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AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION IN *HEVEA BRASILIENSIS*

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ABSTRACT

A method for *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea brasiliensis* has been developed. Two month old callus derived from immature anther was used as the explant. These calli were infected with *Agrobacterium* strain EHA101 harbouring the plasmid vector pDU 96.2144 containing b-*glucuronidase* as the reporter gene, *npt*-II gene for kanamycin resistance as the selectable marker gene and the sequence coding for *superoxide dismutase* under the control of CaMV35S promoter. The explants were initially treated with *Agrobacterium* culture at a density of 10^8 cells/ml for 15 minutes. Further the explants were co-cultured for 3 days at 28°C . The explants were then thoroughly dried with filter paper and transferred to modified Murashige & skoog medium containing 300mg/l kanamycin for the selection of the transgenic cell lines .The transgenic cell lines were confirmed with the positive GUS staining. 3% Transformation frequency was achieved. After the callus proliferation, embryogenesis was obtained in modified MS medium containing spermine (2mg/l) and abscicic acid (0.1mg/l). Regeneration of the plantlets were obtained on transfer of the mature embryos to modified MS medium supplemented with GA_3 (0.2mg/l), kinetin(0.2mg/l) and indole -3-acetic acid (0.1mg/l). Transgenic plantlets exhibited strong GUS activity in the leaves and the roots as indicated by the deep blue colour on staining with X-gluc.

ABBREVIATIONS

GUS - b - Glucuronidase , npt II - Neomycin Phospho transferase II
X-gluc - 5-Bromo,4-chloro, 3-indolyl glucuronide

INTRODUCTION

Hevea brasiliensis, the commercial rubber tree belonging to the family Euphorbiaceae is highly heterozygous in nature. As a perennial tree crop with a long breeding cycle, integration of desired characters through conventional breeding is both time consuming and labour intensive. Fixation of any particular gene requires several generations of crosses & field trials. Therefore, genetic transformation offers a viable alternative approach (Arokiaraj etal, 1994). Over the past decade, the value of introducing foreign DNA into plants has been well documented. Many reports are available about the introduction of a variety of agronomically important traits into plants including genes which confer resistance to pathogens , abiotic stress & herbicide tolerance (Snyder, *et al.*,1999; Aragao *et al.*, 2000). Several methods have been developed for the introduction of foreign genes into various crop species (Jones *et al.*, 1993). Among these methods, *Agrobacterium* mediated transformation system (Hooy Kaas and Schilperoort;1992) is the most popular one since it is an effective vector for genetic transformation of diverse crop species, including cereals, due to its broad host range.

Oxidative damage in plants occurs when the capacity of cellular antioxidant systems are over-whelmed by the 0_2-centered radicals

generated within the cell (Bowler et al 1992; Scandalios 1993; Allen 1995). SOD's are metallo enzymes providing defence against oxidative stress in plants which dismutates two superoxide radicals to produce H_2O_2 & O_2. Enhancing the plants tolerance of oxidative stress would improve its ability to survive combinations of stresses like extreme temperature or drought, high light intensities, ambient ozone or sulphuroxide or pathogens etc (Bowler et.al. 1992) This paper reports an effective method for the *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea brasiliensis* with the plasmid vector containing b- glucuronidase as the reporter gene, npt11 gene for kanamycin resistance as the selectable marker and the sequence coding for superoxide dismutase under the control of *CaMV35S promoter.*

MATERIALS AND METHODS

Plant Material: *Hevea* callus was initiated from immature anthers of the clone RRII 105 in a modified MS medium (Murashige and Skoog; 1962) as reported earlier (Kumari Jayasree et.al. 1999). Two month old anther calli were used in the transformation experiments.

Transformation of *Hevea brasiliensis* anther calli using *Agrobacterium:* *A. tumefaciens* strain EHA 101 harbouring the plasmid vector .pDU 96-2144 were grown on AELB liquid medium. After four hours, the antibiotics , gentamycin (20mg/ml) and kanamycin (50 mg/ml) were added and incubated overnight at 28°C, 250 rpm. The two month old anther calli were treated with the *Agrobacterium* suspension (10^8 cells/ml) containing acetosyringone and betaine-hydrochloride for 10-15 mts. The excess bacterial suspension was blotted dry from the calli using sterile Whatman No.1 filter paper and the calli were carefully transferred to the initiation medium and maintained for 3 days. The calli were then transferred to the selection medium containing Carbenicillin (500 mg/l) & Kanamycin (300 mg/l). Suitable controls (untransformed anther calli) were also maintained. All the cultures were incubated at 25°C in the dark.

