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Molecular analysis of the inheritance and stability of the mitochondrial genome of an inbred line of maize

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Summary. We have investigated the inheritance of the mitochondrial DNA (mtDNA) restriction endonuclease digestion patterns of maize inbred line B37N in individual plants and pooled siblings in lineages derived from five separate plants in the third generation following successive self-pollinations. The restriction fragment patterns of the different mtDNA samples were compared after digestion with five endonucleases. No differences were visible in the mobilities of the 199 fragments scored per sample. Hybridization analysis with two different cloned mtDNA probes, one of which contains homologies to a portion of the S2 plasmid characteristic of cms-S maize, failed to reveal cryptic variation. The apparent rate of genomic change in maize mtDNA from inbred plants appears to be very slow, compared with the faster rates of change seen in maize tissue cultures and with the documented rapid rate of inter- and intraspecific variation for mammalian mtDNA.

Key words: Mitochondrial DNA – Maize – Restriction endonuclease digestion patterns

Introduction

The mitochondrial genome of maize is very large: at least 475 kb of unique sequence based on summation of restriction fragment sizes (Borck and Walbot 1982) and approximately 570 kb based on assembly of restriction fragment cleavage maps (Lonsdale 1984). The genome is hypothesized to be organized into several large chromosomes arising by intramolecular recombination in a "master" chromosome; subsequent intermolecular recombination gives rise to many chromosome types containing partially overlapping sequence composition (Lonsdale et al. 1983). The genomic complexity is reflected in the observation that not all of the restriction fragments are present in equal stoichiometry suggesting that subsets of the genome have been differentially amplified or diluted (Borck and Walbot 1982). Small linear and circular genomes, often termed mitochondrial "episomes" or "plasmids," also exist in all maize lines thus far examined (Kemble et al. 1980, 1983; Weissinger et al. 1982), and their size and concentration relative to the main genome varies considerably.

In addition to the complexity resulting from a large size and a multiple chromosome organization in the maize mitochondrial genome, there are four basic types of mtDNA isolated from male-fertile, normal (N) cytoplasms, and from the three cytoplasmic male-sterile types (T, C or S). Restriction digestion patterns of these four mtDNA types are readily distinguished (Pring and Levings 1978). Borck and Walbot (1982) determined that only 50% of the restriction fragment size classes are conserved in all four mtDNA types and that no more than two-thirds of the fragment size classes are shared between any pair of mtDNA types. Variation in restriction fragment patterns are also found within cytoplasmic types, for example within the C group (Pring et al. 1980). In N cytoplasms, one or more mtDNA fragment differences distinguish modern inbred lines (McNay et al. 1983; Newton and Walbot 1985). McNay et al. (1983) have demonstrated that three restriction pattern classes exist within N in terms of the organization of the "S" region, which shares homology to the S1 plasmid from S type male sterile plants.

The differences in restriction fragment sizes could reflect changes in the distribution of restriction sites on the genome arising from base substitution or more complex genomic rearrangements. However, because the time of divergence of the various cytoplasmic types is unknown, it is impossible to calculate the rate of genomic change in maize mtDNA or to

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understand the relative contributions of base change and genomic rearrangement in the generation of the polymorphisms seen today.

That the maize mitochondrial genome can change rapidly is clear from the appearance of novel mtDNA types in plants regenerated from tissue culture. For example, Gengenbach et al. (1981) detected several changes in restriction fragment sizes in mtDNA of male-fertile plants regenerated after selection for fungal toxin resistance in tissue cultures of T tissue. Chourey and Kemble (1982) demonstrated that the mtDNA in non-regenerable, friable callus of S male-sterile tissue is deficient in the S1 and S2 linear plasmids characteristic of this genotype while the compact callus derived from the same initial culture, as well as plants regenerated from it, still contained the S plasmids. Further evidence that maize mtDNA can change during extended periods of tissue culture was provided by McNay et al. (1984) who compared mtDNA restriction patterns from plants of the Black Mexican Sweet line to those from cell suspension cultures of the same line. Their analysis with two different endonucleases showed that both quantitative and minor qualitative differences in the resultant fragmentation patterns had arisen during the four years of culture, changes they suggested could be due to alterations in the relative copy number of the circular molecules comprising the main mitochondrial genome.

