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Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria

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Summary. The structure of the cytoplasmic genomes in plants has been investigated by using fluorescence microscopy to make moving pictures of ethidium-stained DNA fractionated by pulsed-field gel electrophoresis (PFGE) and emerging from organelles lysed within gelled agarose. For watermelon chloroplasts, PFGE fractions contained linear molecules representing monomeric to tetrameric lengths of the unit genome (155 kilobase pairs, kb) and linear DNA of at least 1,200 kb, whereas circular molecules were clearly identified only with chloroplast agarose inserts. For pea, the oligomeric series extended only to the trimer. Most of the DNA from watermelon mitochondria was in the form of 50–100 kb linear molecules with some DNA of at least 1,200 kb, but no band was seen at the size of this genome (330 kb) and no circular molecules were identified in either PFGE fractions or mitochondria embedded in agarose. Most mitochondrial DNA from cauliflower also consisted of 50–100 kb linear molecules with some much longer linear forms, but no genome-sized (220 kb) PFGE band was evident. The possible relevance of the very long linear DNAs to previous cytological and genetic observations is discussed.

Key words: Chloroplasts – Fluorescence microscopy – Mitochondria – Pulsed-field gel electrophoresis

Introduction

The chloroplast genome ranges in size from 120–220 kilobase pairs (kb) in land plants (Palmer 1985) and from 90 to over 400 kb in algae (Tymms and Schweiger 1985; Manhart et al. 1989). The mitochondrial genome in land plants ranges from 210–2400 kb (Ward et al. 1981; Palmer 1988). Both genomes are present in many copies per cell (Bendich 1987). The structure of the genetic material in chloroplasts, inferred from restriction mapping, consists of a circle (or circles) no larger than the size of the unit genome. Electron microscopy of chloroplast DNA (cpDNA) revealed circular molecules of this size and a small proportion of dimeric circular forms (Kolodner and Tewari 1975a, 1979). From restriction mapping and cosmid clone analysis, circular structures have also been proposed for plant mitochondrial genomes 210–570 kb in size: one circle, or three or more subgenomic circles in dynamic equilibrium (Palmer 1988; Lonsdale et al. 1988). For the mitochondrial DNA (mtDNA), however, none of these large circles has been identified by electron microscopy (Bendich 1985).

Although circular unit-sized genomes can be obtained from chloroplasts, we do not know whether these are the principal forms active in DNA replication, transcription and inheritance. The following considerations are relevant to the question of the structure of the genome within cytoplasmic organelles. (1) A circular restricton map does not necessarily indicate a circular DNA molecule since circular maps would be derived from linear molecules with either circularly permuted sequences (T4 bacteriophage DNA, for example), terminal repeated sequences, or a concatemeric array of monomer units (discussed by Bendich 1985). (2) Observations of plastid nucleoids by fluorescence microscopy using the DNA-binding fluorochrome, DAPI, have revealed contiguous masses far larger than that expected for unit genomes in many algal species (Kuroiwa et al. 1981; Coleman 1985), oats (Hashimoto 1985) and wheat (Miyamura et al. 1986). (3) Some of the cpDNA isolated from spinach (Herrmann et al. 1974; Yoshida et al. 1978; Herrmann and Possingham 1980; Brait et al. 1982) and a liverwort (Herrmann et al. 1980) appeared in electron micrographs as rosette structures consisting of molecules much longer than unit genomes and folded into numerous loops emanating from electron-dense material. (4) The number of units responsible for the segregation of chloroplast and mitochondrial genetic markers is much smaller than the copy number of these genomes (Sager 1977; Forster et al. 1980; Birky 1983). It is thus not clear whether all genomic DNA within chloro-

