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High frequency *Agrobacterium* mediated genetic transformation in rubber tree *via*. vacuum infiltration

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Abstract

In a tree species like *Hevea brasiliensis*, genetic transformation offers a viable approach for crop improvement within a short period. *Hevea* being highly recalcitrant to *in vitro* culture, an efficient transformation protocol is necessary for generating large number of transgenic plants with stable foreign gene expression. After several modifications in the initial transformation protocol, the transformation frequency was increased to 14 per cent using proliferated anther callus. Therefore, vacuum infiltration was attempted for enhancing the transformation frequency. In the present work the conditions for vacuum infiltration *viz*. vacuum pressure and period of infiltration were standardized. Vacuum infiltration at 30 psi pressure for 10 minutes was found to be ideal for getting high frequency transformation in *H. brasiliensis*. Irrespective of the gene constructs experimented, transformation frequency was significantly improved by adopting vacuum infiltration. Employing this technique, the transformation efficiency of MnSOD gene construct with FMV34S promoter could be enhanced from 14 to 50 per cent.

Keywords: Genetic transformation, rubber tree, vacuum infiltration

Introduction

Hevea brasiliensis (Para rubber tree) belonging to the family Euphorbiaceae is the major source of commercial natural rubber mainly because of its abundance in latex, quality and convenience of harvesting. In *H. brasiliensis*, natural rubber (*cis*-1,4-polyisoprene) is synthesized in latex vessels and stored in the form of latex. Advances in molecular and cell biology over the past few decades have led to the development of a wide range of techniques for manipulating genomes. Genetic engineering is one of the approaches that enable the transfer of genes of interest across sexual incompatibility barriers and the success of this technology is determined by the ability to integrate foreign DNA into host genome and the efficiency of regeneration of transformed cells into fully developed plantlets.

Hybridization and selection is the most important method of *Hevea* breeding. Increased crop productivity, disease resistance and improved wood quantity as well as quality, are the primary aims of conventional breeding. In a tree crop like *Hevea*, this involves the process of repeated back-crossing and selection, which will take several years before a set of desirable genes could be transferred. Since *in vitro* plant regeneration system from different explants (immature anther, inflorescence, leaf *etc*.) is available in *Hevea*, and the callus is amenable to *Agrobacterium* infection, genetic transformation offers a viable approach for the transfer of desirable genes within a short period. The first transgenic plant integrated with marker genes was developed by Arokiaraj *et al*. (1994). Later transgenic plants integrated with MnSOD gene under the control of figwort mossaic virus (FMV) 34S promoter was

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produced by Sobha *et al*. (2003) and with cauliflower mossaic virus (CaMV) 35S promoter (Jayashree *et al.*, 2003) with a transformation frequency of 6 per cent. Even after several modifications in the initial transformation protocol, the transformation frequency could be increased to 14 per cent (Sobha *et al*., 2010). Since *in vitro* plant regeneration frequency is low in *H. brasiliensis*, an efficient transformation protocol is necessary for generating large number of transgenic plants with stable transgene expression. Therefore, *Agrobacterium* mediated genetic transformation *via*. vacuum infiltration using proliferated anther callus was attempted with a view to generate large number of transgenic plants from independent transformation events by increasing the transformation frequency.

Materials and methods

Soft friable immature anther derived callus from *Hevea brasiliensis* (clone RRII 105) was used as the target tissue for *Agrobacterium* mediated gene transfer by vacuum infiltration. The target tissue was generated according to the protocol reported earlier (Sobha *et al*., 2010). Five binary vectors harbouring different agronomically important genes such as manganese superoxide dismutase (MnSOD) under the control of (1) CaMV 35S and (2) FMV 34S promoter, (3) sorbitol 6-phosphate dehydrogenase, (4) antisense 1-aminocyclopropane-1-carboxylate (ACC) synthase and (5) 3-hydroxy methyl glutaryl co-enzyme A reductase (*hmgr*I) were used for genetic transformation. The maps of the binary vectors are given in Figure 1 (A-E). In the *hmgr*I gene construct hygromycin phosphotransferase (*hpt*) was used as the plant selectable marker gene and all other binary vectors contained neomycin phosphotransferase (*npt*II*)* as the selectable marker gene. In all the constructs except *hmgr*I, *uidA* (GUS) was used as the reporter gene.