Considering the existence of mtDNA variability found in the four cytoplasmic types of maize, the discovery of rapid mtDNA change in tissue culture, and the proposal of Lonsdale et al. (1983) that maize mitochondrial genomes may exist in multiple forms which arise by intra- and intermolecular recombination, it is important to determine how stable the mtDNA is within the plant. We have examined the inheritance of mtDNA restriction patterns in individuals from the B37N inbred line to determine whether the mtDNA is homogeneous and stable. Continual self-pollination could allow sorting out of any pre-existing variability in the multiple molecular forms. While many generations might be required to sort out pure cytoplasmic types, relatively short time spans might be sufficient to show differences, indicative of variation, in the stoichiometries of restriction fragments. Would we detect changes due to base substitution or rearrangement over a few plant life cycles, as might be predicted from the rapidity of change in tissue culture?

Materials and methods

Our starting material was a random sampling of 10 lb of bulked seed from the inbred B37N line kindly provided by Pioneer Hi-Bred. In 1981 plants grown from many kernels (probably derived from different ears) were self-pollinated. Kernels from several of the progeny ears, chosen at random, were grown in 1982 and again selfed. In 1983, 10 separate lineages were grown. As sources for mtDNA, unfertilized cobs of length 9–14 cm (10–15 g) were taken from individual plants within seven of these families, while cobs were pooled from five plants in each of the remaining three families. We compared these mtDNAs to one another and to mtDNA from a pooled sample of over 300 seedling shoots of the original B37N bulk seed.

MtDNAs were isolated from the cobs following a method similar to that used by Kemble et al. (1980) for shoots of young seedlings. The 1,000×g first pellet was used to prepare "chloroplast" DNA (heavily contaminated with mtDNA in these tissues) after DNase treatment. MtDNAs were restricted with up to 10 Units/μg of BamHI, XhoI, HindIII, PstI or SmaI following the supplier's (BRL) recommended procedures. Agarose gel electrophoresis was conducted in 0.7% agarose, with 40 mM Tris pH 7.8, 5 mM NaAcetate, 10 mM Na_2EDTA buffer, at 0.8–1.0 V/cm for 24 h. Gels were stained with 1 μg/ml ethidium bromide for 20 min and photographed over a 254 nm UV transilluminator with Polaroid 667 film. The gels were acid treated for 10 min in 0.25 N HCl, base treated for 30 min (2 changes) in 1.5 N NaOH, 0.5 M NaCl, and neutralized for 60 min (2 changes) in 3 M NaCl, 0.5 M Tris pH 7.5. DNAs were transferred to nitrocellulose using Southern's method (1975).

Two plasmids, pMZ248 and pMZ37, from a BamHI library of B37N mtDNA in pBR322 (Newton et al., unpublished), were used as hybridization probes. Clone pMZ37 was chosen at random and contains two BamHI inserts of 6.9 and 5.3 kb. The second probe, pMZ248, contains a single 4.6 kb insert which is homologous to a portion of the S2 plasmid of cms-S. Probe DNA was labeled by nick-translation using ^{32}P-dCTP (Amersham) as described by Rigby et al. (1977). Spun-column chromatography on BioGel P10 columns in 1.5 ml Eppendorf tubes was used to remove the unincorporated deoxyribonucleotides. Prehybridization of the nitrocellulose blots was performed at 65 °C for 4 h in 10× Denhardts (Denhardt 1966), 2.5% Sarkosyl, 5× SSPE (Maniatis et al. 1982) with 200 μg/ml denatured salmon sperm DNA. The denatured, labeled DNAs were injected into the hybridization bags and incubated for 24 h at 65 °C. The blots were washed at 45 °C in 1% SDS, 10 mM Tris pH 8.0 for 60 min and then in 10 mM Tris pH 8.0 for 60 min prior to autoradiography.

