Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of maize *

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Summary. Mitochondrial DNA (mtDNA) of the Black Mexican sweet line of maize isolated from tissue cultured cell suspension cultures and young seedlings was examined. Restriction fragments generated by two endonucleases were comparatively analyzed by visualization of ethidium bromide stained gels as well as by membrane hybridization with nick-translated DNA probes of plasmid-like S1 and S2 DNA. Although no major molecular alterations were seen in tissue cultured cells, the samples were clearly not identical. The variation was mainly in the stoichiometry of several restriction fragments. Hybridization analyses with S1 and S2 probes show no evidence of molecular rearrangement in this part of the genome in tissue cultured cells. Minor variations in restriction patterns could reflect alterations in frequency of circular mtDNA molecules, perhaps related to nuclear alterations during the extended period of culture.

Key words: Maize – Mitochondrial DNA – Tissue culture

Introduction

Plant cell suspension cultures are increasingly becoming an important tool in the study of biochemical and molecular processes and somatic cell genetics in plants. Much work has been done using cell suspension cultures from dicotyledonous plants. Tobacco, carrot and soybean are probably the most widely used plant species for such analyses. Monocotyledonous plants, and cereals in particular, have been studied the least. In this group, maize is of special interest because besides being an important crop plant species, it occupies a rather prominent place as a model genetic system among eukaryotes. Maize cell suspension cultures have been reported by several investigators (Sheridan 1975; Potrykus et al. 1979; Chourey and Zurawski 1981) with genotype found to be an important factor in the initiation and final isolation of cell suspension culture. For example, Black Mexican sweet (BMS) line appears to be better suited for this purpose than any other line of maize (Sheridan 1982; Chourey and Zurawski 1981).

The recovery of a friable type of callus from the usual compact and highly organized type of initial callus seems to be a critical step in the isolation of a cell suspension culture. So far, this is the only documented approach to obtain a finely dispersed maize cell suspension culture which can be subsequently used for protoplast culture and regeneration (Chourey and Zurawski 1981). The initial change in the morphology of callus, i.e. compact type to friable type, appears to be the result of a development phase change in the callus growth. The basis of such somatic variation is unknown, and attempts to revert to compact type morphology or to attain plant regeneration have been unsuccessful to date (Chourey, unpublished). Freeling et al. (1976) analyzed these two types of calluses by various parameters. They observed a higher frequency of tetraploid and octaploid cells in friable callus than in the compact callus. Brar et al. (1979) also reported many tetraploid and aneuploid cells in a cell suspension culture of maize. Recently Chourey and Kemble (1982) described a specific type of mitochondrial DNA (mtDNA) variation which was uniquely correlated with the friable callus morphology in maize. This variation was restricted to two plasmid-like DNA species, designated as S1 and S2, which are known to be associated with the S type of cytoplasmic

male sterility in maize (Pring et al. 1977). Six independent isolations of friable calluses from the compact type in *S-cms* genotype showed loss and presumably transposition of S1 and S2 molecules. Such alterations were uniquely associated with friable calluses whereas the compact type subcultures or plants regenerated from such cultures remained unchanged.

Lack of plant regeneration capacity and the abnormalities described above in friable calluses and cell suspension cultures have made it important to examine genomic stability of such tissue culture systems. This communication describes an analysis of mtDNA stability under extended culture of Black Mexican sweet maize as visualized by restriction fragment analyses. Stability of sequences homologous to the S1 and S2 plasmid-like DNAs (Pring et al. 1977) was included because of transpositional capability of these sequences in S cytoplasm male-sterile maize plants (Levings et al. 1980; Kemble and Mans 1983).