The binary vector harboring MnSOD gene with FMV34S promoter was used as the reference gene for optimizing the conditions for vacuum infiltration. The bacterial culture for *Agrobacterium* infection was prepared according to the protocol reported earlier (Dandekar *et al.*, 1989). Approximately 2.0 g of the target tissue was taken in 30 mm sterile glass petri plates containing 2 ml of the *Agrobacterium* culture and subjected to

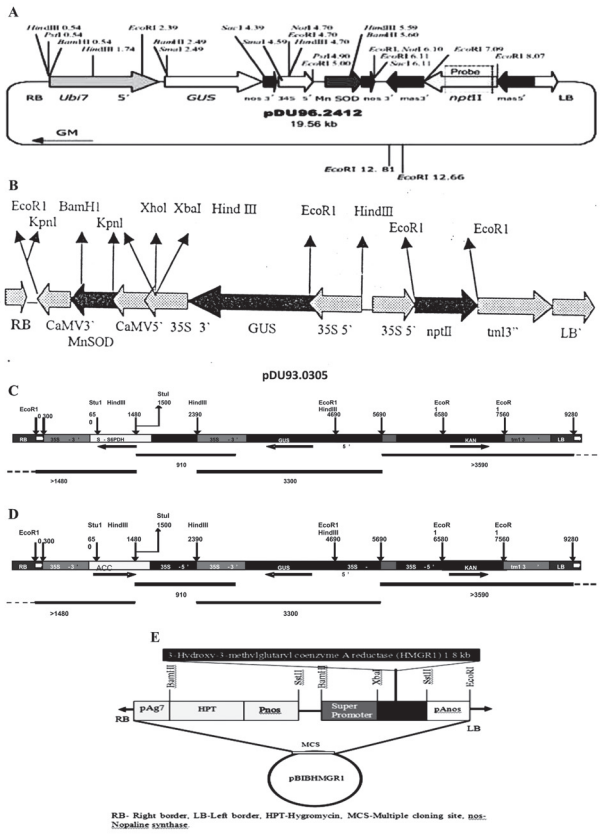

Fig. 1. (A-E): Map of the binary vectors
(A) MnSOD gene construct with FMV 34S promoter, (B) MnSOD gene construct with CaMV 35S promoter, (C) TDNA portion of Sorbitol 6-phosphate dehydrogenase gene construct, (D) TDNA portion of antisense ACC synthase gene construct (E) TDNA portion of the *hmgr* I gene construct

vacuum infiltration. The period of *Agrobacterium* infection standardized for *Hevea* anther callus with MnSOD gene construct under the control of CaMV35S promoter was 10 minutes (Jayashree *et al.*, 2000). For the present work, initially 10 min vacuum infiltration was carried out for identifying the optimum vacuum pressure. Infiltration was carried out at different vacuum pressures ranging from 10- 40 kilopascal (psi). After *Agrobacterium* infection, the excess bacterial culture was removed by drying in a laminar flow hood and then transferred to co-culture medium. After three days of co-culture, the calli were transferred to selection medium containing kanamycin (300 mg L^{-1}) and 500 mg L^{-1} cefotaxime to inhibit the overgrowth of *Agrobacterium* after co-culture and the calli were subcultured at three week intervals in the antibiotic medium for the emergence of transgenic callus lines.

Antibiotic resistant callus lines emerged 40-50 days after *Agrobacterium* infection. A small portion of the callus lines emerged was subjected to GUS histo-chemical staining and all the GUS positive lines were proliferated separately in the callus proliferation medium fortified with 300 mg L^{-1} kanamycin and 500 mg L^{-1} cefotaxime. The transformation frequency was calculated using the formula:

$$\text{Transformation frequency} = \frac{\text{number of proliferating GUS positive callus lines}}{\text{total number of callus clumps subjected to } \textit{Agrobacterium} \text{ infection and cultured per plate}} \times 100$$

After determining the optimum pressure for vacuum infiltration, the experiment was carried out at different time intervals (5-20 min) to determine the optimum period of infection. The conditions for vacuum infiltration was optimized using the MnSOD gene construct with FMV34S promoter, then *Agrobacterium* infection was carried out with the other gene constructs. For the selection of transgenic lines with *hmgr*I gene construct, hygromycin (40 mg L^{-1}) was provided in the selection medium and for the other gene constructs 300 mg L^{-1} kanamycin was added. 500 mg L^{-1} cefotaxime was added in the selection medium for inhibiting the overgrowth of the *Agrobacterium* and three subcultures were made at three weeks interval in the selection medium for the elimination of escapes. Antibiotic resistant transgenic callus lines emerged after the third subculture (Fig. 2. A-E) and the putatively transgenic callus lines were selected after subjecting GUS histo-chemical staining (Fig. 3). The GUS positive callus lines were proliferated separately in the callus proliferation medium fortified with the respective antibiotics. A portion of the proliferated callus lines were selected randomly for determining the presence of transgene by performing PCR using gene specific primers. The PCR positive callus lines were subcultured individually on embryogenic callus induction medium. The embryogenic calli obtained were cultured over embryo induction medium and further to plant regeneration medium.

