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Molecular analysis of plants regenerated from embryogenic cultures of hybrid sugarcane cultivars (*Saccharum* spp.)*

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Summary. The genomic stability of tissue culture regenerants of sugarcane (*Saccharum* spp. hybrids, cvs 'CP72-1210', 'CP68-1067' and 'B43-62') was analyzed by DNA restriction fragment length polymorphism (RFLP). Plants regenerated from calli, cell suspensions, cryopreserved cell suspensions and protoplasts were used. Total DNA isolated from 19 different sources was digested with *Eco*RI, *Hin*dIII, *Bam*HI, *Bam*HI + *Eco*RI and *Pst*I and probed with six known maize mitochondrial genes (*coxI*, *coxII*, *atpA*, *atp6*, *atp9* and *rrn18-rrn5*), three random maize mitochondrial cosmid clones, two random maize chloroplast cosmid clones and a wheat *Nor* locus clone. Hybridization patterns indicated that the variation observed was minor and appeared only in the second-cycle regenerants. No differences were observed among the three cultivars and the regenerants from calli, suspension culture, cryopreserved suspension culture and protoplasts. Mitochondrial DNA (mtDNA) isolated from 'CP72-1210' plants and its embryogenic cell suspensions, and bulk samples from all 'CP72-1210' regenerants pooled together were digested with *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI and *Sal*I and probed with three recombinationally active wheat mtDNA clones, K′, K3 and X2. No variation in the mtDNA restriction patterns was observed between the 'CP72-1210' plants and its regenerants. However, restriction pattern variation was observed only from *Eco*RI digestion, and hybridization patterns of K3, K′ and X2 revealed minor variations in the mtDNA of cell suspensions when compared with the DNA of the 'CP72-1210' plant. Except for a qualitative variation detected by the X2 probe and minor stoichiometric variations detected by the K3 probe, sugarcane DNAs were found to be stable after plant regeneration.

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Introduction

Sugarcane is an economically important polysomatic, highly heterozygous clonally propagated crop that accounts for more than 60% of the world's sugar production. Considerable morphological, cytogenetical and isozyme variation has been reported in tissue culture-derived sugarcane clones (Heinz and Mee 1971). Because of its high genetic heterozygosity, direct gene transfer is an attractive approach for introducing foreign genes into sugarcane. However, all direct gene transfer methods involve tissue culture and can be of practical value only if the genetic fidelity of the transformed cells is maintained during cell proliferation and plant regeneration. Although the generation of variation in cultured cells is well documented, there is increasing evidence that plants regenerated from somatic embryos show very little or no variation (Shenoy and Vasil 1992; Shimron-Abarbanell and Breiman 1991; Morrish et al. 1990; Saleh et al. 1990; Gmitter et al. 1991; Vasil 1988; Kobayashi 1987; Rajasekaran et al. 1986; Swedlund and Vasil 1985).

In vivo, over a long period of time, mitochondrial (mt) DNA may undergo recombination in the repeated sequences resulting in stoichiometric and structural changes in the genome. In vitro culture has also been reported to rapidly induce such changes. This makes the use of mitochondrial probes an attractive tool for the study of variation (McNay et al. 1984). Alterations at the DNA level have been used by many investigators to monitor such variation (Larkin et al. 1989). The most commonly used probes for such studies are of ribosomal

or organellar origin. We have studied changes at the DNA level in regenerants obtained from calli, cell suspensions, protoplasts and cryopreserved suspension cultures of three hybrid cultivars of sugarcane using a wide variety of genetic probes. In addition, DNA isolated from calli and suspension cultures (both embryogenic and non-embryogenic) and from embryogenic calli collected at different time intervals after transfer to regeneration medium were also compared.

Materials and methods

Initiation and establishment of callus cultures

Calli were initiated from immature leaf explants of three hybrid sugarcane clones ('CP72-1210', 'CP68-1067', 'B43-62'), and a cell suspension regenerant of 'CP72-1210' (R1210S1) as described previously (Ho and Vasil 1983a). Plant materials were grown either in the field or in the greenhouse. The outer leaves were removed from mature shoots after surface sterilization with 95% ethanol. The five innermost tightly furled leaves were cut into 2-mm-thick transverse sections and transferred to the surface of a modified semi-solid MS medium (Murashige and Skoog 1962) designated as MS3C (MS basal salts and vitamins, 30 g/l sucrose, 3 mg/l 2,4-D, 50 ml/l coconut water and 3 g/l Gelrite) and incubated in the dark at 28 °C. Both embryogenic and non-embryogenic calli were maintained by subculture onto fresh medium every 4 weeks.

