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Genetics

Sequence homology among different size classes of plant mtDNAs

(supercoiled mtDNA/Southern hybridization/restriction enzyme analysis)

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ABSTRACT **Supercoiled mtDNAs were isolated from tissue culture cells of tobacco, bean, and corn, and the smallest size classes were used to study the relationships among the different size classes of each species through restriction digests and hybridizations. Three of the smallest tobacco mtDNAs [10.1, 20.2, and 28.8 kilobases (kb)], the two smallest bean mtDNAs (1.9 and 3.8 kb), and the two smallest corn mtDNAs (1.5 and 1.8 kb) were extracted from the gels and nick translated. The 10.1-kb tobacco mtDNA hybridizes to all the other tobacco mtDNA size classes and a large percentage of the tobacco mtRNAs. Restriction digests indicate that the 20.2-kb size class is a dimer of the 10.1-kb size class. The 1.9-kb bean mtDNA hybridizes to all but three of the bean mtDNA size classes and hybridizes to two mtRNAs. Restriction digests indicate that the 3.8-kb size class is a dimer of the 1.9-kb size class. The 1.5- and 1.8-kb corn mtDNAs, which do not have any *Hha* I restriction fragments in common, both hybridize to many of the same size classes of the corn mtDNA profile and, in addition, each hybridizes to a few size classes not recognized by the other. The 1.5- and 1.8-kb size classes both hybridize to two RNAs, one of which they appear to have in common. However, with both the 1.9-kb bean mtDNA and the two corn mtDNAs, the molecular sizes of the two RNAs exceed those of the respective DNAs. The possible role and origin of the many size classes are discussed.**

Although the mitochondrial genomes of many animal and fungal systems are well characterized, relatively little is known about the structure and organization of plant mtDNA. Electron microscopic investigations of mtDNA from a variety of systems including maize (1), soybean (2), and tobacco (3) have revealed that plant mitochondria have a heterogeneous population of circular DNA size classes ranging from 0.5 μm to >30 μm. By contrast, animal mitochondria have a single size class that is remarkably similar in size, varying from 5.3 μm in *Drosophila* to 5.0 μm in humans (4). Similarly, within a given fungal line, the mtDNA is generally found as a unique size class that, in the case of *Saccharomyces cerevisiae*, is 21–15 μm (5).

The major stumbling block in the analysis of plant mitochondrial genomes has been the difficulty encountered in isolating the mtDNAs intact. Recently, however, my colleagues and I have reported the isolation of intact supercoiled mtDNA from tobacco plant suspension culture cells (3). These supercoiled mtDNA size classes can be separated on agarose gels, and discrete size classes can be isolated. The availability of purified size classes has made it possible to begin an analysis of the mitochondrial genome organization of tobacco, bean, and corn.

MATERIALS AND METHODS

Chemicals and Enzymes. Chemicals and buffers were obtained from regular commercial sources. Restriction enzymes were purchased from Bethesda Research Laboratories (Rockville, MD), DNAase was from Worthington, and biochemicals and isotopes were from New England Nuclear.

Plant Material. *Nicotiana tabacum*, variety Wisconsin 38, suspension culture cells were grown in Linsmaier and Skoog liquid medium (6) containing indoleacetic acid (3.0 mg/liter) and N^6-dimethylallyladenine (0.3 mg/liter). *Phaseolus vulgaris*, var. Taylor's Horticultural, was the gift of I. Sussex, Yale University. The cells were grown on MB5 medium (7). *Zea mays*, var. Black Mexican, culture was the gift of B. Gegenbach, University of Minnesota. The cells were grown in Linsmaier and Skoog basal liquid medium supplemented with 2,4-dichlorophenoxyacetic acid at 2 mg/liter, thiamine at 10 mg/liter, glutamine at 146 mg/liter, and lysine at 182 mg/liter.

mtDNA. Mitochondria and mtDNA were isolated as described (3). The procedure involves the following: (*i*) cell breakage with a French pressure cell, (*ii*) differential centrifugation to isolate a mitochondrial pellet, (*iii*) treatment with DNase at 100 μg/ml for 30 min at 20°C, (*iv*) a sucrose step gradient to purify the mitochondria, and (*v*) lysis of the purified mitochondria and centrifugation on a CsCl/ethidium bromide gradient.

