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Mitochondrial genome variability in *Sorghum* cell culture protoclones*

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Summary. *Sorghum bicolor* cv NK300 seedlings, a cell suspension culture, and five protoclone suspension cultures were compared for the occurrence of somaclonal variation by analysis of their mitochondrial DNA (mtDNA). Restriction digests of the mtDNA showed qualitative and quantitative variation of restriction fragments. Southern analyses were performed using a 14.7-kb *Eco*RI mitochondrial genome fragment and regions carrying mitochondrial protein coding genes, *atpA, atp6, cob,* and *coxI* as probes. These analyses revealed part of the 14.7-kb *Eco*RI region to be present as a repeat in planta, and to be hypervariable when cells were subjected to protoplast culture. All protoclones differed from each other, from the parental cell suspension culture, and from the seedlings in their mitochondrial genome arrangement. Seedlings of five independent sorghum accessions, unrelated to cv NK300, of diverse geographic origin showed conservation of this mitochondrial fragment. Southern analyses of the mtDNA showed no variation for genomic organization of the region carrying *coxI,* and *atpA* was identical in all the tissue culture lines. The *atp6* gene was present as two copies in the seedlings, and one copy was rearranged upon tissue culture. The region carrying the *cob* gene was also found to be variant between tissue culture and seedling mtDNA. A substoichiometric 3.3-kb *Eco*RI *cob* fragment present in seedlings was amplified in the tissue culture lines. Protoclone S63 differed from the original suspension culture and remaining protoclones in that it had lost the 3.0-kb *Eco*RI band, the most abundant fragment in seedlings. A new set of fragments was detected in this protoclone. Northern analysis for the *cob* gene demonstrated altered transcript size in protoclone S63.

Key words: *Sorghum bicolor* – Protoplast – Mitochondrial DNA – Somaclonal variation

Introduction

Somaclonal variation resulting from plant cell and tissue culture is well documented in both monocotyledonous and dicotyledonous species (reviewed by Larkin and Scowcroft 1981), and has been described at a phenotypic level for sorghum plants regenerated from callus cultures (Bhaskaran et al. 1987; Cai et al. 1990). Our interest was in understanding somaclonal variation by a molecular analysis of events that may be occurring in the tissue culture process. To do this we chose to analyze the mitochondrial genome of protoplast-derived suspension cultures (protoclones) of *Sorghum bicolor* (L.) Moench cv NK300 (Chourey and Sharpe 1985). Variability in the organization of the mitochondrial genome of different sorghum cytoplasms is known for a number of cytoplasmic male-sterile and fertile lines (Pring et al. 1982; Bailey-Serres et al. 1986a, b). Variation in the mitochondrial genome of plants is attributed to rearrangements involving repeat sequences (Lonsdale et al. 1988), amplification of substoichiometric molecules, and duplication or loss of existing genomic arrangements (Small et al. 1987). Such changes have been found in suspension cultures of rice (Chowdhury et al. 1988; 1990; Saleh et al. 1990), tobacco (Grayburn and Bendich 1987), sorghum (Chourey et al. 1986), and *Brassica campestris* (Shirzadegan et al. 1989, 1991), as well as in regenerated plants of maize (Gengenbach et al. 1981) and wheat (Hartmann

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et al. 1989). The rearrangements involve known protein coding regions and other regions of the genome.

However, there are few reports of molecular variation in sorghum as a result of plant tissue culture (Wilson et al. 1985; Zack and Chourey 1985). In this paper we report the characterization of a 14.7-kb *Eco*RI region of the mitochondrial genome of sorghum that was hypervariable in protoclones, and we compare in seedlings, suspension culture, and protoclones the genomic organization of four mitochondrial protein coding genes: *atpA*, which encodes the alpha subunit of the F_1-ATPase; *atp6*, which encodes subunit 6 of the F_0-ATPase; *cob*, which encodes apocytochrome *b* of the bc_1 complex; and *coxI*, which encodes subunit 1 of cytochrome *c* oxidase. In addition, transcript analysis was performed for the *cob* gene.

Materials and methods

Plant material

Sorghum bicolor cv NK300 cell suspension culture and protoclones were obtained and grown as described previously (Chourey and Sharpe 1985). Cultures were harvested during logarithmic phase for isolation of mtDNA. Seedlings of *S. bicolor* cv NK300 were grown in the dark for 5–7 days, and the mtDNA was isolated from the etiolated coleoptiles. Sorghum seeds of geographically diverse germ plasms for seedling mtDNA analysis were kindly provided by Dr. K. F. Schertz, USDA/ARS, Texas A & M University. These accessions were: IS2801C (S.Rhodesia), IS12567C (Sudan), IS12605C (Nigeria), IS12608C (Ethiopia), and IS12685C (India).