Plant expression Vectors: The plasmid vector used for the transformation was pDU 96-2144 containing b- glucuronidase (GUS) as the reporter gene & npt II gene for the selection in plant cells. The plasmid also contain the nucleotide sequence for SOD under the control of CaMV35S promotor

Selection of Transformants and Regeneration into plantlets: The Kanamycin-resistant transgenic lines emerging after 40 days of infection were proliferated by subculturing to the proliferation media which is modified MS medium (Kumari Jayasree et. al. 1999). For the embryo induction modified MS medium with several organic supplements, varying levels of sucrose, growth regulators, phytagel, polyamines etc. were tried. Embryos obtained were matured and germinated into full plantlets. These plantlets were transferred to small polybags for hardening.

X-gluc staining for GUS expression: GUS expression was determined in the leaves of the transgenic plants. Evaluation of GUS activity by histochemical staining was carried out by incubating the leaves overnight in X-gluc

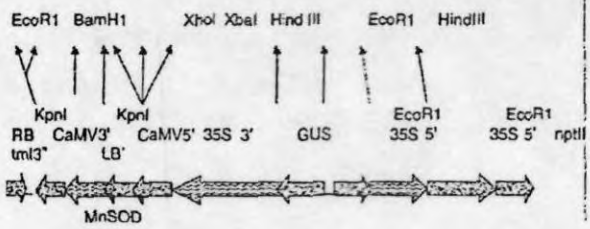
Table 1. Optimised conditions for Hevea transformation

Explant Stage	Infection period	Duration & condition for	Concentration of Antibiotic (mg/l)		Frequency of subculture
		co-culture	Carbenicillin	Kanamycin	
2 month old	10-15 mts	3 days at 28°C	500	300	3 weeks interval

Table 2. Effect of growth regulators on embryo induction, maturation and germination of transgenic callus.

Treatment	Growth Regulators			Mean Percentage		
	K	GA3	ABA	EI	EM	G
T_1	0.1	0.2	0.02	10.00	19.60	4.00
T_2	0.2	0.25	0.05	42.00	40.40	0.80
T_3	0.3	0.3	0.1	29.60	61.20	2.00
T_4	0.4	0.35	0.15	20.40	29.20	1.20
T_5	0.5	0.4	0.2	11.60	11.20	1.20
V.R.				51.35**	116.76 **	4.29*
C.D (5%)				5.48	5.32	1.83

Fig. 1 Plasmid vector pDU 96.2144



solution under darkness at 37C (Jefferson and Wilson,1991)

RESULTS AND DISCUSSION

The transgenic cell lines started proliferating in the selection medium about 40 days after infection. The transformation frequency was found to be 3%. The control explants failed to grow in the presence of Kanamycin. Total 22 lines were isolated and of these lines only two responded to embryogenesis and only from one line full plantlet formation was observed.

The modified MS medium supplemented with casein hydrolysate (300 mg/l), malt extract (100mg/l), coconut water (10%) along with sucrose concentration of 50g/l and spermine (2mg/l) was found to be effective for embryogenesis and plant regeneration. The growth regulator requirement for embryo induction, maturation and germination are summarised in Table 2. The maximum embryo induction frequency (40%) was obtained in a medium, with a growth regulator combination of kinetin (0.2mg/l), GA_3 (0.25mg/l) and ABA (0.05mg/l). The maximum embryo maturation frequency (60%) was observed in the same basal medium but with a different combination of the growth regulators ie, kinetin (0.3mg/l), GA_3 (0.3mg/l) ABA (0.1mg/l). Germination of these embryos were obtained in the growth regulator combination of kinetin (0.1mg/l), GA_3 (0.2mg/l) and ABA (0.02mg/l). The maximum germination frequency obtained was 4%. When the hormonal combination of GA_3 (0.2mg/l), kinetin (0.2mg/l), and IAA (0.1mg/l) were used better germination of the embryos were observed (data not given). The plantlets obtained were transferred to small polybags for acclimatisation.

GUS expression in transgenic leaves: The GUS expression was determined in the leaves of the transgenic plantlets as described by Jefferson et al 1987. The substrate 5-Bromo-4-chloro-3indolyl b D-glucuronide was solubilized to a final concentration of 1mM in a sterile solution of 100 mM sodium phosphate buffer, 0.5mM potassium Ferrocyanide and potassium ferrocyanide trihydrate, 20% (v/v) methanol and 0.3% (v/v) Triton X-100, and added directly to the tissues in the petridish. The dishes were incubated at 37°C overnight before observation.

As control, tissue samples from a plant derived using identical protocols as with the transformed plantlets but omitting *Agrobacterium* at all stages were similarly treated. GUS expression was observed in the leaves of the transgenic plants and was absent in the control plants. More intense blue colouration was observed at the cut edges of

the leaf in the transformed plants.

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