Biotecnología Aplicada, Vol. 9, No. 2, 156-165 (1992)

Regeneration of transgenic sugarcane (*Saccharum officinarum L.*) plants from intact meristematic tissue transformed by electroporation

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Received in February 1992
Approved in April 1992

SUMMARY

We describe an efficient procedure for transformation and regeneration of sugarcane transgenic plants. The methodology was established by using plasmids which confer the GUS and NPT II activities to transform plant cells. When necessary, electroporated material was subsequently grown on a selective medium and led to the regeneration of whole transgenic plants. The transformed plants were tested by histochemical, fluorometric, PCR and Southern blot analysis. In this report, we also promote the electroporation of intact cells as an efficient and attractive method to transform both monocotyledonous and dicotyledonous plants.

RESUMEN

Describimos un procedimiento eficiente para la transformación y regeneración de plantas transgénicas de caña de azúcar. El método se basa en la electroporación de tejido meristemático de dos variedades comerciales de caña de azúcar (POJ 2878 y Ja 60-5). La transformación se realizó usando plasmidios que confieren actividad GUS y NPT II a las células transformadas. Cuando fue necesario, el material electroporado fue subcultivado en medio selectivo hasta la regeneración de plantas transgénicas. Las plantas transformadas se analizaron por los métodos histoquímico, fluorométrico, PCR y *Southern blot*. Los resultados obtenidos promueven el método de electroporación de tejidos vegetales intactos como vía eficaz y atractiva para la transformación de plantas monocotiledóneas y dicotiledóneas.

INTRODUCTION

Agrobacterium tumefaciens, the causative agent of crown gall, has become an established tool in the genetic engineering of plants. Agrobacterium mediated transformation of dicotyledonous plants is well established, and has produced stable transgenic plants (Walden *et al.*, 1988). However, many of the most agricultural important crops are monocotyledonous plants and are difficult to transform by current techniques. Up to date only a few members of the families *Liliaceae* (Bytebier *et al.*, 1987; Hooykaas *et al.*, 1984), Amaryllidaceae (Hooykaas *et al.*, 1984), Iridaceae (Graves *et al.*, 1987), *Discorea* (Shafer *et al.*, 1987) and *Gramineae* (Graves *et al.*, 1986; Raineri *et al.*, 1990) have been reported to be transformable by Agrobacterium

In recent years, the use of alternative techniques for direct introduction of DNA into plant cells has opened new perspectives for transforming many previously inaccessible plants (Potrikus, 1989). These alternative methods involve electroporation of plant protoplasts (Fromm *et al.*, 1985;

1986), polyethylene glycol treatments of protoplast (Sillito *et al.*, 1985; Lörz *et al.*, 1985) and micro-projectile bombardment of plant tissues (Sanford, 1988; Gordon Kamm *et al.*, 1990).

Sugarcane (*Saccharum officinarum L.*), one of the most important crops in the tropical and subtropical countries, is a gramineous plant up to date inaccessible for genetic engineering manipulation. The establishment of an efficient method for direct transformation of sugarcane involves the introduction of foreign DNA into plant cells as well as the regeneration of the transformed cells to a whole transgenic plant. Chen *et al.* (1987) reported transformed sugarcane protoplasts to kanamycin resistance clusters, but failed to obtain transformed embryogenic calli. Regeneration of sugarcane plants from protoplasts was also reported (Srinivasan *et al.*, 1986), but manipulation is difficult and efficiency is low. Transient gene expression in intact and organized leaf tissues from rice, maize, wheat and barley electroporated with both p2 -npt II and p35S-npt II reporter genes was demonstrated by Dekeyser *et al.* (1990). Furthermore, no suitable conditions to obtain transformed calli and regeneration of transgenic plants for these species have been reported so far.

Here we report the successful application of electroporation of intact meristematic tissues for the direct transformation and the regeneration of sugarcane transgenic plants.