DNA Gel Electrophoresis and Transfer. Supercoiled mtDNAs were subjected to electrophoresis in 0.4% or 0.7% agarose gels with continuous buffer circulation (40 mM Tris base/20 mM sodium acetate/2 mM Na_2EDTA, pH 8.0) at 2 V/cm for 24–48 hr. DNAs were extracted from gels by using the freeze technique of Thuring *et al.* (8). Gels were transferred to nitrocellulose as described by Southern (9). Restricted DNA samples were subjected to electrophoresis in 1% gels.

Nick Translations and DNA·DNA Hybridizations. Isolated mtDNA was nick translated by using the Bethesda Research Laboratories nick-translation reagent kit, and the products were extracted with phenol precipitated by addition of ethanol. The specific activity of the samples was 10^7–10^8 dpm/μg of template DNA (1 Bq = 60 dpm). Filter hybridizations were done according to the procedure of Jeffreys and Flavell (10).

RNA Isolation, Electrophoresis, and Transfer. Total tobacco RNA was isolated from cells by using the procedure of Bonnet and Lord (11). mtRNA was isolated from purified mitochondria by phenol extraction followed by ethanol precipitation. The RNA was then treated with RNase-free DNase, followed by phenol extraction and reprecipitation. RNAs were subjected to electrophoresis in 2% agarose/5 mM methylmercuric hydroxide according to the procedure of Bailey and Davidson (12). When the RNAs were to be extracted, low-melting-point agarose (Bethesda Research Laboratories) was used. The RNA bands were cut out, the agarose was melted, and the samples were phenol extracted and precipitated with ethanol. RNA gels were transferred to Schleicher & Schuell Trans-a-Bind paper, the RNAs were hybridized, and the hybrids were washed as described by Alwine *et al.* (13).

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Abbreviations: kb, kilobase(s); MDal, megadalton(s).

Electron Microscopy. Samples were spread by using the aqueous procedure of Ferguson and Davis (14). RFII ϕX174 DNA was used as an internal standard.

RESULTS

The gel electrophoresis patterns of tobacco, bean, and corn mtDNAs from the supercoil region of a CsCl/ethidium bromide gradient (3) are shown in lanes 2, 3, and 4 of Fig. 1. The gel illustrates both the physical heterogeneity of plant mtDNAs and the diversity of size classes among different plants. Also included on the gel (lane 1) is an example typical of the mtDNA extracted from the open-circular/linear region of a CsCl/ethidium bromide gradient.

The tobacco mtDNA gel pattern is relatively simple when compared with those of bean and corn. Depending on the agarose concentration used, one can distinguish 10–12 bands in the tobacco pattern and 20–30 bands in the corn and bean profiles. In the case of the tobacco mtDNA, the actual number of distinct size classes is less than the number of bands visible in the gel because at least the 10.1-kb molecule is present in both a supercoiled and a nicked supercoiled form, giving rise to two bands from this single size class.

The mtDNA pattern of these plants, especially the bean profile (Fig. 1, lane 3), bears some resemblance to an oligomeric series. If the different size classes did constitute a simple oligomeric series, the restriction pattern of the total mtDNA population would also be relatively simple. However, as is shown by the restriction digests of total tobacco, bean, and corn mtDNAs (Fig. 2), this is not the case.

Tobacco mtDNA. Three of the smallest tobacco mtDNAs (10.1, 20.2, and 28.8 kb) were extracted from the agarose gels and nick translated, and each was hybridized to a Southern blot of the total tobacco mtDNA profile. As shown in Fig. 3, each of the size classes hybridizes both to itself and to each of the other bands visible in the ethidium bromide-stained gel, indicating that all the mtDNAs are related to some degree. To evaluate the extent of homology among the three smallest size classes, the nick-translated samples were digested with four restriction endonucleases and compared on an agarose gel (Fig. 4). With all four enzymes, the two smallest size classes have identical restriction patterns, indicating that the 20.2-kb size class is a dimer of the 10.1-kb molecule. Estimates based on summation of the molecular sizes of the *Eco*RI, *Hin*dIII, and