Establishment and maintenance of cell suspension cultures

Suspension cultures were initiated from 10-week-old calli (embryogenic and nonembryogenic) of 'CP72-1210' in 250-ml Erlenmeyer flasks containing 35 ml of MS3C liquid medium (Ho and Vasil 1983b). The flasks were placed on a gyratory shaker (100 rpm) in the dark at 28 °C. During the first few weeks, 20 ml of medium were replaced by an equal volume of fresh medium twice a week. Once a fine suspension was established, it was maintained by transferring 8 ml of the culture to 35 ml of fresh medium every 7 days.

Regeneration of plants

Plants were regenerated from calli and cell suspension cultures on semi-solid (3 g/l Gelrite) regeneration media MSO (MS with 30 g/l sucrose), MSB (MS with 30 g/l sucrose, 0.5 mg/l BAP) or MSSR (MS with 30 g/l sucrose, 0.13 mg/l 2,4-D and 0.25 mg/l each of BAP, kinetin and zeatin). The cultures were incubated in the dark at 28 °C for 2 weeks and then transferred to a 16-h light photoperiod. Individual plantlets were transferred to rooting medium (MS with 30 g/l sucrose, 0.2 mg/l NAA). Well-rooted plants were transferred to 4-inch square pots containing sterile soil and kept on a light cart for 10 days and then finally transferred to gallon containers in the greenhouse. Tissue for future analysis was collected from individual plants approximately 3 months after transfer to the greenhouse. Plant regeneration from cryopreserved cell suspension cultures (Gnanapragasam and Vasil 1990) and protoplasts (Srinivasan and Vasil 1986) has been described previously.

Mitochondrial DNA isolation

MtDNA was isolated from soft internode tissue and immature leaves of the 'CP72-1210' control plant, its regenerants pooled together and its embryogenic cell suspensions as previously described (Chowdhury and Smith 1988). In summary, tissues were ground in a Waring blender using a saline extraction buffer, followed by differential centrifugation to isolate mitochondria. A DNase treatment was used to remove extramitochondrial DNA. MtDNAs were purified by precipitating SDS-protein-carbohydrate complexes with potassium acetate, then precipitating mtDNA with isopropanol followed by phenol, phenol-chloroform and chloroform extraction and a second isopropanol precipitation. Cell suspensions were ground in liquid N_2 before being suspended into the saline extraction buffer.

Isolation of total DNA

Total genomic DNA was isolated either by the Dellaporta et al. (1983) or CTAB (Saghai-Maroof et al. 1984) method from immature tender leaf tissue of greenhouse or field-grown plants, calli, and cell suspensions (Table 1), frozen in liquid nitrogen and stored at −80 °C. Frozen tissue (1 µg) was ground in a cold mortar and pestle. DNA was quantified spectrophotometrically by measuring optical densities at 260 and 280 nm (Sambrook et al. 1989).

Preparation of probes

Probes used in this study came from nuclear and mitochondrial sources. They were prepared by digesting plasmid DNA with appropriate restriction enzyme(s), electrophoresing to separate insert and plasmid DNA and recovering the insert from the gel using NA45 membrane (Schleicher and Schuell) according to the manufacturer's directions. Probes were labelled using [^{32}P]-labelled deoxycytidine 5′-triphosphate (CTP) by the random priming method of Feinberg and Vogelstein (1983). Maize clones containing mitochondrial genes cytochrome c oxidase subunit I (*coxI*, Isaac et l. 1985), subunit II (*coxII*, Fox and Leaver 1981), F1-F0 ATPase subunit 6 (*atp6*, Dewey et al. 1985a), subunit 9

Table 1. Plant materials used for the analysis of sugarcane cultivars

Control	CP72-1210 3(1210PL)	CP68-1067 3(1067PL)	B43-62 3(62PL)
Embryogenic callus (EC)			
2 months old	1	1	1
+6-day regeneration	1		
+14-day regeneration	1		
+23-day regeneration	1		
Non-embryogenic callus (NEC)			
2 months old	1	–	–
Embryogenic suspension (ES)			
9 months old	1	–	–
Non-embryogenic suspension (NES)			
9 months old	1	–	–
Regenerants			
2 months old EC (RC)	7	5	5
9 months old ES (RS)	7	–	–
Cryopreserved suspension (RCR)	5	–	–
RS1 EC (RRS)	5	–	–
ES-derived protoplasts (P)	–	–	4