MATERIALS AND METHODS

Plasmids

Plasmids pBI 221.1 (Jefferson, 1987a) and pGSCGN 2 were used for electroporation (Fig. 1). To generate the plasmid pGSCGN 2, the 3 kb PstI-EcoRI fragment from pBI 221.1 (CaMV 35S promoter, β-glucuronidase (GUS) gene and NOS terminator) was blunted using klenow DNA polymerase and inserted into the XbaI (blunted) site of pGSC-1706 (Plant Genetic System, Belgium). The plasmid pGSCGN 2 includes the NPT II and the GUS genes both under the control of CaMV 35S promoter orientated in opposite transcription direction. Both plasmids were purified by CsCl gradient (Sambrook *et al.*, 1989) prior to electroporation.

Plant material

Basal meristematic tissues (1-2 mm) were excised aseptically from *in vitro* micropropagated plants of sugarcane (*Saccharum officinarum L.*) varieties POJ 2878 and Ja 60-5 (Jovellanos Agricultural Station, Cuba). The differentiated tissues from roots, stems and leaves were removed carefully. The meristematic tissues were incubated in Petri dished with MS liquid medium (Murashige and Skoog, 1962) plus 3-5 mg/L ascorbic acid for 2 hours in the dark at 25°C. The explants were transferred to 96 wells plated for 3 hours containing the electroporation buffer (EPR): 5 mM $CaCl_2$, 10 mM Hepes, 10 % glucose, pH 7.2 (Dekeyser *et al.*, 1990), which was replaced every hour. The EPR buffer was substituted for 200 mL of fresh EPR supplemented with 0.2 M spermidine and 20-30 μg plasmid DNA, The samples were kept in the dark at 4°C for 3 hours. Before electric discharge, 11 mL of sterile 3 M NaCl were added to each sample, increasing its ionic strength.

Electroporation

Electroporation was performed using sterile cuvettes (BIORAD. Richmond, USA) with 0.2 cm path length in a EPE-010 exponential pulse electroporator (Heber Biotec, Havana, Cuba). The pulse discharge was monitored using a voltmeter model FER-30A (CNIC, Havana, Cuba).

Experiments using 220; 440; 660; 880 uF capacitances and 150; 375; 550 V/cm voltages were done to adjust the electroporation conditions. Each treatment was replicated three times. Samples electroporated without plasmid DNA, and non-electroporated containing plasmid samples were used as controls. After electric discharge, meristems were maintained on ice for 10-15 minutes until plating on solid culture medium.

Growth and selection conditions

The electroporated meristems were cultured (Ro) in callus induction medium: MS salts supplemented with 1-3 mg/L 2,4 D; 500 mg/L casein hydrolyzate;