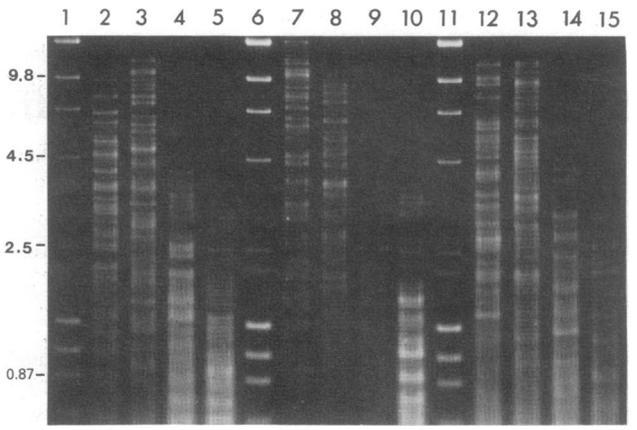

FIG. 2. *Eco*RI, *Hin*dIII, *Hha* I, and *Hae* III restriction digests, respectively, of tobacco (lanes 2–5), bean (lanes 7–10), and corn (lanes 12–15) mtDNAs were subjected to electrophoresis in a 1% agarose gel. Lanes 1, 6, and 11 are a mixture of a *Hin*dIII digest of ϕX174 DNA. Approximate molecular sizes (in kb) of selected λ phage and ϕX174 fragments are shown.

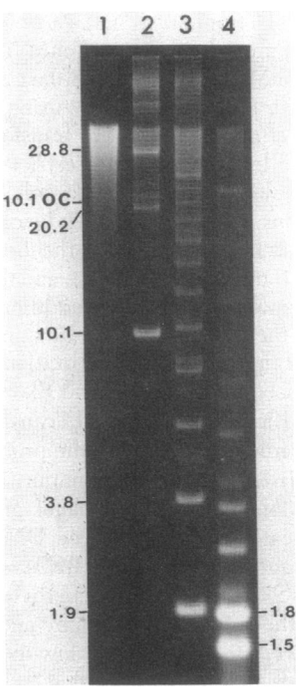

FIG. 1. Tobacco mtDNA from the open-circular/linear region of a CsCl/ethidium bromide gradient (lane 1) and tobacco (lane 2), bean (lane 3), and corn (lane 4) mtDNAs from the supercoil region of CsCl/ethidium bromide gradients were subjected to electrophoresis in a 0.7% agarose gel. The supercoiled forms of the 28.8-, 20.2-, and 10.1-kb tobacco mtDNAs, the 1.9- and 3.8-kb bean mtDNAs, and the 1.5- and 1.8-kb corn mtDNAs are labeled, as is the open-circular (OC) form of 10.1-kb tobacco mtDNA.

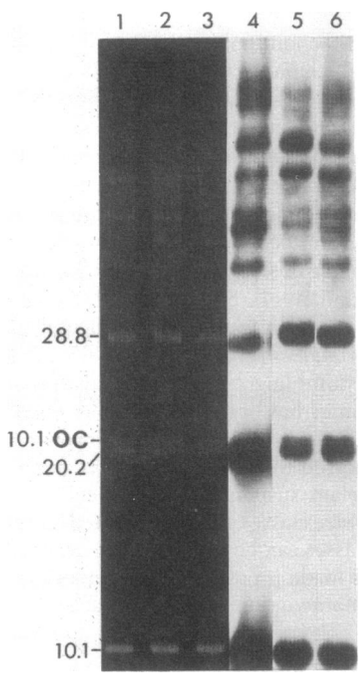

FIG. 3. Hybridization of the 10.1-kb (lane 4), 20.2-kb (lane 5), and 28.8-kb (lane 6) tobacco mtDNAs to a Southern blot of total tobacco mtDNA that had been subjected to electrophoresis in a 0.4% agarose gel. Lanes 1, 2, and 3 are the ethidium bromide-stained gel pattern of tobacco mtDNA. The supercoiled forms of the 28.8-, 20.2-, and 10.1-kb tobacco mtDNAs are labeled, as is the open-circular (OC) form of the 10.1-kb molecule.

