Fig. 5. Physical map of restriction endonuclease fragments of petunia ctDNA, showing the Sal I, Bgl I and Hpa I cleavage sites. Sizes of the numbered fragments are listed in Table 1. The thick lines indicate the positions of the inverted repeat regions.

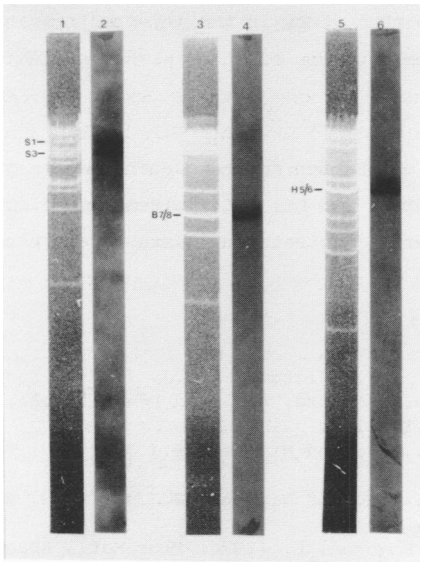

Fig. 6. Hybridization of ^{32}P-labeled rRNA from *E. coli* to restriction endonuclease fragments of petunia ctDNA. Lane 1 and 2, Sal I fragments; 3 and 4, Bgl I fragments; 5 and 6, Hpa I fragments. 1, 3 and 5 are photographs of ethidium bromide stained gels; 2, 4 and 6 are autoradiographs of the transferred fragments on DBM-paper to which ^{32}P-rRNA was hybridized.

Mapping of chloroplast rRNA genes

The major RNAs in the chloroplast ribosomes are 23S, 16S and 5S. Schwarz and Kössel (28) showed that the nucleotide sequence of the 16S RNA gene is strikingly similar for *Zea mays* chloroplasts and *E. coli*. In view of this similarity of rDNA in *E. coli* and chloroplasts, ^{32}P-rRNA from *E. coli* was used as a probe for hybridization with DBM-paper strips containing restriction endonuclease fragments of petunia ctDNA to obtain information on the location of the chloroplast rRNA genes. Fig. 6 shows that *E. coli* rRNA specifically hybridizes with fragments S1 and S3, fragments B7 and B8 and fragments H5 and H6. All these fragments are part of the inverted repeat sequences of petunia ctDNA (Fig. 5). The hybridization data indicate a sequence homology between petunia ctDNA and *E. coli* rDNA. They also strongly suggest that the chloroplast rRNA genes are located within these fragments. Since no hybridization was observed with fragments B3, B9 and B10, the maximum size of the ctDNA region that could be occupied by these rRNA genes is the 5.3 Md dashed region in the inverted repeats (Fig. 5). A

similar location of the rRNA genes in the inverted repeat regions has been found for the ctDNA from a number of other plant species (7, 11, 12, 13, 23).

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REFERENCES

(1) Colijn, C.M., Kool, A.J., Nijkamp, H.J.J. (1979) Protoplasma 99, 335-340.
(2) Seyer, P., Marty, D., Lescure, A.M. and Péaud-Lenoël, C. (1975) Cell Differentiation 4, 187-197.
(3) Colijn, C.M., Kool, A.J. and Nijkamp, H.J.J. (1979) Theor. Appl. Genet 55, 101-106.
(4) Colijn, C.M., Kool, A.J. and Nijkamp, H.J.J. (1980) Manuscript in preparation.
(5) Bedbrook, J.R. and Bogorad, L. (1976) Proc.Natl. Acad. Sci. USA 73, 4309-4313.
(6) Bohnert, H.J., Gordon, K. Driesel, A.J., Crouse, E.J. and Herrmann, R.G. (1978) in Chloroplast Development, Akoyunoglou, G. Ed., Elsevier,Amsterdam.
(7) Jurgenson, J.E. and Bourque, D.P. (1980) Nucl. Acid. Res. 8, 3505-3516.
(8) Koller, B. and Delius, H. (1980) Molec. gen. Genet. 178, 261-269.
(9) Rochaix, J.D. (1978) J. Mol. Biol. 126, 597-617.
(10) Gray, P.W. and Hallick, R.B. (1977) Biochemistry 16, 1665-1671.
(11) Kolodner, R. and Tewari, K.K. (1979) Proc. Natl. Acad. Sci. USA 76, 41-45.
(12) Bedbrook, J.R., Kolodner, R. and Bogorad, L. (1977) Cell 11, 739-749.
(13) Rochaix, J.D. and Malnoe, P. (1978) Cell 15, 681-670.
(14) Rawson, J.R.Y., Kusher, S.R., Vapnek, D., Altom, K.K. and Boerma, C.L. (1978) Gene 3, 191-209.
(15) Schildkraut, C.L., Marmur, J. and Doty, P. (1962) J. Mol. Biol. 4, 430-443.
(16) Ferguson, J. and Davies, R.W. (1978) in Advanced Techniques in Biological Electron Microscopy, Koehler, J.K. Ed., Springer Verlag New York.
(17) Greene, P.J., Heyneker, H.L., Betlack, M., Bolivar, F., Rodriquez, R., Covarrabias, A., Fodor, I., Backman, K. and Boyer, H.W. (1978) Nucl. Acids Res. 5, 2773-2780.
(18) Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
(19) Herrmann, R.G., Whitfeld, P.R. and Bottomley, W. (1980) Gene 8, 179-191.
(20) Kool, A.J., Pols, C. and Nijkamp, H.J.J. (1975) Antimicrob. Agents Chemother. 8, 67-75.
(21) Vereyken, J.M. (1977). Thesis, State University of Utrecht.
(22) Wahl, G.M., Stern, M., Stark, G.R. (1979). Proc. Natl. Acad. Sci USA, 76, 3683-3688.
(23) Whitfeld, P.R., Herrmann, R.G. and Bottomley, W. (1978) Nucl. Acids Res. 5, 1741-1751.
(24) Kolodner, R. and Tewari, K.K. (1975) Biochim. Biophys. Acta 402, 372-390.
(25) Elzen, P.J.M., Konings, R.N.H., Veltkamp, E. and Nijkamp, H.J.J. (1980) J. Bacteriol. (in press).

(26) Bedbrook, J.R. and Kolodner, R. (1979) Ann. Rev. Plant Physiol. 30, 593-620.
(27) Bedbrook, J.R., Beaton, A.R., Bogorad, L., Coen, D.M., Dyer, T.A. and Kolodner, R. (1980) in The plant genome, Davies, D.R. and Hopwood, D.A. Eds., F. Crowe & Sons Ltd., Norwich, England.
(28) Schwarz, Zs. and Kössel, H. (1979) Nature 283, 739-742.