Isolation and analysis of mtDNA and mtRNA

Mitochondrial DNA from cell suspension cultures and seedlings was isolated according to Wilson and Chourey (1984). The mtDNA was digested with restriction enzymes according to the manufacturer's (BRL) specifications. Electrophoresis was performed in 0.8% agarose gels using TAE buffer. Gels were stained with ethidium bromide, 0.5 μg ml^{-1}, for visualization of the DNA. For mtRNA preparation, mitochondria were isolated as for mtDNA preparation, lysed in 6 *M* guanidine thiocyanate (Maniatis et al. 1982), and phenol/chloroform was extracted prior to treating the nucleic acid with RNase-free DNase. The mtRNA was then recovered by precipitation with ammonium acetate and absolute ethanol. RNA was denatured with glyoxyl and electrophoresed in 1% agarose gels using a 10 m*M* Na_2HPO_4 buffer (pH 7.0).

Mitochondrial gene clones

The mitochondrial gene clones used in this study were TA22, which contained a 4.2-kb *Hin*dIII mitochondrial fragment on which the *atpA* gene from cms-T cytoplasm maize was found (Braun and Levings 1985), clone 9C2, which contained a 240-bp *Sau*3A fragment from the *atp6* gene of sorghum (supplied by Dr. D. R. Pring, University of Florida), clone pKMCOXI, which had a 4.3-kb *Eco*RI insert on which the *coxI* gene from sorghum was present (Bailey-Serres et al. 1986b), and clone pK9ECOB, harboring a 3.0-kb *Eco*RI insert containing the *cob* gene from sorghum (supplied by Prof. C. J. Leaver, University of Oxford, UK).

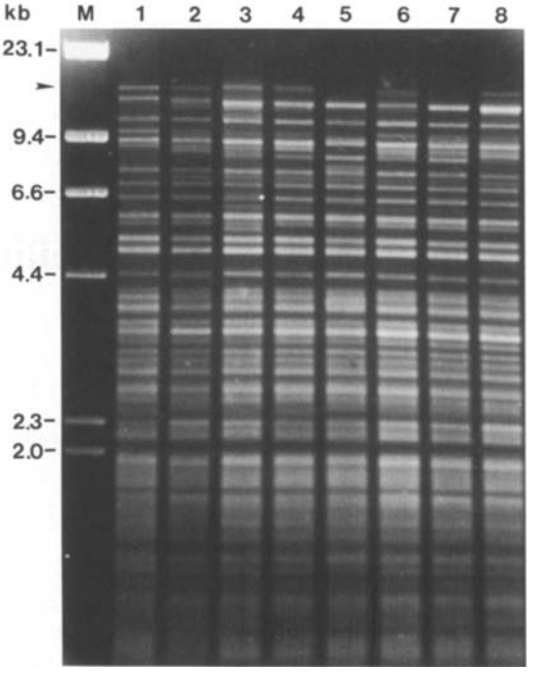

Fig. 1. Agarose gel electrophoretic pattern of *Eco*RI-digested mtDNA from NK 300 seedlings (*1*), NK 300 cell suspension (*2*), protoclones S50 (*3*), S51 (*4*), S63 (*5*), S181 (*6*), S199 (*7*), and S262 (*8*). The *arrow* indicates the 14.7-kb fragment

Cloning mtDNA

*Eco*RI-digested mtDNA was electrophoresed and the 14.7-kb fragment was isolated from the gel (Maniatis et al. 1982). This region was cloned into the plasmid pBR322 (Bolivar et al. 1977). Three unique *Hin*dIII subclones spanning the 14.7-kb fragment were obtained by *Hin*dIII digestion of the pBR322 clone and ligation with *Hin*dIII-digested pUC118. The three subclones were: 14.7a, having a 5.3-kb *Hin*dIII insert; 14.7b, having a 5.1-kb *Hin*dIII insert; 14.7c; having a 2.8-kb *Hin*dIII insert.