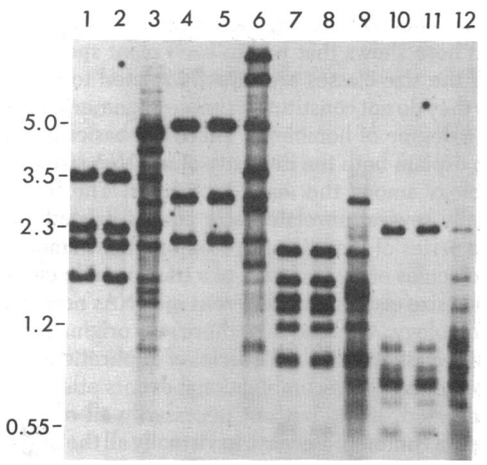

FIG. 4. The *Eco*RI (lanes 1–3), *Hin*dIII (lanes 4–6), *Hha* I (lanes 7–9), and *Hae* III (lanes 10–12) restriction digests of the nick-translated 10.1-, 20.2-, and 28.8-kb tobacco mtDNAs, respectively, were subjected to electrophoresis in a 1% agarose gel. The approximate molecular sizes of several of the fragments are shown.

Hha I restriction fragments are ≈10.1–10.2 kb, in close agreement with electron microscopy measurements of the smallest molecule. The *Hae* III fragments total only 6.3 kb unless one assumes multiplicities of 2 for the top fragment and 3 for the fourth fragment down, in which case the total is also ≈10.1-kb.

Although the digests of the 28.8-kb molecules are more complex than the corresponding digests of the 10.1-kb molecule, they also contain fragments of the same molecular sizes as those found in the smaller molecule. Summation of the fragment sizes gives estimates of ≈34 kb for the *Hin*dIII digest and 32 for the *Eco*RI digest. These estimates are slightly larger than the value derived from electron microscopy measurements, suggesting that there is some heterogeneity within this size class.

Hybridization of the 10.1-kb size class to a blot of seven mtRNAs that had been extracted from a CH_3HgOH-containing gel and rerun along with a sample of total tobacco RNA and total tobacco mtRNA is shown in Fig. 5. There is slight hybridization to the total tobacco RNA, which would include some mtRNA as well as plastid, cytoplasmic, and nuclear RNA, and strong hybridization to the lane of total mtRNA and six of the seven purified RNAs. Only the lowest size class (4S) RNA (band 1), which is presumably composed of tRNAs, failed to hybridize strongly with the 10.1-kb mtDNA (Fig. 5*b*, lane 9). Whether this reflects an absence of tRNA genes in this size class or problems in the transfer or hybridization conditions remains to be seen. In any event, at a first approximation, the 10.1-kb molecule appears to code for a large percentage of the tobacco mtRNAs.

Bean mtDNAs. The two smallest mtDNAs (1.9 and 3.8 kb) of the bean mtDNA profile were also isolated from gels, nick translated, and used in hybridization and restriction digest studies. Fig. 6*a* shows the hybridization of the 1.9-kb bean mtDNA to the total mtDNA profile. The 1.9-kb size class hybridizes to all but three of the ethidium bromide-stained bands, indicating that there is homology among most of the bean mtDNAs. Digestion of the two smallest bean mtDNAs with *Hha* I is shown in Fig. 6*b*. Summation of the molecular sizes of the fragments gives an estimate of 1.9 kb, which agrees well with the electron microscopy measurements of these molecules. The patterns of both size classes are identical, indicating that the 3.8-kb molecule is a dimer of the 1.9-kb size class. Digests of the next two largest molecules also appear to be identical with that of the 1.9-kb size class, suggesting that they may be oligomers of the 1.9-

