Agrobacterium mediated multiple gene integration in *Hevea brasiliensis* Muell. Arg.

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Abstract

Hevea brasiliensis (Euphorbiaceae) is the major source of the world natural rubber. Susceptibility of *Hevea brasiliensis* to various biotic and abiotic stresses and incidence of tapping panel dryness are the major constraints in natural rubber production. Gene stacking permits the integration of multiple genes for complex traits simultaneously into the target plant. In the present study, gene stacking was achieved by repeated genetic transformation for the integration of two genes, manganese superoxide dismutase (*MnSOD*) for abiotic stress tolerance and 3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgr*I) for enhanced latex yield. Initially, using the embryogenic callus derived from immature zygotic embryo as the target tissue, *Agrobacterium* mediated transformation by vacuum infiltration was carried out with the binary vector harboring *MnSOD* gene and *npt*II as the selectable marker gene. High frequency (30%) transformation was obtained and was detected by GUS histochemical staining and PCR using *MnSOD* gene specific primer. This MnSOD transgenic callus was used as the target tissue for the integration of *hmgr*I gene containing *hpt* as the selectable marker gene. PCR using *hpt* gene specific primer was performed for the detection of *hmgr*I gene integration. Somatic embryo induction (32%) was achieved from the multiple gene integrated callus lines in modified MS medium with BA and Kin (0.3 mg l^{-1} each) and GA_3 (0.5 mg l^{-1}). Fifteen percent of the embryos were matured and were further cultured for plant regeneration. This is the first report on multiple gene integration in *Hevea brasiliensis*.

Key words: *Agrobacterium*, Gene Stacking, *Hevea brasiliensis*, *hmgr*I, *MnSOD*

Introduction

Latex, synthesized in the latex vessels of *Hevea brasiliensis* (Euphorbiaceae) accounts for 99 per cent of the world natural rubber. Susceptibility of *H. brasiliensis* to various biotic and abiotic stress factors and incidence of tapping panel dryness are the two major concerns in crop production. *Hevea* being a highly heterozygous perennial tree crop with a long breeding cycle, genetic improvement by conventional breeding is laborious and time consuming. This necessitates the gene integration through genetic transformation in *H. brasiliensis*. One of the major technical hurdles impeding the advancement of plant genetic engineering is the expression/ manipulation of multiple genes in plants for complex traits such as crop yield, stress tolerance, disease resistance, etc. On the research front, a variety of conventional and more novel methods have been employed for introducing multiple genes into plants viz. sequential retransformation, co transformation with multiple plasmids, transformation with single binary vector in which several transgenes are stacked. In *H. brasiliensis,* the first transgenic plant with GUS reporter gene was developed in the year 1994 (Arokiaraj et al.) and the genetic transformation protocols reported so far were focused on the integration of a single gene coding for a particular trait (Sobha et al., 2003; Jayashree et al., 2010; Rekha et al., 2013)

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Oxidative stress is a constant burden to plants resulting from toxic reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals and other toxic oxygen species. Superoxide dismutases (SODs) are metallo enzymes present in most aerobic and anaerobic organisms which catalyzes the spontaneous dismutation of superoxide anions to hydrogen peroxide (H_2O_2) and molecular oxygen (Fridovich, 1986). Integration of *MnSOD* gene would impart abiotic stress tolerance by free radical scavenging. Latex is synthesized in the latex vessels by the mevalonate (MVA) pathway (Lynen, 1969). The first step in mevalonate synthesis is catalyzed by the key enzyme 3-hydroxy-3-methyl glutarylco-enzyme A reductase1 (*hmgr*I). The synthesis involves the condensation of three units of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and continues by reduction to mevalonate followed by two successive phosphorylation of mevalonate and a decarboxylation step to form isopentenyle pyrophosphate (IPP). It is presumed that increasing the copy number of *hmgr*I gene will help in increased latex production. Therefore, *Agrobacterium* mediated genetic transformation was performed in *H. brasiliensis* for the integration of *MnSOD* gene for abiotic stress tolerance and *hmgr*I for enhanced latex biosynthesis.

Materials and Methods

Binary vectors

The MnSOD binary vector was developed in collaboration with Prof. A.M. Dandekar, Department of Pomology, University of California, USA and Rubber Research Institute of India. The vector contains β-glucuronidase (*uidA*) as the reporter gene, neomycin phosphotransferase (*npt*II) as the plant selectable marker gene and *Hevea* manganese superoxide dismutase (*HbMnSOD*) gene under the control of Figwort Mosaic Virus (FMV) 34S promoter.

