AGROBACTERIUM-MEDIATED TRANSFORMATION OF *HEVEA BRASILIENSIS* WITH APPLE cDNA ENCODING SORBITOL-6-PHOSPHATE DEHYDROGENASE

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The increasing demand of natural rubber and land unavailability in traditional regions has been resulted the cultivation of rubber in non-traditional regions. However, the severe agro-climatic conditions in non-traditional regions adversely affected growth and yield of rubber. In this context, scope for the introduction of genes conferring tolerance to abiotic stresses is of paramount importance and hence the study was undertaken. Embryogenic callus derived from immature anther of *Hevea brasiliensis*, clone RRII 105 was used as the target tissue for genetic transformation. Kanamycin sensitivity to embryogenic callus was examined by kill curve test and medium with 100 mgL^{-1} kanamycin completely suppressed the growth of untransformed callus and was selected as the suitable concentration for selection of transformants. Addition of ABA was found to be beneficial for embryogenesis and maximum embryo induction was obtained with 2.0 mgL^{-1}. Among various levels of sucrose (2-10%), 4 per cent sucrose enhanced embryo induction efficiency and gradually decreased by higher levels. Mature embryos get germinated and converted into plantlets. GUS histochemical assay revealed expression of *uid*A gene in transformed callus and embryos which was evidenced by the intense blue colour. Polymerase chain reaction confirmed the presence of S6PDH, *uid*A and *npt*II genes in transformed callus and embryos. The transgenes were also detected in plasmid DNA (positive control), but absent in untransformed callus (negative control).

Keywords: *Agrobacterium tumefaciens*, Genetic transformation, GUS histochemical assay, PCR analysis, Sorbitol-6-phosphate dehydrogenase gene

INTRODUCTION

Hevea brasiliensis (Muell. Arg.), commonly known as rubber tree, is a perennial tree crop belonging to *Euphorbiaceae* family. In India, rubber is traditionally grown in Kerala and Kanyakumari district of Tamil Nadu. However, scope for further expansion of rubber cultivation in these areas is very limited and attempts were thus made to cultivate rubber in the non-traditional regions which are exposed to a wide range of abiotic stresses. Considering the severe climatic constraints and crop loss in these areas, there is an urgent need for the introduction of genes conferring tolerance to abiotic stresses. In *Hevea*, genetic manipulation was first explored with the

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development of transgenic plants integrated with marker genes through *Agrobacterium* mediated genetic transformation (Arokiaraj and Rahaman, 1991; Arokiaraj, 1996). Further efforts were made by Arokiaraj (2000) and Montoro *et al.* (2000; 2003), however, failed to develop transgenic plants. In the recent past, successful integration and production of genetically modified rubber plants with a foreign gene encoding human serum albumin and native SOD genes have also been reported (Arokiaraj *et al.*, 2002; Jayashree *et al.*, 2003; Sobha *et al.*, 2003; Leclercq *et al.*, 2012).

Among the various environmental stresses, drought stress is one of the major abiotic stresses. Plants react to environmental stresses by the accumulation of compatible solutes in cell which has resulted in increased environmental stress tolerance. Accumulation of polyols such as mannitol, sorbitol and myoinositol and their derivatives is considered to be related to drought and salinity stress tolerance in many plant species (Bohnert and Jensen, 1996). Although the exact function of this sugar alcohol is not known, sorbitol may serves as compatible solute under condition of decreasing water, anti-freeze for chilling or as a scavenger of free radicals under oxidative stress. Among the sugar alcohol, sorbitol is the major photosynthetic product in *Rosaceae* family. Sorbitol is synthesized *via* the reduction of glucose-6-phosphate to sorbitol-6-phosphate. NADP dependent sorbitol-6-phosphate dehydrogenase (S6PDH) is the key enzyme in the biosynthesis of sorbitol from glucose -6-phosphate (Tao *et al.*, 1995). The integration of this plant gene alone may be sufficient for the synthesis and increased accumulation of sorbitol in rubber trees which in turn confer abiotic stress tolerance. Therefore, the present study was undertaken with the aim of integrating S6PDH gene to *Hevea* plants through *Agrobacterium tumefaciens* gene transfer method using embryogenic callus.

