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Steffen Backert · Peter Dörfel · Thomas Börner

Investigation of plant organellar DNAs by pulsed-field gel electrophoresis

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Abstract Mitochondrial (mt) DNAs from several higher-plant species (*Arabidopsis thaliana, Beta vulgaris, Brassica hirta, Chenopodium album, Oenothera berteriana, Zea mays*) were separated by pulsed-field gel electrophoresis (PFGE). Hybridization of the separated DNA with mtDNA-specific probes revealed an identical distribution of mtDNA sequences in all cases: part of the DNA formed a smear of linear molecules migrating into the gel, the rest remained in the well. Hybridization signals in the compression zone of the gels disappeared after RNase or alkaline treatment. It was shown that the linear molecules are not products of unspecific degradation by nucleases. All plastid (pt) DNA from leaves of *Nicotiana tabacum* remained in the well after PFGE. Separation of linear monomers and oligomers of the chloroplast chromosomes of *N. tabacum* was achieved by mild DNase treatment of the well-bound DNA. DNase treatment of well-bound mtDNA, however, generated a smear of linear molecules. PtDNA from cultured cells of *C. album* was found after PFGE to be partly well-bound, and partly separated into linear molecules with sizes of monomeric and oligomeric chromosomes. The ease with which it was possible to detect large linear molecules of plastid DNA indicates that shearing forces alone can not explain the smear of linear molecules obtained after PFGE of mtDNA. The results are discussed in relation to the structural organization of the mt genome of higher plants.

Key words Mitochondrial DNA · Chloroplast DNA · Pulsed-field gel electrophoresis · *Chenopodium album*

S. Backert · P. Dörfel[1] · T. Börner (✉)
Institut für Biologie, Humboldt-Universität zu Berlin, Invalidenstrasse 43, D-10115 Berlin, Germany

[1] *Present address*: Botanisches Institut, Ludwig-Maximilians-Universität München, Menzinger Strasse 67, D-80638 München, Germany

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Introduction

The size of the mitochondrial (mt) genome of angiosperms varies spectacularly from about 200 to 2500 kb, and is generally much larger than the mitochondrial genomes from animals, most fungi and algae (Ward et al. 1981; Wolf and Kück 1993; Schuster and Brennicke 1994). The only completely sequenced plant mtDNA is that of the bryophyte *Marchantia polymorpha* which comprises 186.6 kb, and thus is in the size range of the smallest known mt genomes of higher plants. All the genetic information of the *Marchantia* mitochondria is contained in one circular molecule (Oda et al. 1992). Physical mapping of restriction fragments and cosmid clones of higher-plant mtDNA has also led to circular chromosomes. In addition, large, frequently recombining, repeated sequences were observed. According to a widely accepted model, a circular master chromosome exists which is in balance with subgenomic circles. The subgenomic molecules are generated by intramolecular recombination between direct repeats within the master chromosome (e.g. Lonsdale et al. 1984; Palmer and Shields 1984; Palmer 1992). The number of such subgenomic circles should be low if their origin were to depend exclusively on recombination across large direct repeats. If recombination could also occur between smaller repeated sequences the number of subgenomic circles in plant mitochondria may be larger (André et al. 1992). The only reported exception among the angiosperms thus far is the mitochondrial DNA of *Brassica hirta* which lacks extented recombining repeats and is therefore thought to exist only in circles of genome size (cf. Palmer 1992). Pulsed-field gel electrophoresis (PFGE) of maize mtDNA fragmented by rare cutting restriction enzymes did not reveal molecules corresponding in size to putative master chromosomes; only subgenomic molecules were observed (Levy et al. 1991; André and Walbot 1995).

Surprisingly, electron microscopic investigations of higher-plant mtDNA did not usually show circular molecules large enough to represent master chromosomes or even the larger molecules of subgenomic circles. Higher

yields of circular mtDNA falling into various smaller size classes have been obtained only from suspension cultures of higher-plant cells (for review see Bendich 1993). Similar results were reported from studies on the uncut mtDNA from several higher-plant species using PFGE. In addition to a large portion of the DNA retained in the well, they revealed a smear of linear molecules with sizes between 50 and 150 kb and larger linear molecules in the compression zone (Bendich and Smith 1990; Bendich et al. 1993). The much smaller mt genomes of certain fungal species showed a very similar electrophoretic behaviour during PFGE (Maleszka et al. 1991; Bendich et al. 1993). The striking discrepancy between the current model of the structure and organization of the mitochondrial genome based on physical mapping and the results of electron microscopic and electrophoretic studies led Bendich (1993) to the alternative view that linear molecules may represent the major from of DNA within mitochondria.

In contrast to the complex structure of the mt genome, the genome of plastids seems to be more simply organized. Most data support the concept of a circular chromosome bearing all plastid (pt) genes, although experiments with pulsed-field gels have given ambiguous results (Deng et al. 1989; Bendich and Smith 1990). No evidence for subgenomic molecules has been found. However, the pt chromosome of many species may occur in two alternative states due to intramolecular recombination across two large inverted repeats (for review see Palmer 1992).

Here we report the results of the separation of mtDNA of *Chenopodium album* and other higher-plant species by PFGE, and comparable studies with ptDNA from *C. album* and *Nicotiana tabacum.*