Southern and Northern hybridization

DNA was transferred from agarose gel to Nytran (Schleicher and Schuell) membrane using the procedure of Southern (1975). Membranes were prehybridized for 2–4 h at 68 °C in 6 × SSC, 0.05 × BLOTTO (Johnson et al. 1984), and 100 μg ml^{-1} denatured salmon sperm DNA. Hybridization buffer was identical, with the addition of dextran sulphate to 10% w/v, and contained DNA probes labelled with α-^{32}P-dCTP using the random priming method (Feinberg and Vogelstein 1983). Cloned insert DNA was separated from its vector by agarose gel electrophoresis and recovered using the Geneclean system (BIO 101, Inc.) before labelling. Following hybridization for 18 h, membranes were washed first in 2 × SSC, 0.1% SDS at 68 °C for 45 min, and then in 0.3 × SSC, 0.1% SDS at 68 °C for 45 min. Membranes were air dried and autoradiography was performed at −70 °C using Kodak X-ray film. RNA was transferred from the gel to nytran membrane overnight using 20 × SSC. Membranes were then baked for 2 h before use. Prehybridization solution was as above but with 50% formamide, and incubation was at 42 °C for 6 h. Hybridization was performed at 42 °C for 18 h in the same solution with the addition of the labelled probe. Membranes were then treated as above.

Results

Hypervariable region

Visualization of *Eco*RI-digested mtDNA from *S. bicolor* cv NK300 seedlings, cell suspension culture, and five randomly selected protoclones revealed that a large proportion of the mitochondrial fragments was unchanged. However, rearrangements and changes in stoichiometry of certain fragments in the protoclones were detected (Fig. 1). In particular, a 14.7-kb *Eco*RI region was lost in some of the protoclones (Fig. 1).

Use of the cloned 14.7-kb *Eco*RI region as hybridization probe to blots of *Eco*RI-digested mtDNA (Fig. 2A) showed part of it to be present as a repeat in planta, having homology to bands of 14.7 kb and 11.0 kb. When tissue culture cells were examined, additional bands were detected at 13.0 kb, 9.6 kb, and 7.5 kb. In the protoclones major rearrangements were associated with the 14.7-kb fragment. Each protoclone had a unique hybridization pattern, all of which were different from both the original cell suspension culture and the seedlings. Hybridizing fragments ranged in size from 14.7 kb to 4.8 kb. Two protoclones lacked the 14.7-kb hybridizing fragment, as expected from the restriction endonuclease patterns. A 7.5-kb fragment was found in all the tissue culture lines (Fig. 2A). Hybridization of *Eco*RI-digested mtDNA with *Hin*dIII subclones, representing three non-overlapping subsections of the entire 14.7-kb clone (Fig. 2B), are shown in Fig. 2C. Subclone 14.7a and 14.7b each hybridized with both the 14.7-kb and 11.0-kb fragments in the seedling mtDNA, while subclone 14.7c hybridized with the 14.7-kb fragment alone. In tissue culture cells and protoclones each subclone hybridized to multiple *Eco*RI fragments, emphasizing the uniqueness of each protoclone and the original cell culture. Subclone 14.7a hybridized to the 7.5-kb fragment previously found common to all tissue culture lines when the 14.7 kb fragment was used as probe.

Use of the complete 14.7-kb clone for hybridization with *Hin*dIII-digested mtDNA (Fig. 3A) detected three bands of 5.3, 5.1, and 2.8 kb in the seedling sample, corresponding to the *Hin*dIII-subcloned fragments. A high level of variability was again observed in the tissue culture cells and the protoclones. Hybridization with subclone 14.7a detected only a single 5.3-kb band in the seedlings and indicated that this fragment was a repeat, being present as a 5.3-kb *Hin*dIII region in both the 14.7-kb and 11.0-kb *Eco*RI fragments. Subclone 14.7a also recognized multiple *Hin*dIII bands in the tissue culture cells and protoclones. Two of these bands, 5.3 and 3.0 kb, were common to all tissue culture material (Fig. 3B). Subclone 14.7b as probe hybridized to bands of 5.1 and 2.8 kb in seedlings, which suggested that only part of this region was repeated and present on the 11.0-kb *Eco*RI region. A number of extra hybridizing fragments was detected by subclone 14.7b in the different protoclones, but less than that detected when subclone

