J. nat. Rubb. Res., **11(2)**,77-87

Agrobacterium-*mediated Transformation of* Hevea *Anther Calli and Their Regeneration into Plantlets*

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A gene transfer system for Hevea brasiliensis *was established with* Agrobacterium tumefaciens *GV2260 (p35SGUSINT) and LBA4404 (pAL4404/pMON9793). In this system* H. brasiliensis *anther-derived calli were transformed with vectors harbouring the β-glucuronidase* (gus) *gene and the neomycin phosphotransferase* (nptII) *gene. The success of gene transfer was determined by histochemical staining and fluorometric assay for β-glucuronidase activity and enzyme linked immunosorbent assay for detecting neomycin phosphotransferase II protein levels. These independent assays all showed a several-fold increase, compared to control values, in enzyme activity and protein levels in extracts from transformed calli and embryoids of* H. brasiliensis. *The presence of the* gus *gene in transformed plantlets was confirmed by using the polymerase chain reaction protocol as performed on DNA isolated from transformed calli and embryoids.*

Enormous progress has been achieved in the development of gene transfer systems for higher plants. The ability to introduce foreign genes into plant cells and tissues by plant transformation techniques and to regenerate viable, fertile plants have been widely employed for both basic research and introduction of new traits to modify and improve crop plants. Transformation of crop plants is becoming increasingly routine in terms of the number of plant species which can be transformed and the frequency of transformation, resulting in a number of transgenic products which are ready or close to market introduction[1].

The stable introduction of foreign gene into plants represents one of the most significant developments in the advancement of agricultural technology[2]. A prerequisite for the production of transformed plants is the availability of a method to regenerate a complete plant from the transformed cell. This holds for virtually all systems of genetic manipulation. There is, however, increasing support for the hypothesis that not all cells are totipotent but that only certain cells or cell types in each tissue have the capacity to regenerate into complete plantlets. Gene transfer has therefore to be directed to those cells that have regeneration capacity.

The investigations described in the present work focus on *Agrobacterium*-mediated gene transfer system for *Hevea brasiliensis* (rubber

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plant) to generate transgenic rubber plants. *Hevea* genetic transformation would complement plant breeding efforts by increasing the diversity of genes with agronomic importance in the *Hevea* genome. This approach would shorten the time required for selection of hybrids for the advancement of *Hevea* crop potential.

MATERIALS AND METHODS

H. brasiliensis Tissue Culture

Hevea callus was initiated from individual anthers of the staminal column (*H. brasiliensis* clone GL1). The callus was maintained at 25°C in the dark on the initiation medium as described by Chen[3], modified by the addition of adenine sulphate (100 μM) and lysine (620 μM). Anther calli that were four weeks' old were employed in transformation experiments. Subsequently, all the tissue culture procedures essentially followed the protocols as described by Chen[3] with the addition of silver nitrate (58.80 μM) in the differentiation medium and the omission of 5 bromouracil in the developmental medium.

Plant Expression Vectors

Recombinant plasmid pMON9793 (kindly given by C.S. Gasser, Monsanto Company, St. Louis, Missouri, USA) containing the *gus* and neomycin phosphotransferase (*nptII*) genes was transferred from *Escherichia coli* JM83[4] to *Agrobacterium tumefaciens* strain LBA4404 (pAL4404)[5] by triparental mating[6]. *A. tumefaciens* GV2260 (kindly given by Prof. L. Willmitzer, Institut fur Genbiologische, Forschung, Berlin GmbH, Germany) contained p35SGUSINT and *nptII* gene for selection in plant cells.

Transformation of *H. brasiliensis* Anther Calli using *A. tumefaciens*

A. tumefaciens strains, LBA4404[5] harbouring pAL4404 and the binary plasmid pMON9793 and GV2260[7] harbouring p35SGUSINT[8], were grown on Yeast Extract Broth (YEB) medium[9] containing 50 μg ml^{-1} kanamycin overnight at 28°C. Anther calli were gently removed and submerged in an overnight culture of *Agrobacterium* suspension for one min. The excess bacterial suspension was blotted dry from the calli by using sterile Whatman No. 1 filter paper and the calli were carefully transferred to initiation medium for a co-cultivation period of two days. After this period, the calli were transferred to fresh initiation medium containing cefotaxime at 250 μg ml^{-1} and ticar (Beecham Pharmaceuticals, Sussex, UK) at 500 μg ml^{-1}. Suitable controls (untransformed anther calli) were also initiated. All cultures were incubated at 25°C in the dark.