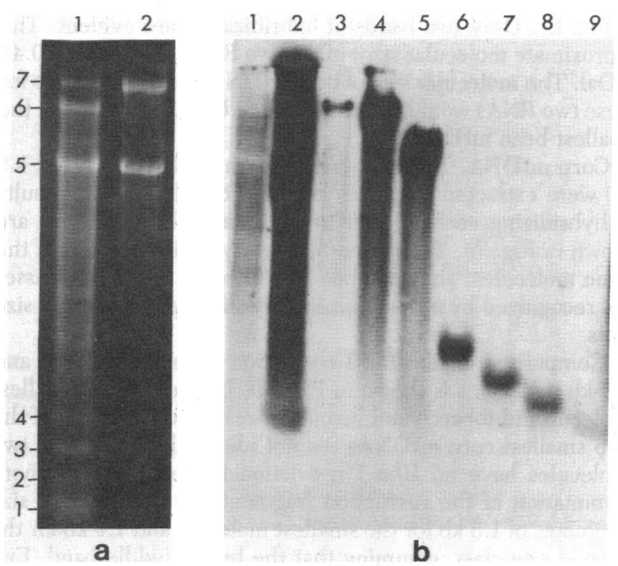

FIG. 5. (*a*) Electrophoresis patterns of total tobacco mtRNA (lane 1) and total tobacco RNA (lane 2) in a 2% CH_3HgOH gel. (*b*) The RNAs from the seven labeled bands shown in *a* were extracted, and ^{32}P-labeled 10.1-kb tobacco mtDNA was hybridized to a blot of a 2% CH_3HgOH gel of total tobacco RNA (lane 1), total tobacco mtRNA (lane 2), and the seven extracted mtRNAs (lanes 3–9).

kb size class as well (unpublished results). As the total bean mtDNA *Hha* I digest is more complex, however, at least a few of the higher molecular weight size classes must contain additional sequences not found among the lower molecular weight molecules.

Hybridization of the 1.9-kb [1.24 megadalton (MDal)] size class mtDNA to a blot of the total bean mtRNA pattern is shown

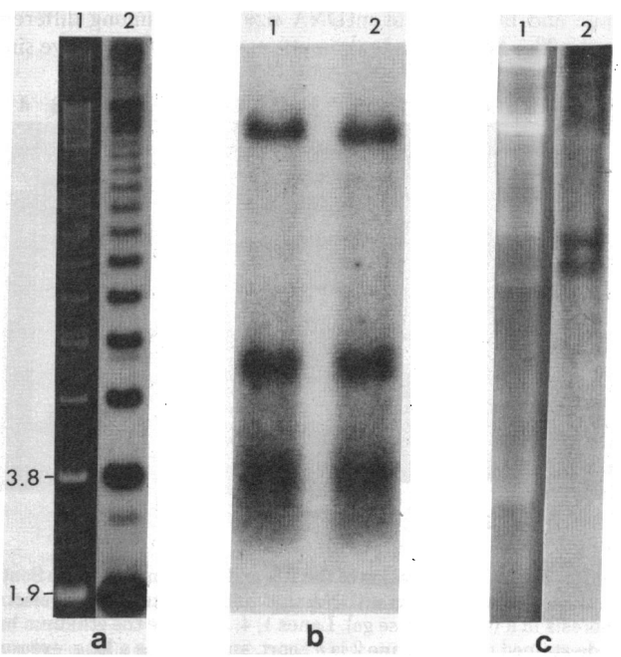

FIG. 6. (*a*) Hybridization of the 1.9-kb bean mtDNA to a Southern blot of bean mtDNA that had been subjected to electrophoresis in a 0.7% agarose gel (lane 2). Lane 1 is the ethidium bromide-stained gel. (*b*) *Hha* I restriction digests of the 1.9- (lane 1) and 3.8- (lane 2) kb bean mtDNAs were subjected to electrophoresis in a 1.5% agarose gel. (*c*) Hybridization of the 1.9-kb mtDNA to a blot of total bean mtRNA that had been subjected to electrophoresis in a 2% CH_3HgOH gel (lane 2). Lane 1 is the ethidium bromide-stained gel pattern of the RNA.

in Fig. 6c. Only two bands of hybridization are evident. The approximate molecular sizes of the two RNAs are 0.48 and 0.44 MDal. The molecular size of duplex DNA required to code for these two RNAs would be 1.84 MDal, 0.6 MDal larger than the smallest bean mtDNA.

Corn mtDNA. The two smallest corn mtDNAs (1.5 and 1.8 kb) were extracted from gels and nick translated. The results of hybridizing each in turn to the total mtDNA pattern are shown in Fig. 7a. The two molecules hybridize to many of the same molecules, and each one hybridizes to a few size classes not recognized by or recognized only slightly by the other size class.

