Agrobacterium Co-cultivation and GUS Gene Expression in Transformed Tissue of Sugarcane (*Saccharum officinarum* L.)

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ABSTRACT

Callus of sugarcane variety Isd 31 was infected with Agrobacterium strain AGL1 (pTab7) using three infection times such as 60, 90 and 120 min. It was found that transformed callus of variety Isd 31 have regeneration ability to produce shoots and roots. Infection time has no effects on the regeneration responses. GUS assay was done among randomly selected calli from 120 min infection time, and showed the highest results and same per cent (100%) of callus was recorded GUS positive. Results indicate possibilities of genetic transformation of foreign genes in sugarcane variety Isd 31 of Bangladesh.

Key words: Sugarcane, *Agrobacterium*, callus, GUS gene expression.

INTRODUCTION

Sugarcane is cultivated on large scale in tropical and subtropical regions as raw materials for sugar and industrial products, such as furfural, dextrans, and alcohol (Martin et al., 1982). Sugarcane represents 65% of the world sugar production (Anon, 1995). Traditional plant breeding techniques, together with chemical, biotechnological approaches, have been extensively used to increase crop yields by selecting improved varieties which are more productive and resistant to diseases and pathogens. Unfortunately some important traits such as resistant to insect pest and to some herbicide appears to be absent from genetic pool of sugarcane cultivars (Arencibia *et al.* 1997). Agrobacterium-mediated genetic transformation may play important role in incorporation of foreign gene for the traits. The first efficient protocol for sugarcane transformation mediated by Agrobacterium tumefaciens was 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, Taiwan. The new method increases possibilities for introducing useful traits into many sugarcane cultivars of economic importance (Arencibia *et al.* 1998). This report demonstrates transient gus gene expression in sugarcane tissue after co-cultivation with *A. tumefaciens*. Agrobacterium-mediated gene transfer is the most widely used method to introduce foreign genes into plants (Horsch *et al.*, 1985; Hooykaas and Schilperoort 1985; Hooykaas 1990), but in general, monocotyledonous are recalcitrant to infection by this bacterium (Hooykaas and Schilperoort, 1992). Agrobacterium-mediated gene transfer has advantages over other transformation methods, as it is simple, inexpensive and easy regeneration systems. Introduced genes are more likely to integrate into the host genome as single copy (thought to reduce co-suppression of genes), rather than multiple transgene integration other associated with direct DNA delivery (Register *et al.* 1994).

MATERIALS AND METHODS

Plant materials and callus culture

Basal meristematic spindle leaf segments from 4-5 months old field grown plant of sugarcane variety Isd 31 were used for callus induction. Spindles were excised and surface sterilized, and explants were placed in MS (Murashige and Skoog, 1962) medium supplemented with 10% coconut water, 30 gml^{-1} sucrose, 6 gm l^{-1} agar and 2,4-D (4 mg l^{-1}). The pH was adjusted to 5.8. Callus cultures were kept in dark at 250C for 21 d. Calli were subcultured on fresh medium at an interval of 14 d.

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Bacterial strain, culture and plasmid

Agrobacterium strain AGL1 containing binary vector pTab7 was used in this trial. The plasmid contains neomycin phosphotransferase (nptII) gene and a ß-glucuronidase (GUS) gene in the T-DNA region, and carried rifampicin and tetracycline resistance gene outside T-DNA region. AGL1 (pTab7) was grown at 280C for 4 d in liquid medium supplemented with rifampicin 20 mg l^{-1} and tetracycline 5 mg l^{-1}. It was shaken at 150 rpm using a horizontal shaker. After 96 h, optical density (OD) of bacterial suspension culture at 600 nm was taken with the help of spectrophotometer, and when optical density (OD) reading was 1.0 then the culture was ready for infection.

Infection and co-cultivation

Infection medium was prepared by adding liquid callus induction medium (without agar) to bacterial suspension culture of strain AGL1 with 1:1 ratio and 2 mgl^{-1} of acetosyringone was added and mixed well. For transformation well organized calli were chosen. The colour of callus was also important consideration because it imparts false results during histochemical GUS test. Calli of the variety Isd 31 were infected with Agrobacterium strain AGL1 (pTab7) using three infection times such as 60, 90 and 120 min. Co-cultivation medium was, MS supplemented with 10% coconut water, 30 gml^{-1} sucrose, 6 gm l^{-1} agar and 2,4-D (4 mg l^{-1}). The pH was adjusted to 5.8. In co-culture medium no antibiotic and no acetosyringone were used. Co-cultivation was done in dark at 280C for 14 d.

Selection and regeneration of transformed callus

Co-cultivated calli were washed one time with 500 mg l^{-1} cefotaxime, and then transferred to selection medium, semisolid medium contained MS salts supplemented with 10 % coconut water, 30 gml-1 sucrose,6 gml^{-1} agar, 2,4-D (4 mg l^{-1}) and 50 mg l^{-1} kanamycin. Selection was done in the dark at 280C for 14 d. The survived calli were then transferred to regeneration medium. Shooting medium composed of MS salts supplemented with sucrose 30 g l^{-1}, agar 6 g l^{-1} and 6-benzyladenine (0.2 mg l^{-1}) + Kinetin (0.2 mg l^{-1}) + indole-3-acetic acid (0.1mg l^{-1}) (Baksha et al., 2002) and incubated at 280C for 4 weeks. When shoots were regenerated transferred to rooting medium composed of MS salts supplemented with sucrose 30 g l^{-1}, agar 6 g l^{-1} and 5.0 mg l^{-1} of NAA as determined by Hossain et al. (1996) and Alam *et al.* (2003) respectively and incubate at 280C for 4 weeks. The total number of plantlets regenerated were scored.