The pBIB HMGRI gene construct used for the integration of *hmgr*I gene was synthesized by (Venkatachalam et al. 2009). The full-length HMGRI cDNA was isolated from *Hevea* tree (clone RRII 105) by a PCR based approach. The total RNA from the latex cells was reverse transcribed and used for PCR amplification of HMGR1 cDNA. The PCR amplified cDNA fragment was gel purified and cloned into pGEM-T vector. After confirming the recombinant clones by colony PCR and by restriction digestion, they were selected for nucleotide sequencing. The full-length HMGR1 cDNA isolated from *H. brasiliensis* (clone RRII 105) was subcloned from pGEM-T to the BamHI and EcoRI sites of the binary plant transformation vector pBIB, placing the HMGR1 cDNA between the super promoter and nos terminator elements. The binary vector contained hygromycin phosphotransferase (*hpt*) as the selectable marker.

Target tissue for MnSOD gene integration

The target tissue for *Agrobacterium* infection was derived from immature zygotic embryo developed by *half ovulo* culture (Rekha et al., 2012). The embryogenic callus obtained was cultured over half strength MS basal medium fortified with 4 g l^{-1} phytagel. Actively growing friable embryogenic callus obtained after two weeks of culture was used as the target tissue.

Bacterial culture

The bacterial culture for *Agrobacterium* infection was prepared according to Dandekar et al. (1989). A single colony of *Agrobacterium* harbouring the MnSOD binary vector was streaked on solid AELB medium supplemented with gentamycin (20 mg l^{-1}) and kanamycin (50 mg l^{-1}) and grown overnight at 28°C. After 24 hours of growth a single colony of the actively growing bacteria was transferred to 10 ml liquid AELB medium containing the above antibiotics in the same concentration. Approximately 1.0 g of the target tissue was taken in sterile (30 mm) glass Petri plates containing 2 ml of the bacterial culture. *Agrobacterium* infection was carried out employing

vacuum infiltration according to the protocol reported earlier (Sobha et al., 2013) and transferred to co-culture medium.

Development of MnSOD transgenic callus

The basal medium composition of the co-culture medium was same as that reported for the transformation of *Hevea* embryogenic callus with osmotin gene (Rekha et al., 2013). Acetosyringone 200 µM, proline and betaine hydrochloride (1 mM each) were added in the co-culture medium. Co-culture was carried out for 72 hours in the dark at 26°C and then transferred to selection medium. The basal medium used for the selection of transformed callus lines was same as that of the co-culture medium, except the addition of antibiotics viz. kanamycin (350 mg l^{-1}) and carbenicillin (500 mg l^{-1}). Twenty four kanamycin resistant callus lines emerged after 40-50 days of culture in the selection medium. They were selected after subjecting a small portion of the callus GUS histo-chemical staining according to Jefferson (1987). The GUS positive callus lines were proliferated individually in the selection medium containing 2,4-D (0.5 mg l^{-1}) and BA (0.4 mg l^{-1}). After three weeks of culture in the proliferation medium, twenty callus lines were proliferated and from this eight callus lines with high GUS expression were selected and DNA was isolated following the protocol reported by Sambrook et al. (1989). PCR was performed for detecting the integration of *MnSOD* gene using the forward primer 5'-ATGGCTCTGCGATCTCTAGTGACCC-3'and reverse primer 5'-TAAGAAGAGCATTCTTTGGCAT-3' and *npt*II gene using the forward primer 5'-GAGGCTATTCGGCTATGACT-3' and reverse primer 5'-AATCTCGTGATGGCAGGTTG-3'.

Integration of hmgrI gene in the MnSOD transgenic callus

From the PCR positive MnSOD transgenic calli, one callus line was selected, proliferated in kanamycin containing medium and used as the target tissue for the integration of *hmgr*I gene. The bacterial culture for *Agrobacterium* infection was prepared in the same manner as reported for MnSOD gene construct, except hygromycin (40 mg l^{-1}) was used for the selection of transformed callus lines. Carbenicellin (500 mg l^{-1}) was used to prevent overgrowth of the *Agrobacterium*. Three subcultures at three weeks interval were made in the selection medium for the elimination of false positives.