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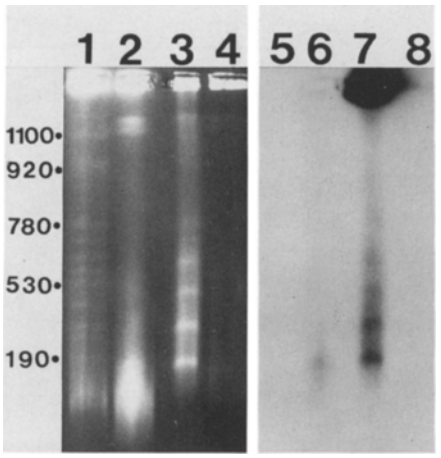

Fig. 1. Size-fractionation of watermelon organellar DNAs and hybridization with a chloroplast gene-specific probe. Ethidium-stained DNA from yeast, mitochondria, chloroplasts and nuclei (*lanes 1–4*, respectively) were blotted and hybridized with a ^{32}P-labelled *rbcL* gene probe, using a 4 h autoradiographic exposure, as shown in *lanes 5–8*, respectively. No additional bands of hybridization were detected with exposures up to 21 h except for a faint signal in lane 6 that corresponds to the diffuse region immediately below the lowest mobility band (the compression zone) in lane 2. Linear DNA lengths in kb are indicated

plasts and mitochondria is circular and whether all the functions served by the genome are conducted from the same structural form of the DNA molecule.

In order to study the structure of the cytoplasmic genomes in plants we have used fluorescence microscopy to make moving pictures of DNA fractionated by pulsed-field gel electrophoresis (PFGE) and as it emerges from lysed organelles. For watermelon chloroplasts, PFGE fractions contained linear molecules representing monomeric to tetrameric lengths of the unit genome and linear cpDNA of at least 1,200 kb, whereas circular molecules were clearly identified only with lysed plastids. Most of the DNA from watermelon mitochondria was in the form of 50–100 kb linears with some mtDNA of at least 1,200 kb, but no circular molecules were identified and no genome-sized (330 kb) band was observed by PFGE.

Materials and methods

Preparation of organelles. Watermelon (*Citrullus vulgaris* Schrad., cv. Dixie Queen), pea (*Pisum sativum* L., cv. Alaska), cauliflower (*Brassica oleracea* L.) and yeast (*Saccharomyces cerevisiae* RM 14-3a) were used. Chloroplasts were prepared by the floatation-sedimentation method of Morgenthaler et al. (1975) with a self-generating (initially 50%) Percoll gradient using cotyledons (watermelon) or leaves (pea) from 10-day old seedlings, grown in vermiculite on 16 h light/8 h dark cycles at 25°C with the final day in darkness. Mitochondria were prepared (Skubatz and Bendich 1990) from dark-grown watermelon cotyledons and cauliflower heads (from a grocery store). Mitochondria from exponentially growing *S. cerevisiae* D273-10B were prepared (Daum et al. 1982) for use in lane 9 of Fig. 2. Chloroplasts and mitochondria were treated with DNAse (100–200 μg/ml) for 1 h on ice and then washed twice (once for yeast). Nuclei were prepared from 13-day old green watermelon cotyledons by the Percoll gradient procedure of Luthe and Quatrano (1980).

DNA fractionation and hybridization. Agarose inserts were prepared (Carle and Olsen 1987) using 200 μg/ml proteinase K and PFGE was conducted (Anand 1986) at 5 V/cm, 120 s pulse time (unless stated otherwise) at 6°C in 1.5% agarose (Standard Low-m_r from Bio-Rad or Low Melting Point from BRL) in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). After irradiation to fragment long DNA (Van Daelen et al. 1989) and blotting to a Nytran filter, hybridization was performed (Church and Gilbert 1984) at 65°C in 0.5 M $NaHPO_4$ with pea *rbcL* (Zurawski et al. 1986) or maize *atp9* (Dewey et al. 1985) probes labelled with [^{32}P]dCTP using a random priming reaction kit from Boehringer Mannheim (FRG). Autoradiography was at −70°C with a Dupont Cronex Lightning Plus intensifying screen and Kodak XAR film. Lambda phage DNA concatemers were prepared according to Bancroft and Wolk (1988).

