Theor Appl Genet (1995) 90: 1169–1173

P. W. J. Taylor · J. R. Geijskes · H.-L. Ko · T. A. Fraser
R. J. Henry · R. G. Birch

Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture

Received: 10 September 1994 / Accepted: 22 November 1994

Abstract Random amplified polymorphic DNA (RAPD) analysis using 10-mer oligonucleotide primers efficiently differentiated sugarcane cultivars and proved suitable for detecting gross genetic change such as that which can occur in sugarcane subjected to prolonged tissue culture, for example in protoplast-derived callus. However, RAPD analysis was not sufficiently sensitive to detect smaller genetic changes that occur during sugarcane genetic transformation. The length of DNA scored for polymorphism per primer averaged 13.2 kb, or 0.0001% of the typical sugarcane genome size of 1.2×10^7 kb (2C). RAPD analysis of sugarcane plants regenerated from embryogenic callus revealed very few polymorphisms, indicating that gross genetic change is infrequent during this tissue culture procedure, although epigenetic effects result in transient morphological changes in regenerated plants. More sensitive variations on the RAPD technique may increase the practicality of DNA-based screening of regenerated plant lines to reveal somaclonal variants.

Key words RAPD · Sugarcane · Embryogenic callus · Genetic transformation · Somaclonal variation

Communicated by Y. Gleba

P. W. J. Taylor (✉)[1] · T. A. Fraser
Bureau of Sugar Experiment Stations, PO Box 86, Indooroopilly, Queensland 4068, Australia

J. R. Geijskes · R. G. Birch
Department of Botany, The University of Queensland 4072, Australia

H.-L. Ko · R. J. Henry
Queensland Agricultural Biotechnology Centre, Gehrmann Laboratories, The University of Queensland 4072, Australia

Present address:
[1] Joint Centre for Crop Improvement Faculty of Agriculture, Forestry & Horticulture, The University of Melbourne, Parkville, Victoria 3052, Australia

Introduction

Genetic variability, termed somaclonal variation, can be generated during tissue culture (Larkin and Scowcroft 1981). The frequency of somaclonal variation may increase with length of time in culture (Scowcroft 1984).

Transgenic sugarcane plants can be regenerated from embryogenic callus transformed using microprojectile bombardment (Bower and Birch 1992). Transgenic sugarcane plants will need to have minimum genetic changes to be used directly as commercial cultivars. Hence, methods are required to detect genetic change in plants regenerated after tissue culture steps required for the transformation process.

Chowdhury and Vasil (1993) found no DNA variation in sugarcane plants regenerated from callus, cell suspension cultures and protoplasts analysed for DNA restriction fragment length polymorphism (RFLP). Similarly, RFLP techniques did not reveal variation among plants regenerated from cell suspension cultures and protoplasts of *Festuca* (Vallés et al. 1993) and rice (Saleh et al. 1990). In contrast, Sabir et al. (1992) detected somaclonal variants of *Beta vulgaris* plants regenerated from callus and leaf discs using isozymes and RFLP analysis, and Muller et al. (1990) used RFLP analysis to detect somaclonal variation in rice plants derived from tissue culture.

Polymerase chain reaction (PCR) techniques were developed in the late 1980s (Saiki et al. 1985) and have been applied widely in the genetic identification of biological samples. PCR requires only a small amount of DNA, which is amplified to produce a specific marker profile for identification of the sample (Welsh and McClelland 1990). Random amplified polymorphic DNA (RAPD) assays utilise arbitrary 10-mer oligonucleotide sequences as primers (Williams et al. 1990). Primers hybridise to two nearby sites in the template DNA that are complementary to the primer sequence. Deletions or insertions in the amplified regions or base changes altering primer binding sites will result in polymorphisms. RAPD markers have been used for distinguishing plant species and cultivars, such as the

cereals (Ko et al. 1994) and *Festuca* (Vallés et al. 1993); for genetic mapping (Williams et l. 1990; Torres et al. 1993; Yu and Pauls 1993); and for analysing the genetic stability of tissue-cultured plants (Vallés et al. 1993). The investigation described in this paper assessed the sensitivity of RAPD assays to detect genetic change in sugarcane plants regenerated from embryogenic callus.

Materials and methods

Two experiments involving different DNA extraction methods and PCR conditions were used to assess the efficacy of RAPD assays to detect genetic change in transgenic and non-transgenic sugarcane plants regenerated from embryogenic callus and in protoplast-derived callus. The different methodologies used in this study reflect two different approaches to RAPD analysis of sugarcane.