Selection of Transformants

After one week, calli were transferred to initiation medium containing 50 μg ml^{-1} kanamycin for selection, and cefotaxime (250 μg ml^{-1}) and ticar (500 μg ml^{-1}) to eliminate *Agrobacterium* infection. The concentrations of cefotaxime and ticar were gradually reduced (200 μg ml^{-1} and 400 μg ml^{-1} respectively after the second transfer, followed by 100 μg ml^{-1} and 200 μg ml^{-1}, respectively after the third transfer) in subsequent sub-cultures at weekly intervals. Calli that were kanamycin resistant were isolated and subcultured separately in fresh initiation medium containing kanamycin and antibiotics to inhibit *Agrobacterium* infection. After 45-50 days, kanamycin resistant calli were selected and transferred to differentiation medium containing kanamycin (100 μg ml^{-1}) and

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cefotaxime and ticar at 100 μg ml^{-1} for a period of two months. Kanamycin resistant embryoids with roots and shoots were finally transferred to developmental medium containing kanamycin at 100 μg ml^{-1} and maintained in this medium (for approximately 2 months) and then transferred to soil conditions.

Enzyme Assays of Plant Tissues β-Glucuronidase (GUS) Assay

GUS activity was determined in *Hevea* calli and embryoids as described by Jefferson et al[10].

Histochemical Analysis of β-glucuronidase Activity

This method was based on the procedure described essentially by Jefferson[11]. Plant tissue was washed with 50 mM sodium phosphate buffer pH 7 and incubated in X-gluc solution at 37°C overnight in the dark. Tissue was examined for blue stained cells 18-36 h after the addition of X-gluc. Photo-graphs of the stained tissue were taken with a Nikon SZM-U binocular microscope and a Nikon F-601 AF camera loaded with Kodacolor Tungsten 160T film.

Protein Extraction and Analysis of Protein Quantitation

The protein concentration in plant extracts was estimated by using the Bio-Rad assay kit (Bio-Rad, Laboratories Ltd., Hertfordshire, UK).

Neomycin Phosphotransferase (NPT II) Assay

This method was based on protocols as described[12,13]. NPT II protein levels in *Hevea* tissue were determined by using an Enzyme Linked Immunosorbent Assay (ELISA) kit (5 Prime-3 Prime, Inc., CP Laboratories, London, UK).

DNA Isolation

Genomic DNA was isolated from transformed and control tissues by using the protease method[14].

PCR Analysis

PCR reaction[15] was carried out in 50 μl containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 200 μM dGTP, *Taq* DNA polymerase (0.5-1.0 U), DNA (2-10 ng) and 100 ng of each primer overlayed with mineral oil. The oligonucleotide primers used were 5'GGTGGGAAAGCGCGTTACAAG3' and 5'GTTTACGCGTTGCTTCCGCCA3' (positions 400-419 and 1599-1579 respectively, in the *gus* gene[15]). *Taq* DNA polymerase was obtained from Promega Corporation, Southampton, UK. The denaturation temperature was 92°C (1 min), annealing temperature was 55°C (1.5 min) and extension temperature was 72°C (2 min). The reaction was programmed for 30 cycles, using a programmable thermal controller (Hybaid Thermal Reactor). DNA was detected after electrophoresis on 1.0% agarose/ethidium bromide gels.

RESULTS

Tissue Culture and Regeneration of Selected Transformants

A. tumefaciens strains LBA4404 (pAL4404/ pMON9793) and GV2260 (p35SGUSINT) were used to transform *in vitro* anther calli of *Hevea*. *Hevea* anther calli transformed with