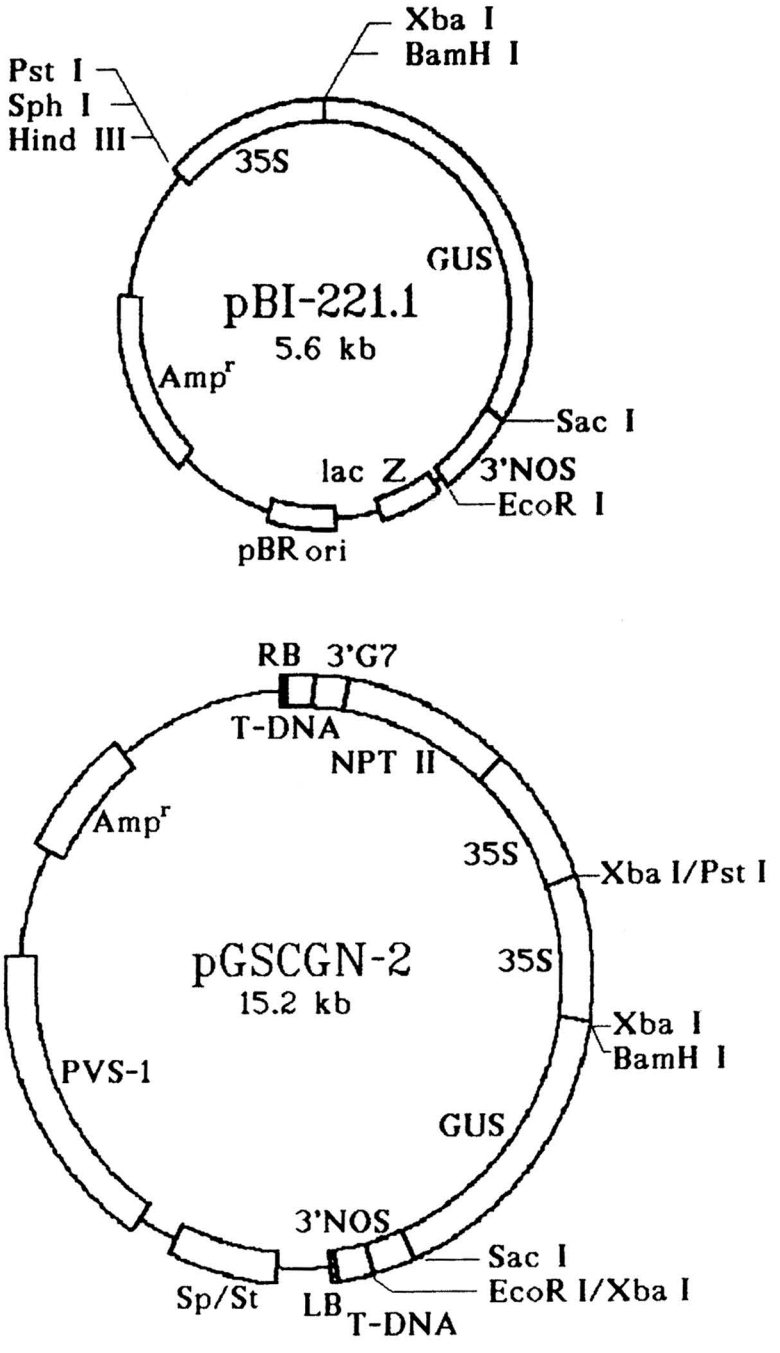

FIG. 1. Maps of the pBI 221.1 (Jefferson, 1987a) and pGSCGN 2 plasmids. 35S: 35S-CaMV promoter; 3'NOS: nopaline synthetase terminator; PVS-1: origin of replication in *A. tumefaciens* (plus stabilization sequence); Sp/St: resistance gene to both spectinomycin and streptomycin; RB and LB: right and left borders of T-DNA (plus adjacent T-DNA regions).

1 mg/L nicotinic acid; 0.8 mg/L thiamine HCl; 0.5 mg/L piridoxine; 100 mg/L myo-inositol; 2 % sucrose; 0.7 % agar, pH 5.6. The Petri dishes with the electroporated explants were maintained in the dark at 25°C.

When experiments were done with the plasmid pBI 221.1, five samples of meristems were harvested at 7 and 21 days for GUS activity determination by a fluorometric assay. The rest of the meristems were cultured during 8-10 weeks until callus formation and transferred to regeneration medium (the same callus induction medium but without 2.4 D) to obtain plants.

For the experiments with pGSCGN 2 plasmid, GUS activity was determined after 7 days in five explants. The rest of the meristems were subcultured (R1) to allow callus formation in the same medium with 200 mg/L kanamycin. After 21 days, the GUS activity was determined in other five explants. The one month old R1 calli were subcultured in the same medium (R2). After one month 10 R2 calli were weekly transfered to a regeneration medium, five of them to a medium supplemented with kanamycin (100 mg/L) and five to a medium without kanamycin. This procedure was performed five times. The regeneration capacity of R2 calli was evaluated.