Fluorescence microscopy imaging. In order to obtain DNA from particular PFGE fractions of low-melting agarose gels, the gel was first placed on its ethidium-fluorescent photograph (at actual size). Then 5–10 μl plugs were removed from the gel and placed on a microscope slide at 55–60°C. A 22-mm coverslip was gently pressed onto the plug until it melted. Watermelon chloroplast agarose (low-melting) inserts (20–25 μl, initially containing 6×10^5 chloroplasts) were stained with 0.5 μg/ml ethidium bromide (EB), destained in 0.5 × TBE and similarly melted for microscopy. For microscopy of watermelon mitochondrial agarose inserts, microbeads (Carle and Olson 1987) were mixed with molten 1.5% agarose in 0.5 × TBE and 0.5 μg/ml EB at 56°C. Video tape recordings were made as described previously (Smith et al. 1989). The images shown in Fig. 3 were made by photographing a video monitor, while the tape was playing, using a shutter speed of 1/15 s. At least 100 DNA molecules were examined from PFGE fractions, and several hundred from agarose inserts, to score the molecules with respect to linear or circular form.

Results

Chloroplasts were prepared from cotyledons of light-grown watermelon seedlings, embedded in agarose, treated with detergent and proteinase K and their DNA was analyzed by PFGE. Four bands were obtained that appear to be the monomer, dimer, trimer and tetramer of the chloroplast genome (Fig. 1, lane 3; Fig. 2, lanes 3 and 4). When pea chloroplasts were similarly analyzed, the series extended only to the trimer (lanes 1 and 2 in Fig. 2). Deng et al. (1989) found a series to the tetramer for spinach and state that cpDNAs exist as multimeric forms for pea, maize, tomato and the green alga *Mesotaenium caldariorum*. The oligomeric forms for watermelon (Fig. 1, lane 7) and pea (data not shown) hybridize with a cpDNA fragment of the *rbcL* gene (encoding the large subunit of ribulosebisphosphate carboxylase). For spinach, the relative molar amounts were calculated to be 1, 1/6, 1/27 and 1/108 for the monomer, dimer, trimer and tetramer lengths of the chloroplast genome, respectively (Deng et al. 1989). The relative intensities (and thus molarities) of the oligomeric cpDNA bands in Figs. 1 and 2 appear to be similar to those for spinach.

For spinach, Deng et al. (1989) could not determine whether the oligomers resolved by FfGE were circular or randomly-linearized molecules. For watermelon, however, two types of evidence indicate that the bands contain linear molecules. First, the migration of these four bands changed with changing pulse times (40, 50, 90 or 120 s intervals between 120° changes in the direction of the electric field) just as did linear yeast chromosomes, such

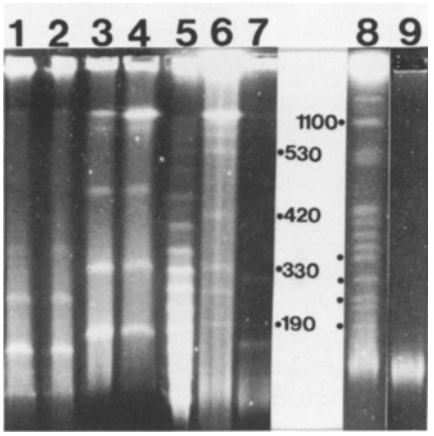

Fig. 2. Size-fractionation of organellar DNAs. Ethidium-stained DNA from agarose inserts of pea choroplasts (*lanes 1 and 2*), watermelon chloroplasts (*lanes 3 and 4*), lambda DNA (*lane 5*), yeast cells (*lanes 6 and 8*), cauliflower mitochondria (*lane 7*) and yeast mitochondria (*lane 9*) is depicted. Pulse times of 50 s (lanes 1–7) or 120 s (lanes 8 and 9) were used. The size in kb of the chloroplast genome monomer calculated from mobility of the trimer or dimer (cauliflower) relative to that of yeast chromosomes was 155 (watermelon), 122 (pea) and 151 (cauliflower, from the apparent cpDNA contaminants). Linear DNA lengths in kb are indicated