RAPD analysis of plants regenerated from embryogenic callus and protoplast callus

Establishment of callus cultures and plant regeneration

Callus was initiated from immature leaf explants of sugarcane (*Saccharum* spp. hybrids) cv 'Q63' cultured on MS medium, and embryogenic callus was selectively subcultured, as described by Taylor et al. (1992a). After 4.5 months of culture, embryogenic callus was transferred to solid MS medium without plant growth regulators and incubated under diffuse light with a 12-h photoperiod at 27°C. Individual plantlets were then transferred to rooting medium (MS with 60 g/l sucrose and 1 mg/l NAA; Taylor and Dukic 1993) before transfer to pots for growth in the glasshouse.

Protoplast callus

Non-morphogenic callus was regenerated from protoplasts isolated from a homogeneous suspension culture of cell line Q63 SP (Taylor et al. 1992a, b). This callus was maintained for more than 2 years by subculture onto fresh medium every 3 weeks.

DNA isolation

DNA was extracted using the modified CTAB method of Graham et al. (1994). Samples for DNA extraction were: (1) the spindle leaves of each of 8 'Q63' plants grown in the glasshouse for 12, 16 and 17 months after regeneration from embryogenic callus; (2) spindle leaves from 5 field-grown plants of 'Q63'; and (3) protoplast-derived callus of Q63 SP. Genomic DNA was quantified spectrophotometrically (Sambrook et al. 1989).

Polymerase chain reaction

A total of 25 arbitrary 10-mer oligonucleotide sequences (Operon Technologies, USA) were screened for amplification of plant genomic DNA fragments. Primers OPB-11, OPC-14, OPC-15, OPD-01 and OPM-01 were used to amplify protoplast-derived genomic DNA. Conditions for DNA amplification were standardised for all primers. Each 25-µl reaction volume contained 30 ng DNA template, 0.8 U *Taq* DNA polymerase (Boehringer Mannheim Biochemica), 0.24 m*M* each of dATP, dGTP, dTTP and dCTP (Promega), 0.2 µ*M* primer and PCR buffer with a final concentration of 10.0 m*M* TRIS-HCl, pH 8.3, 50.0 m*M* KCl, 3.0 m*M* $MgCl_2$, 0.1 mg/ml gelatin.

Amplification was performed in a Perkin Elmer 9600 GeneAmp PCR System and was initiated by a denaturation of 1 min at 94°C, followed by 33 cycles of 10 s each at 94°C, 30 s at 40°C and 1 min at 72°C. The amplification was completed with 1 cycle of 5 min at 72 °C.

Reaction products were resolved by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide and revealed by UV illumination. PCR reactions were repeated to establish reproducibility of results.

RAPD analysis of transgenic plants

Transgenic plants

Transgenic sugarcane plants of cv 'Pindar' containing the neomycin phosphotransferase (*neo*) gene were produced by microprojectile bombardment of 4- to 6-month-old embryogenic callus (Bower and Birch 1992). Untransformed controls were regenerated after the same time in tissue culture.

DNA isolation

DNA was extracted from leaf tissue of 1 control and 11 transgenic 'Pindar' plants 24 months after their regeneration from embryogenic callus and from 1 plant each of the field-grown sugarcane cultivars 'NCo310', 'NG51-142', Pindar, 'Q44', 'Q63' and 'Q87'. DNA was isolated using a modified method of Oard and Dronavalli (1992). Leaf tissue was frozen in liquid nitrogen, ground in 1.5-ml Eppendorf tubes, and the DNA extracted in 0.01 M 2-mercaptoethanol, 0.1 *M* TRIS-HCl, pH 8.0, 0.5 *M* NaCl, 0.05 *M* EDTA at 100°C for 5–15 min. DNA was quantified visually on a 1% (w/v) agarose gel by comparison to λ-DNA standards of known concentration.

Polymerase chain reaction

A total of 41 arbitrary 10-mer oligonucleotide sequences (Operon Technologies, USA) were screened for amplification of genomic DNA fragments. Conditions for DNA amplification were standardised for all primers. Each 25-µl reaction volume contained 20 ng DNA template, 0.17 m*M* each of dATP, dGTP, dTTP and dCTP (Promega) 0.6 µ*M* primer and PCR buffer with a final concentration of 10.0 m*M* TRIS-HCl, pH 8.3, 50.0 m*M* KCl, 4.0 m*M* $MgCl_2$, 0.1 mg/ml gelatin. The reaction mixtures were overlayed with approximately 0.025 ml of paraffin oil. Tth DNA polymerase (1.3 U, Biotech International) was added to each reaction after the initial denaturation step of amplification.

