MATERIALS AND METHODS
MATERIALS AND METHODS

Attempts were made to develop transgenic plants overexpressing laticifer specific \textit{hmgr1} gene in \textit{Hevea brasiliensis}. Experiments were carried out to identify the most efficient \textit{Agrobacterium} strain and suitable target tissues to attain this goal. Parameters influencing somatic embryogenesis and plant regeneration from the transgenic tissues were assessed. Studies were also conducted to determine the gene integration pattern in the plants and to quantify the mRNA and the protein level in the transformants.

3.1a Plasmid vector

The plasmid vector used was pBIB \textit{hmgr1} developed by Venkatachalam \textit{et al.} (2009). The binary vector contained hygromycin phosphotransferase gene (\textit{hpt}) as the plant selectable marker and \textit{hmgr1} gene from \textit{Hevea} under the transcriptional control of a constitutive promoter (super promoter). Institutional Biosafety Committee (IBSC) has approved the initiation of transgenic work using this gene construct and the biosafety aspects were strictly followed as per the safety guidelines of the Department of Biotechnology (DBT). The full-length \textit{hmgr1} cDNA was isolated from \textit{Hevea} tree by a PCR based approach using the primers designed based on the published sequences of \textit{hmgr1} from the Malaysian clone RRIM 600 (Chye \textit{et al.}, 1991; 1992). The \textit{hmgr1} cDNA insert was 1838 bp long containing an open reading frame (ORF) excluding stop codon of 1725 bp, flanked by a 42 bp 5’ untranslated region (UTR) and a 110 bp 3’UTR. The full-length \textit{hmgr1} cDNA isolated from \textit{Hevea brasiliensis} (clone RR1105) was placed between the super promoter and nos terminator elements. The resulting plasmid was designated as pBIB \textit{hmgr1} and the gene fusion details are shown in Fig 3. The binary vector was introduced into three
different Agrobacterium strains and maintained as glycerol stock at -80°C for long-term storage in 70% (v/v) glycerol.

**Fig 3.** Plasmid vector pBIB hmgr1 used for genetic transformation

3.1b. Agrobacterium strains

Different bacterial strains namely LBA 4404, EHA 105 and pGV 1301 were used in the genetic transformation experiments. The Agrobacterium tumefaciens strain EHA 105 is a vir-helper, L-succinamopine type and a Km (S) derivative of EHA 101 (Rm^t) (Hood *et al*., 1993), whereas Agrobacterium tumefaciens strain LBA 4404 is a vir-helper, octopine type which harbors the disarmed Ti plasmid pAL 4404, a T-DNA deletion derivative of pTiAch 5, (Ach5 pTiAch5) Sm/Sp (R) in the virulence plasmid (from Tn 904) (Ooms *et al*., 1982). The third strain, pGV 1301 has a cured Ti plasmid, belonging to the nopaline type of the strain, genotype C58.
3.1c Preparation of antibiotic solutions

The plasmid vector used for the transformation experiment contained genes conferring tolerance to the antibiotics, kanamycin (Kan) and rifampicin (Rif) respectively. The antibiotics were prepared as stock solutions (10 mg / ml), where Kanamycin is water soluble and rifampicin is soluble in methanol. The antibiotics were filter sterilized using 0.2µm Millipore filter and stored at -20°C.

3.2 Antibiotic sensitivity

The hygromycin phosphotransferase gene (hpt) conferring resistance to the antibiotic hygromycin was used for plant selection. This gene isolated from *E.coli* (Gritz and Davies, 1983) has been widely used as the negative selectable marker (Miki and McHugh, 2004). A kill curve experiment was carried out by exposing the target tissues (both clonal and the zygotic) to different levels of hygromycin (10, 15, 20, 25, 30, 35, 40 mg l−1). The basal medium was autoclaved and cooled to 50°C prior to the addition of hygromycin. Ten callus clumps were cultured per plate and replicated five times. The cultures were kept in the dark. The response of the calli on exposure to the antibiotic was scored after one month of culture. The concentration of hygromycin at which the callus proliferation was minimum where 100% of the control calli perished (died) was treated as the optimum concentration for the selection of the transformants.