Materials and methods

Selection of friable callus, initiation, isolation, and maintenance of the cell suspension culture and protoplast culture leading to regeneration of callus in Black Mexican sweet line of maize has been described previously (Chourey and Zurawski 1981). Cells had been in culture for four years when examined. Calli derived from protoplasts have been used to obtain secondary cell suspension cultures and are designated here as protoclones; these cultures had been maintained for two years when assayed. Cell suspension (CS) cultures, either of protoclone origin or of the primary type, were subcultured once every five days, and harvested at log phase stage for mitochondrial DNA isolation.

Methods for isolating maize mitochondria from 5 to 7 day old etiolated coleoptiles and mesocotyls were similar to methods described previously (Pring and Levings 1978). Cell cultures were homogenized in a 15 ml ground glass tissue grinder (B.G. Gengenbach, personal communication) with 3 vol/wt of 0.5 M sucrose, 5 mM Na_2-EDTA, 0.1% BSA and 0.05 M Tris-HCl, pH 7.5. Diethyldithiocarbamate and 2-mercapto-ethanol were added to 5 mM. The homogenate was filtered through cheesecloth and miracloth prior to centrifugation at 1,000 g (max) for 10 min (Kemble et al. 1980). The supernatant was then centrifuged at 16,000 g (max) for 10 min to pellet the mitochondria. The pellets were resuspended with a small paint brush (R.J. Kemble, personal communication) in 0.3 M sucrose, 50 mM Tris-HCl, pH 7.5, and centrifuged for 10 min at 1,000 g. The supernatant was brought to 10 mM $MgCl_2$ and 20 μg/ml deoxyribonuclease I followed by a 60 min incubation at 4 °C. The preparation was underlaid with 0.6 M sucrose, 20 mM Na_2-EDTA (Kemble et al. 1980), 10 mM Tris-HCl, pH 7.5, and centrifuged 20 min at 12,000 g. Pellets were resuspended in the same buffer and washed at 16,000 g for 10 min. Resuspension for lysis was in 50 mM Tris-HCl, pH 7.5, 20 mM Na_2-EDTA; Proteinase K was added to 100 μg/ml and incubated for 60 min at 37 °C. Two extractions with phenol were followed by ethanol precipitation in 0.2 M ammonium acetate and final resuspension in distilled water.

Mitochondrial DNAs were restricted by BamH1 or HindIII according to supplier's recommendations. Agarose gel electrophoresis was conducted in 0.8–1.0% agarose with 30 mM Tris base, 36 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.8. Electrophoresis was conducted at 1.6–2.0 V/cm for 16–18 h at room temperature. Gels were stained with 500 ng/ml ethidium bromide for 1 h, and photographed over 302 nm UV, using Polaroid 55P/N film with Wratten 9 and 23A filters. The gels were then denatured for 30 min in 0.2 N NaOH, 0.6 M NaCl, neutralized for 30 min in 1.0 M Tris base, 0.6 M NaCl, pH 7.5. DNAs were then transferred to nitrocellulose for 16 h by the Southern (1975) procedure.

S1 and S2 DNAs were obtained by preparative gel electrophoresis of undigested S cytoplasm mtDNA. Recovery of the DNAs from gel slices was by the $NaClO_4$ dissolution/glass fiber filtration method as described by Yang et al. (1979). Probe DNA was labeled by nick translation using α-^{32}P-dCTP (Amersham, > 2,000 Ci/mMole) as described by Rigby et al. (1977). Chromatography on a Sephadex G50 column in a 1 ml disposable syringe barrel was performed to remove unincorporated deoxynucleotide triphosphates. Prior to hybridization, the labeled DNAs were denatured in 0.2 M NaOH for 15 min at room temperature and neutralized by the addition of 1/10 volume 2 M HCl.

Prehybridization of nitrocellulose membranes was carried out at 65 °C for 1 h in a hybridization buffer consisting of 10X Denhardt's solution (0.1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.2% BSA) in 3X SSC (0.45 M NaCl, 0.045 M sodium citrate) containing 100 μg/ml of heat-denatured salmon sperm DNA. The denatured, labeled DNAs were injected directly into the hybridization bags which were then incubated for 18 h at 65 °C.