Fluorometric GUS assay

The meristems were harvested 7 and 21 days after electroporation. The GUS expression was tested following the procedure described by Jefferson (Jefferson, 1987b). The fluorescence emission was measured by using an Ultra-Micro-Analytical-System (SUMA^{TM}, CIE, Havana, Cuba) with methylumbelliferone (Sigma, USA) as standard.

Histochemical GUS assay

Calli, roots and leaves from regenerated plants in all cases were tested for demonstration of stable GUS expression by histochemical GUS assay (Jefferson, 1987b). The samples were incubated with the substrate (2 mg/mL) in 96 wells plates for 72 hours at 28°C in the dark.

Molecular analysis of transgenic plants

Transgenic plants were tested by PCR (Frohman *et al.*, 1990) and Southern blot analysis (Southern, 1975). PCR was carried out for the screening of transformed population during the early growth stages. Previously, a small scale DNA extraction method was developed and the conditions for PCR test of transgenic plants were established (Goyenechea *et al.*, 1991). Specific synthesis primers to β-glucuronidase gene were synthesized (5'GTTACGTCCTGTAGAAACCCCAACCC 3' and 5'GTGCGGATTCACCACTTGCAAAGTCC 3'). The reaction mixtures were prepared using 2 μg of DNA, 100 pmol of each specific primer, 250 μM of dNTPs, 250 μM of each dNTP, 50 nM KCl, 10 mM Tris-HCl pH 8.3, 1.5 nM $MgCl_2$, 1.7 mg/mL BSA and 2 U of *T. aquaticus* DNA polymerase I (Heber Biotec, Havana, Cuba) in a final volume of 100 uL. The reaction was overlaid with liquid paraffin and cycled through the following temperature profile; 95°C for 2 min to denature the DNA, 56°C for 1 min for low stringency annealing of primers and 72°C during 2 min for polymerization. The PCR was completed in 30 cycles and the last polymerization was extended for 5 min allowing better termination of the polymerase reaction. PCR products were analyzed by Southern blot (Southern, 1975) using as a probe a 1.8 Kb fragment containing the entire GUS gene. Transgenic plants tested positive by PCR were subsequently grown in greenhouse conditions.

The total DNA transgenic plants were also analyzed by Southern blot, DNA from leaf tissue of both transformed and non-transformed plants was isolated. The leaves were freeze dried, ground in a mortar and pestled until a powder was obtained. DNA was extracted using a method described by Dellaporta *et al.* (1983). Samples with 10 μg of genomic DNA were EcoRI or BamHI-EcoRI digested under standard conditions. Following electrophoresis through 0.8 % agarose gel, DNA was transferred to Hydond-N nylon membranes (Amersham, UK). Hybridizations were done according to the instruction of the manufacturer. The probe consisted of the 1.8 kb BamHI-SacI fragment from pBI 221.1 containing the entire GUS gene. The radioactive probe was prepared by the random primer labeling reaction (Feinberg and Vogelstein, 1984).

RESULTS AND DISCUSSION

Different conditions for electrical field strength (150, 350, 550 V/cm) and capacitance (220, 440, 660, 880 μF) were tested in meristematic tissue of sugarcane varieties POJ 2878 and Ja 60-5 by pulsing plasmid pBI 221.1. The experimental results showed that GUS activity was directly proportional to voltage, but no significative differences among electroporated samples

and negative control were found at capacitance values lower than 660 μF. However, at a field strength of 375 V/cm and capacitance of 880 μF a remarkable GUS activity was observed (figure 2). All subsequent experiments were performed at these optimized electroporation conditions.