3.3 Initiation of Agrobacterium culture

The different *Agrobacterium* strains containing the plasmid vector were taken from the frozen glycerol stock and plated into LB (Luria Bertani) medium containing antibiotics, 50 mg l−1 Kan and 20 mg l−1 Rif for bacterial selection. The culture plates were incubated at 28°C for 2 days. The individual colonies formed in the culture plates were screened for the presence of the insert by colony PCR using specific primers.
3.3.1 Colony PCR

Single colonies were collected from the culture plate with a sterile loop and used for PCR analysis using the promoter specific primer as the forward and the HMGR specific one as the reverse primer. Plasmid DNA was used as a positive control. PCR was carried out using individual colonies as the template, 100 mM each of dATP, dGTP, dTTP, dCTP, 250 nM of each primer, 0.5 µl Taq DNA polymerase and 1.5 mM MgCl₂ in a final volume of 20 µl. The reaction mixture was incubated in a thermal cycler under the following conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature/Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>- 94°C for 10 min</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>- 94°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>- 58°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>- 72°C for 2 min</td>
</tr>
<tr>
<td>3</td>
<td>Repeat step 2</td>
<td>- 35 cycles</td>
</tr>
<tr>
<td>4</td>
<td>Final extension</td>
<td>- 72°C for 7 min</td>
</tr>
<tr>
<td>5</td>
<td>Hold</td>
<td>- 4°C</td>
</tr>
</tbody>
</table>

The amplified PCR products were visualized on a 1.2% agarose gel stained with ethidium bromide using 0.5 x TBE as the running buffer (Sambrook et al., 1989).

3.3.2 Initiation of Agrobacterium culture for tissue infection

Individual colonies of Agrobacterium were grown in liquid LB medium with the respective antibiotics and proliferated on a gyratory shaker, at 250 rpm, overnight at 28°C. The OD of the bacterial culture was measured at A₄₂₀ nm of 0.5 by taking 200µl bacterial suspension from the overnight grown culture and by adding 800 µl LB medium. The bacterial cells were pelleted by centrifugation at 3000g for 10 min and resuspended in the induction medium (IM) to get a density of 10⁸ cells/ml. Modified MS medium was used as the
induction medium (Annexure A) for *Agrobacterium* growth and infection with the target tissue. The pH of the IM medium was maintained at 5.2. The flasks were incubated at 28°C, with shaking at 250 rpm for 4 hrs and used for *Agrobacterium* infection.

**3.4 Source material for genetic transformation**

*Agrobacterium* mediated genetic transformation with the *hmgr1* gene was attempted in rubber with different callus types such as primary callus from immature anthers (Plate 1 A-C) embryogenic callus and embryogenic suspensions derived from the anther tissue and the embryogenic callus obtained along with the developing zygotic embryos.
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Plate 1. (A-C) Explant for culture initiation

A- Inflorescence
B- Immature flower buds at the diploid stage
C- Immature anthers used for callus induction
3.4.1 Callus induction from anther tissue

Young flower buds were collected from 10 year old trees grown in the experimental field of Rubber Research Institute of India. The flower buds were washed thoroughly in running tap water. They were surface sterilized using 0.1 % (w/v) mercuric chloride solution containing 1 to 2 drops of tween 20 for 5 min. The sterilant was removed by thorough washing using sterile distilled water for 3 to 4 times. The immature flower buds were dissected under a stereo microscope to isolate the anthers at the diploid stage (before microsporogenesis) (Plate1 A,B). The dissection was performed in ascorbic acid to prevent oxidation and browning of the tissue. The immature anthers were inoculated in the callus induction medium reported earlier (Kumari Jayasree et al., 1999) with minor modifications in the growth regulator combinations. The basal medium used for callusing was modified MS salts (reducing NH$_4$NO$_3$ level to 1.0 g l$^{-1}$). Alterations were made in the growth regulator combinations by experimenting a factorial trial using 2,4- D (0.5 to 1.5 mg l$^{-1}$) and BA (0.25 to 1.0 mg l$^{-1}$) in presence of NAA (0.5 mg l$^{-1}$). The pH of the medium was adjusted to 5.7 with 1 N potassium hydroxide (KOH) and solidified using 0.2 % phytagel (M/S Sigma Aldrich- USA). The medium was boiled with constant stirring and poured into culture tubes and plugged tightly with cotton plugs (nonabsorbent). The medium was autoclaved at 121°C, 15 lb pressure for 15 min and stored at 26°C.