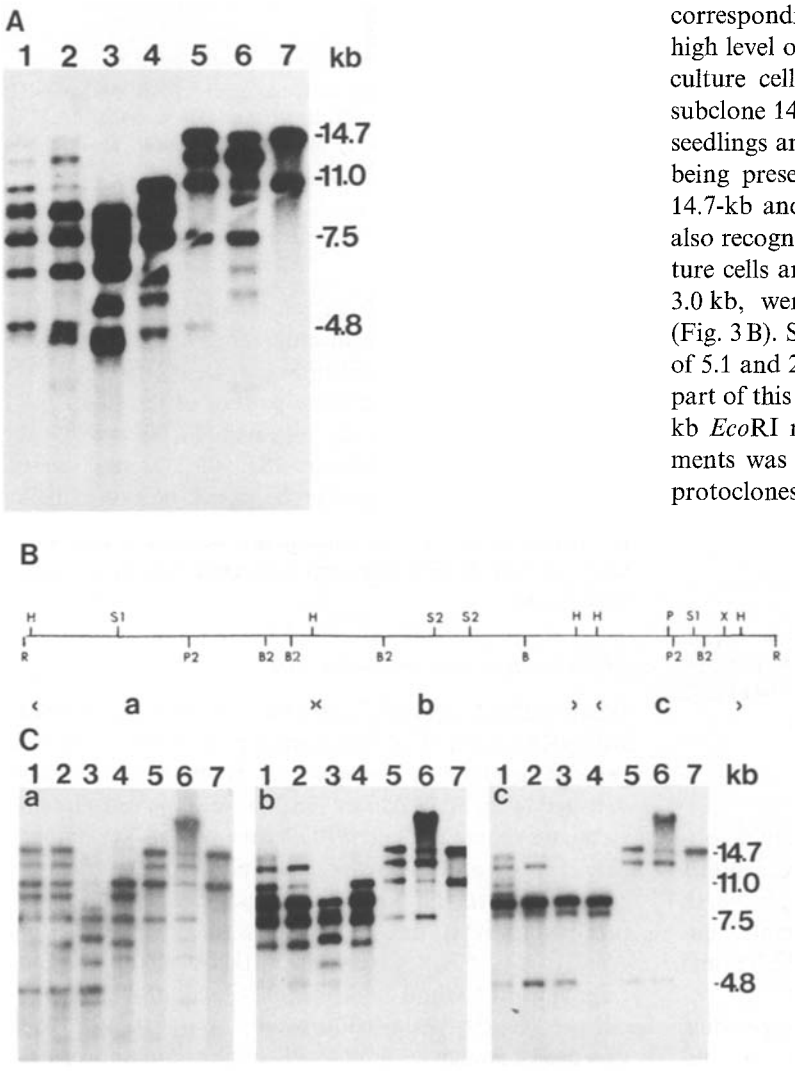

Fig. 2. A Southern hybridization with 14.7-kb *Eco*RI region to *Eco*RI-digested mtDNA from protoclones S50 (*1*), S51 (*2*), S63 (*3*), S181 (*4*), S262 (*5*), NK 300 cell suspension (*6*), and NK 300 seedlings (*7*). **B** Restriction map of the 14.7-*Eco*RI mitochondrial region. **C** Southern hybridization with subclone 14.7a (panel *a*), subclone 14.7b (panel *b*), subclone 14.7c (panel *c*), to *Eco*RI-digested mtDNA of protoclones S50 (*1*), S51 (*2*), S63 (*3*), S181 (*4*), S262 (*5*), NK 300 cell suspension (*6*), and NK 300 seedlings (*7*). Abbreviations for restriction enzymes: *Eco*RI, R; *Hin*dIII, H; *Sst*I, S1; *Pvu*II, P2; *Bgl*II, B2; *Bam*HI, B; *Pst*I, P; *Xho*I, X