Assay for GUS activity

For the confirmation of transformation, histochemical GUS assay was made one week after co-cultivation (Jefferson, 1987). The randomly selected calli from 120 min infection time were plated over sterile discs of paper for drying, and then incubated in X-gluc solution in eppendorf tubes for 24 h at 370C. As Control the same was done with calli without *A. tumefaciens* co-cultivation.

RESULTS AND DISCUSSION

Calli of the variety Isd 31 were infected with *Agrobacterium* strain AGL1 (pTab7) using three infection times such as 60, 90 and 120 min. Calli were chosen for transformation because regeneration of plants from callus was well established and reproducible for sugarcane. Hossain *et al.* (2004) conducted Agrobacterium-mediated genetic transformation experiment using sugarcane callus. Both co-cultivation and selection times were maintained 14 d. Shoots and roots development were obtained within 28 d of culture. The results are shown in the Table 1. Transformed callus of the variety Isd 31 from infection time 60 min showed no regeneration response, and seven plantlets were produced from 90 min infection time and only 3 obtained from 120 min infection time. Total 10 plantlets were obtained using 18 pieces of calli. GUS assay was done among randomly selected callus from 120 min infection time. Callus of the variety Isd 31 from infection time 120 min showed the highest results and same per cent (100%) of callus was GUS positive, and showed blue colour . The results are given in the Table 2 and Figure 1. Matsuoka et al. (2001) reported *Agrobacterium*- mediated genetic transformation in sugarcane variety NiF4, and performed GUS assay successfully. They obtained plants from hygromycin resistant transformed callus. Putative transformation was detected in both transformed callus and plant by GUS assay. Arencibia et al. (1998) assessed transformed callus using histochemical GUS assay and produced first morphologically transgenic sugarcane plant through *Agrobacterium*-mediated genetic transformation. Control callus showed no blue colour after GUS assay. Shoots and roots were successfully produced from transformed callus (Fig 3).

Results indicate possibilities of genetic transformation of foreign genes in sugarcane variety Isd 31 of Bangladesh.

Table 1. Effects of infection, co-cultivation, selection and sub-culture on shooting and rooting media on plant regeneration from callus in varietiy lsd 31 with AGL1 (pTab7).

Variety	Infection time (Min)	No. of callus	Co-cultivation time (d)	Selection time (d)	Shooting time (d)	Rooting time (d)	No. of plants regenerated
lsd 31	60	6	14	14	28	28	-
	90	6	14	14	28	28	7
	120	6	14	14	28	28	3

Table 2. GUS assay of callus of the variety lsd 31 transformed with AGL1 (pTab7).

Variety	Explant	Infection time (min)	No of callus assayed for GUS	No. of callus positive for GUS assay	GUS Positive callus (%)
lsd 31	Callus	120	8	8	100

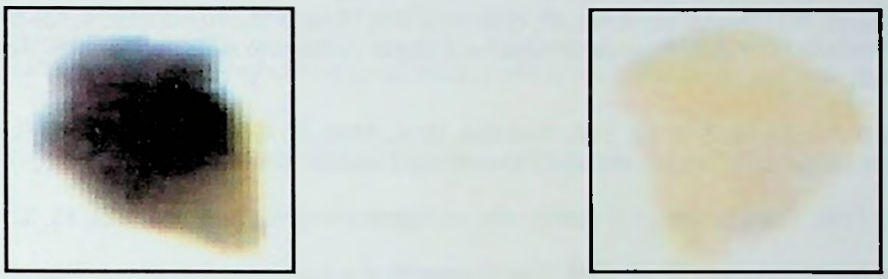

Figure 1. GUS expression in callus of the variety lsd 31 at an infection time 120 min. Transformed callus of the variety lsd 31 (left) and control callus (right).

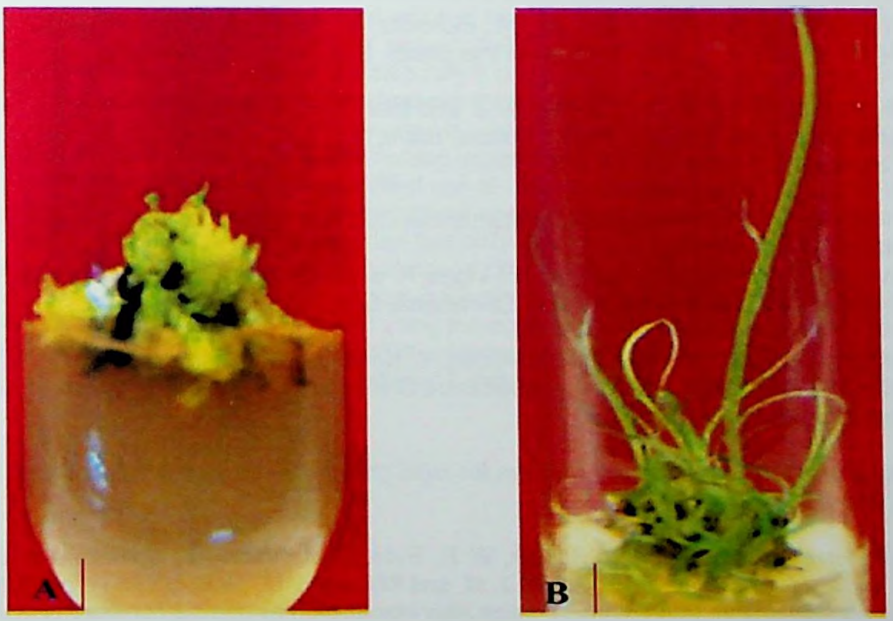

Figure 2. Regeneration of shoots from transformed callus of the variety lsd 31 infected with AGL1 (pTab7) for 90 minutes. A: development of shoots; B: transferred to rooting medium.

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