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Transformation of Foreign Gene in Sugarcane Variety Isd 28 Using *Agrobacterium*-mediated Method

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ABSTRACT

The sugarcane variety Isd 28 and *Agrobacterium* strain LBA4404 containing binary vector pCAMBIA1301 was used in this experiment. The plasmid contains hygromycin phosphotransferase (*hpt*) gene and a ß-glucuronidase (*GUS*) gene in the T-DNA region, and carried neomycin phosphotransferase (*nptII*) gene outside T-DNA region. Unexpanded leaf sheath segments were used as explants for transformation and direct regeneration was obtained after infection, co-cultivation and selection to avoid somaclonal variation. Acetosyringone (2.0 mgl^{-1}) was used to enhance transformation in sugarcane. Infection and co-cultivation was done using direct shoot regeneration medium, MS salts supplemented with 7.5 mgl^{-1} NAA, 0.5 mgl^{-1} Kn, 30 gml^{-1} sucrose and 6 gml^{-1} agar. Shoot and root regeneration medium was used as selection medium contained hygromycin (30.0 mgl^{-1}). Regenerated shoots from transformed explants were rooted in medium composed of MS salts supplemented with sucrose $30gl^{-1}$, agar $6gl^{-1}$ and 5.0 mgl^{-1} of NAA. To observe the transformation, histochemical GUS assay was made after co-cultivation and selection. GUS positive results was showed putative blue colour at surface level as well as cellular level in transformed tissues. Confirmation of transformation was done by PCR method. Results revealed the genomic level transformation of foreign gene in sugarcane variety Isd 28.

Key words: Sugarcane, Transformation, GUS-assay, Foreign gene, Genomic level.

INTRODUCTION

Traditional plant breeding techniques, together with chemical and biotechnological approaches, have been extensively used to increase crop yields by selecting improved varieties which are more productive and resistant to diseases and pathogens. However, conventional sugarcane breeding requires 10-15 years to identify and release a new clone as variety. Unfortunately some important traits such as resistant to insect pest and to some herbicide, appears to be absent from the genetic pool of sugarcane cultivars (Arencibia *et al.*, 1997). Incorporation of desired gene(s) from foreign source is the prime needs to overcome the bottleneck.

Transgenic sugarcane lines resistant to stem-borer attack transferring *Agrobacterium*-mediated gene to plants recently has been reported by Arencibia *et al.* (1997). The first efficient protocol for sugarcane transformation mediated by *Agrobacterium tumefaciens* has been reported by a team of scientists from the Center for Genetic Engineering and Biotechnology, Havana, Cuba and the Institute of Bio-Agriculture, Science Academia Sinica, Nan Kang, Taipei, Taiwan. The new method increases the possibilities for introducing useful traits into many sugarcane cultivars of economic importance (Arencibia *et al.*, 1998).

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Currently the most widely used method for transferring genes into plants is *Agrobacterium*-mediated gene transfer. *Agrobacterium tumifaciens*-mediated transformation must be an optimal system in order to meet up the transformation technology of various genes. Therefore, the present study has been undertaken to transfer foreign gene in sugarcane variety Isd 28 using *Agrobacterium*-mediated genetic transformation.

MATERIALS AND METHODS

Unexpanded leaf sheath segments were excised from spindles of 4-5 months old field grown plant of the sugarcane variety Isd 28 and were used as explants for transformation. Outer whorls of spindles were removed and sterilized in 1% savlon for 10 minutes followed by 3-4 times washed with sterilized distilled water. Then the spindles were immersed in 0.1% $HgCl_2$ for 15 minutes and then washed 3-4 times with sterilized distilled water to remove sterilant. Then the unexpanded leaf sheaths were cut into small pieces (1cmx0.5cm) and used as explants for transformation.

Agrobacterium strain LBA4404 containing binary vector pCAMBIA1301 was used in this experiment. The plasmid contains hygromycin phosphotransferase (*hpt*) gene and a ß-glucuronidase (*GUS*) gene in the T-DNA region, and carried neomycin phosphotransferase (*nptII*) gene outside T-DNA region. LBA4404pCAMBIA1301 was grown at 28^0C for 4 days in liquid medium (LB) supplemented with kanamycin 50 mgl^{-1}. It was shaken at 150 rpm using a horizontal shaker. After 48 hours, optical density (OD) of bacterial suspension culture at 600 nm was taken with the help of spectrophotometer. When optical density (OD) reading was 1.0 then the culture was ready for infection.