MATERIALS AND METHODS

Embryogenic callus, the target tissue for transformation, was induced from immature anther as per the protocol described by Jayasree and Thulaseedharan (2001). In brief, immature floral buds were collected from *H. brasiliensis*, clone RRII 105, growing in the experimental fields of Rubber Research Institute of India. After collection, floral buds were surface sterilized and dissected anthers were inoculated on callus induction medium consisting of modified MS basal salts supplemented with 2.0 mgL^{-1} 2, 4-dichlorophenaxyacetic acid and 0.5 mgL^{-1} Kinetin. Resulting calli were transferred to embryo induction medium containing modified MS basal salts enriched with glutamine (200 mgL^{-1}), casein hydrolysate (400 mgL^{-1}), NAA (0.2 mgL^{-1}), Kn (0.7 mgL^{-1}) and GA_3 (2.0 mgL^{-1}).

In order to test the kanamycin concentration that completely inhibited callus proliferation and differentiation to embryos, a kill curve test was carried out. The sensitivity of kanamycin was determined by culturing embryogenic callus on petri plates containing 20 mL embryo induction medium (devoid of charcoal) along with kanamycin. Kanamycin was filter sterilized and added to the autoclaved medium at concentrations of 0, 50, 75, 100, 150 and 200 mgL^{-1}. Cultures were incubated at 25±2°C under darkness. For control, embryogenic callus was cultured on medium without kanamycin. Five callus clumps were used for each treatment and the experiment was repeated thrice. After 15 and 30 days, cultures were evaluated and observations were recorded.

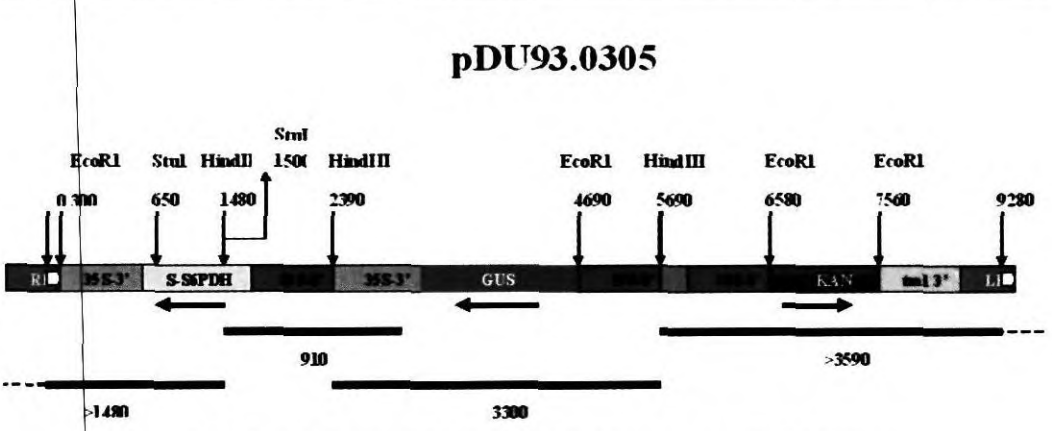

Fig.1. Detailed map of the T-DNA of the binary vector pDU93.0305

Transformation was performed using *A. tumefaciens* strain EHA 101 carrying the plasmid vector pDU 93.0305 containing β-*glucuronidase* (*uid*A) and *neomycin phosphotransferase* (*npt* II) as the reporter and selectable marker genes respectively. The vector also contains an apple cDNA encoding sorbitol-6- phosphate dehydrogenase under the control of CaMV 35S promoter as functional gene (Fig. 1). A single colony of *Agrobacterium* was suspended in 10 mL of AELB (LB medium lacking NaCl) containing 20 mgL^{-1} gentamycin and 50 mgL^{-1} kanamycin (Dandekar *et al.*, 1989). *Agrobacterium* was grown overnight at 28 °C in an orbital shaker at 250 rpm. *Agrobacterium* culture corresponding to 0.5 OD at 420 nm were pelleted at 3000 rpm and resuspended in liquid embryo induction medium to which acetosyringone (20 mgL^{-1}), betaine *HCl* (15.3 mgL^{-1}) and proline (11.5 mgL^{-1}) was added. The bacterial cells were allowed to grow for 4 h under the same condition.