Table 2. Results obtained from 12 probes hybridized to the DNA of sugarcane cultivars and their regenerants digested with restriction endonucleases

Probe	Restriction enzyme	Number of bands	Band sizes (kb)	Variation[a]
coxI	*Eco*RI	6	11.7, 5.1, 3.3, 2.7, 2.2, 1.7	N
	*Hin*dIII	4	10.4, 7.9, 5.1, 4.5, 3.0	N
coxII	*Eco*RI	2	1.64, 1.1	N
	*Pst*I	1	8.23	N
atpA	*Eco*RI	1	4.4	N
	*Hin*dIII	1	3.3	Y (quantitative)
	*Pst*I	1	7.4	Y (quantitative)
atp6	*Eco*RI	1	4.1	N
	*Hin*dIII	1	4.2	Y (quantitative)
	*Pst*I	1	4.1	N
atp9	*Hin*dIII	2	7.1, 3.0	N
18S	*Eco*RI	2	14.33, 8.97	Y (quantitative)
	*Hin*dIII	4	10.65, 10.0, 3.7, 2.9	Y (qualitative)
	*Pst*I	3	8.23, 5.1, 0.96	N
Nor	*Eco*RI (*E*)	1	8.97	N
	*Hin*dIII	1	9.8	N
	*Bam*HI (*B*)	3	10.1, 5.4, 4.2	N
	B+*E*	3	4.9, 3.8, 1.2	N
2A8	*Eco*RI	10	14.0, 8.7, 4.3, 3.5, 2.4, 2.23, 1.9, 1.6, 1.3, 1.0	N
	*Hin*dIII	15	5.9, 5.1, 4.5, 3.8, 3.0, 2.7, 2.5, 2.2, 1.9, 1.5, 1.3, 1.1, 0.9, 0.8, 0.5	N
6B9	*Eco*RI	8	13.1, 5.5, 4.0, 3.7, 2.8, 1.5, 1.0, 0.8	N
	*Hin*dIII	7	8.7, 4.7, 3.8, 3.2, 1.8, 1.4, 1.0	N
	*Pst*I	6	14.8, 11.3, 8.0, 6.3, 3.8, 2.6	N
12B4	*Eco*RI	5	12.5, 4.8, 3.4, 2.9, 1.9	N
CTA5	*Eco*RI	10	5.8, 4.9, 3.3, 3.1, 2.6, 2.0, 1.8, 1.6, 1.2, 0.8	N
	*Hin*dIII	9	10.4, 7.9, 6.2, 4.8, 4.1, 3.0, 2.7, 2.3, 1.5	N
CTB1	*Eco*RI	3	18.8, 2.6, 1.5	N
	*Hin*dIII	7	8.3, 7.1, 4.8, 2.8, 2.5, 1.3, 0.8	N

[a] N = No and Y = yes with respect to the presence of variation

(*atp9*, Dewey et al. 1985b), subunit alpha (*atpA*, Braun and Levings 1985) and 18S-5S ribosomal RNAs (*rrn18-rrn5*, Chao et al. 1984) were provided by Dr. C. S. Levings, III (North Carolina State University, Raleigh, N.C.). Maize cosmid probes (2A8, 6B9, 12B4, CTA5 and CTB1) were provided by Dr. D. R. Pring (University of Florida, Gainesville, Fla.). Wheat mtDNA probes designated as K′, K3 and X2 (Rode et al. 1987) were provided by Dr. A. Rode (University of Paris, Orsay, France). The wheat nucleolar organizer region (*Nor*) locus probe (Gerlach and Bedbrook 1979) was provided by Dr. B. S. Gill (Kansas State University, Manhattan, Kan.).