3.4.2 Initiation of embryogenic callus

The fresh callus obtained from the immature anthers after proliferation remained compact. As the friability of the callus played a significant role in determining the transformation efficiency, attempts were made for the faster conversion of the fresh callus into friable embryogenic callus. Different cytokinins (BA, Kin, zeatin and TDZ) were experimented with NAA for
embryogenic callus emergence among which TDZ, NAA combinations showed positive response (Data not shown). Therefore to expedite the formation of embryogenic callus, varying levels of NAA (0.5-2.5 mg l\(^{-1}\)) was used in combination with TDZ (0.2-1.0 mg l\(^{-1}\)). TDZ is a urea based cytokinin, nondegradable by cytokinin oxidase enzyme. The basal medium used was half strength MS medium additionally supplemented with 0.3 mg l\(^{-1}\) BA. The sucrose concentration of the media was raised to 60 g l\(^{-1}\). The pH of the culture medium was adjusted to 5.7 with 1 N KOH solidified using 0.2 % phytogel. The medium was boiled, poured into the culture tubes and plugged tightly with cotton plugs. Autoclaving was carried out at 121°C, 15 lb pressure for 15 min. After cooling the filter sterilized hormones were added to individual tubes and stored at 26°C. The proliferated immature anther callus was cultured in the media combinations and incubated under dark conditions at 28±1°C, with two to three subcultures at monthly intervals for the emergence of friable embryogenic callus.

3.4.3 Initiation of cell suspensions from the embryogenic cell aggregates

The embryogenic calli derived from the explants were transferred to 100 ml Erlen Meyer flask containing 25 ml ½ MS basal medium supplemented with growth regulators (used for embryogenic callus initiation) and sucrose (60 g l\(^{-1}\)). The suspension cultures were maintained at 25°C in dark on an orbital shaker at 120 rpm. The suspension medium was replaced with fresh media at weekly intervals. The regeneration ability of the suspensions was tested at weekly intervals and the cultures with good regeneration ability were divided and transferred to the same basal media maintained in different flasks for multiplication.
3.4.4 Embryogenic callus of zygotic origin

Immature fruits (8-10 weeks old) were collected from the field grown trees of *Hevea* (clone RRII 105) washed thoroughly in distilled water and dried. Fruits were then dipped in 80% ethanol for 15 minutes and allowed to dry on a sterile Petri plate. The ovules were isolated from the fruits using a sterile knife. The ovules (3 to 4 numbers) from each fruit were cut into two halves and placed on the nutrient medium with the micropylar end touching the medium. The basal medium used for obtaining embryogenic callus was Nitsch basal medium (1969) with sucrose and growth regulators (Rekha *et al.*, 2010). A growth regulator combination of Zeatin, Kin and GA₃ were used for embryogenic callus emergence. The pH of the medium was adjusted to 5.7 with 1N KOH and was solidified using 0.2 % phytagel. Autoclaving was carried out at 121°C, 15 lb pressure for 10 minutes.

3.5 Plant transformation protocol

3.5.1 Development of transgenic cell lines

Different steps involved in *Agrobacterium* mediated transformation of *Hevea brasiliensis* include, *Agrobacterium* infection, co-cultivation and selection.

The callus (≥ 1 g) was suspended in the *Agrobacterium* culture for 10 to 15 min. During this period, the tissues were wounded with a sterile needle to facilitate the exudation of the phenolic compounds which attract the *Agrobacterium* cells towards the target tissue, allowing its easy entry. After the infection, the explants were blotted dry onto sterile Whatman filter paper to remove the excess bacteria. They were then transferred to sterile filter paper placed over the solid co-cultivation medium. The co-cultivation medium (CCM1 in Appendix A) was modified MS with reduced levels of NH₄NO₃ (500 mg l⁻¹) supplemented with phenolic compounds [acetosyringone (20 mg l⁻¹)].