Hybridized membranes were washed twice in 3X SSC for 15 min and once in 0.3X SSC for 15 min (all at 65 °C) and vacuum dried for 1 h at 80 °C. Autoradiography was carried out for various exposure times using Kodak X-Omat AR film and Lightning Plus intensifier screen.

Results

The restriction patterns of mtDNA from etiolated seedlings and cell suspension cultures (primary cell suspension and protoclone derived cells) of BMS line of maize are shown in Fig. 1. BamH1 and HindIII digests, as visualized by UV fluorescence of ethidium bromide stained gels, are in panels A and B respectively. Each restriction digest allows visualization of a minimum of 50 bands on the gels.

BamH1 digests

Comparisons of coleoptile, primary CS, and protoclone mtDNAs showed little qualitative variation as visualized by BamH1 digestion (Fig. 1A). Quantitative variation was observed in a fragment of ca. 9.6 kb, with tissue culture cells yielding apparent higher stoichiometry of the fragment. A similar phenomena was observed at ca. 6.3 kb. Additional stoichiometry variation was found at 5.2–5.8 kb, where several fragments appeared to be amplified in either CS or the protoclone mtDNA; the two cell lines differed in this region. One fragment of ca. 1.9 kb appeared to be amplified in CS cells.
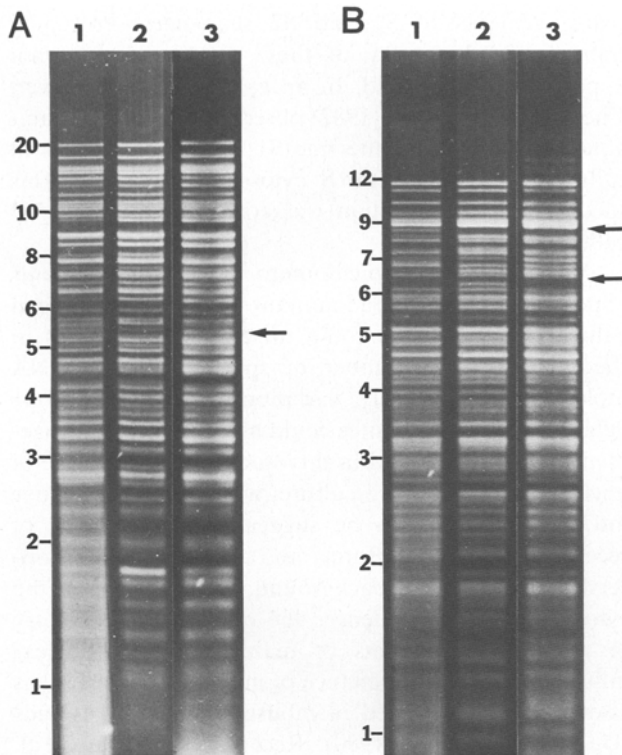
Fig. 1. Agarose gel electrophoresis of Black Mexican sweet mtDNA prepared from *1*) coleoptile, *2*) cell suspension, and *3*) protoclone; digested with BamH1 (A) HindIII (B). *Arrows* indicate the position of qualitative changes among the samples. Numbers are kb values

HindIII digests (Fig. 1 B)

No variation was seen among three samples in the largest sized (12–9 kb) restriction fragments. A fragment at ca. 8.8 kb present in seedling extract, appeared to be missing in tissue cultured cell extracts. The cell suspension extract yielded a fragment at ca. 6.3 kb which was undetectable in coleoptile or protoclone preparations. This fragment was reproducibly missing in protoclone and seedling DNA. This was probably the only difference in this analysis which distinguishes cell suspension from the protoclone extract. These differences were reproducible among different cell culture preparations; coleoptile mtDNA similarly showed no variation among preparations nor differed from several leaf mtDNA samples analyzed.