Comparison of the *Hha* I restriction digests of the 1.5- and 1.8-kb molecules is shown in Fig. 7b. Unlike the two smallest molecules of tobacco and bean, the restriction patterns of the two smallest corn mtDNAs are not identical. In fact, the two molecules have no *Hha* I restriction fragments in common. Summation of the restriction fragments gives molecular size estimates of 1.5 kb for the smallest molecule and 1.9 kb for the second size class, assuming that the broad middle band (Fig. 7b) represents two overlapping fragments. These values are in close agreement with electron microscopy measurements.

Hybridization of the 1.5- and 1.8-kb molecules to a blot of total corn mtRNA is shown in Fig. 7c. The 1.5-kb (0.98-MDal) molecule hybridizes to two RNAs that have approximate molecular sizes of 0.6 and 0.5 MDal. The 1.8-kb (1.17-MDal) mtDNA appears to hybridize to the same 0.6-MDal RNA and to a second RNA having a molecular size of 0.76 MDal. In both cases, the sum of the molecular sizes of the RNAs exceeds the molecular size of the mtDNA.

DISCUSSION

There is considerable mtDNA size heterogeneity within individual plant species as well as substantial differences in the range and frequency of mtDNA size classes among different plants. This situation is in dramatic contrast to the relative simplicity of animal and fungal mitochondrial genomes. The work presented here shows that within each plant species, most, if not all, of the size classes are directly related to one another. Although they do not constitute a simple oligomeric series, most do share a degree of homology. There are basically two possibilities to explain both the diversity of mtDNA size classes and the homology among the many molecules. The first assumes that initially several unrelated size classes existed and either through a series of illegitimate recombinations among the different molecules or by insertion of a transposable element into the various size classes, the different mtDNAs now share a degree of homology. Alternatively, there may originally have been a single size class and through a series of duplications, deletions, rearrangements, and recombinational events other size classes were generated, many but not necessarily all of which share sequences in common. Because in virtually all the other systems studied there is one mtDNA size class that carries the entire mitochondrial genome, it seems more likely from an evolutionary viewpoint that this second alternative reflects the actual course of events.

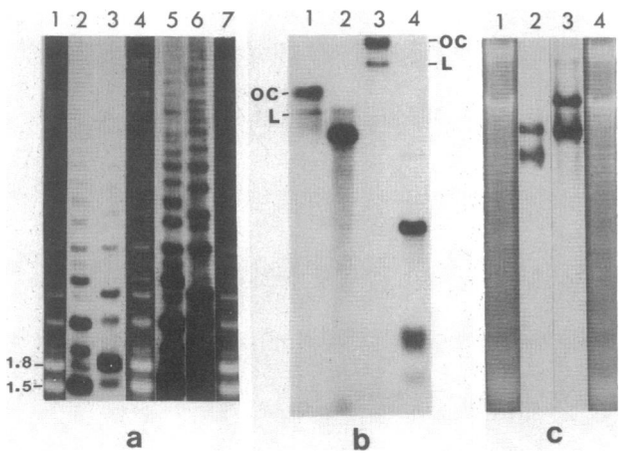

FIG. 7. (a) Hybridization of the 1.5- and 1.8-kb mtDNAs to a Southern blot of the total corn mtDNA that had been subjected to electrophoresis in a 0.7% agarose gel. Lanes 1, 4, and 7 are the ethidium bromide-stained pattern. Lane 2 is a short, and lane 5 is a long, exposure of the 1.5-kb hybridization, and lanes 3 and 6 are hybridizations of the 1.8-kb molecule. (b) *Hha* I restriction digests of 1.5-kb (lane 2) and 1.8-kb (lane 4) corn mtDNAs that had been subjected to electrophoresis in a 1.5% agarose gel. Lanes 1 and 3 are the undigested open-circular (OC) and linear (L) forms of the 1.5- and 1.8-kb molecules, respectively. (c) Hybridization of the 1.5-kb (lane 2) and 1.8-kb (lane 3) molecules to a blot of total corn mtRNA that had been subjected to electrophoresis in a 2% CH_3HgOH gel. Lanes 1 and 4 are the ethidium bromide-stained pattern of the corn mtRNA.