Results

Plant pedigrees

The starting materials in our study were randomly selected kernels of the inbred B37 line with normal cytoplasm (B37N). Relationships among these samples, representing five maternal lineages, are shown in Fig. 1. In 1983 (Year 3, Fig. 1), mtDNA was isolated from

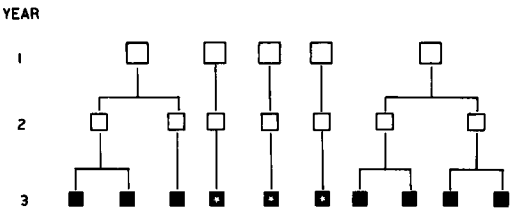

Fig. 1. Line drawing depicting lineages of tested B37N plants. The *black boxes* indicate the 10 separate plants tested. *Starred solid boxes* refer to families where mtDNA was made from a mixture of five plants each. *Unstarred solid boxes* refer to samples from individual plants

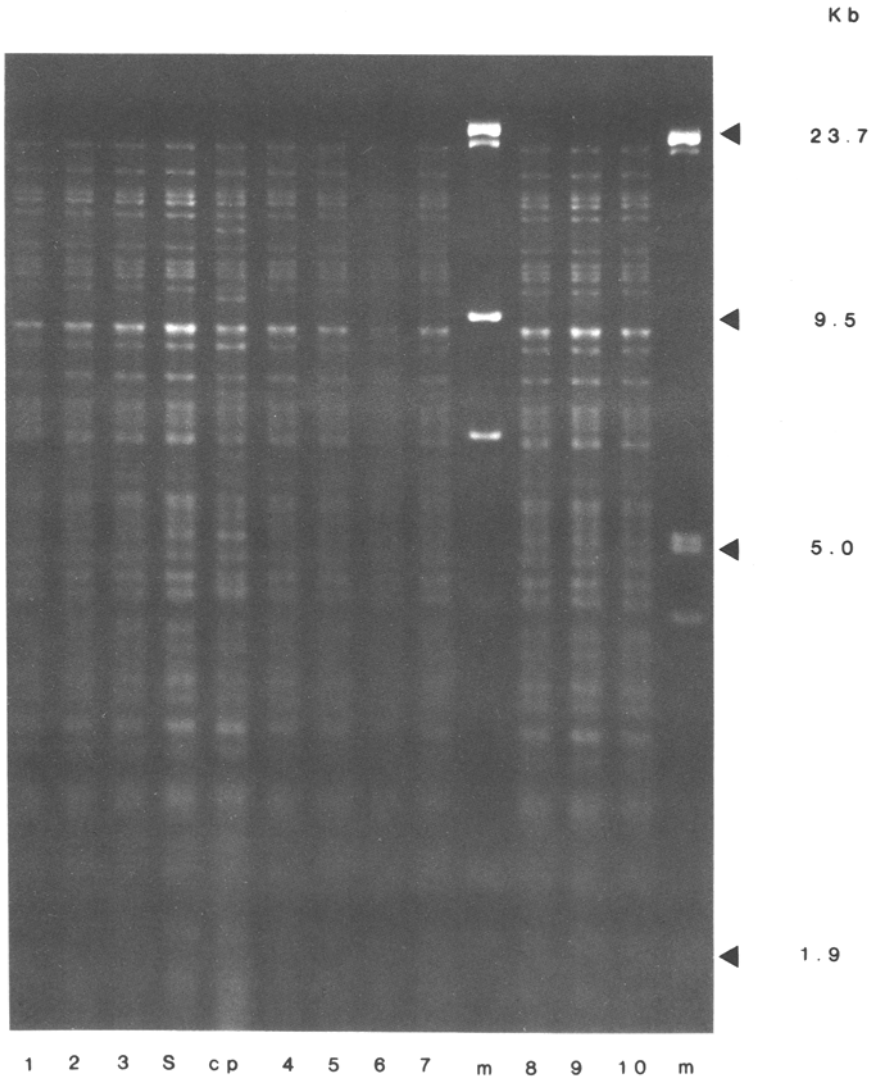

Fig. 2. Bam HI restriction enzyme profiles of mtDNA isolated from plants indicated in Fig. 1. *Lanes 1–7* contain DNA from individual plants; *lanes 8–10* are from pooled samples. *Lane S* is the standard isolated from the original bulk seed of the B37N line. *cp* is the plastid DNA-enriched sample from the B37N standard. *Lanes m* are the molecular weight markers: lambda DNA digested with HindIII (prior to *lane 8*) and double-digested with HindIII and EcoRI (on the *right hand side*)

seven individual third generation plants (solid boxes in Fig. 1) and three sets of pooled third generation plants (starred boxes) to compare with the mtDNA from the original bulk B37N plant material.

MtDNA was isolated from unfertilized ears (cobs), which we find to be an extremely good source of active mitochondria from individual plants (Newton and Walbot, unpublished). Each mtDNA sample was subjected to restriction endonuclease digestion by five enzymes and analyzed following gel electrophoresis. The BamHI restriction digestion patterns of all of the types of mtDNA samples are presented in Fig. 2. A possible source of artifactural variability could be expected from differential contamination of the mtDNA samples with chloroplast DNA (cpDNA). In an attempt to localize the position of plastid DNA restriction fragments, DNA was extracted from the initial 1,000×g pellet. Contrary to our expectations from studies on other tissues, we found that this first pellet includes large numbers of mitochondria, as well as plastids. The BamHI digest of such a plastid-mitochondrial DNA mixture is shown in Fig. 2, lane cp. Both mitochondrial and plastid DNA fragments are readily seen, but the purified mtDNA (all other lanes in Fig. 2) shows only relatively low amounts of certain cpDNA fragments prominent in the cp sample.

Comparison of restriction patterns

Upon visual inspection of the BamHI restriction fragmentation patterns (Fig. 2), no mobility differences could be discerned either between individuals with the same maternal parent or among all of the individuals examined. Further tests with additional restriction enzymes also showed no differences in fragment mobilites. Examples of the PstI, XhoI and HindIII frag-