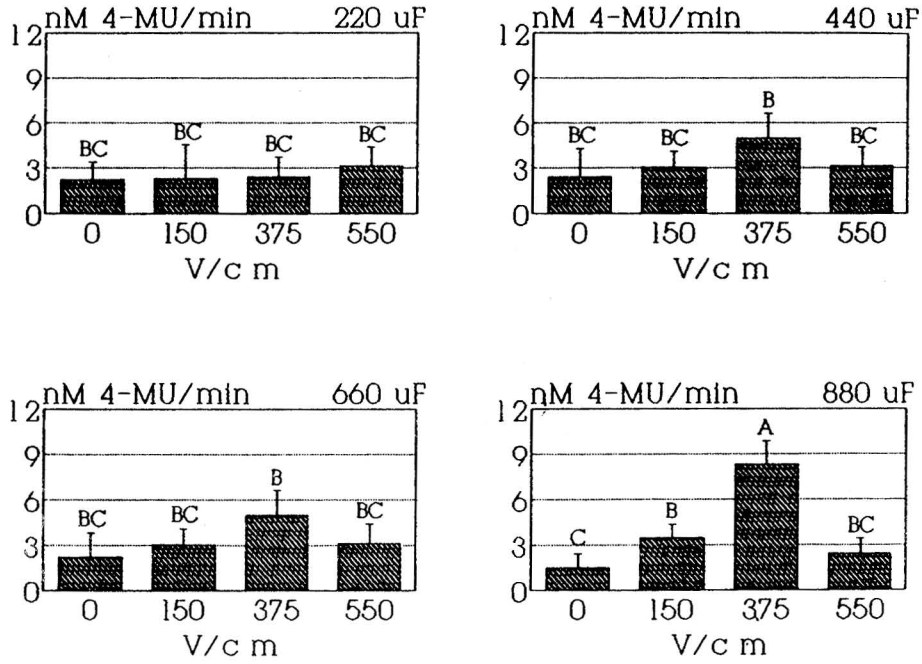

FIG. 2. Influence of voltage and capacitance on transformation efficiency. Electroporated meristems of sugarcane variety POJ 2878 were collected 7 days after electroporation. The expression of the β-glucuronidase gene was assayed by a fluorometric method. The GUS activity was directly proportional to voltage and the best results were obtained at field strength of 375 V/cm and capacitance of 880 μF. These values are the average of 3 independent experiments.

Different levels of GUS activity were found in electroporated meristematic tissue of both commercial varieties POJ 2878 and Ja 60-5. The results of fluorometric assay showed higher levels of methyl-umbelliferone in the case of Ja 60-5 variety after 7 days of electroporation with pBI 221.1 plasmid (figure 3).

We also evaluated the fluorometric GUS activity when either plasmids pBI 221.1 or pGSCGN 2 were pulsed. The major levels were showed in meristematic tissue electroporated with pBI 221.1. The GUS expression values 7 days after electroporation approximately 3 times higher than those obtained when the pGSCGN 2 was pulsed. The electroporated meristems with pGSCGN 2 were subsequently transferred to fresh callus induction medium supplemented with kanamycin (200 mg/L). After two weeks, the second evaluation was carried out and differences among the values obtained in a first evaluation were observed. The relative decrease in GUS activity in meristematic tissues electroporated with pBI 221.1 is a consequence of culture in a non-selective medium, where both transformed and non-transformed cells are able to grow. In contrast, when pGSCGN 2

was pulsed and electroporated tissues were subcultured in selective kanamycin medium, the increase of GUS activity was clearly observed (figure 4).

As a result of a long time study on the regeneration capacities of different explants obtained from various types of sugarcane tissues, we determined that only tissues