Hybridization analyses of S1 and S2 sequence homology

The S1 and S2 plasmid-like DNAs are uniquely associated with the S source of cytoplasmic male sterility (Pring et al. 1977). However, integrated sequences are present in normal, male fertile cytoplasm mtDNA (Thompson et al. 1980; Spruill et al. 1980). Southern blots of gels shown in Fig. 1 were hybridized with nick-translated S1 and S2 to assess possible transposition of these sequences during tissue culture. S1 hybridized strongly to BamH1 fragments of 8.0 and 4.4 kb, with minor homology at 3.9 and 2.1 kb (Fig. 2A). Most
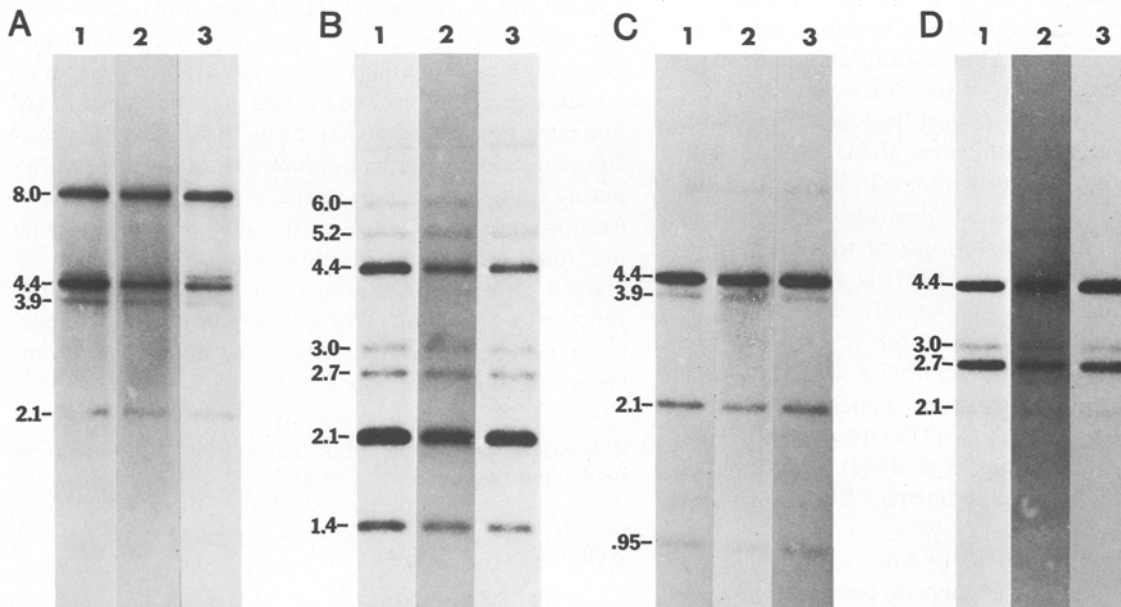
Fig. 2. Hybridization of nick-translated S1 DNA to Southern blots of A) BamH1 and B) HindIII digested mtDNAs from *1*) coleoptile, *2*) cell suspension, and *3*) protoclone. Hybridization of nick-translated S2 DNA to Southern blots of C) BamH1 and D) HindIII digested mtDNAs from *1*) coleoptile, *2*) cell suspension, and *3*) protoclone