Agrobacterium retained the ability to regenerate. Transformation experiments involved co-cultivating *Hevea* anther calli with *Agrobacterium* strains, GV2260 (p35SGUSINT) and LBA4404 (pAL4404/ pMON9793) and then selecting transformants initially in the presence of 50 μg ml^{-1} kanamycin and then later after 23 days in 100 μg ml^{-1} kanamycin. Transgenic calli and embryoids were developed and selected on media containing 100 μg ml^{-1} kanamycin, whilst control *Hevea* anther calli (untransformed anther calli) on kanamycin selection showed no growth within 14 - 21 days. Selection was carried out on 50 μg ml^{-1} kanamycin at the early stages of transformation, since kanamycin resistant microcalli showed slow growth. The above procedure allowed selection and propagation of transgenic calli. Embryoids that were kanamycin-resistant were isolated from kanamycin-resistant calli after 60 days on differentiation media containing kanamycin at 100 μg ml^{-1}. The kanamycin resistant embryoids and those of the controls appeared normal in morphology (round types, globular types, torpedo types and cotyledon types) but the growth and development of the kanamycin resistant embryoids were slower than that of the controls. For this reason, the kanamycin resistant embryoids were kept in the differentiation medium for a period of 75 days before they were transferred to developmental medium containing kanamycin at 100 μg ml^{-1} for the production of plantlets. Three plantlets were obtained from 65 embryoids giving a frequency of 3% plantlet production, derived from anther calli transformed using *Agrobacterium* strain GV2260 (p35SGUSINT). No plantlets were obtained from 38 embryoids using *Agrobacterium* strain LBA4404 (pAL4404/pMON9793).The control and transgenic plantlets were established in soil.

Expression of GUS in Transgenic Calli and Embryoids.

The results obtained from histochemical staining with X-gluc for calli, embryoids and roots are shown in *Figure 1A-1F*.

Detection of GUS Activity by Fluorometric Analysis

The results obtained from histochemical staining with X-gluc of *Agrobacterium* transformed calli and embryoid cultures were confirmed by using the alternative fluorometric assay to detect GUS activity. GUS activity was measured in extracts of kanamycin resistant calli (24 days after transformation), kanamycin resistant embryoids (50 days' old) and the corresponding control cultures . The results are shown in *Figures 2* and *3*. Overall, there was a 200% increase in GUS activity in kanamycin resistant calli from GV2260 (p35SGUSINT) and LBA4404(pAL4404/pMON9793) compared to control calli. GUS activity in kanamycin resistant embryoids showed an overall 340% increase compared to control values. Kanamycin resistant embryoids in general showed a 50% increase in GUS activity in comparison to kanamycin resistant calli.

Expression of the NPT II Protein in Transgenic Calli and Embryoids of *Hevea*

NPT II levels were quantified in kanamycin resistant anther calli (24 days after transformation), kanamycin resistant embryoids (50 days' old), and in control cultures by the ELISA technique. The results of these investigations are shown in *Figures 4* and *5*.

Overall, NPT II protein levels in kanamycin resistant calli were approximately 230% above background control values and averaged around 27 ng NPT II/mg total protein. Kanamycin