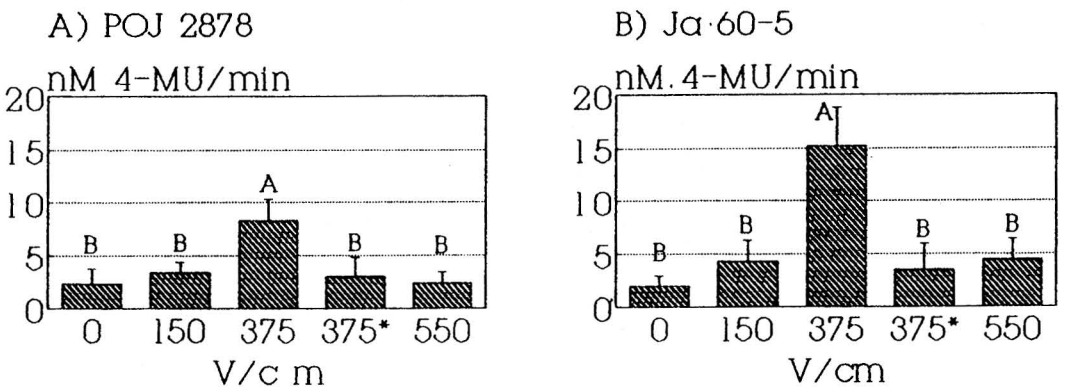

FIG. 3. Expression of the β-glucuronidase gene in electroporated meristems of commercial sugarcane varieties POJ 2878 (A) and Ja 60-5 (B). In both cases the test was carried out 7 days after electroporation by a fluorometric method. The best results for both varieties were obtained at 375 V/cm and 880 μF. These values are the average of 3 independent experiments.

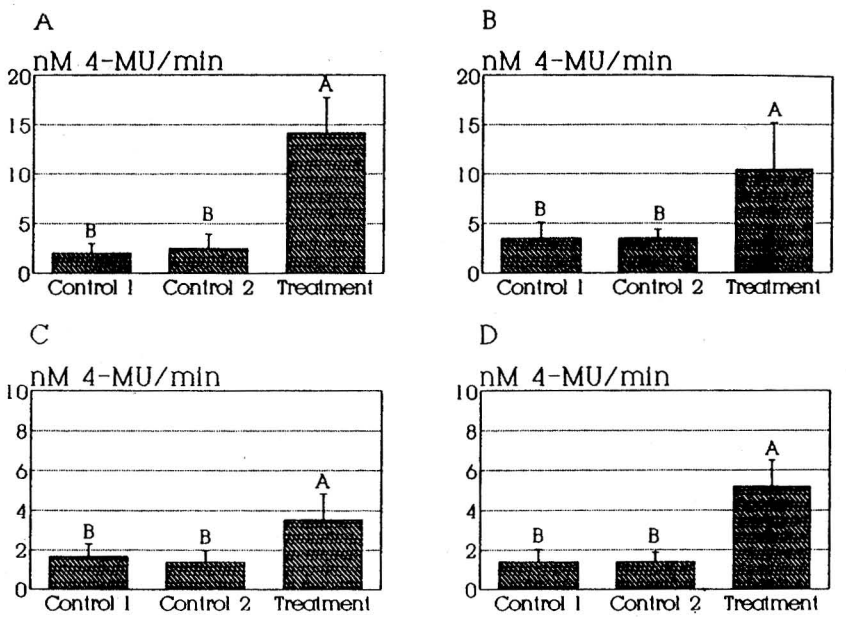

FIG. 4. Expression of the β-glucuronidase gene in electroporated meristems of sugarcane variety POJ 2878. GUS activity assayed 7 days (A and C) and 21 days (B and D) after electroporation in meristems pulsed with plasmid pBI 221.1 (A and B) and pGSCGN 2 (C and D), respectively. The test was performed by a fluorometric method. The relative increase in GUS activity was observed in meristems electroporated with pGSCGN 2 grown in selective kanamycin medium. These values are the average of 3 independent experiments. Electroporation was carried out at 375 V/cm and 800/ μF.

