Supercoiled mitochondrial DNAs from plant tissue culture cells

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ABSTRACT

Supercoiled mitochondrial DNA (mtDNA) has been isolated in preparative amounts from five different plant species and compared on agarose gels. The gels reveal considerable mtDNA size heterogeneity within each species as well as substantial differences in the range and frequency of the mtDNA size classes among the different plants. The two lowest size classes of three of the plants have been extracted and their molecular weights determined. Comparison of two *N. tabacum* W38 culture lines has revealed significant differences in their mtDNA size classes. However, despite the totally different supercoil patterns, these two lines have almost identical restriction digests. The availability of preparative amounts of separated mtDNA size classes will make it possible to carry out a detailed analysis of plant mitochondrial genomes.

INTRODUCTION

Electron microscopic studies from several plant systems including maize (1,2,3), soybean (4) and tobacco (5) have revealed that most plant mitochondria have a heterogeneous population of circular DNA size classes extending from 0.5 μ m to more than 30 μ m in length. Fungal mitochondrial DNAs, on the other hand, are 15 to 25 μ m and animal mtDNAs are only 4 to 6 μ m. How the many size classes of plant mtDNA are related and why plants maintain such large, physically heterogeneous genomes remain largely unanswered questions.

A principal problem in analyzing plant mtDNAs has been the difficulty encountered in isolating significant quantities of the different size classes. With the exception of the small maize mitochondrial DNAs isolated by Kemble and Bedbrook (6) most investigators have been able to obtain only a small percentage of the plant mtDNA in the form of supercoiled or circular molecules, generally only enough for EM analysis. The rest of the mtDNA has been isolated as a mixture of linear and broken linear molecules that has been impossible to resolve into distinct size classes.

In this paper we wish to report our success in isolating 30% of the total plant mitochondrial DNA as supercoiled molecules, from a variety of

different plant cell suspension culture lines. Our results indicate that there is wide variation in the frequency and distribution of different size classes between species and within a species. With the availability of significant quantities of supercoiled mtDNA, it will be possible to analyze in detail how plant mitochondrial genomes are organized in different plants and how the different size classes in a single species are related.

MATERIALS AND METHODS

Chemicals and Enzymes

Chemicals and buffers were obtained from regular commercial sources. Cesium chloride was purchased from Kawecky Brylco and from Bethesda Research Laboratories (BRL). Agarose was obtained from BRL. Restriction enzymes and molecular weight markers were purchased from New England Biolabs, Boehringer Mannheim and BRL. Miracloth came from Calbiochem. Pancreatic DNAase I was supplied by Sigma and Worthington Biochemicals.

Plant Material

The established culture line of Nicotiana tabacum var. Wisconsin 38 came to us from Dr. J. Polacco (University of Missouri, Department of Biochemistry). The cells were grown in Linsmaier and Skoog (?) basal liquid media containing 3.0mg/l of indole acetic acid and 0.3mg/l of 6(8'-dimethylallyl-amino)-purine. Cultures were grown in the dark at 25°C on a gyrotary shaker set at 140 rpm. Cultures were routinely transferred every five days and diluted 1 to 5.

The new line of Nicotiana tabacum, var. Wisconsin 38, was initiated February, 1980, using leaf discs from young leaves of vegetative plants. The cells were grown in a complex medium (RDM) devised by J. Duesing. A detailed description of the medium will be published elsewhere (manuscript submitted for publication). Cultures were grown in the dark as described above and were subcultured 1 to 4 every two weeks.

The Nicotiana rustica cultures which were derived either from leaf or pith tissue were initiated in March of 1980. The cells were grown in the light at 25°C in RDM media. Cultures were transferred 1 to 4 every 2 weeks.

The Datura stramonium, var. white inermis, was an anther-derived haploid cell line initiated from anther filament callus in November, 1979. It was grown on RDM media at 25°C in the light on a gyrotary shaker set at 140 rpm and transferred 1 to 4 every 2 weeks.