Infection medium was prepared by adding liquid direct shoot induction medium (MS + NAA 7.5 mgl^{-1} + Kn 0.5 mgl^{-1} without agar) to bacterial suspension culture (LB + kanamycin 50mgl^{-1}) of strain LBA4404pCAMBIA1301 with 1:1 ratio and 2 mgl^{-1} of acetosyringone was added and mixed well. Sterilized explants of the variety Isd 28 were infected with *Agrobacterium* strain LBA4404pCAMBIA1301 for 60 min. Co-cultivation medium was, MS salts supplemented with NAA (7.5 mgl^{-1}) and Kn (0.5 mgl^{-1}), 30 gml^{-1} sucrose and 6 gm l^{-1} agar. The pH was adjusted to 5.8. In co-cultivation medium no antibiotic and no acetosyringone were used. Co-cultivation was done in 16h light per day at 25-28^0C for 14 days.

Co-cultivated leaf segments were washed with 500 mgl^{-1} cefotaxime, and then transferred to selection medium, semisolid medium contained MS salts supplemented with 7.5 mgl^{-1} NAA, 0.5 mgl^{-1} Kn, 30 gml^{-1} sucrose, 6 gml^{-1} agar and 30 mgl^{-1} hygromycin. Selection was done in the 16 hours photoperiod at 25-28^0C for 4 weeks. The survived explants were then transferred to regeneration medium. Shooting medium composed of MS salts supplemented with sucrose 30 gl^{-1}, agar 6 gl^{-1} and NAA (7.5 mgl^{-1}) + Kn (0.5 mgl^{-1}) and hygromycin (30 mgl^{-1}) and incubated at 25-28^0C for 4 weeks. Regenerated shots were transferred to rooting medium composed of MS salts supplemented with sucrose 30 gl^{-1}, agar 6 gl^{-1} and 5.0 mgl^{-1} of NAA and incubate at 25-28^0C for 4 weeks.

For the confirmation of transformation, histochemical GUS assay was made after co-cultivation and selection (Jefferson *et al.* 1987). The randomly selected tissues were plated over sterile discs of paper for drying, and then incubated in X-gluc solution in eppendorf tubes for 24 hours at 37^0C. As Control the same was done with explants without *A. tumefaciens* co-cultivation.

Plants regenerated from GUS positive explants were used for DNA isolation following the method reported by Hossain *et al.* (2006) to confirm of transformation by PCR method. Primer pairs, Forward 5´-TTT GCA AGT GGT GAA TCC CGA CCT-3´ and Reverse 5´-AGT TTA CGC GTT GCT TCC GCC AGT-3´ (Gambley *et al.*, 1993) were used. PCR amplification was done in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) following the PCR profile of 95ºC for 3 minutes (initial denaturation) followed by 35 cycles of 1 minute denaturation at 95ºC, 1 minute annealing at 54ºC and elongation or extension at 72ºC for 1 minute. After the last cycle, a final step of 5 minutes at 72ºC was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4ºC. PCR reactions were performed on each DNA sample in a 10 μl reaction mixture containing 1.0 μl of 10XA Taq buffer (Tris with 15 mM $MgCl_2$), 1.0 μl of 2.5 mM DNTPs, 2.25 μl of 1.0 μM each of forward and reverse Primer, 0.3 μl of 3U/μl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), , 3 μl of 25 ng/μl genomic DNA and a suitable amount (0.2 μl) of sterile deionized distilled water. After amplification 2 μl loading dye was added to the amplification product for separation using 1.4% agarose gel (containing Ethidium Bromide 0.8 μgml^{-1}) electrophoresis. Electrophoresis was performed at 100V for 2.5 hours. DNA ladder 100 bp (Bangalore Genei Pvt. Ltd., India) was run alongside the reactions. Expected amplified DNA fragment was observed on UV-transilluminator in Gel Documentation System (uvitech, DBT-2000LS), printed and saved as soft copy for lateral use.