Amplification was performed in a Perkin Elmer 480 Thermal Cycler and was initiated by a denaturation of 5 min at 93°C, followed by 33 cycles of 10 s at 93°C, 30 s at 37°C, and 1 min at 72°C. The amplification was completed with 1 cycle of 5 min at 72°C.

Reaction products were resolved by electrophoresis on 2.0% (w/v) agarose gels, stained with ethidium bromide and revealed by UV illumination.

Results

RAPD analysis of plants regenerated from embryogenic callus and protoplast callus

All the plants regenerated from embryogenic callus of cv 'Q63' showed some morphological variation compared to the field-grown source plants of the same cultivar. This variation consisted of profuse tillering, twisting of the leaf lamina giving a crinkled appearance and in several plants the spindle leaf was enclosed within an elongated leaf sheath. However, propagation of single-bud cuttings from these plants resulted in plants with typical morphology for cv 'Q63'.

Table 1 Arbitrary 10-mer primers used in RAPD analysis of sugarcane plants and protoplast-derived callus

Primer[a]	Sequence 5′–3′	Number of scorable bands[b]	
		Experiment 1	Experiment 2
OPB-02	TGATCCCTGG	4	5
OPB-03	CATCCCCCTG	0[c]	9
OPB-05	TGCGCCCTTC	0[c]	14
OPB-06	TGCTCTGCCC	0	7
OPB-08	CTCCACACGG	0	6
OPB-11	GTAGACCCGT	10	8
OPC-13	AAGCCTCGTC	9	15
OPC-14	TGCGTGCTTG	10	11
OPC-15	GACGGAGCAG	15	10
OPC-16	CACACTCCAG	8	5
OPD-01	ACCGCGAAGG	14	12
OPC-05	TGAGCGGACA	5	16
OPD-08	GTGTGCCCCA	0	11
OPE-01	CCCAAGGTCC	0	12
OPE-02	GGTGCGGGAA	0	14
OPE-05	TCAGGGAGGT	7	19
OPE-08	TCACCACGGT	0	9
OPM-01	GTTGGTGGCT	8	–[d]
OPM-05	GGGAACGTGT	6	–
OPM-06	CTGGGCAACT	12	–
OPM-13	GGTGGTCAAG	4	–

[a] Primers from Operon Technologies, USA
[b] Number of bands scored for cv 'Q63' in experiment 1, and for six cultivars in experiment 2. Amplification conditions for each experiment are described in the text
[c] Amplified product was present, but bands were faint and not consistent
[d] Primer not tested

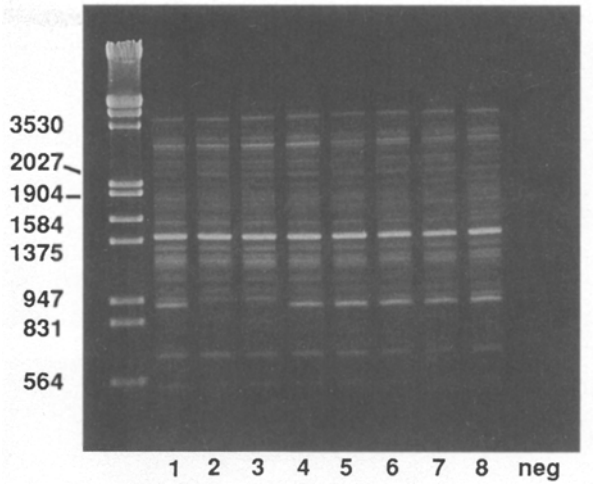

Fig. 1 Amplification of genomic DNA of 8 plants of sugarcane cv 'Q63' regenerated from embryogenic callus with primer OPM-06 (Operon Technologies). Approximate fragment sizes are shown, determined from *Eco*R I/*Hin*d III-cut λDNA on the same gels. The negative control (*neg*) is PCR amplification without DNA