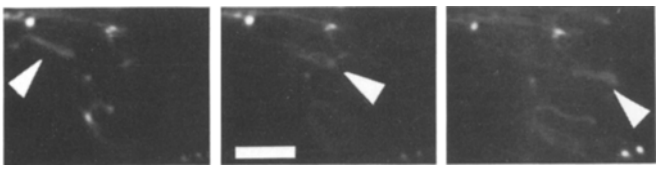

Fig. 3. DNA released from agarose inserts of watermelon chloroplasts. Images spaced by 2 and 6 s, respectively, are shown from a video tape recording of ethidium-stained DNA. The *arrowed molecule* moved toward the right (anode), opened to reveal its circular structure, migrated further and became immobile. The *two dots* (*lower right*) are unobstructed ends of immobile molecules mostly out of focus. The bar equals 10 microns

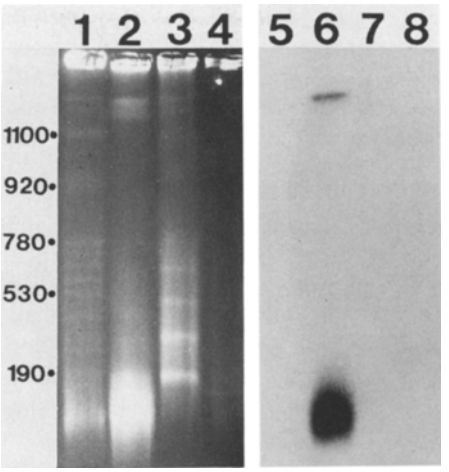

Fig. 4. Size-fractination of watermelon organellar DNAs and hybridization with a mitochondrial gene-specific probe. Conditions and lane designations are the same as for Fig. 1 except that an *atp9* gene probe was used and a 5 h exposure is shown. No additional bands of hybridization were detected with exposures up to 135 h

that their sizes estimated from mobility relative to the yeast molecules did not change with changing pulse times. In contrast, the apparent size of a 66 kb supercoiled plasmid DNA, pSM409 (Smith and Bendich 1990), calculated from its mobility relative to that for linear yeast molecules, was 410 kb with 40 s pulse time, 530 kb (60 s) and 850 kb (120 s). The migration of pSM409 and the watermelon oligomers is not that expected for relaxed or nicked circles since open circles of 30 kb and 85 kb in size do not migrate far in these PFGE conditions (Beverley 1988). Secondly, direct observations in the microscope of each of the four bands showed them to contain linear molecules since their appearance was like that of linear yeast chromosomes (Smith et al. 1989) and linear DNA from bacteriophage G (Schwartz and Koval 1989) rather than that of circular molecules. The pea oligomeric bands also contain linear cpDNA because their migration relative to that of linear molecules does not change with changing pulse times (40–120 s).

We previously used fluorescence microscopy to study plasmid pSM409 after it was trapped in gelling agarose (Smith and Bendich 1990). Some molecules become caught on several obstructions (agarose fibers) and are held in an open circular form even in the presence of an electric field. Due to the presence of ethidium bromide and intense illumination, the plasmids viewed in the microscope may be relaxed from their supercoiled form. Most molecules, however, are evidently hooked on a single obstruction since they appear as lines extending downfield with about twice the fluorescence intensity of linear molecules. The unobstructed end of the line has a bright head that wiggles (from Brownian motion) and the line pivots through 180° about its anchor when the polarity of the field is reversed.