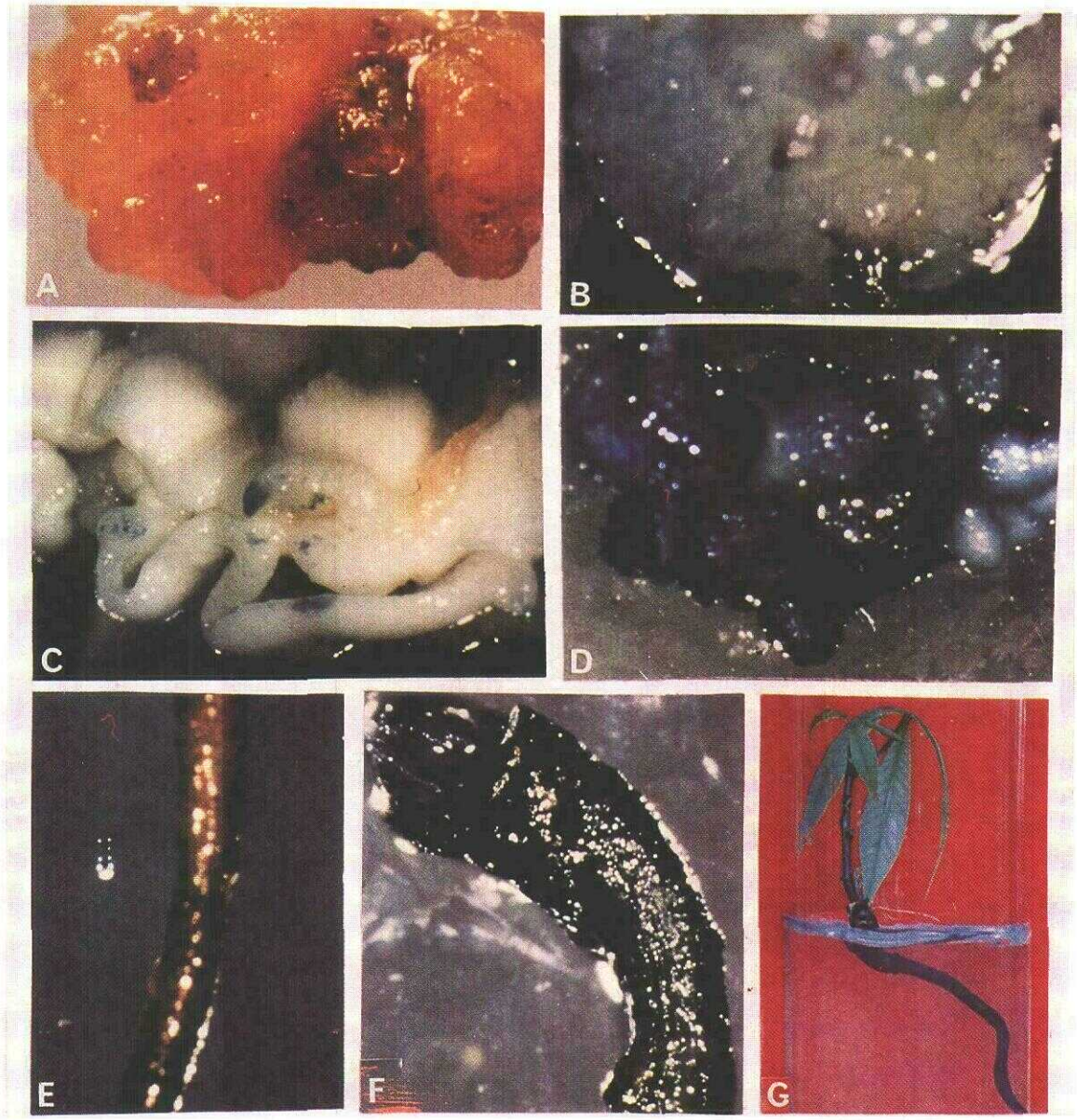

Figure 1. GUS activity after treatment with the chromogenic substrate X-gluc at different stages in the transformation and regeneration of Hevea *plant* via Agrobacterium *GV2260 (p35SGUSINT). (A) Control calli, 24 days after transformation; (B) kanamycin resistant calli, 24 days after transformation with GV2260 (p35SGUSINT); (C) control embryoid (50 days old), (D) kanamycin resistant embryoid, 50 days old; (E) control root; and (F) kanamycin resistant root from kanamycin resistant embryoid; (G) kanamycin resistant plantlet (designated M1) derived from GV2260 (p35SGUSINT) transformed anther calli, 165 days after transformation.*

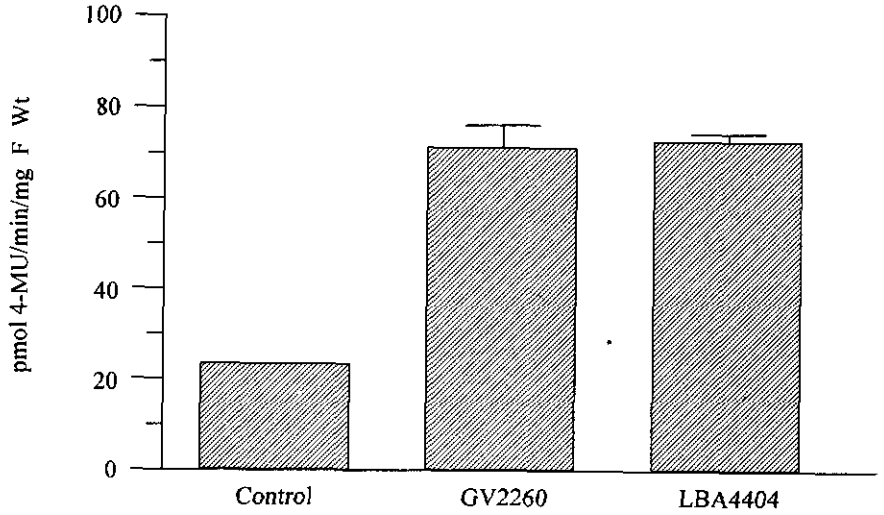

Figure 2. GUS activity in control calli and kanamycin resistant anther calli (24 days after transformation) with Agrobacterium *strains GV2260 (p35SGUSINT) and LBA4404 (pAL4404/pMON9793). The results are presented as mean ± s.d.; n = 3 independently transformed anther calli. GV2260 refers to GV2260 (p35SGUSINT) transformants, LBA4404 refers to LBA4404 (pAL4404/pMON9793) transformants, and control refers to untransformed calli.*