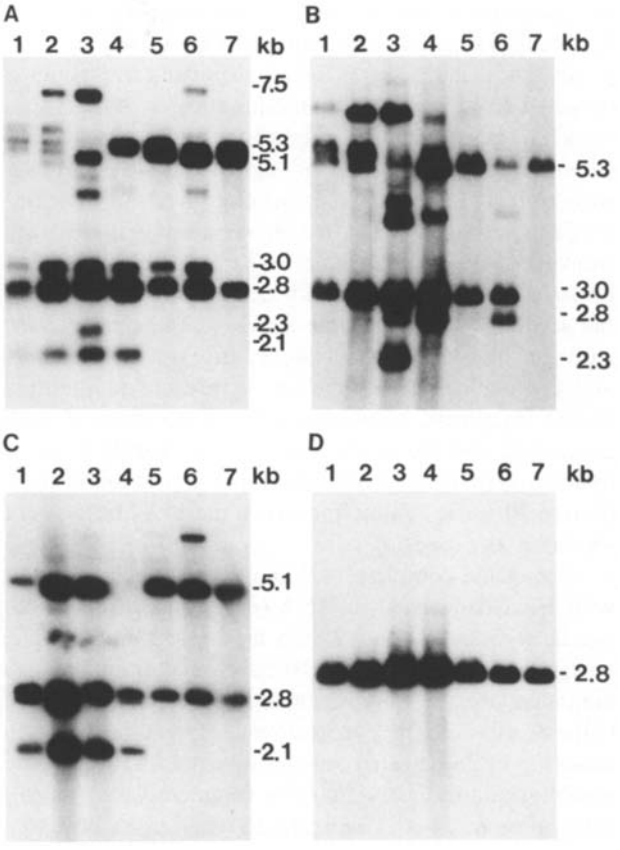

Fig. 3A–D. Hybridization with **A** 14.7-kb *Eco*RI region; **B** subclone 14.7a; **C** subclone 14.7b; **D** subclone 14.7c; to *Hin*dIII-digested mtDNA of protoclones S50 (*1*), S51 (*2*), S63 (*3*), S181 (*4*), S262 (*5*), NK 300 cell suspension (*6*), and NK 300 seedlings (*7*)

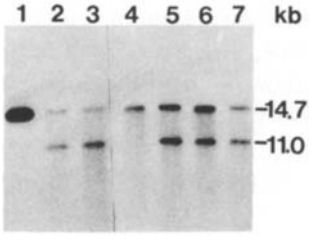

Fig. 4. Hybridization with 14.7-kb *Eco*RI region to *Eco*RI-digested seedling mtDNA of 14.7 clone (*1*), NK 300 (*2*), IS2801C (S. Rhodesia) (*3*), IS12605C (Nigeria) (*4*), IS12685C (India) (*5*), IS12567C (Sudan) (*6*), and IS12608C (Ethiopia) (*7*)

14.7a was used as the probe. The 2.8-kb fragment detected by subclone 14.7b was present in all the samples (Fig. 3C). Subclone 14.7c hybridized to a single 2.8-kb band in the seedlings and all other mtDNA samples; this region appeared to be invariant in the cell culture line and the protoclones (Fig. 3D).

To test whether or not hypervariability corresponding to the 14.7-kb *Eco*RI fragment was also present in natu-

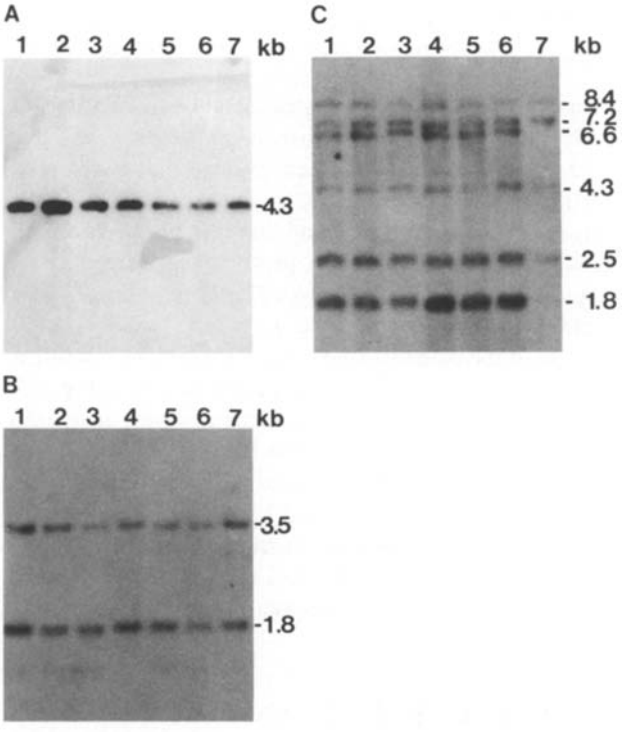

Fig. 5A–C. Hybridization with **A** *coxI* clone pKMCOXI; **B** *atpA* clone TA22 to *Eco*RI-digested mtDNA; and **C** TA22 to *Hin*dIII-digested mtDNA of protoclones S50 (*1*), S51 (*2*), S63 (*3*), S181 (*4*), S262 (*5*), NK 300 cell suspension (*6*), and NK 300 seedlings (*7*)

ral population of sorghum, we examined seedling mtDNA of five cultivars from different geographic sources, and results are presented in Fig. 4. Of the five cultivars tested, four showed the same pattern of the two *Eco*RI hybridizing fragments as observed for *S. bicolor* cv NK300. Only one cultivar, IS12605C from Nigeria, showed a loss of the smaller fragment; however, unlike the protoclones no rearrangements were detected. The large 14.7-kb *Eco*RI fragment was conserved in all cultivars tested.

