Production of transgenic sugarcane (Saccharum officinarum L.) plants by intact cell electroporation

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Summary. We describe an efficient procedure for genetic transformation of commercial sugarcane varieties POJ 2878 and Ja 60-5. The transformation protocol is based on electroporation of a plasmid conferring GUS activity into cell clusters isolated from embryogenic calli. Six to eight weeks after electroporation, Ja 60-5 plants regenerated from electroporated tissues were tested and confirmed to be transgenic using histochemical glucuronidase and Southern hybridization analysis. Electroporation of intact cells is an efficient and reproducible method for sugarcane transformation and may also be useful for transformation of other plants.

Key words: sugarcane - electroporation - GUS - stable transformation

Abbreviations: GUS = β-glucuronidase; CAT = chloramphenicol acetyl transferase; PCV = packed cell volume; PCR = polymerase chain reaction; DTT = dithiotreitol; Hepes = N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; NOS = nopaline synthase; 4-MUG = 4-methylumbelliferyl β-D-glucuronide.

Introduction

Many different techniques exist for the introduction of naked DNA into plant cells. These methods include electroporation (Rhodes et al. 1988) or polyethylene glycol treatment (Datta et al. 1990), particle bombardment (Gordon-Kamm et al. 1990), and silicon carbide fibers (Kaeppler et al. 1990).

Sugarcane (Saccharum officinarum L.) is one of the most important crops in the tropical and subtropical countries, but reports of gene transfer using this plant have been extremely limited. Chen et al. (1987) transformed sugarcane protoplasts via polyethylene glycol treatment and regenerated kanamycin-resistant cell clusters, but transformed embryogenic calli and plants were not obtained. Srinivasan and Vasil (1986) and Chen et al. (1988) regenerated sugarcane plants from protoplasts but transformation procedures were not utilized in these studies. Recently, using particle bombardment, Franks and Birch (1991) successfully transformed sugarcane cell suspensions and embryogenic calli and later reported recovery of transgenic sugarcane plants (Bower and Birch 1992).

Electroporation of intact plant cells has been suggested as an alternative method for plant transformation. Although electroporation has been used successfully with sugarcane protoplast systems and transient activity of the GUS and CAT genes in sugarcane protoplasts has been obtained (Rathus and Birch 1992) the feasibility of gene transfer into walled cells by an electric pulse was in doubt (Potrykus 1990). Transient expression of the GUS and CAT genes in electroporated meristematic tissues of rice, maize, wheat, barley (Dekeyser et al. 1990) and sugarbeet (Lindsey and Jones 1987) has been reported but stable integration of the introduced foreign genes was not shown and transgenic plants were not recovered. However, D'Haulin et al. (1992) reported the transformation and recovery of transgenic maize plants applying the electroporation to immature zygotic embryos and embryogenic type I calli after enzymatic or mechanic treatment. Recently, transgenic sugarcane plants have been obtained following electroporation of intact meristematic tissues of in vitro grown plants (Molina et al. 1992; Arencibia et al. 1992). We report here the electroporation of intact plant cells from embryogenic calli as an efficient method for obtaining transgenic sugarcane plants.

Materials and methods

Plant material. Spindles were excised from 6 months old field-grown
sugarcane plants. Explants were disinfected by a 1 minute dip in absolute ethanol followed by flaming the tissue on a Petri dish. The external leaves were removed and 1 cm sections containing the basal leaf and meristematic zones were excised and placed on callus induction medium (P+) containing MS salts (Murashige and Skoog 1962), 100 mg/l myo-inositol, 0.8 mg/l thiamine-HCl, 500 mg/l cascin hydrolysate (OXOID, U.K.), 4 mg/l 2,4-D (SIGMA, USA), 20 g/l sucrose, 7 g/l agar-agar (SIGMA, USA) at pH 5.6. After one month of culture in the dark at 25°C, the induced friable calli were transferred to fresh medium. Indifferenciated tissues for electroporation were obtained from two to three month old friable embryonic calli of the commercial sugarcane varieties POI 2878 and Ja 60-5.