Many molecules, scored as circes by the above criteria (immobile line with a wiggling bright head), have been observed in chloroplast agarose inserts. Their length is usually about 2–3 times that of the 66 kb plasmid under similar conditions. We estimate that between 20 and 80% of the molecules are circular, although no attempt was made to determne this value more accurately. Occasionally, a molecule will become dislodged from its anchor, migrate a short distance in the electric field and again become stuck on a new anchor point. Figure 3 shows such a molecule that appeared fleetingly as an open circle; apparently several agarose fibers hooked it at the same time. We conclude that chloroplast genome-sized circles are common in the agarose inserts but do not migrate in the prominent PFGE bands. The circles presumably remain in the well of the gel.

The size of the mitochondrial genome of watermelon is 330 kb (Ward et al. 1981) but no prominent mtDNA band of this size was evident when mitochondrial agarose inserts were analyzed by PFGE using pulse times of 120 s

(lane 2 in Figs. 1 and 4; Fig. 4 lane 6), or 30, 40 and 80 s (data not shown). Most of the mtDNA migrated as linear molecules between about 50 and 100 kb, as estimated with respect to the migration of linear yeast chromosomes and linear lambda phage DNA concatemers. As with the linear cpDNA molecules, the size estimates for these mtDNAs did not change with changing pulse times. Numerous linear molecules, but no obviously circular forms, were observed by fluorescence microscopy of both the 50–100 kb region (gel not depicted here) and mitochondrial agarose inserts. The DNA from the inserts that enters the gel hybridizes with a mtDNA fragment containing the *atp9* gene (encoding subunit 9 of the mitochondrial ATPase) as seen in lane 6 of Fig. 4, but some cpDNA contaminates the preparation since bands the size of the monomer and dimer hybridize with the *rbcL* probe (Fig. 1, lane 6). For cauliflower mitochondrial agarose inserts, most of the DNA migrated in the 50–100 kb region using pulse times of 50 s (Fig. 2, lane 7) or 120 s (data not shown) with apparent monomer and dimer cpDNA contaminants, but no prominent band was evident at the linear size of the mitochondrial genome (220 kb) or either of its predicted subgenomic circles (49 kb and 170 kb; Palmer 1988). The smear of fluorescence in total DNA from yeast at about 50–100 kb (lane 1 in Figs. 1 and 4; lanes 6 and 8 in Fig. 2) is mtDNA since this smear is the only DNA evident in the gel when DNAse-treated mitochondria are analyzed by PFGE (Fig. 2, lane 9). It contains linear DNA because its migration relative to that of linear molecules does not change with changing pulse times (30–120 s) and its appearance is clearly linear by fluorescence microscopy.

In addition to the DNA near the bottom of the gel, a much more slowly-migrating band was found near the top of the gel for both chloroplast and mitochondrial agarose inserts (lanes 2 and 3 in Figs. 1 and 4). These "bands" may not be comprised of uniformly-sized molecues but instead represent the "compression" zone containing molecules greater than about 1,200 kb. Direct observation with the fluorescence microscope of DNA from these bands (from gels not depicted here) shows linear molecules longer than 1,200 kb, in additon to shorter molecules evidently produced by shearing during spreading of the remelted agarose beneath the coverslip. Thus its low mobility is not due to circulariy, unusual DNA conformation or bound impurity, but rather to a very high molecular weight. These slowly-migrating bands contain cpDNA (Fig. 1, lane 7) and mtDNA (Fig. 4, lane 6), respectively, because they hybridize with organellar gene probes. A slowly-migrating fluorescent band in the compression zone was also observed for agarose inserts of pea chloroplasts and cauliflower mitochondria (Fig. 2), although no attempt was made to examine those bands with the fluorescence microscope.

The diffuse region of fluorescence immediately below the compression zone band in lane 2 of Figs. 1 and 4 was seen in seven gels made with the standard (not low-melt) agarose, but was not seen in the three gels made with low-melt agarose (including the one used to visualize the mtDNA greater than 1,200 kb). It was not seen with chloroplasts from green cotyledons using either type of agarose. This diffuse region does not hybridize with the *atp9* gene probe (Fig. 4, lane 6), but shows faint hybridization with the *rbcL* probe (legend to Fig. 1). It is evidently not mtDNA, but may be a circular form of cpDNA.