Mitochondrial protein coding genes

When mtDNA samples digested with *Eco*RI were probed with pKMCOXI (Fig. 5A), carrying *coxI* mitochondrial gene of sorghum, a single 4.3-kb *Eco*RI fragment was detected in the *S. bicolor* cv NK300 seedling, cell suspension, and protoclones. The *atpA* gene was also examined (Fig. 5B, C) and no major genomic variation was observed between the NK300 cell suspension culture and protoclones when mtDNA was digested with *Eco*RI or *Hin*dIII (Fig. 5B, C). Seedling mtDNA lacked *Hin*dIII fragments of 6.6 and 1.8 kb (Fig. 5C), all of which were detected in the tissue culture lines. No variation was observed between seedlings, cell suspension, and proto-

clones when *Eco*RI digests were examined. When the probe for the *atp6* gene was used for hybridization to *Bam*HI-digested mtDNA (Fig. 6A), two *Bam*HI fragments of 5.0 and 6.5 kb were detected in seedling mtDNA, an arrangement common to sorghum cytoplasms having two copies of *atp6*. The cell suspension and protoclones retained the 5.0-kb fragment, but instead of the 6.5-kb fragment, had a 4.0-kb band in all lines examined. Protoclone S63 was also unique in having a third hybridizing 7.0-kb *Bam*HI fragment (Fig. 6A). The single 3.1-kb fragment detected with the *atp6* probe in all samples when mtDNA was digested with *Eco*RI indicated that a core sequence common to both copies in seedlings was maintained in the rearranged form of the gene, even in S63 which had the extra 7.0 kb *Bam*HI fragment (Fig. 6B).

When mtDNA was digested with *Eco*RI and probed with pK9ECOB, fragments of 3.0 and 3.3 kb were detected in seedling mtDNA (Fig. 6C, lane 7). The 3.3-kb fragment was present in substoichiometric quantities; however, in all tissue culture lines this fragment was amplified and present in amounts equal to that of the 3.0-kb fragment. The 3.0-kb fragment was not present in protoclone S63; instead, two more fragments were found at 4.0 and 4.8 kb (Fig. 6C, lane 3). Based on this polymorphism among the protoclones, it was decided to examine mitochondrial transcripts with the *cob* probe (Fig. 6D). Comparison of seedling, cell suspension, and protoclone S63 showed that the *S. bicolor* cv NK300 seedling and cell suspension had an identical pattern of transcription, with a major transcript at 2,400 nt and a minor transcript at 3,000 nt. In protoclone S63 a sinlge major transcript was detected at 2,200 nt (Fig. 6D). It seems that the rearrangements associated with *cob* in this protoclone resulted in an altered pattern of transcription.