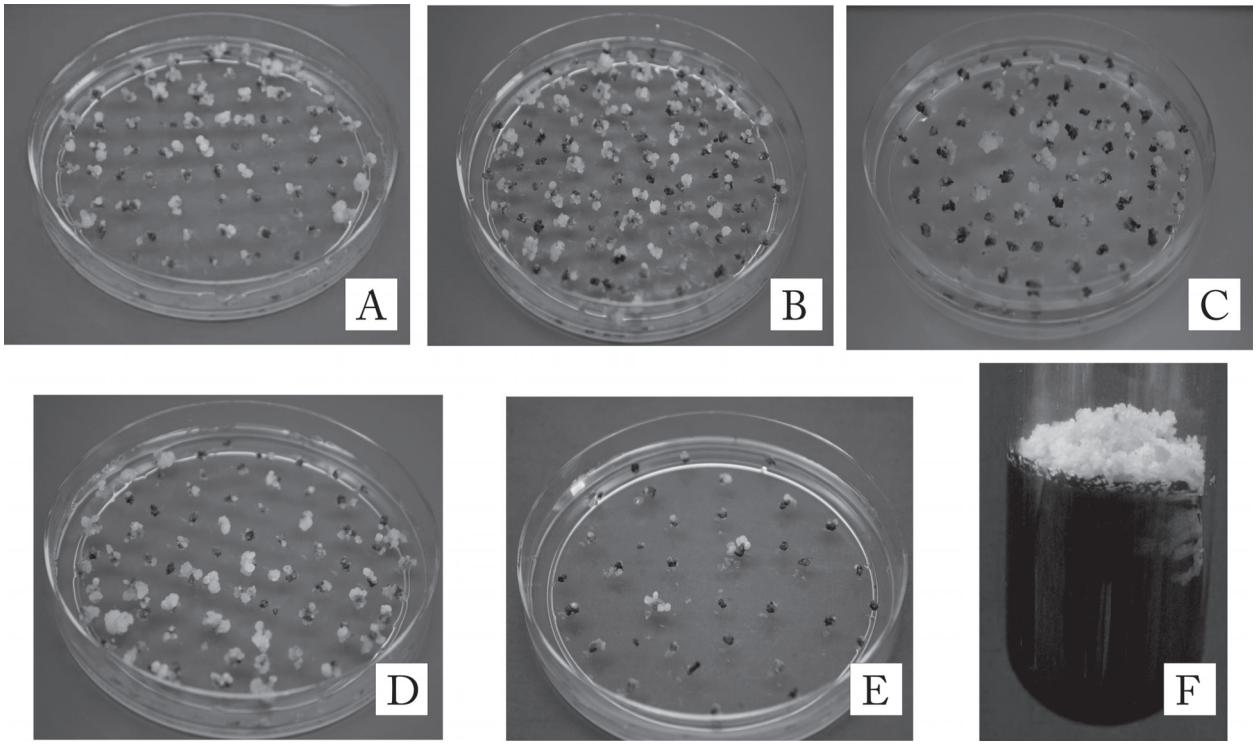

Fig. 2. (A-F): Emergence of transgenic callus lines with different gene constructs
(A) MnSOD with FMV 34S promoter, (B) MnSOD with CaMV 35S promoter, (C) Sorbitol 6 phosphate dehydrogenase, (D) Antisense ACC synthase, (E) *hmgr*I, (F) Embryogenic callus derived from MnSOD (34S) transgenic callus