Restriction endonuclease digestion, blotting and hybridization of DNA

The mitochondrial and genomic DNAs were digested with restriction endonucleases using 4 units of endonuclease per microgram DNA for 4–6 h with the manufacturer's recommended buffer and digestion conditions. About 2 and 6 µg of mitochondrial and genomic DNAs, respectively, were loaded per lane onto 0.8% agarose gels using TPE buffer (80 m*M* *TRIS*-phosphate and 2 m*M* EDTA, pH 8.0) and electrophoresed overnight at 2 V/cm. To facilitate fragment-size calculations, *Hin*dIII-digested lambda DNA molecular size markers were included. The gels were stained for 45 min in 0.5 µg/ml ethidium bromide, destained for 10 min in deionized water and photographed using 300 nm UV illumination. The migration distance of the size markers was measured. Southern blots were prepared by transferring the DNA to nylon membranes (Hybond-N, Amersham) as described by Sambrook et al. (1989) and covalently binding the DNA to the membrane using UV irradiation. The blots were hybridized overnight at 65 °C in a buffer (7% SDS, 0.5 *M* sodium phosphate, 1% BSA) washed twice in 3 × SSC and once in 0.3 × SSC (15 min each) and autoradiographed using Kodak XAR-5 X-ray film with variable exposures.

Results

Assessment of tissue culture-induced variability using total DNA

Total DNA was isolated from only one tiller of each plant. One gram of immature leaf tissue yielded approximately 0.3 mg DNA, and 1 g of calli or suspensions, approximately 0.5 mg DNA. A summary of the hybridization patterns resulting from the 12 probes hybridized to the blots containing total DNA digested with

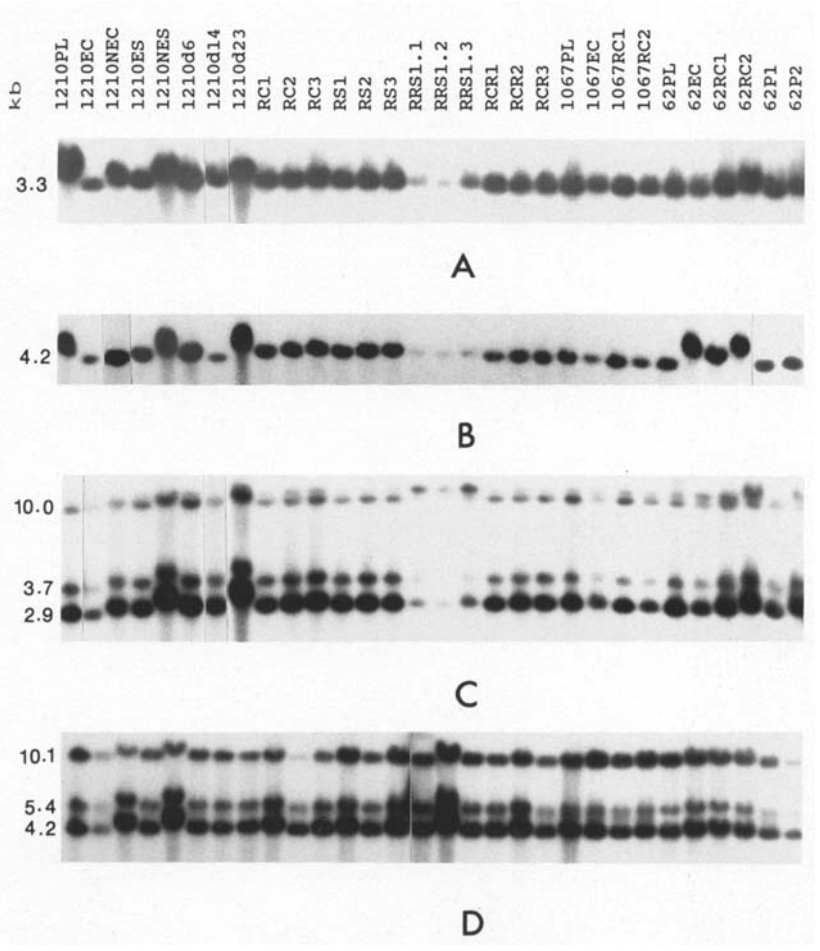

Fig. 1 A–D. Hybridization patterns of the total DNAs of sugarcane materials (listed in Table 1) digested with restriction endonucleases *Hin*dIII hybridized by *atpA* (**A**), *atp6* (**B**), 18S (**C**), and *Bam*HI hybridized by *Nor* (**D**)