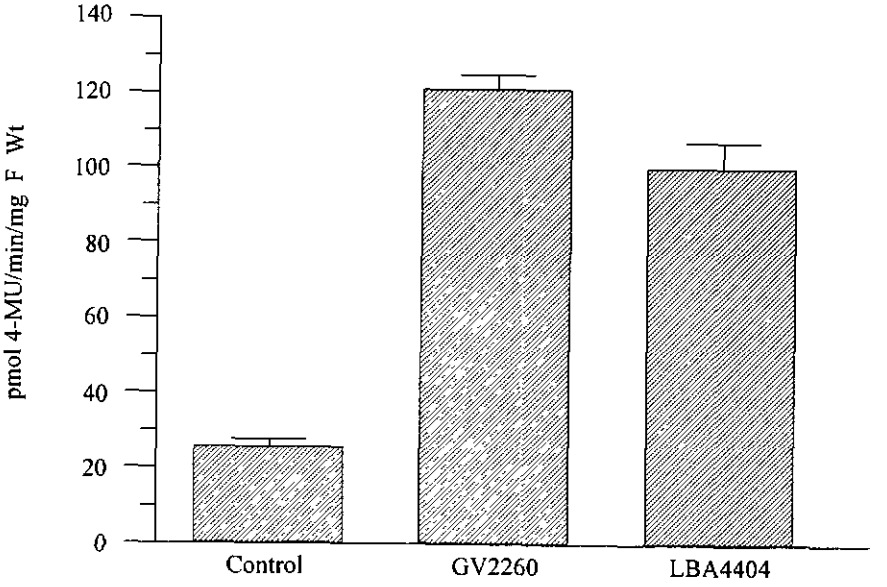

Figure 3. GUS activity in control embryoids (50 days old) and kanamycin resistant Hevea *embryoids (50 days old) initiated from anther calli transformed with* Agrobacterium *strains GV2260 (p35SGUSINT) and LBA4404 (pAL4404/pMON9793). The results are presented as mean ± s.d.; n = 3 embryoids (ca. 100 mg fresh weight) from independently transformed anther calli. GV2260 refers to GV2260 (p35SGUSINT) transformants, LBA4404 refers to LBA4404 (pAL4404/pMON9793) transformants, and control refers to untransformed embryoids.*

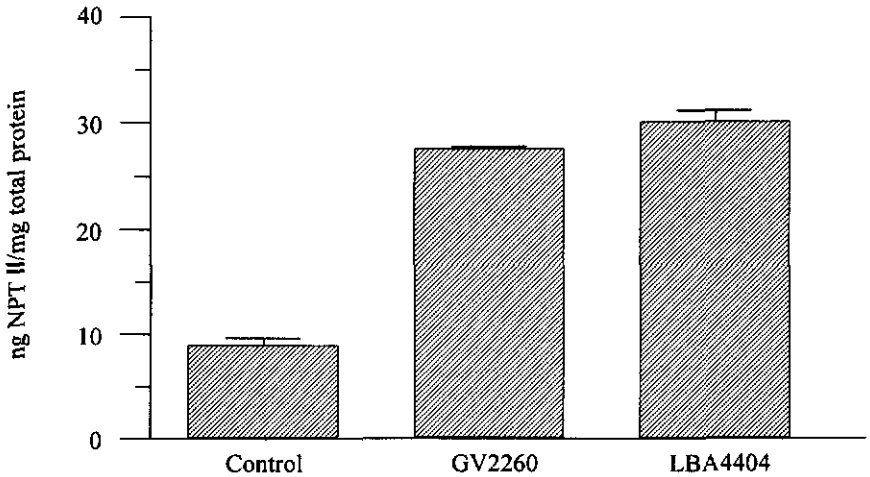

Figure 4. NPT II protein levels in control calli and kanamycin resistant anther calli (24 days after transformation) generated by transformation of anther calli with GV2260 (p35SGUSINT) and LBA4404 (pAL4404/pMON9793) Agrobacterium *strains. The results are presented as mean ± s.d.;n = 3 independently transformed anther calli. GV2260 refers to GV2260 (p35SGUSINT) transformants, LBA4404 refers to LBA4404 (pAL4404/pMON9793) transformants, and control refers to untransformed calli.*