Friable yellow embryogenic callus was immersed in the bacterial culture for 10 min. After infection, the tissues were removed and blotted dry with sterile filter paper. Co-cultivation was done on agar solidified embryo induction medium supplemented with acetosyringone (20 mgL^{-1}), betaine HCl (15.3 mgL^{-1}) and proline (11.5 mgL^{-1}) for 3 days under darkness at 25±2°C. Following co-cultivation, the explants were dried with sterile filter paper and cultured on selection medium consisting of aforementioned embryo induction medium with kanamycin (200 mgL^{-1}) and cefatoxime (500 mgL^{-1}). Kanamycin resistant callus emerged from each independent lines were selected. Transformed callus was transferred onto selection medium containing 100 mgL^{-1} kanamycin for further proliferation and embryo induction. Separate experiments were carried out to test the influence of ABA and sucrose on embryo induction. Proliferating callus was placed on medium with 5 concentrations of ABA (0, 0.25, 0.5, 1.0 and 2.0 mgL^{-1}) and 5 levels of sucrose (2, 4, 6, 8 and 10%) and observations were made visually. Cotyledon stage embryos were kept for maturation and further transferred for plant regeneration. For experiments with ABA and sucrose, five replications were made for each treatment and experiments were repeated three times. To analyse the effect of ABA and sucrose, analysis of variance was performed and the mean number of embryos were compared with ANOVA test.

Histochemical assays with transformed callus and embryos along with untransformed callus were analysed for GUS activity as described by Jefferson *et al.* (1987). Tissues were incubated overnight at 37 °C in a solution of 2 mM X-Gluc. To confirm the presence of transgenes in putative transformants, PCR analysis of the genes was performed. Plasmid DNA was used as positive control and DNA from untransformed callus was used as negative control. Genomic DNA was extracted from randomly selected transformed callus and embryos and untransformed callus following the protocol of Doyle and Doyle (1990). For the detection of *uid* A gene, forward primer 5'-TAGAGATAACCTTCACCCGG-3' and reverse primer 5'-CGCGAAAACTGTGGAA TTGA-3' were used. For *npt*II gene, 5'-GAGGCTATTCGGCTATGACT-3' and 5'-AATCTCGTGATGGCAGGTTG-3'were used as forward and reverse primers. The S6PDH gene was amplified by using 5'-GCGACTCTCTCGTCAAATTCTGTAT-3'(forward) and 5'-TTATGCATACACGTCT AAGCCCCAAGT-3'(reverse) primers. PCR reaction was carried out with 20 µl volume containing 100 µM of each dNTP's 250 nM of each primers, 0.75 unit Taq polymerase, 20 ng template DNA in a thermal cycler. For PCR analysis, the reaction conditions were initial denaturation at 93 °C for 2 min followed by 36 cycles of 92 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min with a final extension for 10 min at 72 °C. Amplified PCR products were analysed in 1.5 per cent agarose gel electrophoresis.

RESULTS AND DISCUSSION

Callus was induced from immature anther at 40-50 days of culture on callus induction medium. Friable yellow callusfirst turned to brown upon subculture to embryo induction medium, and subsequently highly friable embryogenic callus was produced from the brown callus. This embryogenic callus produced was maintained by serial subculture onto fresh medium in an interval of 50 days and was used as the target tissue for genetic transformation.