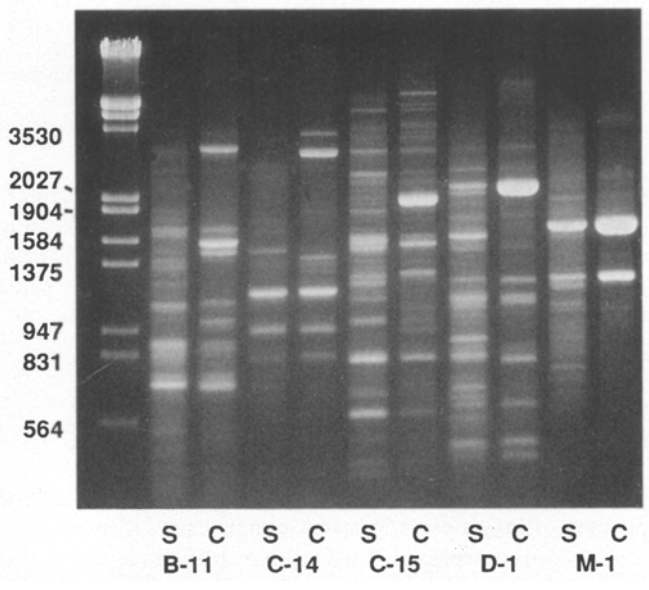

Fig. 2 Amplification of genomic DNA of a plant regenerated from embryogenic callus (*S*) and protoplast-derived callus (*C*) of sugarcane cv 'Q63' with primers OPB-11, OPC-14, OPC-15, OPD-01 and OPM-01 (Operon Technologies). Approximate fragment sizes are shown, determined from *Eco*RI/*Hin*dIII-cut λDNA on the same gels

RAPD analysis resolved 112 scorable markers from 13 of the 25 primers screened (Table 1). Primers produced between 4 and 15 amplification products, which ranged in size between approximately 400 and 3800 bp. The primers that did not produce scorable markers either produced faint or non-consistent amplification products, or no amplification product.

PCR amplification products for the 8 regenerated plants were identical to those of the field-grown plants, except with primer OPM-06 where the 940-bp fragment was missing from 2 of the plants (Fig. 1, lanes 2, 3). The polymorphism in these two plants was consistent in three DNA extractions over a 5-month period and after the plants were vegetatively propagated.

Protoplast-derived callus of the Q63 SP cell line showed a high degree of genetic change relative to the source cultivar, with polymorphisms being detected by primers OPB-11, OPC-14, OPC-15, OPD-01 and OPM-01 (Fig. 2).

RAPD analysis of transgenic plants

Analysis of the genomic DNA of field-grown plants of six sugarcane cultivars using 41 primers resolved 204 scorable markers, from 17 primers (Table 1). Primers produced between 5 and 19 amplification products, which ranged in size between approximately 220 and 1930 bp. Consistent banding profiles were obtained for replicates of each cultivar of sugarcane.

A selection of 6 primers (OPC-13, OPC-14, OPC-15, OPC-16, OPD-01 and OPE-08) was sufficient to reveal multiple polymorphisms between all tested cultivars. For example, primers OPC-13 and OPC-15 produced a distinct pattern of amplification products for each cultivar (Fig. 3).

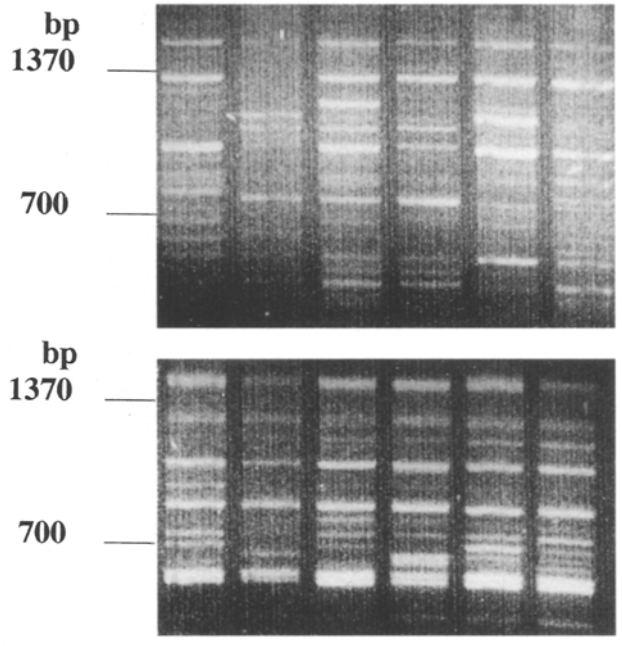

Fig. 3 Amplification of genomic DNA of 6 sugarcane cultivars with 2 RAPD primers: *top panel* primer OPC-13, *bottom panel* primer OPC-15 (Operon Technologies). *Lanes* from *left to right* are from sugarcane cvs 'Q44', 'Q63', 'NCo310', 'Q87', 'NG51-142' and 'Pindar'. Approximate fragment sizes are shown, determined from *Bst*EII-cut λDNA on the same gels

PCR amplification products from the 17 useful primers determined above were identical for the 1 non-transgenic and 11 transgenic plants of cv 'Pindar' regenerated from embryogenic callus, and the field-grown plants of the same cultivar.