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betaine hydrochloride (153.6 mg l\(^{-1}\)) and proline (115.5 mg l\(^{-1}\)] and growth regulators. The growth regulators used were 2,4-D (1.0 mg l\(^{-1}\)) and BA (0.5 mg l\(^{-1}\)). The basal medium was sterilized by autoclaving and cooled to 50\(^\circ\) C. The phenolic compounds along with the growth regulators were filter sterilized using a 0.2 \(\mu\)m Millipore filter. They were then added to the cooled basal medium and poured into sterile petri plates and used for co-culturing. The co-cultivation was performed for 3 days in dark and the plates were incubated at 26 \pm 2\(^\circ\) C. After the period of co-cultivation, the infected callus was dried using a sterile whatman filter paper and transferred to the fresh selection medium. The basal medium for selection was the same as that used for co-cultivation (SM in Appendix A) with antibiotics cefotaxime (500 mg l\(^{-1}\)) and hygromycin (the optimum concentration from the kill curve). The antibiotic cefotaxime (500 mg l\(^{-1}\)) was added to prevent bacterial overgrowth. The selection media contained a growth regulator combination of 2, 4-D (0.2 mg l\(^{-1}\)), Kin (0.5 mg l\(^{-1}\)) and BA (0.5 mg l\(^{-1}\)). The petri plates were sealed with parafilm and incubated in the dark at 25\(^\circ\)C. The infected calli were subcultured into fresh selection media at monthly intervals until hygromycin resistant transgenic calli emerged from the cultures.

The frequency of transformation was assessed as,

\[
\frac{\text{The no. of transgenic cell lines emerged}}{\text{Total no. of callus clumps cultured}} \times 100
\]

The resistant cell lines were subcultured for proliferation and the transgene integration was confirmed by PCR analysis.

3.5.1.1 Transformation efficiency- influence of the Agrobacterium strain and the target explants

The efficiency of transformation usually varies with different Agrobacterium strains and the target tissues used and therefore studies were
done with three different strains (LBA 4404, EHA 105 and pGV 1301) and different target explants. After identifying the best strain and the target explant, experiments were done to improve the transformation frequency.

3.5.1.2 Treatments to improve the frequency of transformation

The following methods were tried to improve the transformation efficiency in *Hevea* cells

- Explant pre-treatment with anti-necrotic mixture
- Use of L-cysteine and AgNO₃ in the co-cultivation medium
- Incubation temperature
- Desiccation of the explants

3.5.1.2.a Pre-treatment with anti-necrotic agents

Experiments were carried out to improve the efficiency of transformation in *Hevea* by adopting changes during various phases of genetic transformation. Pre-treatment of the target tissue with the anti-necrotic mixture was given to improve the viability of the explants during transformation. The anti-necrotic mixture contained ascorbic acid (15 mg l⁻¹), L-cysteine (40 mg l⁻¹) and AgNO₃ (2.0 mg l⁻¹). The explants were dipped in this mixture for a period of 1 to 10 hrs. They were then dried on a sterile whatman filter paper and infected with the *Agrobacterium* culture for 10 to 15 min. After infection, the callus was blotted dry on a sterile filter paper and placed over the co-cultivation medium and incubated for 3 days at 28°C.

3.5.1.2.b Desiccation

The suspension cell aggregates were infected with the *Agrobacterium* strain EHA 105. *Agrobacterium* culture (100 µl) was pipetted over the tissue and wounded with a sterile scalpel blade for 10 minutes. The infected tissue was
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transferred to a sterile petri plate and placed in a sealed desiccator containing calcium nitrate for a period of 1-10 hrs. After subjecting the infected tissues to different desiccation periods, they were subcultured to the co-cultivation medium (CCM1) at 28°C for 3 days and later to the selection medium (SM). The putatively transgenic cell lines emerging in the selection medium (SM) were isolated and subcultured for proliferation.

3.5.1.2.c Addition of thiols and AgNO₃ in the co-cultivation medium

Components that have a beneficial role on enhancing the efficiency of transformation were included in the co-cultivation medium. The co-cultivation medium, CCM1 was additionally supplemented with varying concentrations of L-cysteine (0, 100, 200, 300 mg l⁻¹) and AgNO₃ (0, 20, 40, 60 μM) separately. The infected tissues were co-cultured in the modified medium for three days and subsequently transferred to the selection medium.