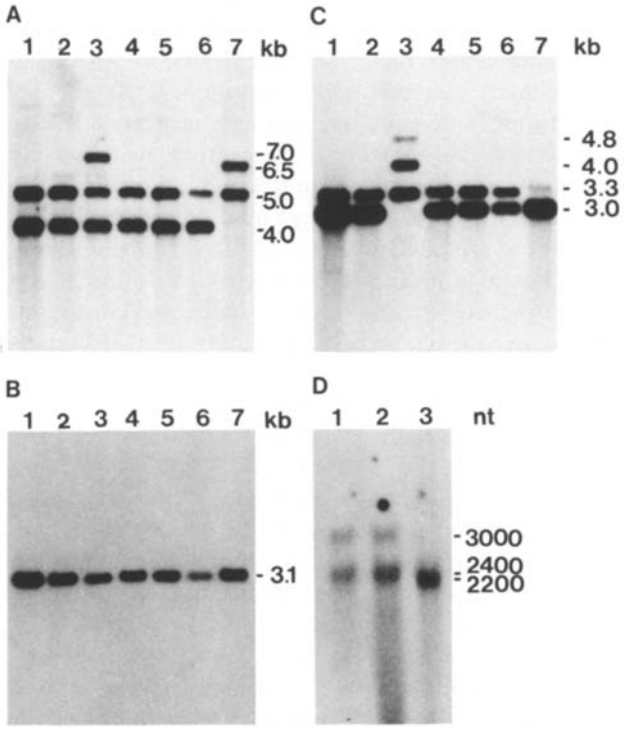

Fig. 6A–D. Hybridization with *atp6* clone 9C2 to **A** *Bam*HI-digested mtDNA; **B** *Eco*RI-digested mtDNA; *cob* clone pK9ECOB to **C** *Bam*HI-digested mtDNA of protoclones. *Lanes* include: S50 (*1*), S51 (*2*), S63 (*3*), S181 (*4*), S262 (*5*), NK 300 cell suspension (*6*), and NK 300 seedlings (*7*). **D** Northern blot hybridization with *cob* clone pK9ECOB to mtRNA of NK 300 seedlings (*1*), NK 300 cell suspension (*2*), and protoclone S63 (*3*)

Discussion

Mitochondrial genome organization of *S. bicolor* cv NK300 seedlings, cell suspension culture, and protoclones was examined by viewing restriction endonuclease-digested mtDNA in ethidium-bromide-stained gels and by Southern hybridization analyses. Loss or rearrangement as well as changes in the stoichiometry of certain regions of the mitochondrial genome were detected by comparison of the restriction profiles of the different samples. An *Eco*RI fragment of 14.7 kb was missing in two of the protoclones. This was further characterized by cloning and restriction mapping as well as using it as a hybridization probe in Southern analyses. When the complete 14.7-kb *Eco*RI fragment and the three subclones (a, b, and c), which spanned the entire 14.7-kb region, were used for hybridization analyses, an increase in the number of bands recognized was detected in the protoclones relative to the parental seedling and cell suspension mtDNAs. Part of the 14.7-kb *Eco*RI region was present as a repeat in the mitochondrial genome of the seedlings, and was very active in generating altered genome organization when cells were subject to protoplast isolation and subsequent culture. This was based on the increase in number of hybridizing fragments in the original suspension culture and the protoclones, and the loss of some regions in certain protoclones. Among the five protoclones tested no two cell suspension cultures had the same genomic arrangement for this region. However, some hybridizing fragments were common to all tissue culture lines. This is probably among the highest levels of variation in the mitochondrial genome of protoclone cultures. Similar results concerning high level of variability in mitochondrial genome were reported previously in tissue culture cells of Black Mexican Sweet maize (McNay et al. 1984), in regenerated potato protoclones (Kemble and Shepard 1984), and in long-term rice cell suspenion cultures (Chowdhury et al. 1988, 1990; Saleh et al. 1990). In *Brassica campestris* both stoichiometric changes and the appearance of novel restriction fragment swere detected in 2-year-old cell suspension culture

(Shirzadegan et al. 1989), as well as in a group of protoclones (Shirzadegan et al. 1991). However, there are no reports of high levels of variation associated with a single mitochondrial region, as reported here for the 14.7-kb *Eco*RI region.

It should be noted however that the hypervariability of the 14.7-kb region of the mitochondrial genome described here was specific to stress in the tissue culture environment of these cells, as no variation was detected in sorghum accessions of diverse geographic origin. All five accessions were similar to NK300, except one, which showed only the largest of the two fragments (Fig. 4). The loss of the 11.0-kb fragment in this accession, IS12605C, was not accompanied by any genomic rearrangements. It was concluded on this basis that the region of the mitochondrial genome homologous to the 14.7-kb fragment was stable and conserved in planta. The involvement of repeat sequences in generating mitochondrial genome diversity is well documented (Lonsdale et al. 1988). It has been suggested that there are mitochondrial sequences which are more susceptible to rearrangement and which are primarily responsible for the variation seen in the genome (Kemble and Shepard 1984; Brears et al. 1989; Shirzadegan et al. 1989). The 14.7-kb *Eco*RI region of *S. bicolor* cv NK300 seems to behave in such a manner under tissue culture conditions. We believe that much of the variation seen in the protoclones is a consequence of the protoplast isolation, since such high levels of variation were not detected for this region of the mitochondrial genome in cell clone suspension cultures (Chourey et al. 1986) derived from the same parental NK300 cell suspension culture as the protoclones studied in this report.