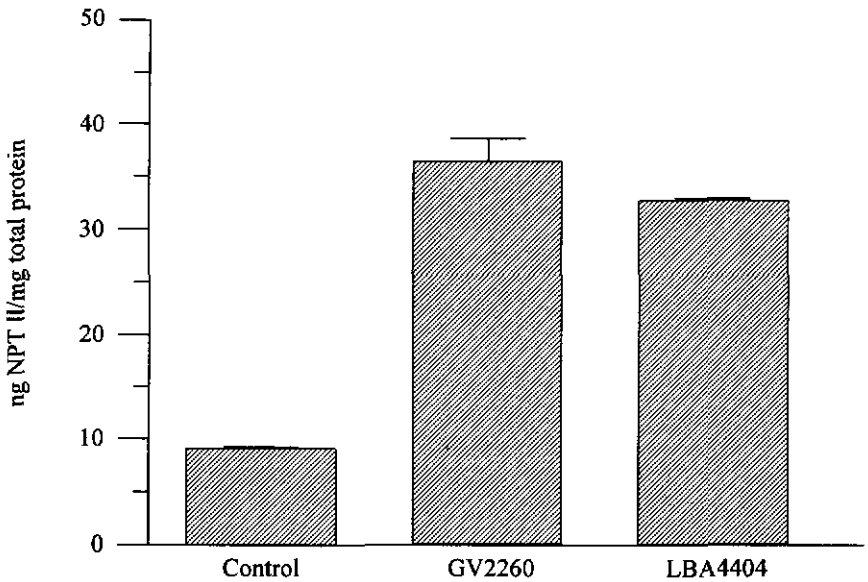

Figure 5. NPT II protein levels in control embryoids and kanamycin resistant embryoids (50 days old) initiated from anther calli transformed with Agrobacterium *strains GV2260 (p35SGUSINT) and LBA4404 (pAL4404/pMON9793). The results are presented as mean ± s.d.;n = 3 embryoids (*ca. *100 mg fresh weight) from independently transformed anther calli. GV2260 (p35SGUSINT) transformants, LBA4404 refers to LBA4404 (pAL4404/pMON9793) transformants, and control refers to untransformed embryoids*

resistant embryoids on average displayed a 310% increase compared to control embryoid values and averaged around 33 ng NPT II/mg total protein. Overall NPT II protein levels ranged from 27 to 33 ng NPT II/mg total protein in transformed *Hevea* tissues.

Detection of Gus Gene in Transformed Tissues

A direct check for the presence of transferred *gus* reporter gene in *Hevea* tissues and in a transgenic plant (*Figure 1G*) was performed using the PCR technique[15]. After 30 cycles of amplification, with as little as 2 ng template DNA a single band was visible on agarose/ ethidium bromide gels (*Figure 6*). This amplified fragment was identical in size to that predicted for the internal *gus* gene fragment[15].

DISCUSSION

Successful *Hevea* transformation is dependent upon: (a) gene transfer into target tissue, and (b) regeneration of transformed tissue into a plantlet through tissue culture. A variety of *Hevea* explant materials have been tested by different research groups for their ability to regenerate into plantlets by *in vitro* culture[3]. The target tissue of choice for *Hevea* is embryogenic callus tissue (initiated from *Hevea* GL1 anther because of its high propensity to form embryoids). Through tissue culture methodologies already in place, the transgenic calli can subsequently be developed into embryoids and complete plantlets. The results presented in this study show that *Agrobacterium* co-cultivation can deliver foreign DNA into the cells of *Hevea* as determined by reporter gene assays, the recovery of kanamycin-resistant transformants and the use of PCR technique.