RESULTS AND DISCUSSION

In the present investigation, *Agrobacterium* strain LBA4404pCAMBIA1301 and sugarcane variety Isd 28 were used. Unexpanded spindle leaf sheath segments were used as transformation explants. Results of transformation, regeneration of transformed explants, bioassay, histochemical GUS-assay and finally molecular assay by PCR were discussed as follows.

After infection for 60 minutes explants were co-cultivated for 14 days. GUS assay was done among randomly selected leaf sheath segments after co-cultivation and selection. GUS-assay revealed prominence positive results showing putative blue colour in 88.9% selected leaf sheath segments (Fig. 1, 2 and 3). Unexpanded spindle leaf segments of sugarcane variety Isd 28 were used for transformation using inducing chemical acetosyringone. Acetosyringone (2.0 mgl^{-1}) was used during infection time in medium. Perhaps the use of acetosyringone enhanced the transformation and explants showed good GUS positive results. The importance of acetosyringone for transformation of monocot as sugarcane were described by Alt-morbe *et al.* (1989); Bidney *et al.* (1992); Hoekema *et al.* (1993); Hiei *et al.* (1994); Komari *et al.* (1996); Nauerby *et al.* (1997); Klee (2000). They studied the transfer of T-DNA and its integration into the plant genome is influenced by several *A. tumefaciens* and plant tissue specific factors. These include plant genotypes, explants, vectors-plasmid, bacterial strains, addition of vir-gene inducing synthetic phenolics compounds, culture media composition, tissue damage, suppression and elimination of *A. tumefaciens* infection after co-cultivation. Southgate *et al.* (1996) reported that

supplemented of *Agrobacterium* culture with appropriate antibiotics and 100 µM acetosyringone increase transformation efficiency in monocot. Stachel *et al.* (1985) reported that *A. tumefaciens* infects only wounded, actively dividing plant cells, which excrete phenolic compounds, such as acetosyringone and hydroxy-acetosyringone. These phenolics act both as chemo-attractants for *Agrobacterium* and inducers of the virulence genes (Stachel *et al.*, 1985).

Regeneration responses were showed good results after transformation. After transformation 66.7% of explants produced shoots. All shoots produced roots in the rooting medium (Figure 4).

For the confirmation of transformation at genomic level, genomic DNA was isolated from the GUS positive putative transgenic plantlets regenerated from unexpanded leaf sheath segments of variety Isd 28 after transformation with LBA4404 pCAMBIA1301, using a method already established with simple modification from the method of Aljanabi *et al.*, 1999. Genomic DNA from non-transformed plants was also isolated. The purity of total DNA was checked for PCR operation by running in 1.0 % electrophoresis gel. By viewing on Gel documentation system DNA was selected for PCR amplification. The GUS gene in genomic level was detected by amplifying using primer pair reported by Gambley and co-workers (1993). Primer pair amplifies 600 bp DNA segment from GUS gene. The transformed plantlets amplified DNA segment of the expected size 600 bp by the primer pair for the GUS fragment at the same position. No band was produced in DNA isolated from the non-transformed sugarcane control plants. The PCR results were shown in fig. 5. Matsuoka *et al.* (2000) conducted *Agrobacterium*-mediated transformation in sugarcane. Hygromycin-resistant, GUS-positive calli were selected from the infected cell suspension culture. Hygromycin-resistant, GUS-positive shoots were regenerated from the selected calli. The *hpt* gene was detected from these calli and shoots by PCR analysis.

PCR amplification of selected segments from GUS reporter gene was done using DNA isolated from transformed regenerated plantslets in Isd 28. Selected amplified DNA band was found against GUS primer pair. Thus the foreign gene was stably transferred to the genome of sugarcane variety Isd 28.

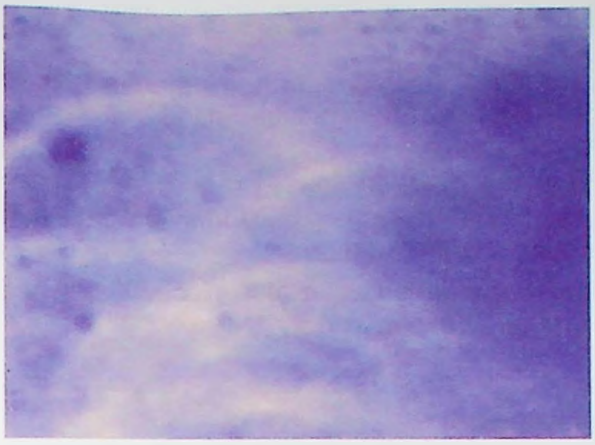

Figure 1. GUS expression showing blue colour of Isd 28 in slice of transformed leaf segment at surface level.