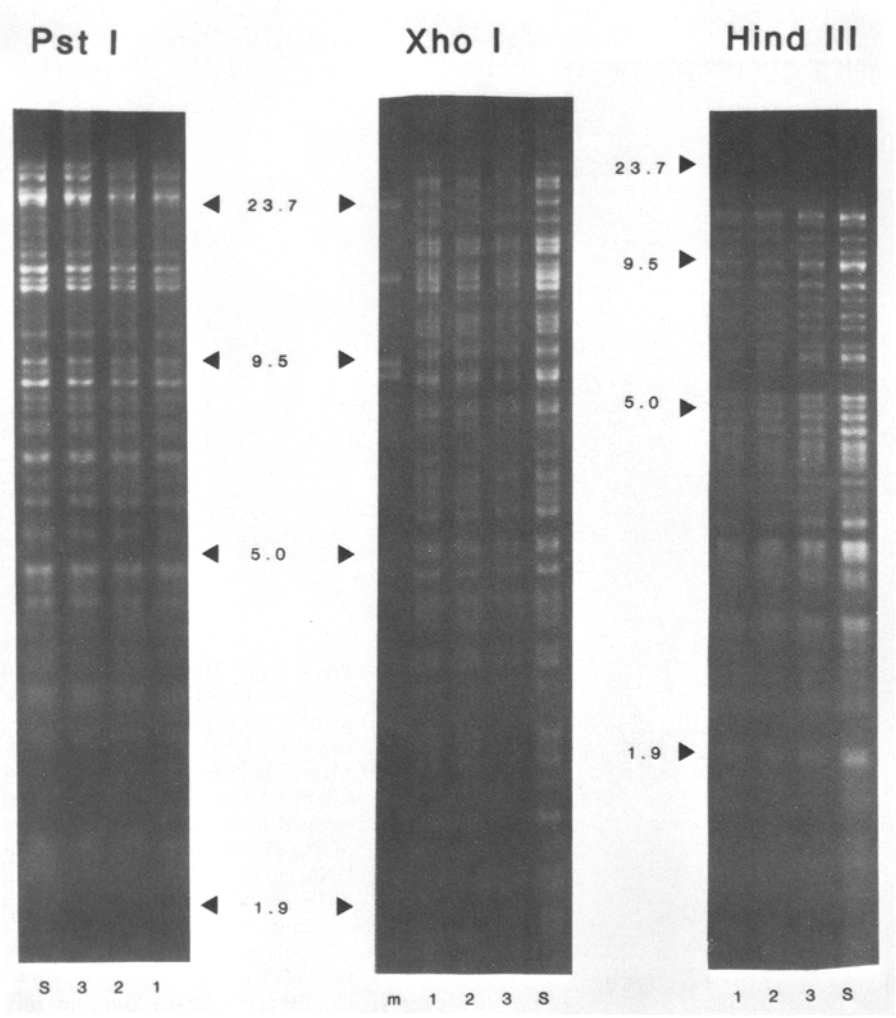

Fig. 3. Additional restriction digests of the mtDNA samples. *Lanes 1–3* are the same mtDNAs from individual plants as in Fig. 1, each digested with PstI, XhoI, and HindIII. *Lane S* is the standard as described above. *Lane M* is lambda DNA digested with SmaI. The numbers on the sides of the figure refer to the size of the marker DNA fragments, in kilobases

mentation patterns are shown in Fig. 3. (Complete data are not shown for all enzymes and all samples.) A total of 199 restriction fragments were resolved from each individual mtDNA sample in the five enzyme survey. The stained gels gave good resolution of fragments between 26 and 1.5 kb. Based on standard curve measurements, a change of 75 bp in the 1.5 kb range and changes of 500 bp in the 26 kb region of the gels would have been distinguishable (Southern 1979). Therefore, if rearrangements, large deletions or insertions were occurring, they would have been seen in this survey.

This analysis also represents a test for base substitutions which create or destroy restriction endonuclease recognition sites in the mitochondrial genome resulting in the gain or loss of restriction fragments. Five enzymes with different six-base recognition sequences were used, including an enzyme with a GC-rich recognition site (SmaI) and one with