The Solanum dulcimer culture was the kind gift of Dr. P. Bhatt, Biology Department, Yale University. The cells were grown on the revised

tobacco medium of Khanna and Staba (8) supplemented with 1.1mg/l 1,4-dichlorophenoxy acetic acid. Myo-inositol was reduced from 55g/l to 2g/l. Cells were grown in the light at 25°C on a gyrotary shaker (140 rpm) and transferred every week 1 to 4.

The Phaseolus vulgaris, var. Taylor's Horticultural, culture was the kind gift of Dr. I. Sussex, Biology Department, Yale University. The cells were grown on Gamborg's media (9) at 25°C in the dark on a gyrotary shaker at 140 rpm. The culture was transferred 1 to 4 every ten days.

The Zea mays, var. Black Mexican (BM), culture was the kind gift of Dr. B. Gegenbach, Department of Agronomy and Plant Genetics, University of Minnesota. The cells were grown in Linsmaier and Skoog (7) basal liquid media supplemented with 2,4-D to 2mg/l, thiamine 10mg/l, glutamine 146mg/l, and lysine 182mg/l. The cells were grown in the light on a gyrotary shaker at 140 rpm. Cultures were transferred 1 to 4 every 2 weeks.

Mitochondrial Isolations

Mitochondria were isolated using the French pressure cell procedure reported elsewhere (5).

Mitochondrial DNA Isolations

Mitochondrial DNA was isolated from purified mitochondria on CsCl-ethidium bromide gradients as previously reported (5). The supercoiled and open circular/linear bands were collected, the ethidium bromide extracted and the samples dialyzed. The absorbance at 260nm and 280nm were measured and the DNA ethanol precipitated. The percentage of DNA occurring in the supercoiled band ranged from 22 to 40 percent of the total mtDNA with an average of 30 percent.

Gel Electrophoresis and Restriction Enzyme Analysis

Mitochondrial DNAs extracted from the linear/open circular or supercoil region of a CsCl-ethidium bromide gradient were electrophoresed in 0.4 or 0.7% agarose gels using a BRL Horizontal gel apparatus with continuous buffer circulation (0.04M Tris, 0.02M Na acetate, 2mM Na₂EDTA at pH 8.0). Electrophoresis was at 2 volts/cm for 24 to 48 hours.

DNA samples (5µg or less) were digested with 10 units of enzyme under the conditions described by the suppliers of each enzyme for 2 hours at 37°C. The reactions were terminated with a stop solution of Na₂EDTA, SDS, bromophenol blue and glycerol. Samples were electrophoresed in a 1% agarose gel using the conditions described above.

Electron Microscopy

DNAs were extracted from agarose gels using the freeze-squeeze tech-

nique of Thuring et al. (10). Samples were spread for electron microscopy using the aqueous procedure described by Ferguson and Davis (11). RFII ϕ X174 DNA (5386 base pairs) was used as an internal standard. Grids were examined using a Philips 300 electron microscope. Photographs were taken, enlarged using a projector, traced, and measured.

RESULTS

A comparison of the mtDNA removed from the open circular/linear region with that from the supercoiled region of a CsCl-ethidium bromide gradient reveals some striking differences as shown in Figure 1. The open circular/linear mtDNA profiles of N. tabacum, P. vulgaris, and Zea mays (lanes 1 through 3, respectively) are essentially identical, all of them running as broad, diffuse bands. However, the supercoiled mtDNA profiles of these same plants (lanes 4 through 6, respectively) are significantly different among the three plant species in both the distribution and frequency of the various size classes. The P. vulgaris and Z. mays mtDNAs exhibit a large number and a wide range of size classes. In the case of P. vulgaris the different sizes are present in roughly equal concentration while in Z. mays the two smallest molecules are present in substantially higher concentration than all the others. By comparison, the N. tabacum mtDNA is composed of a relatively small number and narrow range of size classes. It is possible that some of the bands present may be open circular forms of supercoiled mtDNA molecules that were nicked during isolation. Extraction and EM analysis of each of the bands is presently underway and should answer this question.