Materials and methods

Plant material. Mitochondria were prepared from: *Chenopodium album* suspension culture C.9.1. (Knösche and Günther 1988; Dörfel et al. 1989), *Beta vulgaris* L. suspension culture HS (CMS cytoplasm, obtained from K. Zoglauer, HU Berlin, Germany), *B. vulgaris*, taproot tissue (var. *crassa* line B 111, obtained from A. Weihe, HU Berlin, Germany; var. *altissima*, bought at a local market), *Zea mays* seedlings (line BEKE 246, Saat- und Pflanzgut, Quedlinburg, Germany), *B. hirta* seedlings (line "Kastor", obtained from D. Enderlein, Saatgutstation Christenfeld, Germany), *Arabidopsis thaliana* suspension culture and *Oenothera berteriana* callus culture (both cultures were obtained from A. Brennicke, Institut für Genbiologische Forschung GmbH, Berlin, Germany). Cells from the suspension and callus cultures were harvested 6–7 days after transfer to new medium, in a phase of active growth and division. Etiolated seedlings from *Z. mays* and *B. hirta* were grown in moist vermiculite and harvested 10 days after germination and growth in the dark. Non-green plastids were prepared from *C. album* suspension culture C.9.1. Chloroplasts were isolated from mature leaves of *N. tabacum* (line W-38, obtained from R. Breitfeld, Institut for Genbiologische Forschung GmbH, Berlin, Germany) grown at 16 h light, 8 h dark and, before harvesting, 1 day in the dark. *Escherichia coli* strain C 2110$^+$, containing the megaplasmid pSM409 of 66 kb (obtained from A.J. Bendich, University of Washington, USA), and *Bacillus subtilis* strain 168 trp C2, containing plasmid pSE411 of 32 kb (obtained from R. Borriss, HU Berlin, Germany), were used to isolate supercoiled marker plasmids by the method of Birnboim and Doly (1979).

Preparation of mitochondria and mitochondrial DNA. We checked several methods for the preparation of mitochondria (Leaver et al. 1983; Levy et al. 1991; Meißner et al. 1992) with the aim of isolating mtDNA as pure and undegraded as possible (Backert unpublished). Although DNase treatment was found to be necessary to avoid contamination by nuclear DNA, for most studies we selected a procedure without DNase-treatment of mitochondria to rule out the possibility of DNA degradation by added nuclease. We found it useful to start with protoplasts as source of mitochondria to increase the proportion of high-molecular-weight (hmw) mtDNA in the preparation. If not otherwise indicated, we prepared mitochondria and mtDNA in the following way: about 300 g of cells, calli, cut seedlings, or tissue were gently shaken in 300 ml of a solution containing 1% cellulase, 2% pectinase and 0.1% driselase (Serva, Heidelberg, Germany) in 0.5 M mannitol, 5 mM morpholinoethane sulphonic acid and 3 mM $CaCl_2$ for 4–5 h. Protoplasts were collected by centrifugation and disrupted by the addition of a 1.5 vol of lysis solution (50 mM TRIS-HCl, pH 8.0, 50 mM EDTA, 10 mM monothioethylene glycol, 0.1% BSA). The following steps for the isolation of mt fractions were carried out at 4 °C with pre-cooled solutions. Mitochondria were obtained by differential centrifugation (Wilson and Choury 1984). We refer to this fraction as "crude" mitochondria. In certain cases the mt fractions were treated by DNase I, followed by centrifugation on a discontinous sucrose gradient and collection of the purified mt fractions from the 52/30% interphase as described by Schuster et al. (1988). We refer to this fraction as "purified" mitochondria. Mitochondria were lysed by adding 0.1 mg/ml of proteinase K and 1% sarcosyl (final concentrations) and incubated for 1 h at 55 °C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Contaminating RNA was removed by treatment with a DNase-free ribonuclease coktail (RNase A and a non-specific RNase from *Aspergillus oryzae*) according to the instructions of the supplier (Stratagene, La Jolla, USA). Supercoiled, open circular, and linear mtDNA forms were isolated by centrifugation in CsCl-ethidium bromide gradients as described by Brennicke and Blanz (1982).

Preparation of plastids and plastid DNA. All preparations were carried out at 4 °C with pre-cooled solutions. About 200 g of suspension-cultured cells from *C. album* and the same amount of leaves from *N. tabacum* were homogenized with a mortar and pestle in grinding buffer (380 mM sorbitol, 25 mM TRIS-HCl, pH 8.0, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol) and filtered through cheesecloth and miracloth. Non-green suspension-culture plastids from *C. album* were obtained following the procedure of Siemenroth et al. (1981). Chloroplasts from *N. tabacco* leaves were fractionated on a 80–35% percoll step gradient, adapted from Klein and Mullet (1986).

Enzymatic digestions. MtDNA in solution was digested with restriction enzymes under the conditions recommended by the suppliers (Amersham-Buchler, Braunschweig, Germany, Stratagene). To digest DNA (about 2–3 µg) and RNA embedded in low melting point (LMP)-agarose, the plugs were first equilibrated several times in 20 ml of TE (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA) for 2 h at 4 °C, followed by another 2 h in 20 ml of 1 × enzyme buffer. Enzymatic digestions were carried out in 100 µl of 1 × enzyme buffer for different times at 37 °C as described in "Results".

PFGE. Crude and purified fractions of mitochondria and plastid fractions were immobilized in 0.5% LMP-agarose (Sea Plaque GTG agarose; FMC Bioproducts, Rockland, USA), lysed and washed as described by Kenwrick et al. (1987). Before PFGE the plugs were equilibrated for 30 min in electrophoresis buffer. Hmw mtDNA and ptDNA were resolved in FastLane agarose of FMC Bioproducts with the "Transverse Alternating Field Electrophoresis" (TAFE) system of Beckman (Palo Alto, USA) or the CHEF-DR II system of BioRad (Richmond, USA). Details of PFGE conditions are given in the figure legends.

Elution of mtDNA from PFGE-gels. MtDNA was separated on a 1% LMP-agarose CHEF-gel (conditions as in legend of Fig. 2). Pieces

of agarose containing well-bound DNA, or the fraction of molecules of 40–200 kb, were cut out and digested with gelase following the instructions of the supplier (Biozym, Hameln, Germany). DNA was purified by extraction with phenol/chloroform (1:1) and precipitated with ethanol.

Agarose-gel electrophoresis. Conventional agarose-gel electrophoresis and electrophoresis in "Eckhardt"-gels (Eckhardt 1978) were performed in the "DNA Sub Cell-apparatus" of Bio-Rad. After electrophoresis the gels were stained with ethidium bromide (0.1 mg/ml) for 30 min. The DNA was visualized on a UV-transilluminator (λ=312 nm).