an AT-rich recognition site (HindIII). The nucleotide sequence of 1,194 bp (199 restriction sites × six base recognition sequence) were examined: a large sample compared to other studies of restriction site polymorphism in mtDNA, but only 0.2% of the maize mitochondrial genome. No changes in any of these restriction recognition sites were detected over the three generations represented in this experiment.

If the mitochondrial genome consists of multiple, large chromosomes plus plasmids, sorting out or differential replication could occur over several plant life cycles to produce mitochondria containing different representations of the total genomic complexity. Such processes would result in changes in the relative stiochiometries of restriction fragments representing regions of the different classes of chromosomes. Such a result is found in mtDNA isolated from tissue cultures, as discussed in our introduction. However, close examination of the restriction fragmentation patterns of mtDNA from plants revealed no changes in relative

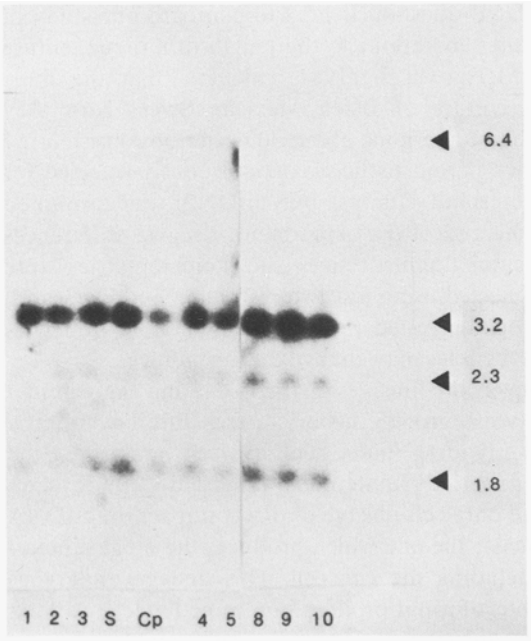

Fig. 4. Hybridization of nick-translated clone pZM248 to Southern blots of PstI digests. *Lanes 1–7* are mtDNAs from individual plants; *lanes 8–10* are the pooled samples. *Lane S* is the B37N mtDNA standard. *Lane Cp* is the plastid DNA-enriched sample