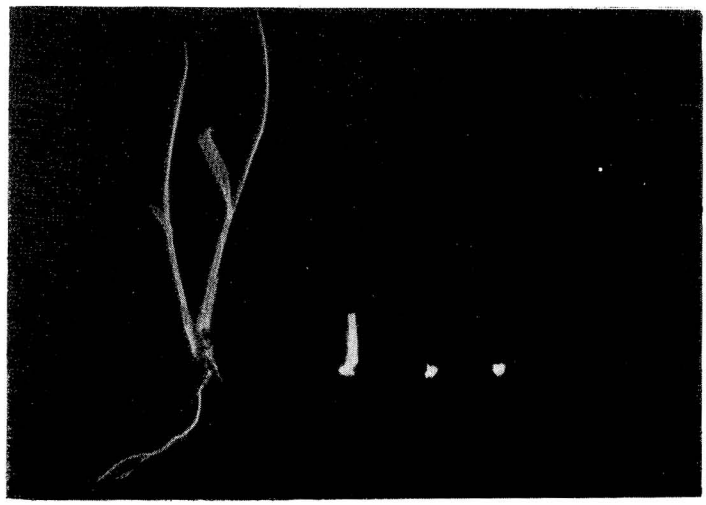

FIG. 5. Gross anatomy of a 15 day old *in vitro* micropropagated sugarcane plant and explant from meristematic region used in electroporation procedure.

localized in the central basal part of *in vitro* growing plants can produce an embryogenic calli (figure 5). About 50 % of explants from these tissues are able to develop embryogenic calli, and then, only 20 % can regenerate whole plants, although from one callus several plants can be formed.

The histochemically selected calli were subcultured in the same callus induction medium and its regeneration capacity evaluated by weekly transferring of 5 calli to the regeneration medium. The results showed that only calli grown for 4 or 5 weeks on callus induction medium were able to regenerate whole plants. During this critical period, the somatic embryogenesis could be induced in the transformed calli tissues. Some transgenic calli were regenerated in kanamycin free medium and other in kanamycin selective medium (100 mg/L) and mosaic plants were recovered showing GUS activity only in part of the tissues. In this case the transformation took place in an unpredictable number of cells, thus explaining the possibility of the regeneration of mosaic plants from cell clusters formed by both transformed and non-transformed cells. Whole transgenic plants expressing GUS activity were also regenerated. These plants were able to grow in selective medium containing kanamycin (100 mg/L).

The control cultures of non-transformed calli grew well in selective kanamycin medium at concentration levels up to 200 mg/L, although whole plants were not regenerated from these calli. Obviously, the kanamycin is an inadequate marker for early and accurate selection in callus cultures of sugarcane, contrasting with the inhibitory effect on regeneration. Similar results were reported by Dekeyser *et al.* (1989) and Hauptam *et al.* (1988) for some *Gramineae* including rice and sugarcane.

PCR test and Southern blot analysis evidenced the incorporation of the GUS gene in the genome of transgenic sugarcane plants (figure 6). The integration pattern showed the presence of the GUS gene in multiple copies, some of them without rearrangements (figure 7).

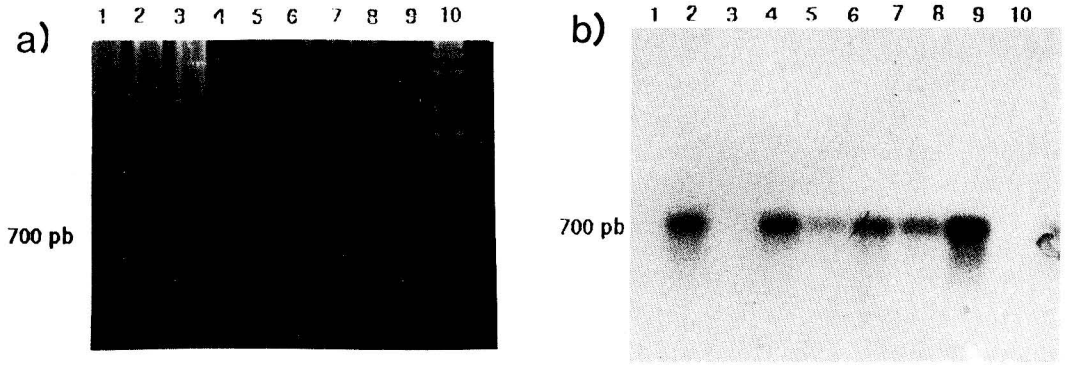

FIG. 6. a) PCR test of total DNA from transgenic sugarcane plants; b) Southern blot analysis of PCR products. Lane 1: Non-transformed plant of commercial variety Ja 60-5. Lanes 2 to 8: Transformed plants recovered from electroporated meristems. Lane 9: Molecular weight markers (lambda DNA PstI digested). Lane 10: PCR of DNA from a transgenic tobacco plant containing the GUS gene (positive control).