Southern blotting and hybridization. After electrophoresis the DNA was blotted by alkaline transfer to Zeta-Probe GT membranes according to the instructions of the supplier (BioRad). Alternatively, gels were prepared for in-gel hybridization by incubation for 20 min in 0.5 N NaOH, 0.15 N NaCl, followed by neutralization in 0.5 M Tris-HCl, 0.15 N NaCl for another 20 min (Beyermann et al. 1992). Afterwards the gels were dried for 2 h at 60 °C on a gel dryer (model 583; BioRad). Filters and dried gels were hybridized overnight in 6–8 ml of 7% SDS/250 mM NaH_2PO_4, pH 7.2, at 65 °C (filter) or 42 °C (dried gels), with mtDNA- or ptDNA-specific probes in hybridization tubes from Schott (Mainz, Germany). The following probes were used for hybridization: *cox II* from *Zea diploperennis* (Gwynn et al. 1987) *cob* from *O. berteriana* (Schuster and Brennicke 1985), *rrn26* from *O. berteriana* (Manna and Brennicke 1985), CsCl-gradient-purified total mtDNA from *C. album*, the 1.3-kb plasmid mp1 from mitochondria of *C. album* (Dörfel et al. 1991; Dörfel et al. in preparation), *rbcL* from rice (Hiratsuka et al. 1989). Radioactive labelling was performed with the rediprime kit and 1.85 MBq of α-^{32}PdCTP provided by Amersham. Afterwards, unincorporated dNTPs were removed by a chromatography step using a Sephadex G50 column (Pharmacia, Uppsala, Sweden). After stringent washing, filters and dried gels were exposed to Hyperfilm-MP X-ray films (Amersham-Buchler). Alternatively, hybridizations were evaluated and the relative amounts of DNA in the various zones of the gels were calculated by cutting out and measuring in a LS 6000 SC liquid scintillation counter in 4 ml of Ready Safe scintillation fluid (Beckman).

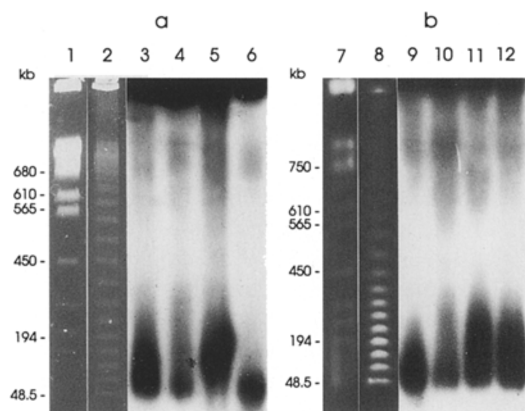

Fig. 1 a, b Autoradiograph of mitochondrial nucleic acids from higher-plant species separated on TAFE-gels. Crude mt fraction were prepared by "osmotic shock" (see Materials and methods): *lane 3, A. thaliana; lane 4, O. berteriana; lane 5, Z. mays; lane 6, B. hirta; lane 9, B. vulgaris* (var. *crassa*); *lane 10, B. vulgaris* (var. *altissima*); *lane 11, C. album; lane 12, B. vulgaris* (HS). Chromosomes of *S. cerevisiae* (*lane 1, 7*) and phage λ-concatemers (*lanes 2, 8*) purchased from BioRad served as size markers. PFGE conditions: 0.8% agarose in 0.5×TBE; 200 V; 13 °C; pulse time in **a**: 40 s for 26 h; pulse time in **b**: 30 s for 7 h (1st step) and 50 s for 18 h (2nd step). Dried gels were hybridized with mt-specific probes (*cob, cox II* and *rrn26S* genes)

Results

PFGE reveals a smear of linear mtDNA molecules

Crude mitochondrial fractions were prepared from protoplasts of *O. berteriana*, *Z. mays*, *B. vulgaris*, *B. hirta*, *C. album* and *A. thaliana* and their DNAs were separated in TAFE-gels. In order to visualize only mtDNA, and not contaminating plastid or nuclear DNA, the gels were hybridized with a combined probe of mt genes (Fig. 1). Although the mt genomes of the investigated plant species are different with respect to size and proposed organization (Brennicke 1980; Lonsdale et al. 1984; Brears and Lonsdale 1988; Palmer and Herbon 1987; Dörfel et al. 1989; Unseld et al. 1993) their migration during electrophoresis was nearly identical. In all lanes, mtDNA was visible as a diffuse zone starting at the well and extending to an area corresponding to linear marker molecules of about 40 kb. The strongest hybridization signals were always seen in a region of the gel which contains linear molecules with a mass of about 40 kb to 200 kb. Additionally, diffuse bands corresponding to linear molecules of approximately 700–1100 kb could be observed around the compression zone of TAFE-gels. Linear molecules too large for being resolved in the gel are presumed to migrate to this position (e.g. Bendich et al. 1993). Bands were always seen in the compression zone of ethidium bromide-stained gels. However, they could be detected by in-gel hybridization with mtDNA-probes only when the length of alkaline treatment applied to denature the DNA was less than 20 min, and disappeared after longer treatment (20 to 40 min) or after alkaline Southern transfers. These signals probably represent RNA molecules, since no bands appeared in the compression zone at about 800 kb when the DNA was treated with DNase-free RNases. No other influence of RNase treatment on the distribution of molecules after electrophoresis was detected (Fig. 2).