Plasmid. The plasmid pBI 221.1 (Jefferson 1988) which contains the GUS gene under the control of the CaMV 35S promoter and the polyadenylation signal of the Agrobacterium tumefaciens NOS gene was used for electroporation studies. The plasmid was purified by CsCl gradient and its final concentration was estimated both spectrophotometrically and by agarose gel electrophoresis (Maniatis et al. 1982).

Electroporation. Approximately 3 g fresh weight of calli were placed in 50 ml liquid P+ medium in an erlenmeyer flask and agitated for 3 days on a rotatory shaker (150 rpm) in the dark at 25°C. The supernatant was removed daily and fresh medium was added. Prior to electroporation, the disaggregated calli were filtered through 100 μm, 500 μm and 297 μm mesh polypropylene filters (SPECTRUM, USA). Cell cluster fractions between 297-500 μm and 500-1000 μm were collected and resuspended in 50 ml of the electroporation buffer (EPR) (Dekeyser et al. 1990): 4 mM CaCl₂, 10 mM Hapes (SIGMA, USA), 10 % glucose, pH 7.2. Purified clusters were agitated on a rotatory shaker at 150 rpm in the dark at 25°C and the EPR buffer was replaced every hour. After 3 hours, the cell aggregates were collected by centrifugation at 650 rpm for 10 min and resuspended in 15 ml of EPR buffer containing 0.2 M spermidine (SIGMA). Aliquots of 1 ml (PCV: 0.12 ml; fresh weight: 22.0 g) were mixed with 150 µg of plasmid DNA in microcentrifuge tubes. Samples were kept at 4°C in the dark for 3 hours, and 55 µl of 3M NaCl (final concentration 156 mM) were added to each tube just prior to the electric discharge and transferred to sterile cuvettes (BIORAD, USA) with 0.4 cm path length. Electroporation was performed in an electroporator providing an exponential pulse EPE-010 (CIGB, CUBA). The pulse discharge was monitored using a voltmeter model FER-30A (CNIC, CUBA). Field strength values of 600, 675, 750, 800, and 850 V/cm and capacitances of 440, 660, and 880 μF were evaluated. After electroporation, samples were maintained on ice for 10-15 minutes. Aliquots of 0.5 ml were plated on 2 cm diameter sterile filter paper disks (Whatman 3MM) which were placed on solidified culture medium P+ for subsequent culture in the dark at 25°C. Control treatments included samples which were electroporated without plasmid and non-electroporated samples incubated with plasmid DNA. The filter paper disks with electroporated material were subcultured on fresh medium every two weeks.

Fluorometrical and histochemical GUS assays. GUS assays were performed according to Jefferson (1988) with minor modifications for the histochemical method. Samples were incubated in a filter-stereilized X-Gluc solution containing 4 mM X-Gluc (5-Bromo-4-Chloro-3-indolyl-β-D-Glucuronide, SIGMA, USA), 0.6 mM Potassium ferricyanide (MERCK, FRG), 0.6 mM Potassium ferrocyanide (MERCK, FRG) and 10 mM DTT (SIGMA, USA) in 50 mM sodium phosphate buffer (pH 7.2) for 24 hours at 28°C in the dark. Leaf material was rinsed in 70% ethanol after staining to improve contrast.