3.5.1.2.d Incubation temperature

The target material after Agrobacterium infection was subjected to varying temperature regimes namely 4°C, 20°C and 28°C during co-cultivation. The co-culture medium was prepared with and without acetosyringone. The infected tissues were cultured in these media combinations and co-cultured for three days. After the period of incubation, they were transferred to the selection medium (SM), and kept under dark at 25°C. The combined effect of acetosyringone and incubation temperature was assessed.

3.5.2 Multiplication of the cell lines

Though putatively transgenic cell lines could be obtained from all the explants used as target tissues, the frequency was higher with the embryogenic suspension cultures and using the callus of zygotic origin. Based on the friability and texture of the proliferated callus further experiments were carried out with embryogenic suspension cultures and using the callus of zygotic origin.
The basal medium for proliferation was CCM$_2$ (Appendix B) fortified with growth regulators 2, 4-D (0.1 - 0.6 mg l$^{-1}$), NAA (0.1- 0.5 mg l$^{-1}$) and Kin (0.2 - 0.6 mg l$^{-1}$). The proliferation was carried out in presence of hygromycin. The untransformed control callus was maintained separately.

### 3.6 Molecular characterization of the transgenic callus

#### 3.6.1 Design of specific primers

The gene specific as well as the promoter specific primers was designed to amplify the integrated transgene in the transgenic cell lines. The software primer 3 was used to design the forward and reverse primers. The cDNA sequence information of the $hmgr1$ gene published in the NCBI database (Accession No. AY706757) (Venkatachalam et al., 2009) was used for designing the gene specific forward and reverse primers respectively. The promoter specific primers were based on the published sequence of the super promoter (Ni et al., 1995). Marker specific primers were designed based on the $hpt$ gene sequences available in the pBIB vector. The primer sequences and their $T_m$ values are given below.

- **Super promoter** primer  5’- CGGAATGCGCGACTAGAGCC-3’ $T_m$ = 68 (4 GC+2 AT)
- **$hmgr$ reverse** primer   5’- GACATATCTTTGCTGGATCTGT-3’ $T_m$ = 62
- **$hmgr$ Forward** primer 5’- CGCGTCGGCGACTAGAGCC -3’  $T_m$ = 66
- **$hmgr$ Reverse** primer 5’- GCAAGTTGAGTTCCACCTC -3’  $T_m$ = 58
- **hpt forward** primer  5’- CGATTGCGTCGCATCGAC 3’    $T_m$ = 58
- **hpt reverse** primer   5’- CGTGCACAGGGGTGCACG 3’    $T_m$ = 60

The synthesized primers were suspended in TE buffer (10:1) (Appendix C) and then diluted in sterile double distilled water to get a concentration of 100 pico mol/µl. The primer stocks were stored at -20° C.
3.6.2 Genomic DNA isolation

The genomic DNA was extracted from the transformed as well as untransformed callus according to the modified protocol of Doyle and Doyle (1990), the CTAB method.

- Two gram of the tissue was ground in liquid nitrogen to a fine powder using a mortar and pestle.

- The ground tissue was homogenized with 20 ml CTAB (2X) extraction buffer

<table>
<thead>
<tr>
<th>2X CTAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% CTAB</td>
</tr>
<tr>
<td>0.1 M Tris HCl (pH 8.0)</td>
</tr>
<tr>
<td>20m M EDTA (pH 8.0)</td>
</tr>
<tr>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td>0.1% β-mercaptoethanol</td>
</tr>
<tr>
<td>1% polyvinyl polypyrrolidone</td>
</tr>
</tbody>
</table>

- The samples were incubated at 65°C for 30 minutes in a 50 ml centrifuge tube.

- After incubation, the mixture was centrifuged at 8000 rpm for 10 to 15 min at room temperature and the supernatant was carefully transferred to a fresh tube.

- To the supernatant collected in the tube, equal volume of Tris saturated phenol: chloroform (1:1) was added and mixed gently by inverting the tube.