One of the important factors deciding the success of transformation is the type and concentration of antibiotics used for the selection of transformants. Generally, kanamycin was used to select transformed cells and plants (Uchimiya *et al.*, 1989). But the sensitivity of the tissue is highly variable to this antibiotic and hence it is important to determine the lethal dose of kanamycin with embryogenic callus also. In the present

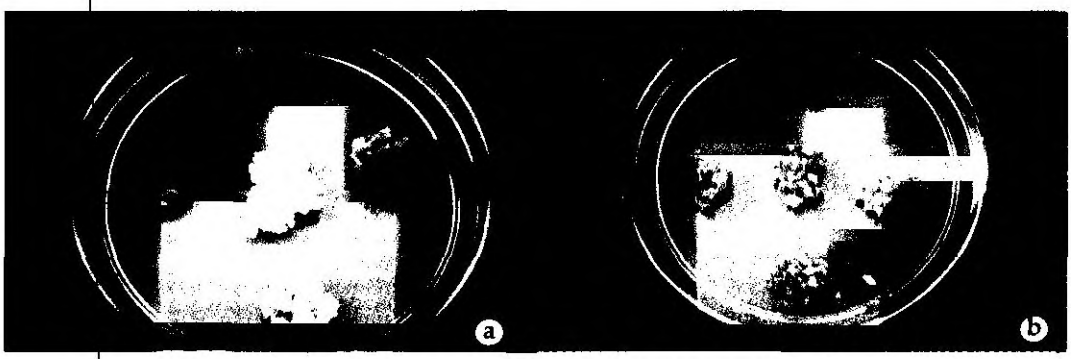

Fig. 2. Embryogenic callus showing kanamycin sensitivity at a. 50 mgL^{-1} b. 100 mgL^{-1}

study, proliferation of control callus started after two weeks of culture, whereas it was delayed further with the addition of kanamycin. After culture of yellow embryogenic callus on medium supplemented with 50-75 mgL^{-1} kanamycin, no significant change in colour was observed in the first 15 days. However, when the callus was maintained upto 30 days, some callus clumps slightly turned brown (Fig. 2a) and at this concentration, survived callus underwent proliferation and further embryo differentiation. This indicates that kanamycin at 50-75 mgL^{-1} were ineffective for selection of transformed callus. However, at higher concentration (100 mgL^{-1}), full tissue necrosis was observed within 30 days and caused total inhibition of callus proliferation and embryo induction (Fig. 2b). Similar level of total growth inhibition was also obtained with 150-200 mgL^{-1} kanamycin in the medium (Table 1). In transformation experiments, occurrence of escapes is a major problem. Hence to minimise any escapes, in the present study, 200 mgL^{-1} kanamycin was used (only in the first subculture) for selection of putative transformed callus and then reduced to 100 mgL^{-1}. In *Hevea*, Jayashree *et al.* (2003) and Sobha *et al.* (2003) also employed kanamycin for the selection of transformants and a higher level of kanamycin (300 mgL^{-1}) was used with two month old anther somatic callus as target tissue. This difference may probably due to the tissue sensitivity to kanamycin. Since embryogenic callus is highly friable and in single cell nature, unlike the compact nature of two month old anther callus, in the present investigation, a low level kanamycin was required. In *Podophyllum hexandrum* Royle, a perennial medicinal plant, kanamycin at 150 mgL^{-1} as effectively employed for the selection of transformed callus and embryos using highly friable embryogenic callus as the target tissue (Rajesh *et al.*, 2013).

Table 1. **Response of embryogenic callus to kanamycin**

Kanamycin mgL^{-1}	Callus proliferation	Mean no of embryos
0	+++	24.27 ± 0.64
50	+	15.20 ± 0.64
75	+	11.38 ± 0.61
100	-	0
150	-	0
200	-	0
CD (P≤0.05)		1.8

Callus proliferation is expressed as
- = no proliferation, + = 1-25%, ++ = 25-50%, +++ = 50-75%

Fifteen days after culture on kanamycin containing selection medium, viable infected callus clumps were transferred to fresh selection medium while cultures with bacterial overgrowth were discarded. At two months of culture, kanamycin resistant callus emerged from each line were selected. Each putative transformants were then get proliferated (Fig. 3a) and somatic embryos were induced (Fig. 3b) from the proliferating callus. In the present study, ABA showed a positive response on embryogenesis. Among different concentrations, a gradual increase in embryo induction was observed with an increasing concentration of ABA (0.25-2.0 mgL^{-1}). As shown in Table 2, maximum number of embryos was induced on medium supplemented with 2.0 mgL^{-1} ABA. Similarly, as reported by Cailloux *et al.* (1996), a high ABA concentration (2.6 mgL^{-1}) in combination with high sucrose enhances embryogenesis and maturation of somatic embryos of *Hevea*. In contrast, a low concentration of ABA was found to be effective for embryogenesis (Jayashree *et al.*, 2003) whereas no influence on embryo maturation (Sushamakumari *et al.*, 2000). This variation may be attributed to the