In the following studies we performed the alkaline denaturation of mtDNA exclusively under conditions which led to the disappearance of hybridization signals in the compression zone. The signals obtained after hybridization with mtDNA appeared as diffuse zones over a large size range independent of the conditions used for the preparation of the mitochondria, the treatment of the DNA, or electrophoresis in pulsed-field gels (compare Figs. 1, 2, 3, 6, 8). Under the same conditions the linear molecular-weight markers were separated as sharp bands. We never detected distinct bands which could represent the complete mt genome (master chromosome) or discrete subgenomic circles. Regardless of the experimental conditions, a substantial amount of the DNA remained in the well of PFGE-gels (Figs. 1, 2, 3, 6, 8). The use of other systems (rotating-gel electrophoresis, field-inversion gel electrophoresis, data not shown), did not produce significantly different pictures as far as the estimated molecular weights of

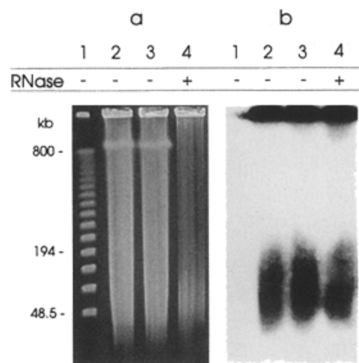

Fig. 2 Ethidium bromide-stained gel (**a**) of nucleic acids from *C. album* mitochondria and autoradiograph (**b**) of mtDNA of *C. album* separated on a CHEF-gel. Nucleic acids were prepared from purified mitochondria, embedded in LMP-agarose and treated with 15 ml of a solution containing 1% Triton-X-100, 20 mM EDTA and 2 units of RNase block II (Stratagene) at 37 °C to inhibit RNase activity in the mt lysate (1st step). After 1 h incubation the solution was replaced by a lysis solution containing SDS, EDTA and proteinase K for the degradation of proteins (2nd step). Nucleic acids prepared by both steps are displayed in *lane 2* and nucleic acids prepared exclusively by the 2nd step are displayed in *lane 3*. Mt plugs (lysed by both steps) were treated with 10 units of DNase-free ribonuclease in a total volume of 100 µl TE for 30 min at 37 °C to remove contaminating RNA (*lane 4*). The DNA size marker was the λ-ladder in *lane 1*. After ethidium bromide staining the gel was treated with 0.5 N NaOH/1.5 N NaCl for 40 min followed by neutralization and gel drying. PFGE running conditions: 1% agarose in 0.5×TBE, 13 °C, 6 V/cm, 18 h, incl. angle = 120 °; pulse times: 0.35–59 s, ramping factor **a** = linear. The hybridization probe was purified mtDNA from *C. album*

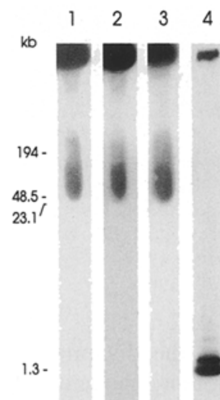

Fig. 3 Autoradiographs of mtDNA from *C. album* separated on a TAFE-gel. The lanes were probed with the mt genes *coxII* (*lane 1*), *rrn26S* (*lane 2*), *cob* (*lane 3*) and plasmid mp1 (*lane 4*). PFGE running conditions: 1% agarose in 0.5×TBE, 150 V, 13 °C, pulse times: 8 s for 2 h (1st step), 15 s for 5 h (2nd step) and 30 s for 8 h (3rd step). Molecular-weight markers are indicated along the right margins and were derived from a λ-ladder and the low-range PFGE-marker of New England Biolabs

migrating mtDNA molecules are concerned. Moreover, the observed distribution of mtDNA was not influenced by the probe used for hybridization: a combined probe of three mt genes (Fig. 1), total mtDNA (Fig. 2), or the genes *coxII*, *cob* and *rrn26* used as single probes (Fig. 3). These surprising results indicate that sequences of these genes are part of linear molecules of all sizes between about 40 to at least 200 kb and are present in the mtDNA fraction remaining in the well. We also used mt plasmid (mp1) as probe which is amplified relative to hmw mtDNA in *C. album* (Dörfel et al. 1989). This plasmid was found to hybridize preferentially to linear and circular molecules of the expected size of 1.3 kb (Fig. 3, lane 4). As with other probes, mp1 revealed a strong signal with well-bound DNA. A smear of hybridization signals, probably corresponding to linear molecules upt to a size of about 10 kb, was only observed after longer exposure.

The presence of a band corresponding to open circles of mp1 demonstrated that at least small circles could migrate into the gel. In order to see if larger circles could also be separated under our conditions, we subjected to PFGE the plasmid pSM409 from *E. coli* as supercoiled mono-, di- and tri-mers (66 kb, 132 kb, 198 kb) as well as the *B. subtilis* plasmid pSE411 (32 kb). The circular plasmids migrated only a short distance into the gel or were retained in the well (data not shown). Previous reports also demonstrated that larger circular DNA molecules (especially open circles) cannot be separated by PFGE (Levenne and Zimm 1987; Beverley 1988; Bendich and Smith 1990; Naravanan et al. 1993), supporting the view that at least the migrating part of the mtDNA represents linear molecules.

Supercoiled and open circles, however, can be separated by electrophoresis in agarose gels under conventional, non-pulsed field conditions. We separated supercoiled DNAs of known size (from 2.1 to 198 kb) in Eckhardt-gels together with purified mtDNA of *C. album* isolated from CsCl-gradients (Fig. 4). No mtDNA was found in the fraction of supercoiled DNA even after very long exposure, except in the case of the small circular plasmid mp1, which was partially converted into open circles during extraction (Fig. 4, lane 1). Part of this plasmid and all larger molecules of the mtDNA were found in the fraction of linear molecules and open circles. In contrast to the situation with PFGE, a few bands were visible in the range of low-molecular-weight molecules which might represent open circles. The hybridization signals formed a smear. Most of the mtDNA was observed in a region of the gel containing molecules corresponding to linear size markers of about 10 kb to 150 kb (Fig. 4, lane 2). The smaller size of these molecules compared to mtDNA prepared in LMP-agarose plugs (Fig. 1) can be attributed to breakage of mtDNA during preparation from CsCl-gradients by shearing forces. Virtually no mtDNA remained in the well using this approach.

Thus, neither PFGE nor electrophoresis in Eckhardt-gels revealed molecules with sizes of the master chromosome or large subgenomic linear or circular molecules.