- Centrifuged the sample at 8000 rpm, room temperature and the aqueous phase was collected. The organic phase containing the denatured proteins was discarded.
• To the aqueous phase, 40 µl of RNase A (Appendix C), from a stock solution (10 mg/ml) was added and incubated at 50°C for one hour. The RNA in the sample was degraded by adding DNase free RNase (10mg/ml)

• Equal volume of chloroform was added to the sample and centrifuged at 8000 rpm at room temperature for 10 minutes. This step was repeated twice.

• The aqueous phase was collected leaving behind the organic phase. To the aqueous phase, 0.6 volume ice-cold isopropanol was added to precipitate the DNA and mixed thoroughly.

• The samples were incubated at -20°C for 30 minutes to precipitate the DNA.

• The mixture was centrifuged at 8000 rpm at 4°C for 15 minutes to pellet the precipitated DNA.

• The supernatant after the centrifugation was discarded and the pellet was washed with 70% ethanol for 5 minutes.

• Finally the pellet was air-dried and suspended in TE buffer.

• The DNA samples were stored at -20°C.

3.6.2.1 Quantification of the DNA

The quality of the genomic DNA was assessed electrophoretically by separating them on an agarose gel (0.8%). Later the quality of the genomic DNA was detected using a UV-spectrophotometer (Beckman, USA). The ratio of absorbance at 260 nm and 280 nm was measured (260/280) to know the purity of the DNA. A ratio between 1.8 to 2.0 indicates good quality DNA
without protein contamination. Quantification of the DNA was made using the formula

\[
1 \text{ O.D at 260 nm} = 50 \text{ ng } /\mu\text{l}
\]

Thus the O.D of each DNA sample at 260 nm was measured and quantified accordingly.

### 3.6.3 Plasmid isolation- Alkaline Lysis method

The plasmid isolation from the *Agrobacterium* culture was performed using the alkaline lysis method of Birnboim and Doly (1979). The isolated plasmid was used as the positive control in the subsequent experiments.

- One loop of the bacterial culture from the glycerol stock was inoculated in 5 ml of liquid LB medium containing the antibiotics Kan 50 mg l\(^{-1}\) and Rif 20 mg l\(^{-1}\).
- 5 ml of the overnight grown *Agrobacterium* culture was pelleted at 4000 rpm, 10 min at room temperature.
- The pellet was resuspended in 0.3 ml of solution I (GTE) and transferred to microcentrifuge tube containing 3 µl of RNase A and vortexed.

#### Solution I (GTE)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris HCl (pH 8.0)</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM (pH 8.0)</td>
</tr>
</tbody>
</table>

Autoclaved at 121°C, 15 lb and cooled before use

- To the tube, 0.3 ml of the freshly prepared solution II (lysis solution) was added and mixed gently. The sample was then incubated at room temperature for 5 min.
Solution II (Lysis solution)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>0.2 M (freshly diluted from 10M stock)</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
</tr>
</tbody>
</table>

- To this tube 0.3 ml of cold, solution III was added and mixed thoroughly by vortexing. Incubation was carried out at room temperature for 5 min.

Solution III

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M Potassium acetate</td>
<td>60 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>11.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>28.5 ml</td>
</tr>
<tr>
<td>Autoclaved at 121°C, 15 lb before use</td>
<td></td>
</tr>
</tbody>
</table>

- The samples were then centrifuged at 12000 rpm for 5 min. This step was repeated twice.

- The supernatant was transferred to clean tubes

- To the supernatant, 630 µl of cold isopropanol was added and microfuged at 12000 rpm for 10 min.

- The pellet was washed with 70% ethanol and air dried.

- The pellet obtained was resuspended in 50 µl of TE buffer.

3.6.4 PCR analysis of the transformed cell lines

The template DNA was used for PCR amplification of hmgr1 gene. PCR analysis was carried out using promoter specific forward and hmgr specific reverse primers designed to amplify the hmgr1 gene fragment of approximately 1.9 kb and also with the marker specific primers to amplify the hpt gene fragment of size 602 bp. The forward and reverse primers for amplifying the hmgr1 gene were 5’- CGGAATGCCTGACGCTCC -3’ and 5’-GACATATCTTTGGGCTGATCTGT-3’ respectively. The sequences of the
marker specific primers were mentioned in section 3.6.1. Plasmid DNA was used as a positive control whereas DNA from the untransformed calli served as the negative control. PCR reaction was carried using the components described in Table.1.