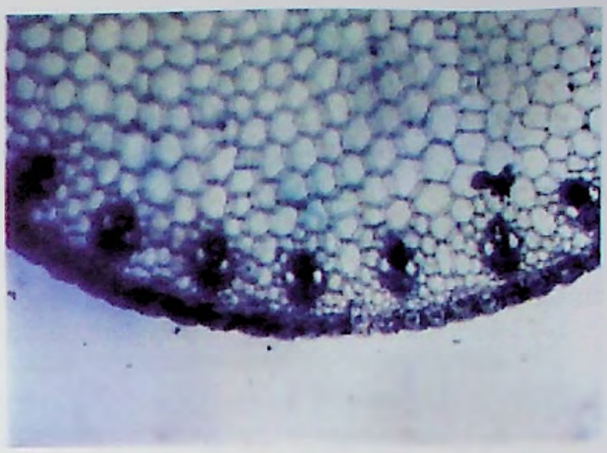

Figure 2. GUS expression showing blue colour of Isd 28 in the cross section of transformed leaf segment at cellular level.

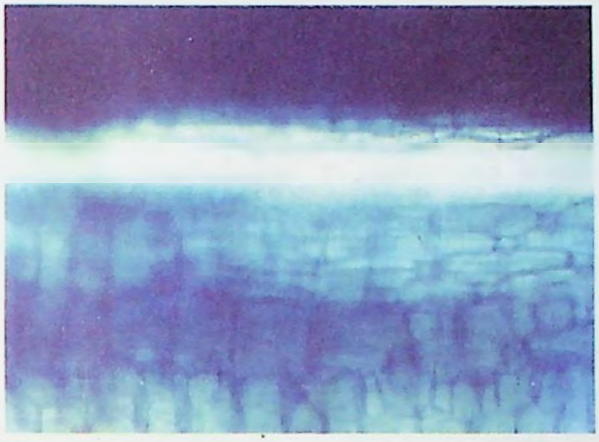

Figure 3. GUS expression showing blue colour of Isd 28 in longitudinal section of transformed leaf segment at cellular level.

Figure 4. Regeneration of shoots from transformed leaf segment of Isd 28 infected with LBA4404 (pCAMBIA 1301) 60 minutes.

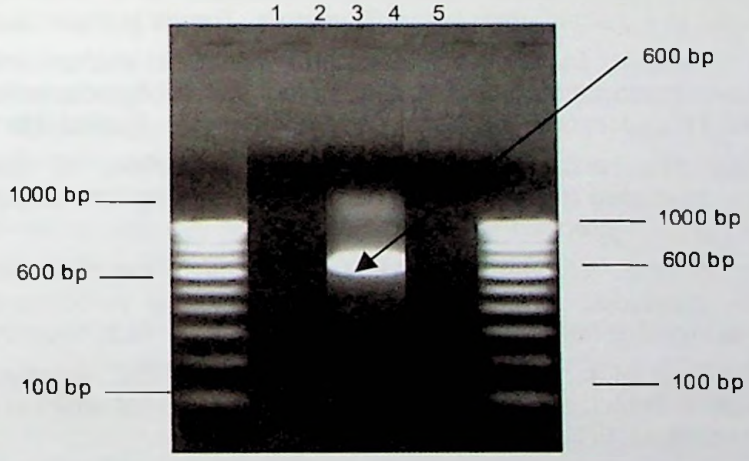

Figure 5. Detection of GUS gene by PCR of genomic DNA isolated from transgenic sugarcane variety Isd 28. Lane 1, 100 bp ladder; Lane 2, blank; Lane 3, genomic DNA isolated from transformed variety Isd 28; Lane 4, genomic DNA isolated from non-transformed sugarcane variety Isd 28; Lane 5, 100bp ladder

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