Molecular analysis for the detection of multiple gene integration

The hygromycin resistant callus lines emerged after 50-60 days of culture in the selection medium were selected and proliferated individually in the proliferation medium containing hygromycin (40 mg l^{-1}). Untransformed callus was also cultured as the negative control. DNA was isolated from all the hygromycin resistant callus lines and from the negative control callus. Plasmid DNA was isolated from the MnSOD and HMGRI binary vectors following the alkaline lysis method. Multiple gene integration was detected by PCR analysis using *MnSOD*, *npt*II and *hpt* gene specific primers. The *MnSOD* and *npt*II genes were amplified using the primers reported earlier. Presence of *hpt* gene in all the callus lines was assured by PCR using *hpt* gene specific forward primer, 5'-CGATTGCGTCGCATCGAC-3'and reverse primer 5'-CGTGACACCCTGTGCACG-3'. The PCR conditions were: initial denaturation at 94°C for 4 min, denaturation at 92°C for 1 min, annealing at 55°C for 1 min, extension 72°C for 1 min and final extension at 72°C for 10 min. The reactions were performed for 36 cycles and the amplification was visualized by agarose gel electrophoresis of the PCR product.

Transgenic plant regeneration via somatic embryogenesis

The *MnSOD* and *hmgr*1 gene integrated callus lines

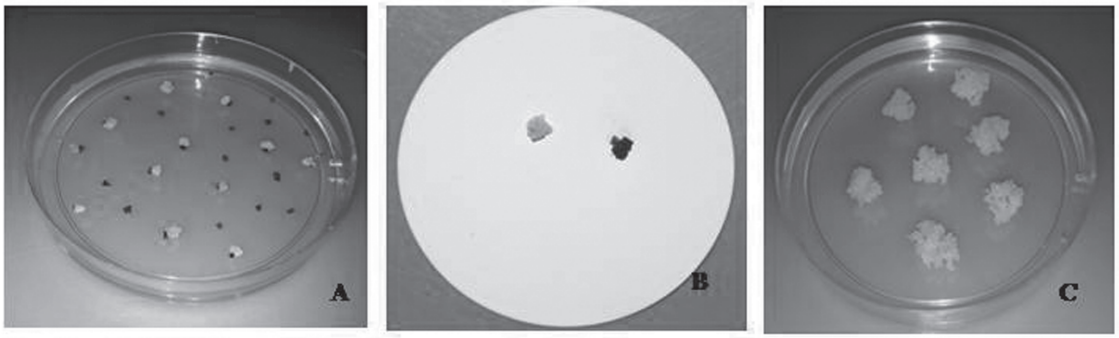

Figure1.A Emergence of putatively transgenic callus *Figure1*.B. GUS histochemical staining *Figure1*.C. Transgenic embryogenic callus

and untransformed calli were proliferated individually in the proliferation medium for thirty days and then transferred to embryo induction medium. Modified half MS basal medium fortified with growth regulators was used for embryo induction. A factorial experiment was carried out with benzyl adenine BA (0.0- 0.5 mg l^{-1}) in combination with gibberellic acid (GA_3) (0.0-1.0 mg l^{-1}) in presence of 0.3 mg l^{-1} kinetin (Kin) for identifying the optimum concentration of the growth hormones required for efficient embryo induction. The somatic embryos upon three subcultures over the same medium developed into cotyledonary stage embryos. These embryos were transferred to maturation medium reported earlier for the maturation of MnSOD transgenic embryos. The mature embryos obtained were transferred to plant regeneration medium.

Results and Discussion

Agrobacterium mediated dual gene integration was successfully carried out in *Hevea* through repeated transformation. Transgenic cell lines were developed, proliferated and embryos were induced. The gene integration was confirmed in the cell lines through PCR analysis. The different steps involved in the process are described below.