The two smallest size classes of the tobacco, bean and corn mtDNAs were extracted from agarose gels and the molecules measured in the electron microscope (see Table I). In the case of both N. tabacum and P. vulgaris, the larger of the two mtDNA molecules is twice the size of the smaller one suggesting that the larger molecule may be a dimer of the smallest size class. This is clearly not the case with the two smallest corn mtDNAs which differ by only 0.3 kilobases.

The 10.1 kb size class is sufficiently large to code for many of the N. tabacum mitochondrial RNAs. However, neither the P. vulgaris 1.9 kb molecule nor the Z. mays 1.8 kb mtDNA are large enough to code for more than a few RNAs. Thus either these small mtDNAs represent a subset of a larger molecule that is capable of coding for all the mitochondrial RNAs or the mitochondrial genomes of these plants are distributed over several size classes.

Figure 1. Electrophoresis of mtDNA from *N. tabacum* (lanes 1, 4), *P. vulgaris* (lanes 2, 5) and *Z. mays* (lanes 3, 6) tissue culture cells in 0.7% agarose. Lanes 1 through 3 are mtDNAs extracted from the open circular/linear region of cesium chloride/ethidium bromide gradients and lanes 4 to 6 are mtDNAs extracted from the supercoiled region.

The availability in our laboratory of several culture lines from Solanaceous species other than *Nicotiana tabacum* made it possible for us to compare the distribution of mtDNA size classes among species within a family and between different culture lines of the same cultivar. As can be seen in

Table I

Plant	Two Smallest Size Classes
<u>N. tabacum</u> , var. W38 established line	10.1 kilobases $\pm$ 0.2 20.2 kilobases $\pm$ 0.3
<u>P. vulgaris</u> , var. T.H.	1.9 kilobases $\pm$ 0.2 3.8 kilobases $\pm$ 0.2
<u>Zea mays</u> , var. B.M.	1.5 kilobases $\pm$ 0.2 1.8 kilobases $\pm$ 0.2

Supercoiled mtDNA of N. tabacum, P. vulgaris, and Z. mays was electrophoresed in a 0.4% agarose gel. The two lowest bands of each plant mtDNA were extracted, nicked, and the molecular weights of open circular molecules were measured and compared with ϕ X174 RFII DNA, an internal marker.

Figure 2, the supercoil pattern of the established N. tabacum W38 line (lane 3, Figure 2) is similar to the Solanum dulcimera profile (lane 5) and the faint pattern of Datura stramonium mtDNAs (lane 6), while the N. rustica pattern (lane 2) resembles that of the new N. tabacum cell line (lane 4). Surprisingly though, the supercoiled mtDNA populations of the old (lane 3) and recently initiated (lane 4) N. tabacum W38 culture lines are dramatically different both in the range and frequency of their size classes. To see whether the differences in the supercoil patterns reflected a significant difference in sequence organization, we compared the restriction digest patterns of the mtDNA from the old and new cell lines. As can be seen in Figure 3, the restriction patterns are very similar. In fact, with the exception of one or two fragments, they are identical. Thus, although substantial differences exist in the size classes of mitochondrial DNA between the old and new culture lines, the basic sequence organization is essentially the same.

In Podospira anserina there are significant alterations in the mitochondrial DNA patterns as the cultures reach a stationary phase and begin to senesce (12). Similarly, different size classes of mtDNA in S. cerevisiae appear to be dependent on the growth phase of the cells, the maximum range of sizes being present during late log phase of growth (13). To see whether a similar change in the frequency or distribution of mtDNA molecules might occur in plant suspension culture cells, we examined the mtDNA population at different stages of cell growth. Figure 4 shows the fresh and dry weight changes of the established N. tabacum W38 line over a 15 day period. The pattern of the supercoiled molecules isolated from cells harvested after 4, 8, 12 or 15 days in culture are shown in Figure 5. There is no detectable alteration in the distribution or frequency of the supercoiled mtDNA population as the

Figure 2. Electrophoresis of mtDNA from different Solanaceous culture lines in 0.7% agarose. Lane 2 is the mtDNA of *N. rustica*, lane 3 the mtDNA of the established *N. tabacum* W38 line, lane 4 the mtDNA of a recently-established *N. tabacum* W38 line, lane 5 is the mtDNA of *S. dulcimer* and lane 6 is the mtDNA of *D. stramonium*. Lanes 1 and 7 are a mixture of a Hind III digest of lambda DNA and a Hae III digest of ϕ X174.

cells move from log phase growth to a stationary and senescing culture.