fragment stoichiometries comparing the individual seedling mtDNA preparations to pooled samples or to the original B37N parent.

Hybridization patterns of mtDNA samples

Although the lack of change in the relative mobilities of restriction fragments is a good indication that there are no novel fragments, the visual staining method would be unlikely to detect fragments present at 10% or less of the stoichiometry of neighboring fragments. In a restriction digest as complex as that of maize mtDNA, minor fragments may also be hidden by co-migration with other, non-homologus fragments. To test whether specific regions of the genome had undergone any significant rearrangement or alterations of endonuclease recognition sites, nitrocellulose blots of the gels described above were hybridized with radioactively-labeled cloned B37N mtDNA. Clone pZM37 carries 2 BamHI inserts of approximately 6.9 and 5.3 kb, and pZM248 has a single 4.6 kb BamHI insert, which represents sequences homologous to the S2 plasmid of S-type male sterile maize in the main mitochondrial genome of normal lines. An example of the pZM248 hybridization to a Pst 1 digest is shown in Fig. 4 (complete data set of hybridizations of each probe to each restriction digest is not shown). Each probe consistently hybridized only to the expected fragment size classes in each restriction digest. Furthermore, the pattern of hybridization with each sample was identical.

Data analysis

Our search for restriction fragment polymorphism or stoichiometric variation in B37N mtDNA failed to detect any changes in mtDNA organization in five maternal lineages over three generations. As the original 1981 plants represented a random sampling from a large collection of kernels of the inbred line and were unlikely to have shared the same female parent, our samples actually encompass more than three generations of maternal divergence. Because we found no changes we cannot calculate a rate of base substitution or genome rearrangement for maize mtDNA. However, our data can be used to set an upper limit to the generation of restriction site polymorphism that could arise as base changes in the endonuclease recognition sites we scored. The model of Nei and Li (1979) for estimating DNA divergence in a population assumes that random base-pair substitutions occur. If we assume, for the sake of calculation, that one fragment out of the 199 assayed did change (gained or lost a restriction site) in one of the mtDNA lineages examined we can calculate the upper limit to the rate of base-pair change as follows:

$(N_{xy}/N_o)^2 = e^{-2\lambda rt}$ where N_{xy} is the number of fragments in common at time t (198); N_o is the number of fragments in common at time 0 (199); $2\lambda t$ is the rate of nucleotide substitution per nucleotide per unit time; and r is the number of bases recognized by the restriction endonucleases used (six bases). We calculate a rate ($2\lambda t$) of 8.4×10^{-4} (variance 0.5×10^{-4}) base changes per nucleotide in three generations. Assuming a 600 kb mitochondrial genome size including the main chromosomes and plasmids, we calculate that this rate of substitution represents a change of *less than* 504 nucleotides (variance 30 bases) over three generations, or 160 nucleotides per generation.

Discussion

Given the proven variability of maize mtDNA in tissue culture lines of the N, T and S cytoplasmic types, we hypothesized that continuous self-pollinations of plants within a maize line should eventually allow detection of mtDNA restriction fragment polymorphisms and stoichiometric differences among the fragments. We failed to find any fragment

differences with our methods. This finding must be tempered by two considerations. First, the methods used produced a survey of the genome using convenient tests and were by no means conclusive. A better, but as yet unattempted method for plant mtDNA, would be to sequence various parts of the mitochondrial genome looking for specific base changes and rearrangements each generation. Second, our results apply only to B37N. Because the exact maternal ancestry to modern maize lines is unknown, and we do not understand the manner in which nuclear genes influence mtDNA composition (Borck and Walbot 1982), it is impossible at present to know whether B37N is a particularly stable genotype while the types examined in culture are particularly prone to variation.