Separation of plastid DNAs by PFGE

Using similar methods, we prepared plastids from tobacco leaves and cultured cells of *C. album* and separated their DNA by PFGE. The aim of this experiment was to see if large circular DNAs could be isolated from plant cells under our conditions and if DNA with the size of the plastid chromosome was detectable by PFGE. DNA of untreated *Nicotiana* chloroplasts remained in the well (Fig. 5,

lane 2 b). The chloroplasts were not treated with DNase before extraction of DNA. Therefore, preparations of ptDNA were contaminated with nuclear DNA. The two bands observed in the compression zone of the ethidium bromide-stained gel most probably represent contaminating nuclear DNA (Fig. 5, lane 2 a). These bands did not hybridize with ptDNA (Fig. 5, lane 2 b). Treatment of the DNA with low concentrations of DNase I led to the migration of part of the ptDNA into the gel, forming a smear of linear molecules corresponding in size to linear marker molecules of about 50 kb to 150 kb. In addition, distinct bands of obviously linear molecules of about 160, 320 and 460 kb were clearly visible (Fig. 5, lanes 3, 4. It should be noted that ptDNA and size markers may move slightly differently in the gel due to different sizes of the agarose plugs containing the DNAs). These bands represent monomers, dimers and trimers (tetramers were also visible on the autoradiograph after longer exposure) of the complete chloroplast chromosome which has a size of 155.8 kb (Shinozaki et al. 1986). Increasing concentrations of DNase I reduced the amount of well-bound DNA and at the same time increased the smear of ptDNA migrating into gel. The smear extended to regions containing smaller linear molecules (below 100 kb). First the multimers, and with increasing DNase concentration also the monomers, of the plastid chromosome disappeared.

Comparable results were obtained with ptDNA prepared from *C. album* cells, the only difference being that part of the DNA, including linear monomers and multimers of the chromosome, migrated into the gel without DNase I treatment (data not shown for purified ptDNA; for ptDNA combined with mtDNA see Fig. 6).

These findings are best explained by the assumption that untreated ptDNA is trapped in the agarose as large circles which cannot move into the gel. Mild DNase I treatment linearizes the chromosomes and their multimers which can now be separated and move exactly to the position expected from their size. Further treatment with DNase I cuts all circles in the well and consequently virtually all ptDNA enters the gel, the average size of the linear molecules becoming smaller. Moreover, these results demonstrate that high-molecular-weight organellar DNA could be isolated and studied by PFGE under the conditions used in our experiments.

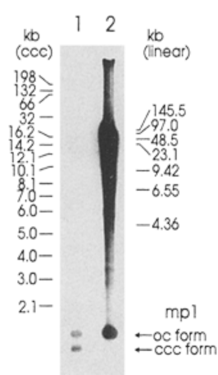

Fig. 4 Autoradiograph of electrophorectically separated mtDNA fractions from *C. album* collected from a discontinous CsCl-ethidium bromide gradient (*lane 1*, lower band – supercoiled DNA; *lane 2*, upper band – nicked circular and linear DNA). Supercoiled markers (left margin) were plasmid pSM409 from *E. coli* (66 kb, 132 kb, 198 kb), the 32-kb plasmid pSE411 from *B. subtilis*, as well as a supercoiled ladder (Gibco Brl, Gaithersburg, USA). A low-range PFGE-marker was used as standard for linear molecules (right margin). DNA was electrophoretically separated on a Eckhardt-gel: 0.5% agarose in 0.5×TBE, 4 °C, 140 V, 7 h. The hybridization probe was mtDNA from *C. album*. The small plasmid mp1 is marked by arrows.

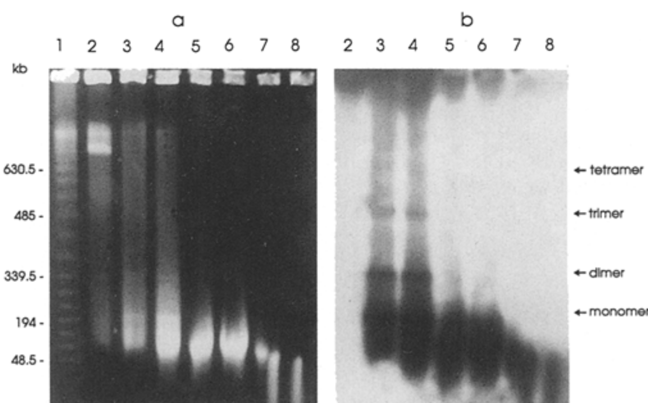

Fig. 5 Ethidium bromide staining (**a**) and autoradiograph (**b**) of ptDNA from tobacco separated on a TAFE-gel: *lane 1*, concatemers of phage lambda; *lane 2*, untreated ptDNA; *lanes 3–8*, DNase I-treated embedded ptDNA (1 h, 37 °C), series of dilutions in 100 µl of low-buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT): 0.5×10^{-6} units (*lane 3*), 1×10^{-6} units (*lane 4*), 0.5×10^{-5} units (*lane 5*), 1×10^{-5} units (*lane 6*), 0.5×10^{-4} units (*lane 7*), 1×10^{-4} units (*lane 8*). PFGE conditions: 0.8% agarose in 0.5×TBE, 200 V, 13 °C, pulse times: 40 s for 10 h (1st step) and 60 s for 14 h (2nd step). The hybridization probe was *rbcL*

Nucleases are not active during the preparation of mtDNA

Nuclease action during the preparation of mitochondria and DNA isolation could be responsible for the large portion of linear molecules seen in electron microscopy and PFGE. DNase treatment was omitted during isolation of mitochondria; however, nucleases derived from mitochondria or other parts of the cells could nick and linearize DNA circles during preparation and further degrade the resulting linear molecules. Therefore, we tested our mitochondrial preparations for the influence of externally added and potential internal nucleases.