either *Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI or *Bam*HI + *Eco*RI is presented in Table 2. None of the probes detected DNA sequence differences between the control plants from the three cultivars tested. Similarly, no difference was observed between the DNA restriction patterns of plants regenerated from callus, cell suspension, protoplast or cryopreserved cells. However, quantitative variation was observed between the DNA of R_0 (from callus, cell suspension cultures, protoplasts or cryopreserved cells) and second-cycle (plants regenerated from a regenerant) plants for probes *atpA* and *atp6*, and qualitative variation was observed for the 18S probe (Fig. 1 A–C). A 10.0-kb band observed in all the lines was absent in the second-cycle regenerated plants, and a 10.65-kb band present in the second-cycle regenerated plants was either absent or much fainter in all other lines (Fig. 1). No variation was observed between the DNA restriction patterns of embryogenic and non-embryogenic calli and suspensions. In addition, no variation was observed between the DNAs of the calli collected at different time intervals after transfer to regeneration medium. No variation was observed between regenerants from different sources (calli, suspension, protoplast or cryopreserved cells) or within sources. Since very little or no variation was revealed in the sugarcane materials tested in the present investigation by the organelle DNA probes, a total of ten enzyme combinations were tested on the 'CP72-1210' control plant DNA to identify an enzyme or enzyme combination that would produce the maximum number of well-separated bands when hybridized by the wheat *Nor* locus DNA probe. Only *Bam*HI and *Bam*HI + *Eco*RI enzymes produced more than one well-separated band. Therefore, in addition to *Eco*RI- and *Hin*dIII-digested blots, *Bam*HI and *Bam*HI + *Eco*RI blots containing sugarcane DNAs (listed in Table 1) were also hybridized with the *Nor* locus probe. No variation was observed (Fig. 1 D).

Restriction patterns of mtDNAs

MtDNAs isolated from the 'CP72-1210' source parent, 1210ES and R1210 (regenerants pooled together) were digested with *Sal*I, *Bam*HI, *Eco*RI, *Pst*I and *Hin*dIII and their restriction patterns (Fig. 2) were compared visually. The number of sugarcane mtDNA restriction fragments ranges from 30–50 for the five restriction enzymes used. The restriction patterns of 'CP72-1210' and R1210 were

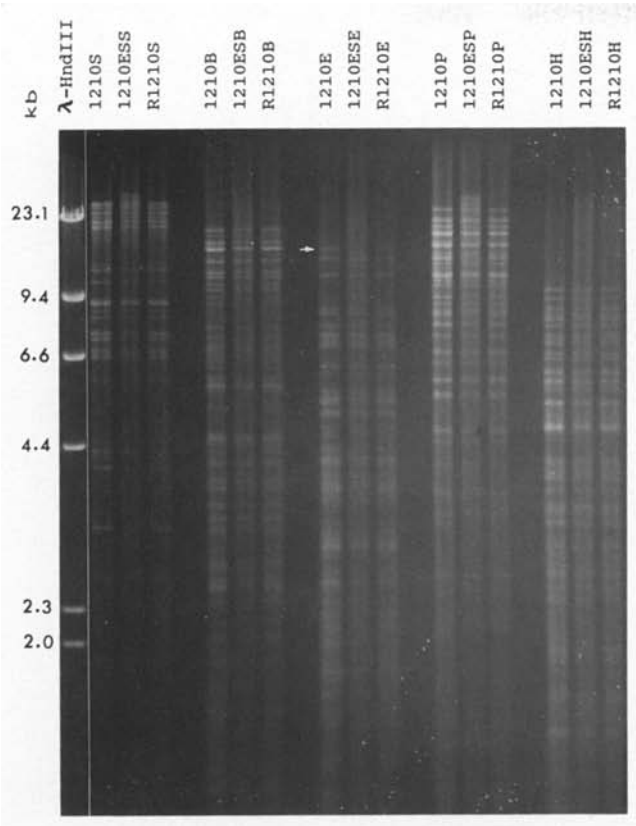

Fig. 2. Restriction patterns of mtDNAs from the 'CP72-1210' (*1210*) source parent, the embryogenic suspension culture (*1210ES*) and the bulk regenerants (*R1210*) digested with *Sal*I (*S*), *Bam*HI (*B*), *Eco*RI (*E*), *Pst*I (*P*) and *Hin*dIII (*H*). *Arrow* indicates the band difference. Lambda DNA digested with *Hin*dIII was used as size markers