Transformation of Hevea callus with MnSOD gene

From the *Agrobacterium* infected callus with the *MnSOD* gene construct, twenty four kanamycin resistant callus lines were emerged after 40-50 days of culture in the selection medium (Fig. 1A). A transformation frequency of 30 per cent was obtained. The putative transgenic callus lines were

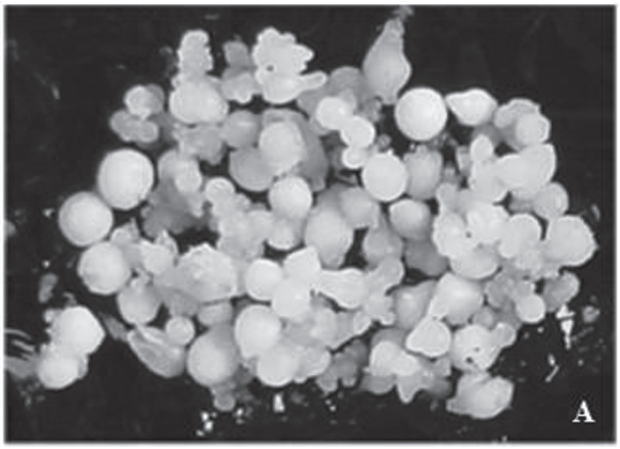

Figure 2.A Emergence of somatic embryos

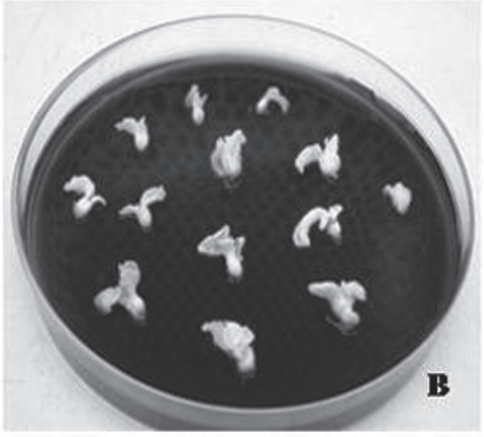

Figure 2. B Cotyledonary stage embryos

Table 1. Effect of BA and GA_3 on the number of embryos induced in presence of 0.3 mg l^{-1} Kin (Observation over 12 callus clumps with transformed means in parenthesis, r-3, analysis - arcsine transformation

Concentration of hormones (mg l^{-1})		BA				
		0.1	0.2	0.3	0.4	0.5
GA_3	0.0	0.0 (1)	5.5 (2.37)	6.9 (2.80)	5.5 (2.37)	1.3 (1.69)
	0.25	5.5 (2.37)	12.5 (3.64)	15.2 (4.02)	11.1 (3.44)	5.5 (2.37)
	0.50	13.8 (3.82)	23.6 (4.96)	31.9 (5.74)	27.7 (5.35)	11.1(3.44)
	0.75	13.8 (3.82)	20.8 (4.66)	19.4 (4.50)	16.6 (4.12)	8.3 (2.75)
	1.0	9.7 (3.18)	13.8 (3.82)	11.1 (3.44)	6.9 (2.8)	5.5 (2.37)
				SE (0.48)		

tested for GUS histo-chemical staining and the GUS positive callus lines were selected (Fig. 1B). The GUS positive callus lines were proliferated individually in the selection medium containing 2,4-D (0.5 mg l^{-1}) and BA (0.4 mg l^{-1}). Out of the twenty four GUS positive callus lines cultured, twenty callus lines were proliferated.

DNA was isolated from randomly selected eight GUS positive callus lines and PCR was performed using the standard procedure. DNA isolated from the untransformed callus was used as the negative control and the plasmid DNA isolated from the MnSOD binary vector was taken as positive control. With *npt*II gene specific primer, an 800 bp fragment was amplified in all the callus lines tested as well as in the positive control (binary vector control), but it was absent in the untransformed control callus (Fig. 3A). Similarly with *MnSOD* gene specific primer, 700 bp band was amplified in the positive control as well as in all the callus lines tested and this corresponds to *MnSOD* transgene, but no amplification was obtained for the untransformed control callus (Fig. 3B). The PCR results assured the presence of *MnSOD* and *npt*II genes in the transgenic callus lines selected.