One positive outcome of our study is that it illustrates the high stability of the mtDNA of the B37N genotype. Consequently bulked seed samples are likely to contain the same mtDNA composition throughout the population, and it is unnecessary to check individual plant mtDNA genotypes to insure uniformity. Differences in restriction patterns do occur within N cytoplasms from different inbred lines (McNay et al. 1983; Newton and Walbot 1985). Such differences among N types as well as between N, T, C and S may reflect rare events resulting in genomic rearrangements and the consequential change in the sizes and stoichiometries of several restriction fragments.

A rapid rate of mtDNA change has been found in studies of animal populations (Avise et al. 1979; Shah and Langley 1979). The rate of base substitution in mtDNA in a survey of populations of higher primates was 3.2×10^{-2} per year (Ferris et al. 1981). The origin of replication region, the "D" loop, is particularly variable and changes in this region can be used to distinguish individuals within a population (Upholt and Dawid 1977). Furthermore, Hauswirth and Lapis (1982) reported restriction fragment variation in a single maternal lineage of Holstein cows, with shifts between genotypes occurring within two generations. It is rather surprising that the 16 kb mitochondrial genome of mammals can be more variable from one generation to the next than the many times larger maize genome.

The detection of changes in maize mtDNA in tissue culture but not in our survey of mtDNA extracted from intact plant tissue, raises the question of how material in culture differs from that in a whole plant. The results of Chourey and Kemble (1982) studying S mtDNA demonstrated that two different callus morphological states differ in the state of the mtDNA. The callus incompetent to regenerate into plants had lost both S plasmids, while the callus capable of giving rise to plants retained the whole mtDNA genome including the S plasmids. However, loss and gain of restriction fragments in the main mitochondrial chromosomes in cultures of the T genotype was not associated with the capacity of these cultures to regenerate (Gengenbach et al. 1981).

A related question is how to compare our study of three plant generations to the length of a tissue culture period. McNay et al. (1984) calculate that the four-year-old culture of Black Mexican Sweet corn they studied had undergone about 300 cell divisions. During the culture period tissue was continuously selected for growth in small clumps, but mtDNA was examined only at the end of the experiment. Because differences in restriction fragment sizes and stoichiometries were readily detected using just two restriction endonucleases, the authors suggested that the relative stoichiometries of mtDNA circles may change during culture.

However, the lineages giving rise to the egg within a plant have a growth history quite different from a regularly dividing tissue culture cell mass. As the mtDNA is strictly maternally inherited (Conde et al. 1979), the only cell lineage of direct impact on mtDNA inheritance is the one which produces the megagametophyte, including the egg cell. This lineage undergoes two periods of rapid proliferation punctuated by a long period of quiescence. Extrapolating from the data of Coe and Neuffer (1978), there are approximately 50 cell generations from one zygote to the next (Walbot, unpublished), thus three plant generations represents approximately 150 cell divisions. We do not know if there is selection within the meristem for cells containing mtDNA of a particular type; that is, whether there is a higher likelihood that cells containing the most typical steady-state concentration of the various mtDNA chromosomes and plasmids will give rise to the egg cells.

The mitochondrial genome of B37N inbred maize appears to be very stable in the plant, with the same genomic constitution inherited from one generation to the next. The genome is hypothesized to consist of multiple chromosomes and plasmids in varying stoichiometry with the possibility for the generation of many chromosomal types by recombination (Lonsdale et al. 1983). However, we observe that the mtDNA remains in a steady-state in terms of the sequence complexity and distribution among the multiple chromosomes. This observation also suggests that selection for a specific genomic constitution exists. This selection could take the form of nuclear genes which act to regulate the replication and assortment of mitochondrial genomes as well as selection at the phenotypic level for mitochondria, and ultimately cells, which contain a balanced complement of mitochondrial genes.

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