Discussion

We have identified three forms of cpDNA: linear molecules representing monomeric to tetrameric lengths of the unit genome, much longer linears, and circular molecules. The first two forms were designated as linear DNAs because of their electrophoretic properties in pulsed-field gels and their appearance in moving pictures (Movies) made with the fluorescence microscope. The third form was not clearly identified as a band on a gel, but was seen to be circular in Movies of the contents of chloroplast agarose inserts. It is not evident why the circular cpDNA (monomer size of about 150 kb) did not usualy enter the gel since *E. coli* plasmids of about 100 kb and 200 kb did migrate in our gels as sharp bands (unpublished results).

In contrast to the situation with chloroplasts, in which the oligomeric series of gel bands clearly corresponds to multiples of the unit genome, no band of mtDNA represented the size of the mitochondrial genome. Most of the mtDNA from watermelon and cauliflower migrated as linear molecules between about 50 and 100 kb, with no obvious bands at the size of these genomes (330 kb and 220 kb, respectively). The same size range was also observed for mtDNA from the yeast *S. cerevisiae* which does include its genome size, although no distinct band within this smear was evident. Linear, but no circular, molecules were seen in Movies of mtDNA from the 50–100 kb region of gels for each of these species and of watermelon mitochondrial agarose inserts (occasional circular molecules could have escaped our notice, and cauliflower and yeast agarose inserts were not so examined). We thus conclude that, as is the case with older methods for mtDNA extraction, mostly or only linear DNA is produced from plant or yeast mitochondrial agarose inserts despite the fact that these genomes have circular restriction maps (Bendich 1985). Broken-stranded recombination intermediates, tenuously held in recombination apparatuses, may prevent the isolation of mitochondrial genome-sized circles by any method (Bendich 1985). The yeast *Torulopsis glabrata* has only a 19 kb mitochondrial genome, yet its mtDNA comigrates in PFGE with that of *S. cerevisiae* at 50–100 kb (Skelly and Maleszka 1989). For plants and yeasts the size of the mitochondrial genome evidently has little influence on the size of extractable mtDNA molecules.

The linear form of cpDNA and mtDNA, much longer than the unit genome, was found in the compression zone of pulsed-field gels. Movies of watermelon DNA from this zone show linear molecules at least 1,200 kb in length, using the relationship of 3 kb per micron of DNA. These linear molecules are surely much longer (by 2 to 3-fold, we estimate) since they were not stretched by a high voltage gradient during their measurement and its elasticity causes unstretched DNA to contain far more than 3 kb per micron of ethidium-fluorescent fiber (Smith and Bendich

1990). Kolodner and Tewari (1975b) proposed a roling-circle phase for cpDNA replication and it is possible that the long linear molecules are replication intermediates.

Continuous DAPI-fluorescent fibers, many times the size expected for unit genomes, have been observed in chloroplasts of many algal species (Kuroiwa et al. 1981; Coleman 1985). Although it is possible that individual monomeric or oligomeric genomes are so aligned in plastids as to give the appearance of thin fibers, long linear molecules, such as those in the compression zone, seem a more likely source for these cytological observations.

The rapid segregation of phenotypic markers suggests that the number of chloroplast genetic units is low (Sager 1977; Forster et al. 1980), whereas the number of copies of this genome is typically greater than 100 per plastid and several thousand per cell (Bendich 1987). The number of units directing the heredity of mitochondrial genomes is also low (Birky 1983) despite a high genome copy number (Bendich 1987). Theories to explain this discrepancy between the genetic and physical estimates of ploidy have invoked clustering or preferential replication for some of the many equivalent copies of the genome (VanWinkle-Swift 1980; Birky 1983). It is possible, however, that a form of the genome represented by the linear DNA in the compression zone is the vehicle of inheritance for cytoplasmic genes rather than the far more numerous small circular molecules that have been described for the past two decades.

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