Discussion

RAPD analysis using arbitrary 10-mer oligonucleotide primers efficiently differentiates sugarcane cultivars. The level of polymorphisms between the cultivars tested in this study indicates that distinction between any two cultivars of sugarcane should be possible with a small number of appropriate primers. This level of polymorphism is not surprising given that sugarcane cultivars are highly heterozygous, polyploid, interspecific hybrids obtained by outcrossing of diverse parents (Sreenivasan et al. 1987).

RAPD analysis also revealed gross genetic change in protoplast-derived callus of line Q63 SP, with all of the 5 primers tested revealing polymorphisms. This callus had been in tissue culture for over 2 years and had lost the ability to differentiate shoots and regenerate plants. It is likely that this cell line has undergone rearrangement, mutation and the loss of chromosomes (Karp et al. 1987).

The morphological changes observed in 'Q63' plants regenerated from embryogenic callus did not persist through vegetative propagation, indicating that these changes were epigenetic. Tissue culture conditions are expected to lead to peculiar patterns of gene expression in plant cells, which may cause some transient phenotypic changes in regenerated plants. The uniformity of RAPD patterns in these plants and the parent cultivar is consistent with this explanation.

However, RAPD analysis failed to reveal any polymorphism in transgenic plants of sugarcane cv 'Pindar' regenerated from embryogenic callus after insertion of the *neo* gene. It is thought that somaclonal variation may be less frequent in plants regenerated from young embryogenic cultures than from other tissue culture systems because the necessity for somatic embryo formation from a single cell selects against many genetically altered cells (Vasil 1988). Chowdhury and Vasil (1993) also found no variation detectable by RFLP analysis in sugarcane plants regenerated from embryogenic cultures. In contrast, RFLP analysis revealed substantial variation in plants regenerated from non-embryogenic tissue cultures of *Beta vulgaris* and *Oryza sativa* (Sabir et al. 1992, Muller et al. 1990). Thus, the transgenic sugarcane plants examined here could have little genetic change other than the insertion of one or a few copies of the *neo* gene revealed by Southern analysis (Bower and Birch 1992).

Under the experimental conditions for experiment 2 described above, effective RAPD primers produced an average of 12 scorable PCR products with an average size of 1.1 kb. Thus, the length of DNA scored for polymorphism per primer averaged 13.2 kb, or 0.0001% of the typical sugarcane genome size of 1.2×10^7 kb (2C). Even in this portion of the genome, DNA base changes, base substitutions, short deletions or short insertions will not result in detectable polymorphisms unless they occur within a primer binding site. It follows that unless a very large number of primers are used, RAPD analysis will lack the sensitivity to reliably detect small genetic changes due to somaclonal variation.

The single polymorphism revealed in plants of sugarcane cv 'Q63' regenerated from 4.5-month-old embryogenic callus could indicate: (1) a cell line which has undergone a substantial genetic change that does not interfere with regeneration, (2) a chance coincidence of a minor mutation with an OPM-06 primer site; or (3) an OPM-06 amplicon spanning a highly mutable genomic site. The failure of other primers to reveal the change and the same polymorphism in two separate regenerants are consistent with the third possibility. This possibility is interesting because the identification of such amplicons would allow the selection of primers with the highest probability of detecting genetically altered lines. It will be necessary to screen more regenerated plants and more primers to test this possibility.

Recent modifications to the basic RAPD approach may reduce the number of primers required to provide a reasonable sensitivity of detection of genetic change. For example, DNA amplification fingerprinting or DAF (Caetano-Anollés et al. 1991) uses shorter primers to generate many potential amplicons for comparison, predigestion with a restriction enzyme to eliminate many shared potential amplicons, polyacrylamide gels for improved resolution of

bands and silver staining for increased sensitivity of detection. These modifications make the technique somewhat more technically demanding, but the increased sensitivity should make DNA-based screening for somaclonal variants more practical, particularly if combined with primers shown to amplify mutable regions of the plant genome.

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