Growth and selection conditions. During optimization of electroporation conditions, 150 μl of X-Gluc solution were added to each filter 10 days following electroporation. After 48 h incubation, blue zones were counted under a stereo-microscope. ANOVA and DUNCAN statistical analysis were done by using the NCSS program version 5.1 (Dr. Jerry L. Hintze, 865 East 400 North Kaysville, Utah 84037, USA). Histochemical staining was performed 4 weeks after electroporation for production of transgenic plants. GUS-positive transformed cell clusters were rescued from the GUS assay and transferred to fresh P+ medium for two to four additional weeks. Putative transgenic calli were then placed on regeneration medium P+ (P+ medium without 2,4-D and supplemented with 30 g/l sucrose) at 25°C and 16 hours light photoperiod under 2000 lux light intensity. After 6 weeks, the 0.5 to 1 cm regenerated shoots were separated and individually cultured on the same medium until they reached 3 to 4 cm length. At this point, some plantlets were directly transferred for rooting to a medium containing MS salts, 100 mg/l myo-inositol, 0.8 mg/l thiamine-HCl, 5 mg/l naphthaleneacetic acid (SIGMA, USA), 30 g/l sucrose, 6 g/l agar-agar (SIGMA, USA), pH 5.6 and one month later analyzed by the histochemical GUS assay and PCR. Other plantlets were vegetatively micropropagated for three subcultures on a medium containing MS salts, 100 mg/l myo-inositol, 0.8 mg/l thiamine-HCl, 0.3 mg/l 6-benzylaminopurine (SIGMA, USA), 1.3 mg/l indole-3-acetic acid (SIGMA, USA), 0.86 g/l kinetin (SIGMA, USA), 30 g/l sucrose, 500 mg/l cascin hydrolysate (OXOID, U.K.), 6 g/l agar-agar (SIGMA, USA), pH 5.6 and then transferred to the rooting medium. Plantlets containing roots and shoots were analyzed after an additional four weeks culture on solid MS medium by Southern hybridization analysis and GUS assays.

Molecular analysis of transgenic plants. Total genomic DNA was isolated from leaf tissue of transformed and control plants. The leaves were freeze-dried and ground in a mortar and pestle. DNA was extracted using the Delligporta et al. (1983) method. PCR analysis (Saiki et al. 1988) was performed using as primers 26-mer oligonucleotides homologous to the positions +3 to +28 (5'-GTTACGTCCTGTAGAAACCCCAACCC-3') and +719 to +694 (5'-GTCGGGATTTCACCTTGAAA-3') of the GUS gene (Jefferson 1988). The PCR reaction mixtures were prepared in volumes of 10 µl containing 2 µg of total plant DNA, 0.2 µM of each specific primer, 250 µM of each dNTP, 10 µl of 10X PCR buffer (Frohman 1990) and 2 units of Taq DNA polymerase (ENZBIOT, CUBA). The reaction was overlaid with paraffin oil and cycled through the following temperature profile: 2 min. 94°C, 1 min. 56°C and 2 min. 72°C. The final incubation at 72°C was extended for an additional 3 minutes to allow better termination of the polymerase reaction. The PCR was completed in 30 cycles and the PCR products were analyzed by Southern hybridization analysis. For genomic Southern hybridization analysis, 10 µg of total DNA was digested with either BamH I or both BamH I and Sac I. Digested DNAs were separated on a 0.8% Tris-borate EDTA agarose gels run at 40 V overnight. Gel treatment, DNA transfer to Hybond-N membrane, fixation of the DNA samples and hybridization were performed as recommended by AMERSHAM (U.K.). The radioactive probe was prepared by the random primer method (Feinberg and Vogelstein 1983) with α-32P dATP. The hybridization probe was the 1.87 kb BamH I/Sac I fragment from pH 221.1 containing the entire GUS gene.

Results

Preparation of cell culture

To obtain callus cultures with a large number of embryogenic cell clusters, it was important to use calli cultured for no longer than 2 or 3 months and to change daily the P+ liquid medium during callus disaggregation for the three days prior to electroporation. The size of the cell cluster to be electroporated was also important. Cell clusters between 297-500 μm were transformed with a higher frequency (Fig. 1) but regenerated plants less efficiently than the large cell clusters. Cell clusters ranging from 500-1000 μm were selected for experiments to obtain transgenic plants.