For this purpose, we prepared a combined fraction consisting of mitochondria and plastids by a slightly altered scheme of differential centrifugation. Cell debris was removed by centrifugation at 1000 *g*. Both types of organelles (supernatant) were pelleted by one centrifugation step at 18 000 *g*. This fraction was incubated in the presence and absence of 100 µg/ml of DNase I and 10 mmM of $MgCl_2$ for 1 h at 4 °C before purification in a sucrose gradient followed by immobilizing and lysis in LMP-agarose. The resulting DNA patterns on a CHEF-gel are displayed in Fig. 6 (lane 2, DNase I treated; lane 3, without

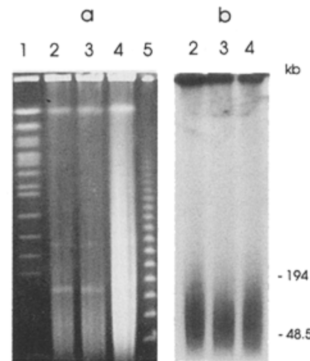

Fig. 6 Ethidium bromide-stained agarose gel (**a**) of DNA samples from *C. album* and autoradiograph (**b**) of mtDNA displayed on a CHEF-gel. Separated DNA fractions: *lane 2*, DNA of purified mitochondria and plastids (including DNase I treatment as described in the text); *lane 3*, DNA of crude mitochondria and plastids (without DNase I treatment); *lane 4*, total DNA of embedded protoplasts. Chromosomal DNA from *S. cerevisiae* (*lane 1*) and a lambda ladder (*lane 5*) were used as size markers. Running conditions were: 0.9% agarose in 0.5×TBE, 13 °C, 6 V/cm, 27 hourse, incl. angle = 120 °, pulse times: 2–140 s, ramping factor **a** = linear. The hybridization probe was mtDNA from *C. album*

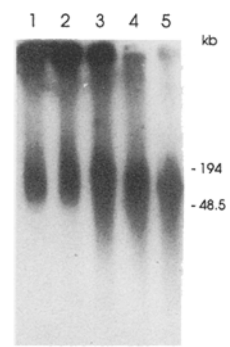

Fig. 8 Autoradiograph of mtDNA from *C. album* separated on a TAFE-gel. *Lane 1*, untreated mtDNA; *lanes 2–5*: DNase I treated mtDNA plug samples (1 h, 37 °C) starting with 1×10^{-6} units (*lane 2*), 0.5×10^{-5} units (*lane 3*), 1×10^{-5} units (*lane 4*) and $0.5-10^{-4}$ units (*lane 5*). PFGE conditions: 0.8% agarose in 0.5×TBE, 150 V, 13 °C, pulse time: 30 s for 16 h. The hybridization probe was mtDNA from *C. album*. Molecular-weight markers are indicated along the right margin and were dervied from λ-concatemers

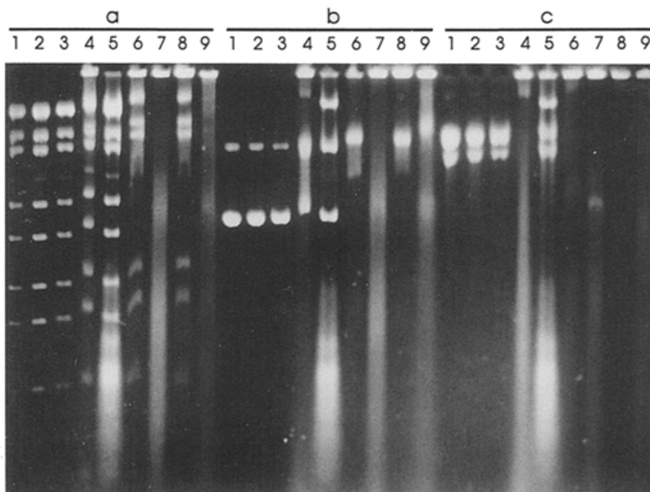

Fig. 7 Ethidium bromide-stained agarose gel of electrophoretically separated marker DNAs (*a* λ-DNA/*Eco*T14I, *b* pBR322, *c* M13 ssDNA) incubated (1 h, 37 °C) in the following solutions/fractions (20 µl total volume): H_2O *lane 1*; 1×PBS buffer, *lane 2*; lysis solution, *lane 3*; purified mitochondria fraction of *C. album* (not treated with DNase I, 1.5 mg/ml mt proteins), *lane 4*; mt fraction + lysis solution, *lane 5*; mt fraction + 1% Triton-X-100, *lane 6*; mt fraction + 10 mM $MgCl_2$ + 1% Triton-X-100, *lane 7*; mt fraction + 5 mM EDTA + 1% Triton-X-100, *lane 8*; mt fraction + 5 mM $MgCl_2$ + 1% Triton-X-100, *lane 9*

DNase I treatment). Total DNA of embedded protoplasts from *C. album* (lane 4) shows a smear with stronger signals in the compression zone and in the well. The bands visible in lanes 2 a and 3 a of about 160 kb and 320 kb represent linear genome-sized and dimeric forms of ptDNA. About 65% of ptDNA remained in the well. In addition, a band in the compression zone is seen in the ethidium bromide-stained gel (Fig. 6 a) which disappeared after alkaline denaturation (Fig. 6 b and cf. Fig. 2). Hybridization with mtDNA revealed the same distribution pattern of DNA molecules as in previous experiments (Fig. 6 b). In all DNA-samples (lanes 2–4) about 45% of the mtDNA signal remained in the well and 55% was observed in the fast-migrating zone of 40–200 kb.

Additionally, we checked mitochondrial lysates for the presence of internal nuclease activities. Incubation of linear double-stranded DNA (phage lambda DNA), supercoiled and open circles (pBR322) as well as single-stranded (ss) DNA (M13), with PBS buffer and lysis solution as a control (without mitochondria) revealed no unspecific nuclease activity (Fig. 7, lanes 2, 3). Incubation of the marker molecules with intact mitochondria, but without nuclease-inhibiting lysis solution, led to the disappearance of ssDNA which might be caused by binding of the DNA to the mt membrane or by a ssDNA-specific nuclease activity that could not be removed completely during the preparation (lane 4 c). Ds linear and circular DNAs were degraded in mitochondrial lysates in the presence of 10 mM of $MgCl_2$ (lanes 7 a, b), but not when $MgCl_2$ was replaced by 5 mM of EDTA (lanes 8 a, b). However, if we applied conditions comparable to those used in all our preparations for PFGE (mitochondria combined with lysis solution containing EDTA, SDS and proteinase K), no nuclease activity was observed (lanes 5 a, b, c). These results demonstrate that internal nucleases were present, but not active, during the preparation of mtDNA.