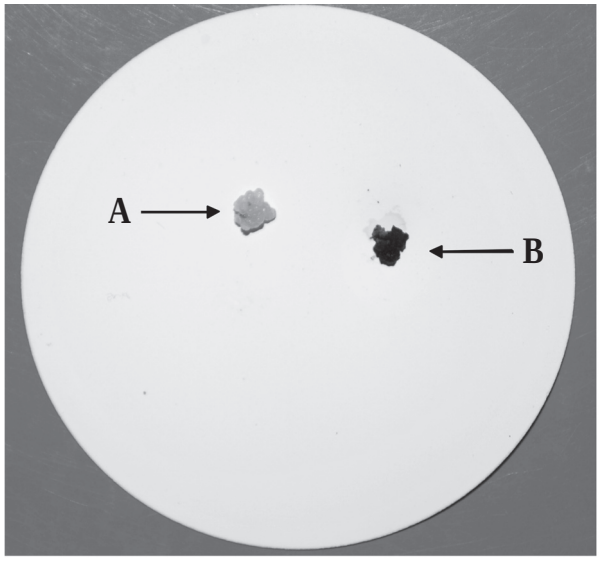

Fig. 3. GUS histo-chemical staining of the transgenic callus
A : Non transgenic, B: GUS positive

Results and discussion

Agrobacterium tumefaciens mediated gene transfer assisted by vacuum infiltration has been successfully employed in several crops *viz*. wheat (Cheng *et al*., 1997), cotton (Ikram- Ul-Haq. 2004), bean (Liu *et al*., 2005), coffee (Canche-Moo *et al*., 2006), banana (Subramanyam *et al*., 2010), but in *Hevea*, this was attempted for the first time. In the present experiment, the conditions for vacuum infiltration were standardized using the MnSOD gene construct with FMV34S promoter. In the optimization experiment, different vacuum pressures were applied keeping the period of infection fixed (10 min) and the results are summarized in Table 1. The transformation frequency was calculated from the number of proliferating callus lines developed from the *Agrobacterium* infected callus clumps cultured in each petriplate containing the selection medium. In all the gene constructs except with the one carrying *hmgr*I gene, GUS gene with a catalase intron is also present as reporter gene. In all the proliferating cell lines in the selection medium GUS expression was also observed confirming the presence of transgene. When the vacuum pressure was 10 psi, the transformation efficiency was not significantly improved. As the vacuum pressure was increased to 20 and 30 psi, the transformation efficiency was significantly increased. There are reports that the application of vacuum pressure allows the infiltration medium, including the transformation vector to relocate in to the plant tissue. Further, it was reported in the genetic transformation of winter jujube that the application of vacuum pressure using a vacuum pump creates a negative pressure environment that results in an increase in the effective *Agrobacterium* volatilization, a condition conducive to the transfer of a foreign gene into plant cells (Gu *et al*., 2008). From these reports, it was concluded that the increase in transformation frequency was due to the application of vacuum pressure. Moreover, it was observed that the unit of vacuum pressure applied had a crucial role in determining the transformation frequency. As the pressure was increased to 40 psi, the frequency was decreased. This may be due to cell rupturing in consequence of the high pressure applied and this was evident from the viability of the infected callus. This callus after co-culture became brownish black within two weeks and finally dried up in the selection medium. Therefore, vacuum infiltration at 30 psi was fixed as the optimum pressure for getting high frequency transformation.

Table 1. Effect of vacuum pressure on transformation frequency

Sl. No	Vacuum pressure (Kps)	Transformation frequency (%)
1	0	14 (21. 84)
2	10	18 (25. 08)
3	20	30 (33. 13)
4	30	50 (45. 00)
5	40	12 (20. 15)
	CD (5%)	4.31

Arcsine transformed values are given in parenthesis

Similar improvement in transformation frequency was reported in *Medicago truncatula* by Trieu *et al*. (2000). The transformation frequency using seedlings as the target tissue was increased to 76 per cent by employing vacuum infiltration. In *Pinus radiata* also *Agrobacterium* mediated genetic transformation frequency was enhanced to 55% through vacuum infiltration (Charity *et al*., 2002). Similarly in cotton, Ikram-Ul-Haq (2004) carried out *Agrobacterium* mediated gene transfer *via* sonication and vacuum infiltration with two month old embryogenic calli and observed that the transformation frequency was enhanced from 20 to 46.6 per cent. *Agrobacterium* mediated genetic

transformation assisted by vacuum infiltration using leaf as source material was evaluated as a fast method to get genetically modified *Coffea canephora* plantlets (Canche-Moo *et al*., 2005). Employing this technique, 33 per cent transgenic coffee embryos could be produced in two months. Oliveira *et al*. (2009) developed an improved method for the *Agrobacterium* infiltration of epicotyl segments of 'Pineapple' sweet orange (*Citrus sinensis* L) and 'Swingle' citrumelo sweet orange and the transformation frequency was increased. The highest transformation frequencies of 8.4 per cent for Pineapple and 11.2 per cent for Swingle citrumelo were obtained when the explants were subjected to a combination of sonication for 2 seconds followed by 10 min vacuum infiltration. An efficient transformation protocol was also reported in banana by Subramanyam *et al*. (2010) *via*. sonication and vacuum infiltration. Similarly in *Arabidopsis*, transformation efficiency enhancement was achieved by vacuum infiltration using surfactant application and apical inflorescence removal (Dehestani *et al*., 2010). All these reports suggested that *Agrobacterium* mediated genetic transformation frequency could be significantly enhanced by adopting vacuum infiltration technique.