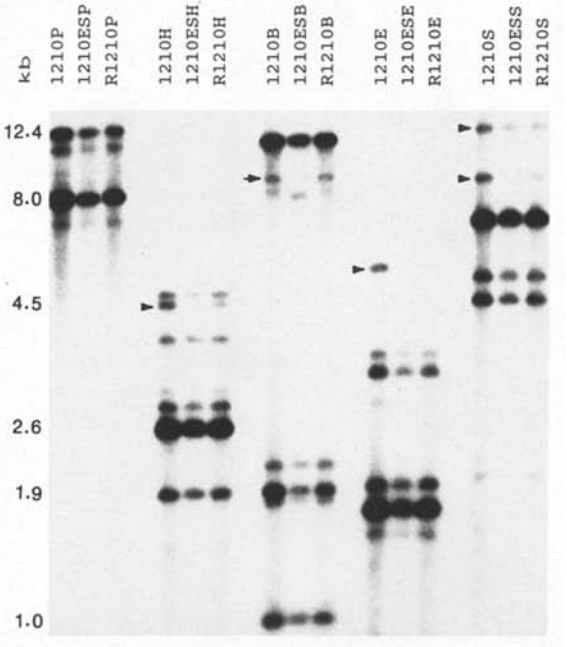

Fig. 3. Autoradiogram of the Southern blots consisting of mtDNAs from *1210*, *1210ES* and *R1210* digested with *Pst*I, *Hin*dIII, *Bam*HI, *Eco*RI and *Sal*I hybridized by the wheat probe K3. *Arrow* indicates the restriction fragment length polymorphism or fragment intensity difference

identical for all of the five enzymes tested and those of 'CP72-1210' and 1210ES were identical for four of the five enzymes. For the *Eco*RI pattern, a 15-kb fragment present in 'CP72-1210' was absent in 1210ES. To test the quality of mtDNA (isolated as part of the total DNA), one lane of parental (source) mtDNA (isolated by using mtDNA isolation procedure) was always included in total DNA gels. The results showed that in every instance the migration pattern was the same, although in some cases the fragment in the mtDNA lane migrated slightly faster than the total DNA.

Hybridization patterns of mtDNA blots

Hybridization patterns of the K3, K′ and X2 probes hybridized to the blots containing *Pst*I-, *Eco*RI-, *Bam*HI-, *Hin*dIII- and *Sal*I-digested mtDNAs are shown in Figs. 3, 4 and 5, respectively. The K3 probe hybridized to a number of fragments in each of the blots of different enzyme digests. No variation was observed in the hybridization patterns of *Pst*I-digested DNA. A 4.5-kb fragment present in the 'CP72-1210' *Hin*dIII blot was missing in 1210ES and present at a much lower intensity in R1210. For *Bam*HI, a 8.7-kb fragment present in 'CP72-1210' and R1210 was absent in 1210ES. A 5.2-kb fragment present in the 1210 *Eco*RI blot was absent in 1210ES and present at a very low intensity in R1210. Similarly, for *Sal*I, a 8.7-kb fragment present in 'CP72-1210' was absent in 1210ES and present at a much lower intensity in R1210, and a 12.4-kb fragment present in 'CP72-1210' showed a faint band in 1210ES and R1210. The K′ probe hybridized to many fragments in each of the restriction digests and except for a 7.7-kb fragment present in 'CP72-1210' and R1210 and absent in 1210ES, no other differences were observed. The X2 probe hybridized to a number of fragments in each of the restriction digests, and the only difference was a 9.5-kb fragment present in 'CP72-1210' that was not seen in 1210ES and R1210.

Discussion

Results of the present investigation show that no major variation occurred in sugarcane mtDNA as a result of in