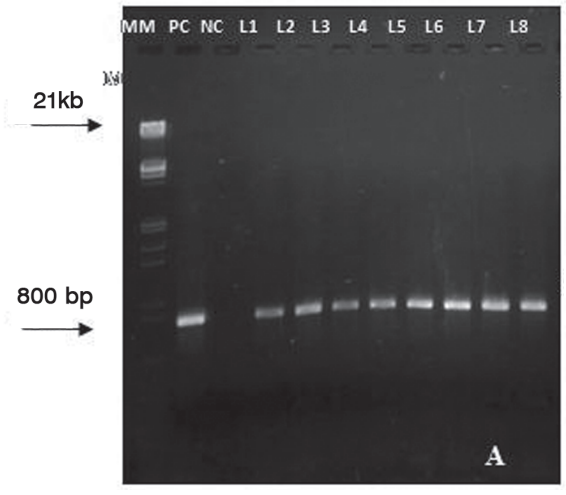

Figure 3. A. PCR amplification with *npt*II gene specific primer

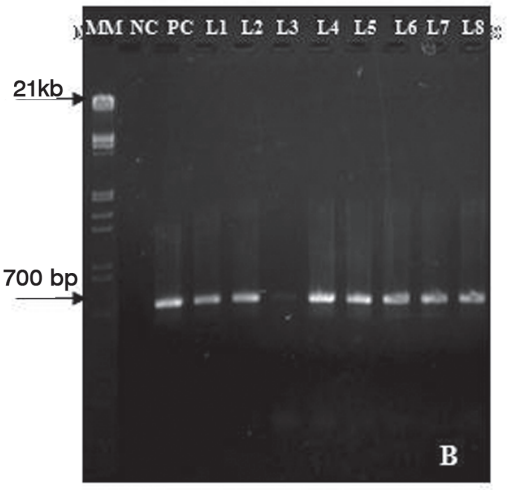

Figure 3.B . PCR amplification with *MnSOD* gene specific primer

Integration of *hmgr*I gene in the MnSOD transgenic callus by repeated transformation

One MnSOD transgenic embryogenic callus (Fig. 1C) was selected and used as the target tissue for the integration of *hmgr*I gene by repeated transformation employing vacuum infiltration. Eleven hygromycin resistant callus lines emerged after 50-60 days of culture in the selection medium. These lines were selected and proliferated individually in hygromycin containing medium. Proliferated callus was obtained only from eight callus lines. PCR was performed using the DNA isolated from these callus lines using *hpt* gene specific primer. A 600 bp fragment corresponding to the cDNA sequence coding for the *hpt* gene could be positively amplified from the entire callus lines tested as well as from the positive control. This band was absent in the untransformed control callus (Fig. 4A). Since *hmgr*I gene is already present in the *Hevea* genome, presence of *hpt* gene is taken as the proof for the *hmgr*I gene integration. PCR was also performed for detecting the presence of *MnSOD* and *npt*II transgenes in the *hpt* PCR positive transgenic callus lines obtained by repeated genetic transformation (Fig. 4B).

The multiple gene integrated transgenic embryogenic callus, upon periodic subculture at 60 days interval on modified half MS medium with growth regulators BA, Kin and GA_3, induced embryos (Fig. 2A), after the third subculture. The embryo induction frequency was scored visually and the results are given in Table 1. An embryo induction frequency of 32 per cent was obtained in modified half MS medium containing BA and Kin (0.3 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). It was also noticed that the percentage of embryo induction was less compared to that was observed with MnSOD transgenic callus which was 52 per cent (Sobha et al., 2003) or *hmgr*1 transgenic callus (80%) as reported by Jayashree et al. (2010). One of the possible reasons for the reduction in embryo induction may be due to the presence of the two antibiotics (kanamycin and hygromycin) used for the selection of transgenic callus lines. It is also suggested that the integration of two genes with different promoters might have influenced somatic embryogenesis. The somatic embryos obtained after subculture over the same medium, developed into torpedo and cotyledonary stage embryos. Cotyledonary stage embryos with normal development (Fig. 2B) were transferred to maturation medium reported earlier for the maturation of MnSOD transgenic embryos. Fifteen percent of the embryos matured and were transferred to plant regeneration medium. Medium manipulations have to be made for getting better