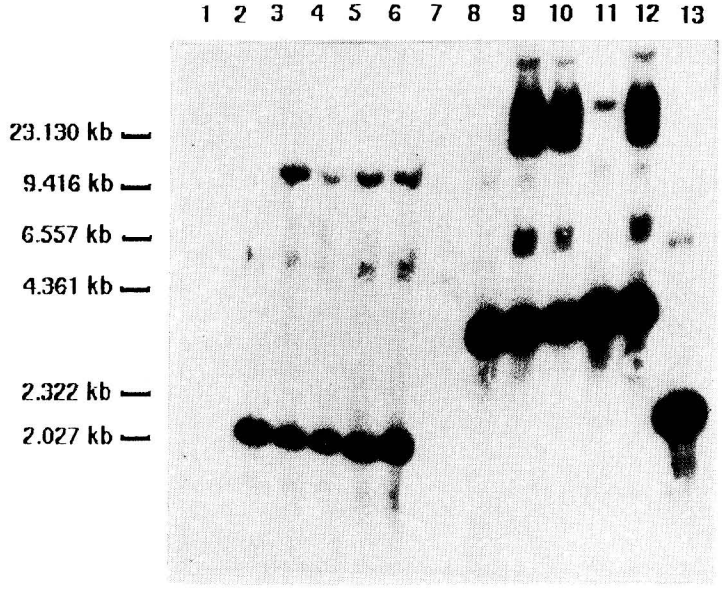

FIG. 7. Southern blot analysis of DNA from regenerated transgenic plants. Leaf DNA was digested by EcoRI-BamHI or EcoRI. The double digestion releases the complete 1.8 kb fragment covering the β-glucuronidase coding sequence incorporated into the plant genome without rearrangements (Lanes 2 to 6). The EcoRI digestion shows the integration pattern of inserted genes into the plant genome (Lanes 8 to 12). Lane 1: Molecular weight markers (lambda DNA HindIII digested). Lane 7: DNA of non-transformed plant SC-Ja 60-5 (negative control). Lane 13: 15 pg of the 1.8 kb GUS-fragment (positive control).

We established a suitable electroporation, growth and selection conditions to deliver DNA into intact sugarcane cells from meristematic tissues and to obtain transgenic plants. The use of meristematic tissue for intact cell electroporation has some disadvantages. The meristem is a tissue composed by several heterogeneous cell layers. This fact allows low transformation efficiency and potentially increases the obtainment of mosaic plants. For these reasons it may be more advantageous to electroporate embryogenic cell suspensions. Lindsey and Jones (1987) reported CAT activity in sugar beet suspension cultures electroporated with CAT gene, though efficient transformation levels were only showed after enzymatic treatment. We introduced foreign gene into intact plant cells without previous enzymatic treatment. Moreover, callus formation and plant regeneration has been obtained from electroporated sugarcane intact cells from embryogenic calli (Arencibia *et al*, manuscript in preparation).

These results constitute an important achievement toward the development of transgenic sugarcane varieties and widely extend the possibilities for genetic manipulation of economically important crops.

ACKNOWLEDGEMENTS

This project was partially supported by the UNIDO/UNESCO/UNDP Regional Program for Biotechnology in Latin-american Countries. We are grateful to A. Hernández for the design and construction of electroporator equipment EPE-010, Sugarcane commercial varieties POJ 2878 and Ja 60-5 were kindly provided by Dr. A. Chinea and Dr. G. Pérez

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