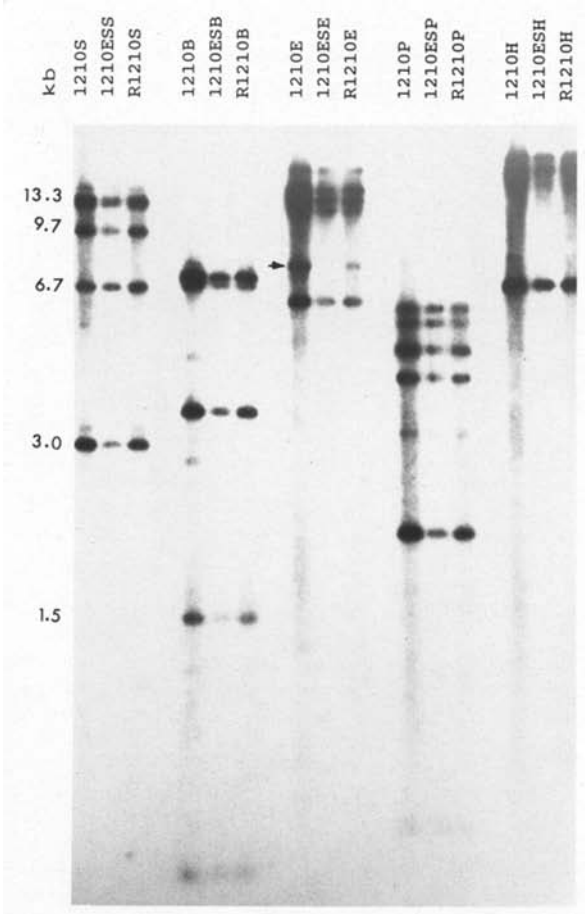

Fig. 4. Autoradiogram of the Southern blots consisting of mtDNAs from *1210*, *1210ES* and *R1210* digested with *Sal*I, *Bam*HI, *Eco*RI, *Pst*I and *Hin*dIII hybridized by the wheat probe K′. *Arrow* indicates the restriction fragment length polymorphism or fragment intensity difference

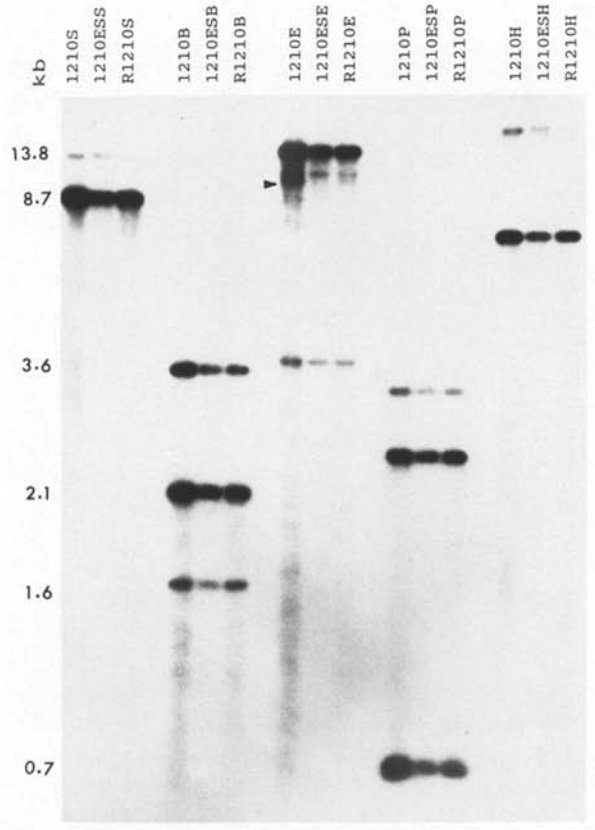

Fig. 5. Autoradiogram of the Southern blots consisting of mtDNAs from *1210*, *1210ES* and *R1210* digested with *Sal*I, *Bam*HI, *Eco*RI, *Pst*I and *Hin*dIII hybridized by the wheat probe X2. *Arrow* indicates the restriction fragment length polymorphism or fragment intensity difference

vitro culture. Similar results have been reported in rice (Saleh et al. 1990), in wheat (Breiman et al. 1989; Rode et al. 1985; M. K. U. Chowdhury, V. Vasil and I. K. Vasil, unpublished results) and in barley (Shimron-Abarbanell and Breiman 1991; Karp et al. 1987). Shimron-Abarbanell and Breiman (1991) assessed tissue culture-derived genetic variability among normal (green) regenerants of *Hordeum marinum* using multiple analytic tools, which included molecular probes (*Nor* rDNA, cDNA of B and C hordein, alpha-amylase gene, coding sequences of mitochondrial *atp9*, *atp6*, *atpA*, *coxI*, *coxII* and *cob*), and biochemical assays (SDS-PAGE analyses of hordein B and C and activity assays of alpha-amylase). They reported relative stability when assayed with coding sequences and by biochemical analyses. Saleh et al. (1990) showed an indistinguishable *Bam*HI restriction profile among the mtDNAs of var 'Taipei 309' from leaves, a 2-month-old totipotent cell suspension culture and a 19-month-old suspension culture that had lost its protoplast regeneration ability. Breiman et al. (1989) examined the *Nor* locus of four wheat lines and their regenerants and observed variability in only one of the four lines, which was variable both for the source parents (cv 'ND7532') as well as for the regenerants. Karp et al. (1987) observed genetic stability in 41 out of 42 barley regenerants from immature embryos. Regenerated plants were studied cytologically and their seed progenies analyzed for the isozymes esterase and glutamate-oxalate transaminase, ribosomal DNA spacer length polymorphism and hordein patterns on SDS-PAGE. Rode et al. (1985) compared the organelle DNAs of wheat source parent (cv 'Moisson') and its dihaploids and observed no variation in their restriction patterns.