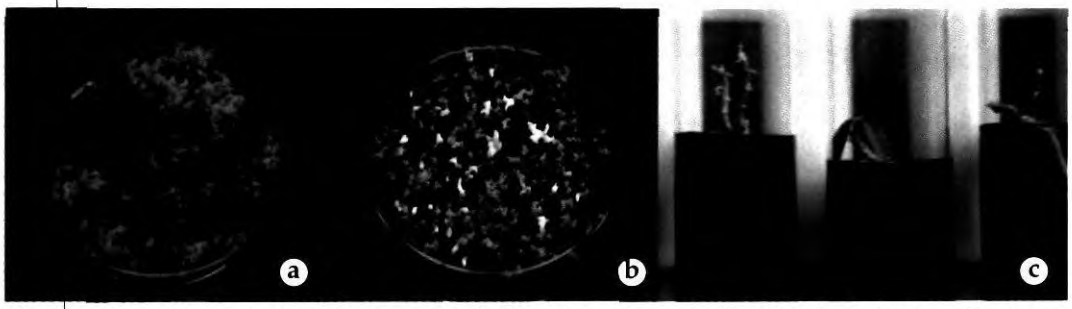

Fig. 3. Stages of transgenic plant recovery a. Proliferation of transgenic callus; b. Induction of embryos c. Plantlet conversion

explant specificity, basal medium components and presence of type and concentrations of other growth hormones of each medium. Table 3 explains the influence of sucrose on embryogenesis from callus integrated with S6PDH gene. Among the 5 levels tested, higher embryo induction was achieved with four per cent sucrose. When increasing the sucrose level to 6-8 per cent, embryo formation was decreased and almost an inhibitory effect was obtained on medium enriched with 10 per cent sucrose. Sucrose is the commonly used carbohydrate in *in vitro* culture and its promotive effect on embryogenesis has been reported by many investigators. In earlier studies on *Hevea*, callus transformed with MnSOD gene, embryos were induced on medium with 5 per cent and three per cent sucrose, respectively (Jayashree *et al.*, 2003; Sobha *et al.*, 2003). However, several workers also employed higher sucrose (7-8%) for somatic embryogenesis from normal callus (Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000; Kala *et al.*, 2005). In the current study, we examined the optimum sucrose level required for embryogenesis and though embryo formation was obtained with 6-8 per cent, an enhanced embryogenesis was noticed on four per cent sucrose containing medium. In tobacco transformed with apple cDNA encoding S6PDH, when the gene was stably integrated, sorbitol was synthesised (Tao *et al.*, 1995). In the present study also, during transformation with S6PDH gene, sorbitol may be synthesised or it created some kind of stress effect in callus which in turn along with sucrose might have created a high osmoticum in the medium and thus at lower levels of sucrose in the present

Table 2. **Influence of ABA on embryo induction**

ABA (mgL^{-1})	Mean no of embryos
0	20.13±0.60
0.25	23.33±0.63
0.50	25.40±0.77
1.0	28.27±0.71
2.0	36.27±0.67
CD ($p \leq 0.05$)	1.92

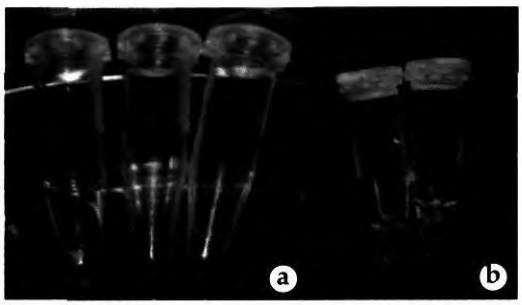

Fig. 4. Histochemical staining of GUS gene a. Transformed callus b. Untransformed callus

Table 3. **Influence of sucrose on embryo induction**

Sucrose (%)	Mean no. of embryos
2	22.27±0.64
4	34.33±0.77
6	27.00±1.06
8	20.13±0.79
10	*8.33±0.65*
CD (p≤0.05)	2.25

medium may sometimes enhanced embryo induction. After maturation, cotyledon stage embryos germinated and subsequently converted into plantlets (Fig. 3c).