The duration of vacuum infiltration employed was further optimized by keeping vacuum pressure constant (30 psi). It was observed that the transformation frequency was not significantly increased when vacuum infiltration was carried out for 5 minutes. As the infection period was increased to 10 minutes, high frequency transformation (50%) was obtained (Table 2). However, it was noted that increasing the infection period to 15 minutes, the transformation efficacy was decreased. On further increasing the infection period to 20 minutes, the transformation frequency was decreased to 6 per cent and the percentage of viable callus was reduced. This was in concurrence with the earlier report that the duration a plant part or tissue is exposed to vacuum infiltration is critical since prolonged exposure causes hyperhydricity. Moreover, it was observed that extended exposure of the callus to *Agrobacterium* led to excessive overgrowth of the *Agrobacterium* over the target tissue and the recovery of the infected callus was difficult. All these factors might have contributed to the drastic reduction in transformation frequency (6%) at 20 minutes vacuum infiltration. Similar results were reported in the genetic transformation of *Pennisetum glaucum* (Jha *et al*., 2011). The *Pennisetum glaucum* shoot apices when subjected to vacuum treatment with *Agrobacterium* for more than 30 min, the explant tissues were completely colonized by the *Agrobacterium*, making it more difficult to eliminate it in the recovery and subsequent stages resulting in loss of shoot apices leading to reduction in transformation frequency. Finally, for *Hevea* proliferated anther callus, 10 minutes vacuum infiltration at 30 psi was fixed as the optimum conditions for getting high frequency transformation. Employing this technique, the transformation frequency for MnSOD gene construct with FMV34S promoter was enhanced from 14-50 per cent. The transformation frequency for sorbitol 6-phosphate dehydrogenase gene was also enhanced to 50 per cent. Whereas, for MnSOD with CaMV35S promoter and antisense ACC synthase gene constructs, the frequency was 40 per cent and for *hmgr*I gene construct the frequency was increased to 30 per cent.

Table 2. Effect of period of infection on transformation frequency

Sl. No	Period of infection	Trans. Frequency (%)
1	0	14 (21. 94)
2	5	26 (31. 50)
3	10	50 (45. 00)
4	15	18 (25. 03)
5	20	6 (6. 05)
	CD (5%)	4.06

Arcsine transformed values are given in parenthesis

The presence of transgene in the putatively transgenic callus lines were observed by PCR reaction carried out with gene specific primers. When PCR was performed with the DNA isolated from the two MnSOD transgenic callus (FMV34S and CaMV35S promoter), using MnSOD gene specific primer, a 700 bp band was amplified in all the GUS positive callus lines and this band corresponds to the MnSOD transgene (Fig. 4. A & B). When PCR was performed with the DNA isolated from sorbitol 6- phosphate dehyrogenase and antisense ACC synthase transgenic callus lines using *npt*II gene specific primer, a 800 bp band was amplified in all the callus lines tested (Fig. 4. C & D). With *hmgr*I gene construct, PCR amplification

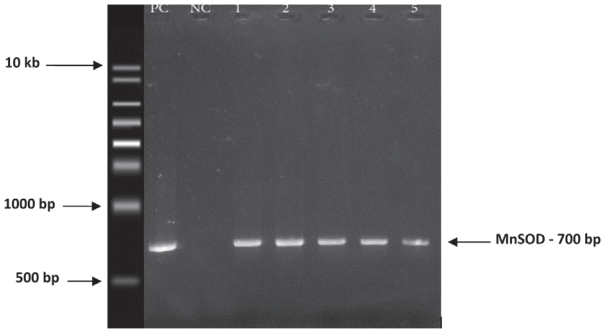

Fig. 4-A. PCR amplification of MnSOD (34S promoter) callus DNA with MnSOD gene specific primer