It has been shown that in regenerants from embryogenic calli there is a strong selection in favor of plant regeneration from cytogenetically normal cells (Swedlund and Vasil 1985). Nevertheless, many reports document morphological, chromosomal, and biochemical

variation, and DNA restriction fragment length polymorphisms among plants regenerated in vitro (see review by Larkin et al. 1989). It is clear that the presence or absence of variation is dependent on the source of explants and the method of regeneration (somatic embryogenesis versus organogenesis) or due to the use of regenerants originating from different sources (calli, cell suspension and protoplast). The interpretation of data on variation may be sometimes complicated by the fact that in many instances variation is perpetuated from the source parent. Breiman et al. (1989) reported that *Nor* region variability among tissue culture-derived wheat plants is not necessarily related to in vitro culture but can be explained, at least partially, by perpetual *Nor* region heterozygosity. Similarly, in *Pennisetum americanum* (Morrish et al. 1990) and sugarcane (Heinz and Mee 1971) a positive correlation was shown between the presence of inherent somatic or chromosomal variation in the original explant and its expression in the regenerants.

Although it can be argued that tissue culture did not produce significant variation, one must keep in mind that a number of other factors may account for the genomic stability observed in the present experiments: (1) the probes used were not suitable to detect somaclonal variation. (2) Sugarcane organelle DNA is less vulnerable to an in vitro culture effect. (3) Somatic embryos from cells with major changes in the coding regions of important gene(s) may not survive, resulting in a positive selection for regeneration from normal cells. It should be pointed out, however, that four of the six mt gene probes used in the present investigation had been previously used to detect variation in rice suspension cultures (Chowdhury et al. 1990). The effectiveness of known mt gene probes to study variation has also been reported in rice by Saleh et al. (1990). The *Nor* locus DNA, consisting of either a coding sequence and/or a non-coding sequence, has been used in many studies to detect tissue culture-derived variation (May and Appels 1987; Brettell et al. 1986; Rode et al. 1987; Breiman et al. 1989). In addition, a very large number of probes were used in the present investigation in comparison to most other studies. It seems safe to conclude, then, that the regeneration of plants from somatic embryos may be the most likely cause of genomic stability in the tissue culture-derived plants.

Sugarcane is polysomatic (a chromosomal mosaic) and vegetatively propagated. Morphological and cytological variations, therefore, are common in plants grown in vivo and in vitro. In the investigation reported here we used DNA restriction fragment length polymorphism techniques to compare the original donor plant with regenerants produced from different sources in the same genetic background, e.g. from calli, cell suspensions, and protoplasts. DNAs from 19 different sources were hybridized with 12 probes of different sources and origin. This may allow some minor changes in the DNA to escape detection, but any major changes should be detectable. In some species, a specific part of the genome may become unstable as a result of in vitro culture and be expressed as a hypervariable segment in calli and in regenerants. For example, in the wheat mitochondrial genome, Rode et al. (1987) reported two to three such hypervariable segments covering less than 1% of the genome, and most of their later publications were based on the results obtained from the hybridization patterns of those probes. However, none of the important mitochondrial genes were located in those segments. Minor variation detected with the known mt genes in the present investigation agrees with the wheat data obtained by A. Rode (personal communication). We have also used wheat hypervariable sequences as probes to assess in vitro culture effects in sugarcane. Variation was observed to a lesser degree and most of it in 1210ES. This suggests that such hypervariable sequences are also induced in the sugarcane mt genome as a result of tissue culture, but to a lesser extent than in wheat. To look for comparable hypervariable sequences in sugarcane regenerants it is necessary to have a complete mt genome library and individual clones hybridized to the parental and regenerant mtDNAs. The most likely explanation for the lack of variation seen in sugarcane regenerants may be the use of embryogenic, rather than organogenic, cultures, which are less prone to genetic changes and produce true to type plants because of stringent selection in favor of normal cells during somatic embryo formation (Vasil 1988).

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