DNase treatment of well-bound mtDNA

Although a certain amount of DNA also remains in the well in the case of the molecular-weight markers, the proportion of well-bound DNA to migrating DNA is much higher with mtDNA (but less compared to ptDNA, Fig. 5). According to the results obtained with large plasmids and ptDNA, one could assume that the well-bound mtDNA may contain circles representing the master chromosome and subgenomic circles of a limited number of distinct size classes. We made several attempts to observe distinct linear mtDNA bands after mild DNase I treatment. The ef-

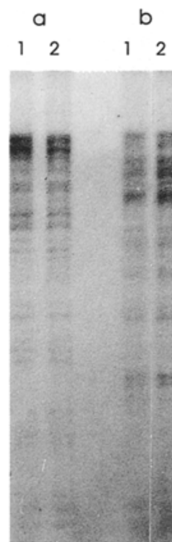

Fig. 9 a, b Autoradiograph of digested and electrophoretically separated mtDNA from *C. album*. MtDNA remaining in the well (*1*) and mtDNA migrating into the gel (*2*) was eluted from the gel and digested by endonuclease *Eco*RI (**a**) and *Bam*HI (**b**). The hybridization probe was mtDNA of *C. album*

fect of DNase I treatment was a decrease, and finally the loss, of well-bound DNA accompanied by an increasing amount of linear DNA migrating into the gel (Fig. 8, lanes 2–5). The average molecular weight of migrating DNA decreased with increasing DNase I concentration, exactly as observed for ptDNA. After longer incubation the signals of mtDNA disappeared completely (data not shown). However, distinct bands have never been observed for mtDNA of *C. album* (Fig. 8) or any other species examined (data not shown). This result suggests that the well-bound fraction of mtDNA, in contrast to ptDNA, does not contain larger amounts of circular molecules belonging to distinct size classes.

In a further attempt to elucidate the nature of mtDNA fractions separated in PFGE-gels, the well-bound DNA and DNA from the 40–200-kb zone were eluted out of the agarose gel and digested by the restriction endonucleases *Eco*RI and *Bam*HI. The patterns of bands visible over a background smear after hybridization with mtDNA were identical for both types of DNA indicating that the DNA in the well and the DNA migrating in the gel contain the same nucleotide sequences in the same proportions (Fig. 9).

Discussion

In agreement with previous studies (Bendich and Smith 1990; Levy et al. 1991; Bendich et al. 1993), our data demonstrate that the mtDNAs of higher plants, including *C. album*, *B. hirta* and other species, migrate as a smear of linear molecules into pulsed-field gels and a major part remains well-bound. Another region of the gel, the compression zone, was also stained by ethidium bromide. This zone seems to contain RNAs rather than large linear DNA molecules (cf. Bendich et al. 1993), as DNase-free RNase and alkaline treatment removed all bands from this region.

According to the results of physical mapping, plant mtDNA should consist of a circular master chromosome in balance with subgenomic circular molecules. In the case of *B. hirta*, no other circles but the master chromosome should be present (cf. Palmer 1992). The smear of linear molecules obtained after PFGE of mtDNA (including mtDNA of *B. hirta*) could be explained by the degradation of the master chromosome and of the subgenomic circles by the action of nucleases and/or shearing forces during the preparation of DNA. Undegraded circular DNA would then compose the well-bound DNA. Several studies, including the present one, have demonstrated that large circular molecules do not migrate into pulsed-field gels. We performed a series of experiments to investigate whether nucleases or shearing forces are active during our preparation of mtDNA. We observed 5′–3′ and 3′–5′ exonucleases in lysed mitochondria of *C. album* during previous studies (Meißner et al. 1993 and unpublished results). Although DNases were also found to be present in the preparations of mitochondria used in the present study, we were able to demonstrate that their activity is completely inhibited under the conditions applied for the preparation of mtDNA (cf. Figs 6, 7). Moreover, we could take a small circular mt plasmid as an internal control for the action of nucleases during the complete procedure of mtDNA isolation starting with the preparation of protoplasts and mitochondria. The presence of a large portion of circular, even supercoiled, plasmid DNA and of oligomeric forms of the plasmid (data not shown) in the mtDNA preparations clearly indicates that the action of unspecific nucleases cannot serve as an explanation for the appearance of large amounts of linear DNA molecules with highly variable size.

Mammalian mtDNA is known to contain ribonucleotides (Lonsdale and Jones 1978; Brennicke and Clayton 1981; Margolin et al. 1981). If this is also true for plant mtDNA, RNases could cleave circular molecules into linear fragments. Neither RNase treatment nor the application of RNase inhibitors had any influence on the migration behaviour of plant mtDNAs in PFGE (Fig. 2). Thus RNases are probably also not responsible for the appearance of large amounts of linear mtDNA.