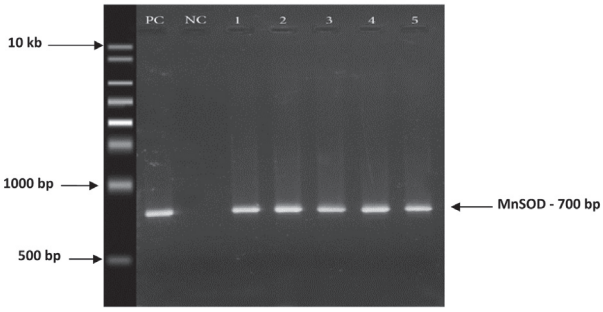

Fig. 4-B. PCR amplification of MnSOD (35S promoter) callus DNA with MnSOD gene specific primer

using hygromycin phosphotransferase (*hpt*) primer, a 600 bp band was amplified in all the callus lines tested (Fig. 4. E). This result along with the GUS activity observed confirms the presence of transgene.

The PCR positive transgenic callus lines were cultured separately on embryogenic callus induction medium at two months interval. After the third subculture in the same medium, some of the transgenic callus became friable with brownish black colour. This calli on further subculture over medium

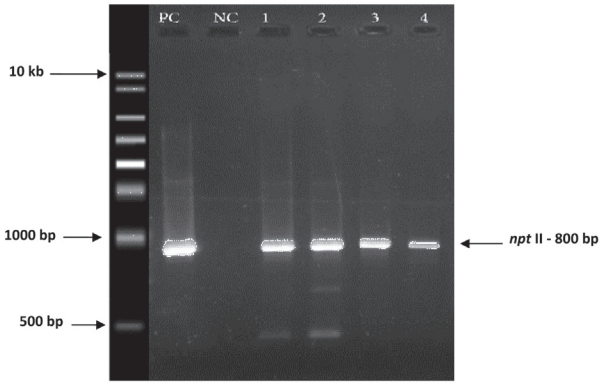

Fig. 4-C. PCR amplification of ACC callus DNA with *npt*II gene specific primer

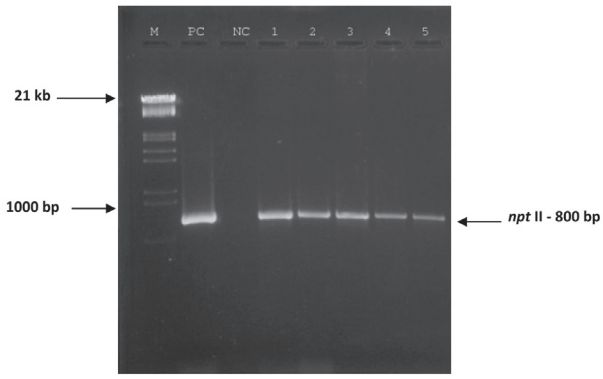

Fig. 4-D. PCR amplification of sorbitol 6-phoaphate dehydrogenase DNA with *npt*II gene specific primer

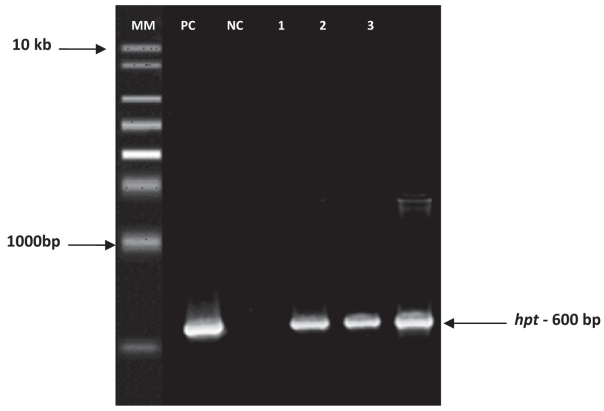

Fig. 4-E. PCR amplification with *hmgr* I callus DNA with *hpt* gene specific primer

containing 0.2 mg L^{-1} ABA and 6 per cent polyethylene glycol, embryogenic callus was obtained from six MnSOD transgenic callus lines. This embryogenic calli on further subculture over embryo induction medium, globular stage embryos were obtained (Fig. 2. F) and the embryos were cultured for plant regeneration. Embryogenesis is awaited from the transgenic callus lines obtained from other gene constructs.

Conclusions

In the present study the parameters for *Agrobacterium* mediated gene transfer in *H. brasiliensis* using proliferated anther callus *via.* vacuum infiltration was optimized. This is the first report on high frequency gene transfer in *Hevea* employing vacuum infiltration. This system can further be utilized for gene transfer to intact explants such as leaf disc, anther and ovule. The added advantage of this technique is that it can also be

used for studying promoter efficiency *via*. transient assay.

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