Variability in the location of *coxI* in the mitochondrial genome of different sorghum cytoplasms has been reported (Bailey-Serres et al. 1986a). Usually the gene is found on a 4.3-kb *Eco*RI fragment; in certain cytoplasms such as 9E and IS7920C it has been found on a 10.4-kb *Eco*RI fragment. This alternate configuration results in altered transcript size which codes for a larger protein product. Leaver et al. (1985) suggest that this difference in genomic location was generated via intragenomic recombination. Multiple genomic environments are also detected for *coxI* in maize cms-S cytoplasms and are believed to be caused by recombination events involving the S plasmids and regions of homology to them found in the 5′ region of the *coxI* gene (Leaver et al. 1985). Further evidence of variation for *coxI* in rice cell suspension cultures has been reported (Chowdhury et al. 1990). Despite such variability we have not detected variability among the samples tested for the *Eco*RI region carrying the *coxI* gene.

Little variation was detected for the mtDNA region carrying *atpA*. It seems that following the initial variation found in the cell suspension cultures, no further changes occurred in the protoclones. Variation in genomic location and copy number of *atpA* has been reported for different cytoplasms in sorghum (Bailey-Serres et al. 1986a). In long-term rice cell suspensions the *atpA* gene is found to be stable (Chowdhury et al. 1990).

For the regions carrying the *atp6* and *cob* genes the situation differed. The *atp6* gene was present in two copies in *S. bicolor* cv NK300 seedlings, an arrangement common to a number of different sorghum cytoplasms (Pring et al. 1988), and we detected variation upon tissue culture. One copy of the gene was rearranged. Further change was noted for one of the protoclones where an extra copy of the gene was found. Pring et al. (1988) report that at least five repeats exist in certain sorghum mtDNA, one of which is close to the *atp6* gene. A recombinationally active repeat has been reported to be associated with *atp6* gene in wheat where all four products of recombination could be detected (Bonen and Bird 1988). Such an arrangement might be responsible for the variation we detected in the tissue-cultured lines via intragenomic recombination. The *atp6* gene is also variable in one of six rice suspension culture lines (Chowdhury et al. 1990).

In a previous study of *cob* in sorghum, a single 3.0-kb *Eco*RI fragment was detected in the five cytoplasms examined, a result similar to that for *S. bicolor* cv NK300, although no substoichiometric 3.3-kb fragment was reported previously (Bailey-Serres et al. 1986a). Amplification of this 3.3-kb *cob* fragment to equimolar level of the 3.0-kb copy in tissue culture may have resulted in recombination events giving rise to the rearrangement seen in one of the protoclones. From this and other studies it is clear that the mitochondrial genome can undergo rearrangement, especially when subject to tissue culture process. The four protein coding genes examined in this study were expected to be under functional constraints and to remain unaltered. The results from this study show that protein coding regions can be altered in the tissue culture process. Such variation can have an effect on gene expression as shown by the altered transcript pattern detected for *cob* (Fig. 6D). Whether this results in altered protein product, as seen for *COXI* in sorghum (Bailey-Serres et al. 1986b), is not known. These variants for regions carrying *atp6* and *cob* provide material for further studies on aspects of transcription of these mitochondrial genes in sorghum.

The involvement of the nucleus in determining mitochondrial genome structure needs to be considered when evaluating our results. It has been demonstrated that the nuclear genotype is a determining factor for the frequency of reversion to fertility of cms-S maize and also influence the copy number of S episomes found in this cytoplasm (Laughnan et al. 1981). Small et al. (1988), analyzing cytoplasmic revertants to fertility from a number of CMS-S maize genotypes, suggest that the nuclear geno-

type and types of events leading to reversion are linked. Similarly, fertility restoration by the nuclear gene *Rf* in *Phaseolus vulgaris* is accompanied by loss of at least 25 kb of the mitochondrial genome, part of which contains unique sequences (Mackenzie and Chase 1990). Nuclear genotype is also reported to influence quantitative differences found in mtDNA fragments of alloplasmic cultivars of *Nicotiana* (Hakansson et al. 1990). The protoclones and the original cell suspension culture used in this study were derived from a single immature embryo of NK300 cultivar (Chourey and Sharpe 1985). The long-term (5–7 years) maintenance of these cultures in tissue culture environment is associated with a slight variation in chromosome number among these protoclones (Chourey et al. 1986); it is possible that the rearrangements detected in the mitochondrial genome of the various cell lines are related to their individual chromosomal backgrounds.

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