GUS histochemical assays revealed that the transgene was successfully integrated in *Hevea* genome which was evidenced by the intense blue colour. Upon X- Gluc staining, embryogenic callus and embryos were stained positive for GUS activity (Fig. 4a), while untransformed callus failed to show blue colouration (Fig. 4b). Transformation was confirmed by PCR analysis using specific primers for S6PDH, *uid*A and *npt*II genes. PCR analysis revealed a 360 bp S6PDH fragment in transformed callus and embryos. The presence of *uid*A gene was confirmed by the amplification of 700 bp DNA fragment while presence of *npt*II gene was detected by the amplification 800 bp bands in transgenic callus (Fig. 5a lanes 2-4) and embryos (Fig. 5a lanes 5-7). All three DNA fragments were amplified in the positive control, but, absent in the untransformed control (Fig. 5b).

CONCLUSION

In conclusion the present investigation reports for the first time, the successful integration of S6PDH gene in embryogenic callus of *Hevea* for the popular Indian rubber clone RRII 105 *via A. tumefaciens* mediated transformation. Kanamycin at 100 mgL^{-1} was found to be the optimum antibiotic concentration with embryogenic callus as the target tissue. Addition of 2.0 mgL^{-1} ABA enhanced embryo induction efficiency. Medium supplemented with four per cent

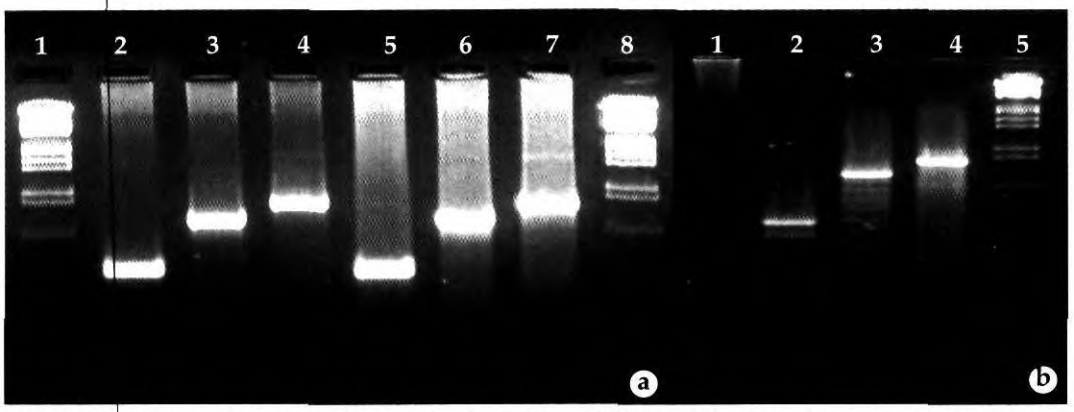

Fig. 5. Molecular confirmation of transgenes by PCR. a. PCR amplification of transgenes in callus and embryos, Lanes 1&8 - Lambda marker (0.5-21kb); 2-4 - Amplification with S-6-PDH, *GUS* and *nptII* genes in callus; 5-7 - Amplification with S-6-PDH, *GUS* and *nptII* genes in embryos.
b. PCR amplification of transgenes in -ve and +ve control; Lanes 1 - Untransformed callus (-ve control); 2-4 - Amplification with S-6-PDH, *GUS* and *nptII* genes in plasmid (+ve control); 5 - Lambda marker (0.5-21kb)

sucrose was found to be the optimum for embryogenesis from callus transformed with S6PDH gene. Embryo germination and subsequent plantlet regeneration was achieved with mature embryos. Expression of *uid* A gene and integration of transgenes in transformed callus and embryos were confirmed histochemically and by PCR analysis.

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