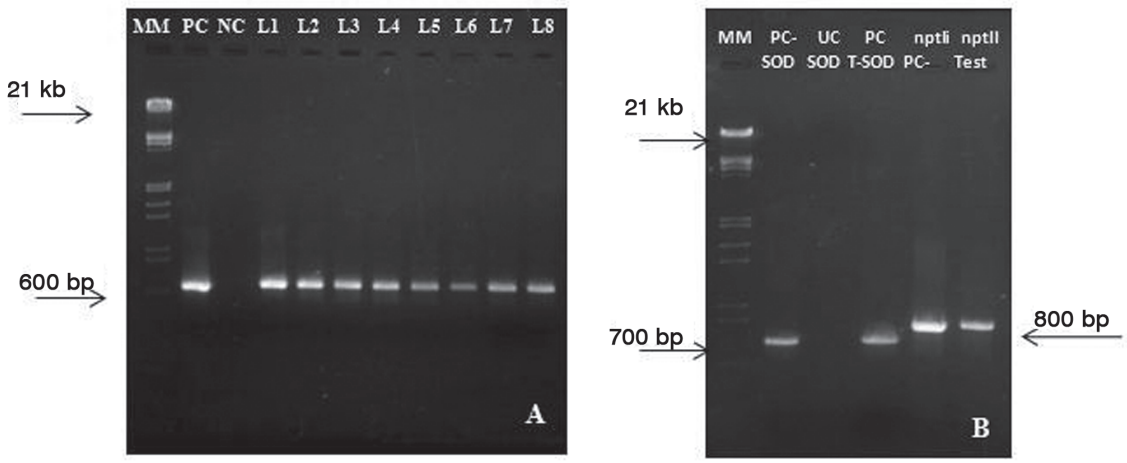

*Figure 4*A. PCR amplification with *hpt* gene specific primer *Figure 4.* B PCR amplification with *Mn SOD and npt*II gene specific primer

embryo maturation and further plant regeneration.

Conventional and more novel methods have been employed for introducing multiple genes in annual crops like rice, wheat, barley, etc. Transgenic golden rice was developed by engineering the provitamin A (â-carotene) biosynthetic gene into carotenoid free rice endosperm (carotene) (Ye et al., 2000). Kexuan et al. (1999) attempted multiple gene integration in rice plants using particle bombardment. They developed transgenic rice plants by simultaneously introducing the rice *Xa21* gene effective against bacterial blight disease and the Galanthus nivalis agglutinin (*gna*) gene against sap sucking insect pests specially the brown plant hopper. Later, Rao et al. (2011) reported multiple gene integration in rice by sequential re-transformation. Rice chitinase (*chi*II) and tobacco osmotin (*ap24*) genes, which cause disruption of fungal cell wall and cell membrane, respectively, were stacked in transgenic rice to develop resistance against the sheath blight disease. The homozygous marker-free transgenic rice line CoT23 which harbored the rice *chiII* transgene was sequentially re-transformed with a second transgene *ap24* by co-transformation using an *Agrobacterium tumefaciens* strain harbouring a single-copy cointegrate vector pGV2260:pSSJ1 and a multi-copy binary vector pBin19" npt II-ap24 in the same cell. Hygromycin phosphotransferase (*hpt*) gene was used for the selection of transgenic cell lines and also *GUS* reporter gene.

In usual crop improvement programmes, one gene coding for a particular trait is introduced in one variety and another gene for another trait is inserted in another variety. By conventional cross pollination, both the genes will be introduced in some of the offsprings by recombination and such plants could be selected after molecular analyses. In a tree crop like *Hevea* which has a long breeding cycle, integration of multiple genes through conventional methods will take several years. Therefore, the present approach of repeated *Agrobacterium* mediated genetic transformation is the best. The integrated *MnSOD* gene will enhance the abiotic stress tolerance and *hmgr*I gene will enhance the latex yield. The methodology for single gene integration has been well standardized in *Hevea* and transgenic plants were developed (Sobha et al., 2003 Jayashree et al., 2003*;* Rekha et al., 2013). However, the process of developing transgenics with single gene integration itself is lengthy and cumbersome and further incorporation of multiple genes through crossing and selection from the segregating populations is very difficult in *Hevea*. Hence the approach of developing transgenic cell lines with multiple genes by any means and developing plants from the lines is a viable option.

In the present study, gene stacking was achieved by the integration of two genes viz. manganese superoxide dismutase (*MnSOD*) for abiotic stress tolerance and 3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgr*I) gene for enhanced latex yield, by repeated genetic transformation. Transformation frequency of 30 per cent was obtained with MnSOD gene construct and this transgenic callus was used as the target tissue for the integration of *hmgr*I gene. PCR amplification with *MnSOD*, *npt* II and *hpt* gene specific primers was performed for assuring the integration of MnSOD and *hmgr* I genes. Somatic embryo induction and maturation frequencies of 32 and 15 percentage, respectively were obtained and plant regeneration is awaited. The transgenic plants with the integration of gene for multiple traits are expected to show abiotic stress tolerance as well as enhance latex yield, which is the major objective in *Hevea* crop improvement. This is the first report of multiple gene integration in *Hevea.*

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