In the case of tobacco, it is initially tempting to speculate that the essential mtDNA is the 10.1-kb molecule, which hybridizes to the other size classes and seems to code for most of the tobacco mtRNAs. However, unless the tobacco mitochondrial genome codes for fewer proteins than all the other systems studied or it has overlapping reading frames or uses the coding capacity of both strands, it is simply too small to code for more than three or four proteins in addition to three rRNAs and 20 tRNAs. Forde *et al.* (15), while studying corn mitochondrial proteins, identified 18 polypeptides in the 8,000–54,000 dalton range. Although they have not demonstrated that all these peptides are unique or functional, one would still expect from the work with fungal and mammalian mitochondria that at least 8–10 unique polypeptides would be coded for by plant mitochondria. Since the 20.2-kb molecule is a dimer of the 10.1-kb size class, the next candidate for the basic mtDNA would be the 28.8-kb molecule. This molecule contains all the restriction fragments found in the 10.1-kb molecule, and if the additional fragments are unique and not rearrangements of sequences found in the 10.1-kb molecule, then it is possible that it could be the master mtDNA for tobacco. Preliminary digests of some of the higher molecular weight size classes suggest that each successively larger molecule possesses most, if not all, of the sequences present in the smaller size classes, in addition to new ones. If this proves true for all the larger size classes, then there must be a single tobacco size class carrying all the tobacco mitochondrial genes.

Clearly the 1.5- and 1.8-kb corn mtDNAs cannot code for all the corn mtRNAs. In this case, one would have to look to one of the larger molecules to which both these small classes hybridize to find a unique size class capable of coding for all the corn mitochondrial genes. Neither can the bean 1.9-kb molecule code for all the bean mtRNAs. Again, one would have to look to the larger molecules to find a master bean mtDNA. However, the situation may be more complex because there are three size classes to which the 1.9-kb bean mtDNA does not hybridize. Either there is a larger molecule that has sequences in common with these three molecules and the other size classes, or there are at least two unique classes of bean mtDNAs.

If there is a single molecule carrying all the mitochondrial genes of a plant, then what role if any is played by the large number of other size classes? One possibility is that they provide an additional means of gene regulation. The reiterated genes may be analogous to amplified sequences, allowing plant mitochondria to increase the rate of synthesis or maintain higher concentrations of specific RNAs of their corresponding proteins.

Different size classes or specific sequences might vary in different cell types, at different stages of development, or in response to different stimuli. Gene amplification has been described in several eukaryotic systems and tandem duplication leading to gene amplification has been demonstrated in bacteria. Plant mitochondrial genomes may simply represent another example of this type of gene regulation.

Alternatively, the many size classes may be defective molecules analogous to the mtDNAs found in the petite mutants of yeast. But unlike yeast, which is a faculative aerobe in which defective mtDNAs can replicate freely to produce petite mutants, plant cells would need to maintain a complete copy of their genome for survival. The stopper mutants of *Neurospora*, which are characterized by irregular periods of growth and nongrowth, may represent an analogous situation. Lambowitz and his colleagues (16) have found that the predominant mtDNA species from five stopper mutants contained deletions of up to 27 out of 60 kb. However, these same mutants also contained low levels of less defective mtDNAs, and the authors speculated that defective mtDNAs can continue to exist as long as low levels of relatively intact mitochondrial genomes are retained to sustain growth.

As shown in *Results*, both the bean and corn mitochondrial genomes have size classes that apparently carry only fragments of one or two genes. The presence of partial genes suggests that at least some and perhaps many of the size classes present in plant mitochondrial genomes are defective molecules. If many of the mtDNAs are indeed simply defective copies, there would be no selective pressure to maintain specific size classes and one might expect variations in the supercoil population over time or between different lines of the same species. Comparison of the supercoiled mtDNA pattern of the tobacco cell line used in this study with another independently initiated cell line of the same variety shows substantial differences in the range and frequency of size classes (unpublished results). However, despite these differences, the mtDNA restriction patterns of the two lines are nearly identical, suggesting that as long as certain basic sequence information is maintained, the organization of plant mitochondrial genomes into particular size class profiles may be relatively unimportant.

Plant mitochondrial genomes may be neither as genetically heterogeneous nor as complex as the physical heterogeneity and the apparent restriction fragment complexity might suggest. They may in fact be derived from a single molecule, and the other size classes may simply be amplified genes or defective mtDNAs differing in the fraction of the genome they possess.

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