DISCUSSION

We have demonstrated in this paper that it is possible to obtain signi-

Figure 3. Gel electrophoresis of restriction digests of mtDNA from the new *N. tabacum* W38 cell line (lanes 2, 4, 6 and 8) and the established *N. tabacum* W38 cell line (lanes 3, 5, 7 and 9). Lanes 2 and 3 are Bam HI digests of the mtDNA, lanes 4 and 5 are Hind III digests, lanes 6 and 7 are Sal I digests, and lanes 8 and 9 are Pst I digests. Lanes 1 and 10 are mixtures of Hind III digests of λ DNA and Hae III digests of ϕ X174 DNA. The approximate molecular weight of some of the fragments is given in kilobases.

ficant quantities of intact supercoiled mtDNAs from a wide range of plant cells in suspension culture. Since we use buffers and protocols similar to those used by others, we think the principal reason for our success may lie in the choice of tissue culture cells rather than whole plant tissue. Cultured cells are more uniform than whole plant tissue and can be disrupted more easily, presumably with less damage to the mitochondria. Cultured cells can also be manipulated easily and as we have shown (5), they are suitable for labeling mtDNA *in vivo*. We have not been any more successful than other investigators in our attempts to isolate significant quantities of supercoiled molecules from whole plants or plant parts and thus can not compare the size distribution of mtDNAs in whole plants with what we obtain from the same species in culture. However, restriction digests of the mtDNA from whole

Figure 4. Cells were harvested at the time points indicated, filtered and weighed to obtain a fresh weight measurement. The filtered cells were then dried for 2 days and reweighed to obtain a dry weight value.

plants and tissue culture cells are essentially identical (unpublished results), suggesting that the basic sequence organization is the same in both. Certainly the physical heterogeneity we have observed in culture has been reported in isolations of mtDNA from plant tissue so that physical heterogeneity is not an artifact of growth in culture.

We have also shown that there is no alteration in the supercoiled mtDNA pattern as W38 tobacco cells progress from log-phase growth into a stationary and senescing state. Neither have we observed any alteration in the size class of supercoils isolated from our older W38 culture over the past 18 months. However, as noted in the Results section, the distribution of size classes in the newly-established cell line is substantially different from that in the older line although the restriction patterns are similar. The reason for this difference is unknown. It may reflect plant to plant variation or mtDNA rearrangements that have occurred in culture. It is not unusual for plant cells in culture to undergo substantial changes in chromo-

Figure 5. A 0.7% agarose gel of *N. tabacum* supercoiled mtDNA isolated from cultures (1) 4 days, (2) 8 days, (3) 12 days and (4) 15 days after subculturing.

some number over time and in fact, we have found that the older W38 line has undergone an increase in chromosome number from 48 to approximately 100 in the four years this line has been in culture. The yield of mitochondria and mtDNA is five times higher in the older cell line, an increase possibly related to the change in genome size, and it may be that the chromosomal changes have influenced the mtDNA organization as well. Other culture lines of W38 are presently being established and mtDNA from each of these lines will be isolated and compared with the two lines described in this paper.

Our results demonstrate that there is significant variation among the five plant species examined, both in the range and the frequency of the size classes of mtDNAs. This diversity is in dramatic contrast to the situation found in animal systems where the mtDNA in all species is about 16 kilobases in size. Why plant mitochondria maintain such an array of different size DNAs is unknown. The genome may be dispersed among the many size classes or the different molecules may simply be the result of recombinations and rearrangements of a single unique size class. We are now in a position to begin answering these questions since we are able to isolate substantial quantities of plant mtDNAs intact and resolve the size classes on agarose gels. Future

papers will investigate the relationship of the different size classes to one another and the organization of plant mitochondrial genomes in detail.

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