Initially, transformed anther calli were selected on kanamycin at 50 μg ml^{-1}, since the antibiotic has been shown to be toxic to certain plant cells at higher concentrations[16] such as 100 μg ml^{-1}. The successful use of kanamycin selection depends on early selection pressure of 50 μg ml^{-1}, even though this level of antibiotic causes a slight inhibition of growth of callus material. As selection was one week after co-cultivation, the production of escape embryoids under kanamycin selection was restricted. Non-transformed calli did not grow on media containing kanamycin. Subsequently, selection was carried out at 100 μg m^{-1} to prevent kanamycin 'escapes' or chimeras. The production of 'escape' calli or embryoids under kanamycin selection is not unusual. It has been suggested that such 'escapes' arise from non-transformed cells that are protected from the selective agent by the transformed cells owing to cross-protection of non-transformed cells[17]. Detection of NPT II protein levels in the transgenic tissues confirmed that kanamycin has successfully selected transformed rubber cells from a mixed population of transformed and untransformed cells. Antibiotics (cefotaxime and ticar) were continuously incorporated into the medium up to the embryoid stage to stop bacterial growth of the transformed tissues as removal of antibiotics at this stage can cause bacterial infection. It may be possible that a small number of surviving bacteria could inactivate the kanamycin in the medium, thereby protecting the *Hevea* cells. At the plantlet stage, the antibiotic ticar was not found to be detrimental to *Hevea* plantlet growth and development.

Three plantlets were obtained from calli transformed with Agrobacterium GV2260 (p35SGUSINT) while no plantlets were obtained from calli transformed with *Agrobacterium* LBA4404 (pAL4404/ pMON9793) and the presence of the *gus* gene in one of the plantlets was confirmed by using the PCR technique. A 1.2 kb fragment that was identical in size to the *gus* gene was

Figure 6. Detection of the gus *gene by PCR of genomic DNA isolated from a regenerated* H. brasiliensis *plantlet. Lane 1: BRL 1 kb ladder. Lane 2: 2.0 ng DNA isolated from a single leaf of a control* Hevea *plantlet. Lane 3: 2.0 ng DNA from a transformed callus (selected on kanamycin). Lane 4: 2.0 ng DNA from a transformed embryoid (selected on kanamycin). Lane 5: 2.0 ng DNA isolated from a single leaf of a transformed* Hevea *plantlet (selected on kanamycin).*

amplified. It should be emphasised that we consistently obtained negative results with control tissues by the same PCR conditions. As the frequency of embryoid production from *Agrobacterium* LBA4404 (pAL4404/pMON9793) transformed cultures was about 58% from that obtained from *Agrobacterium* GV2260 (p35SGUSINT) transformed cultures, the potential to obtain plantlets from LBA4404 (pAL4404/pMON9793) transformed embryoids were significantly reduced.

NPT II protein levels in transformed embryoids were slightly higher compared to those in transformed calli. Overall, there was a 230% increase of NPT II protein levels in transformed calli compared to control values and this averaged from 26 - 28 ng NPT II per mg total protein (*Figure 4*). This compares to a 310% increase of NPT II protein levels in transformed embryoids compared to control values and this averaged from 32–35 ng NPT II per mg total protein (*Figure 5*).

The results of the experiments on histochemical staining for β-glucuronidase activity in calli, embryoids and roots of transgenic *Hevea* as shown in *Figure 1A–1F* suggest that they were positive for GUS. Since the binary vector p35SGUSINT in the *Agrobacterium* GV2260 contains an intron within the *gus* gene, the possibility of GUS expression having arisen by *Agrobacterium* contamination of the *Hevea* tissues is ruled out. In transgenic tissues containing this chimaeric gene, the intron must be spliced thus giving rise to GUS enzymatic activity. The intron-containing *gus* gene was thus used as an optimised marker gene in rubber transformation experiments.

It is well known in conventional breeding that if the number of characters sought for in *Hevea* increases, then a large population size would be required for selection to be effective. For *Hevea*, owing to its perennial nature and low fruit-set, population of large families is often impracticable. Effective introduction and selection of multiple characters could be better attained *via* genetic transformation, thus shortening the selection period.

ACKNOWLEDGMENT

Many thanks to Datuk Dr Abdul Aziz bin Sheikh Abdul Kadir, the Director of the Rubber Research Institute of Malaysia, for his kind permission to publish this article. This work was supported by a research grant from the British Council, Kuala Lumpur, Malaysia.

Date of receipt: April 1996
Date of acceptance: July 1996

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