normal cytoplasm mtDNAs carry major homology to S1 at 6.9 kb, (Lonsdale et al. 1981) but Black Mexican is unique in that the 8.0 kb fragment replaces a 6.9 kb fragment (McNay et al. 1983). Homology at 4.4 kb is a major fragment carrying homology to S2, and is hybridized by virtue of the presence of a ca. 1,400 bp repeat characteristic of the S1 and S2 DNAs (Kim et al. 1982). No differences were detected among coleoptile, CS, or protoclone lines. HindIII digestion and hybridization data (Fig. 2B) similarly showed no variation among preparations. Fragments at 2.2 and 1.4 kb are internal HindIII digestion products of S1 (Kim et al. 1982), and homology at 4.4 kb demonstrates the presence of the 1,400 bp repeat in the S2 region. Other HindIII fragments are unmapped. Hybridization of S2 to BamH1 and HindIII digests (Fig. 2C, D) showed no variation among preparations. Homology at 4.4 kb in each case represents a major S2 fragment and homology at 2.7 kb in HindIII digests represents a fragment contiguous to the 4.4 kb fragments. No homology of S2 was detected at 8.0 kb in BamH1 digests, the major S1 fragment. The S1 region of all normal cytoplasms examined to date does not include the 1,400 bp repeat characteristic of the free S1 molecule (Lonsdale et al. 1981; McNay et al. 1983); Black Mexican, although unique among normal cytoplasms, similarly does not carry this repeat in the S1 region.

Discussion

This investigation of mtDNA stability in maize tissue culture cells examines possible similarities with two preceding examples of mtDNA alteration, associated with phenotypic change. MtDNA alterations are associated with the conversion of the disease-susceptible T male-sterile cytoplasm to disease resistance and male fertility upon regeneration from tissue culture with (Gengenbach et al. 1981) or without (Brettel et al. 1982) disease toxin as a selection pressure. Spontaneous reversion of S male-sterile cytoplasm to male fertility intact plants (Laughnan et al. 1981) is associated with mtDNA rearrangements apparently mediated by sequences homologous to S1 and S2 plasmid-like DNAs (Levings et al. 1980; Kemble and Mans 1983). Normal, male-fertile cytoplasms of maize share homology to the S1 and S2 DNAs (Thompson et al. 1980; Lonsdale et al. 1981; McNay et al. 1983), including the line utilized in these experiments, Black Mexican sweet.

Hybridization experiments to date indicate stability of integrated S1 and S2 sequences during prolonged cell suspension culture of Black Mexican sweet. Therefore tissue culture of this line, which results in alterations of restriction patterns, does not a priori include

rearrangements of S1 and S2 sequences. Presumed transpositional activity of these sequences does not appear to be expressed. In an earlier study, however, Chourey and Kemble (1982) observed loss and presumable transposition of the free S1 and S2 sequences in callus derived from the S cytoplasm genotype. This specific type of alteration was correlated with altered callus morphology.

Variation in the stoichiometry of certain fragments appears to be the predominant effect of prolonged culture. Such changes could arise from alterations in the relative copy number of specific circular DNA molecules. Alternatively, variations such as mutation/deletion in restriction sites could also lead to rearrangements such as observed in this study. A possible role of nuclear changes during culture, which could influence mtDNA patterns, can be suggested on the basis of recent observations. Borck and Walbott (1982) observed that nuclear background, in addition to the cytoplasmic type, influences the relative stoichiometry of restriction fragments of maize mtDNA. Nuclear influence on the maintenance of mtDNA sequences has also been demonstrated in mouse-human cell hybrids (De Francesco et al. 1980). Recently Laughnan et al. (1981) demonstrated that the copy number of S1 and S2 DNAs is under nuclear control. Stoichiometry variation observed here could be related to the altered ploidy levels reported in the nucleus of tissue cultured cells (Brar et al. 1979; Freeling et al. 1976).

The release of a high level of genetic variability in tissue culture regenerated plants is a documented but poorly understood phenomenon (Shepard et al. 1980; Larkin and Scowcroft 1981). In this study, cells were not subjected to selection pressure to retain morphogenetic potential, and were exposed to a perpetual selection pressure to obtain cell aggregates of small number. This selection has been maintained for over 300 generations, and random variations/mutations during this period may have a high probability of fixation and propagation in the population. Although the total number of visually altered fragments remained low, modifications of the mitochondrial genome are evident. Whether any functional importance can be associated with these alterations is unknown at present.

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