We were able to isolate undegraded large circular DNA in high amounts from bacteria and plastids, including plastids from cell suspension cultures of *C. album* which also served as source of mtDNA. Obviously, shearing forces did not destroy the large ptDNAs; even multimers were observed. Our results of the electrophoretic separation of ptDNA by PFGE are in agreement with the results of physical mapping. Mapping data support the view that ptDNA exists as circular chromosomes containing the complete set of genes (e.g. Shinozaki et al. 1986; Hiratsuka et al. 1989; Palmer 1992). Recently the existence of oligomeric forms of the chloroplast chromosome was reported (Deng et al. 1989; Bendich and Smith 1990). In agreement with these studies, we found mono-, di-, tri-, and tetra-mers of the plastid chromosomes of *N. tabacum* and *C. album*. These oligomers migrate in their linear configuration into pulsed-field gels. Chloroplast DNA from *N. tabacum* leaves left the well only after treatment with DNase I (Fig. 5) whereas

about 35% of the ptDNA of *C. album*, including linear chromosomes and their oligomers, migrated into the gel without DNase treatment (Fig. 6 and unpublished results). It is not clear yet why we obtained slightly different results for *Nicotiana* and *Chenopodium*. The difference could find its explanation in the different source of plastids (leaves vs cultured cells) or in the different types of plastids employed (chloroplasts vs unpigmented plastids). We cannot rule out the possibility that part of the ptDNA of *C. album* exists in vivo usually as linear molecules. The important difference to the situation with mtDNA is, however, that large amounts of ptDNA having exactly the expected size could be isolated and separated in PFGE.

The fact that chromosome-sized linear molecules (and their oligomers) could also be mobilized from the well-bound fraction by DNase treatment only in the case of ptDNA (but not of mtDNA) does not support the idea that larger quantities of circular master chromosomes and subgenomic circles belonging to a *few* distinct size classes are retained in the well. If the well-bound DNA were to consist mainly of circular molecules then the circles should be of extremely variable size. The identity of restriction fragment patterns obtained from the zone of migrating DNA and of well-bound DNA demonstrates that the well-bound DNA contains the same (presumably all) sequences of the mt genome as the migrating portion. Therefore, the difference between both fractions of mtDNA is most likely due to a difference in their structural organization.

Taken together, our results support the general consensus that the ptDNA exists *in organello* as circular molecules of genome size and are in agreement with reports on the existence of oligomeric forms (Deng et al. 1989; Bendich and Smith 1990). However, our data cannot easily be brought into agreement with the proposed organization of mtDNA as a balance between a master chromosome and a few subgenomic circles. Though the appearance of large proportions of linear molecules is certainly surprising, we found it especially striking that: (1) the linear molecules appear as a continous smear containing molecules of a large size range (most of them between about 40 kb and 200 kb), (2) molecules of *all* sizes hybridize with *all* mtDNA probes (i.e. sequences of each gene are present in molecules of all size classes; an exception is the plasmid mp1), and (3) the restriction fragment patterns of the well-bound and the migrating fraction of mtDNA were identical both qualitatively *and* quantitatively. While the first observation (linear molecules with sizes between 40 kb and 200 kb) could still be explained by a kind of ordered and/or controlled cleavage of large circular or linear molecules, the two latter observations are most easily explained by a random cleavage and/or degradation of larger molecules.

Thus, a model of the organization of mtDNA compatible with all our data has to take into account the following facts. First, linear molecules arise by a more or less random process. Second, the random process is not the activity of nucleases during the preparation of mtDNA. Third, the random process cannot be identical with shearing forces during the preparation of mtDNA provided that mtDNA is similarly organized as ptDNA and is represented by circular molecules belonging to only a few size classes. Furthermore, it is not known exactly what kind of molecules are present in the well-bound fraction. The existing data on this fraction led us assume that it contains large circular molecules or more complex structures such as catenanes, replication and recombination intermediates, or other complex molecules.

Our data cannot rule out the possibility that mtDNA exists in vivo as circular molecules. If the mt genome were to be represented *in organello* exclusively by circular DNAs then the observed linear DNAs would have to be products of degradation during preparation. In this case, the circles must be organized differently from the chromosomes of plastids and one has to propose the existence of extremely fragile structures that break at more or less random sites, preferably every 40 to 200 kb. The only known peculiarity of plant mtDNA which distinguishes if from ptDNA is its high recombinational activity. One could hypothesize that a majority of the mtDNA participates in recombination events, thereby forming fragile structures. The subgenomic DNA molecules of plant mitochondria might also form complex structures similar to the network of catenanes in kinetoplasts of trypanosomes (Borst and Hoeijmakers 1979; Shapiro 1993) or else may be bound to membranes. Association of DNA with membranes occurs in the mitochondria of several groups of organisms (Albring et al. 1977; Shearman and Kalf 1977; Kawano and Kuroiwa 1985; Echeverria et al. 1991). Such structures may be preferentially destroyed during the isolation of DNA. Furthermore, much more different subgenomic molecules might exist in plant mitochondria than is assumed on the basis of physical maps. In addition to larger repeats, there are numerous shorter repeats in mtDNA (Unseld et al. 1993) which might be used for homologous intra- and inter-molecular recombination, though less frequently than the larger repeats (André et al. 1992). Infrequent recombination between numerous short repeats would increase the number of different subgenomic molecules and could generate the so-called sublimons, a class of molecules occurring at extremely low quantity in plant mitochondria (Small et al. 1989).

Our data do not rule out the possibility that the observed linear molecules are products of rolling-circle replication (cf. Bendich 1993). In this case, one has to assume that the linear replication products would be (mostly) concatemers. In contrast to the situation known from plasmids and the phage genomes replicating via sigma-like intermediates, cleavage of the concatemers of mtDNA has to occur not a specific sites but at more or less random sites, preferably every 40–200 kb.

In conclusion, the data presented in this paper demonstrate that master chromosomes and distinct subgenomic circles of the mt genome are not detectable by methods which easily reveal the existence of monomeric and oligomeric forms of plastid chromosomes. Our data show further that the observed linear molecules cannot be products of unspecific degradation by nucleases. They rule out that linear molecules are generated during preparation by shearing forces destroying circular molecules of a *few* dis-

tinct size classes. Our data suggest a more complex organization of the mt genome. They indicate the involvement of a random process in the generation of the observed linear molecules, e.g. by degradation of highly fragile complex molecules or by a specific mode of rolling-circle replication. We are currently investigating the mtDNA of *C. album* by a combination of two-dimensional gel electrophoresis and electron microscopy in order to determinate whether the prevailing linear molecules are products of fragile complex molecules or of rolling-circle replication.

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