Table.1 PCR reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>2.0 µl</td>
<td>50 ng</td>
</tr>
<tr>
<td>Reaction buffer</td>
<td>2.0 µl</td>
<td>1 X</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2.0 µl</td>
<td>100 µM of each dNTPs</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.0 µl</td>
<td>10 pmol</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.0 µl</td>
<td>10 pmol</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase</td>
<td>0.15 µl</td>
<td>0.75 U</td>
</tr>
<tr>
<td>Sterile D.W</td>
<td>12.85 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

The reaction mixture was incubated in a thermal cycler (Perkin Elmer 480, Foster City, Calif) under the following conditions.

- **Step 1** Initial denaturation - 94°C for 4 min
- **Step 2** Denaturation - 94°C for 1 min
  - Annealing - 58°C for 1 min
  - Extension - 72°C for 2 min
- **Step 3** Repeat step 2 - 35 cycles
- **Step 4** Final extension - 72°C for 7 min
- **Step 5** Hold - 4°C

The PCR amplified products were visualized on a 1.2% agarose gel stained with ethidium bromide using 0.5x TBE as the running buffer.
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(Sambrook et al., 1989). Gel images were captured using ‘EDAS 290’ (Electrophoresis Documentation and Analysis System- Kodak, USA). Molecular weight of the amplified products was determined using Kodak ID Image Analysis software.

3.7 Somatic embryogenesis from the transformed cell lines

The proliferated antibiotic resistant cell lines from the suspension cultures and from the embryogenic cells of zygotic origin were cultured for somatic embryogenesis and plant regeneration. Factors influencing embryogenesis and transgenic plant regeneration were analyzed under different sections.

3.7.1 Effect of basal salts on somatic embryogenesis

The influence of basal medium on somatic embryo induction was investigated employing different media viz., MS (Murashige and Skoog, 1962), modified MS denoted as AG₁ and WPM (Lloyd and McCown, 1980) (Appendix B). AG₁ medium was a modified version of MS, obtained by lowering the level of ammonium nitrate and magnesium sulphate with a rise in the potassium nitrate concentration. This was done based on earlier reports that lower levels of ammonium nitrate favoured somatic embryogenesis in tree crops. The basal medium was supplemented with sucrose 50 g l⁻¹ and growth hormones (2,4-D 0.1 mg l⁻¹, BA 0.3 mg l⁻¹ and GA₃ 0.5 mg l⁻¹). The pH of all the media were adjusted to 5.7 and autoclaved at 121°C, 15 lb pressure for 15 min. After autoclaving, the phytohormones were filter sterilized and added to the medium and poured into culture tubes. The major and minor salts used in the culture media were summarized in the Appendix B.

3.7.2 Addition of polyamines on somatic embryogenesis

The effect of exogenously applied polyamines on somatic embryo induction (AG₁) was evaluated in the present study. The three polyamines
namely putrescine, spermine and spermidine (1.0-5.0 mg l$^{-1}$) were individually added to the embryo induction medium and the effect was monitored. The responding chemical was identified and used along with higher concentrations of sucrose in the subsequent experiment.

3.7.3 Somatic embryogenesis influenced by addition of amino acids

The influence of amino acids on embryo induction was assessed in the embryo induction medium fortified with phytohormones (2,4-D 0.1 mg l$^{-1}$, BA 0.3 mg l$^{-1}$ and GA$_3$ 0.5 mg l$^{-1}$). Varying levels of amino acids, L-asparagine (50-400 mg l$^{-1}$), L-arginine (100-500 mg l$^{-1}$), L-alanine (50-200 mg l$^{-1}$), serine (10-50 mg l$^{-1}$), L-proline (50-250 mg l$^{-1}$) and L-glutamine (100-1000 mg l$^{-1}$) were incorporated in the embryo induction medium individually. The best amino acid and its optimum concentration was assessed and later used in combinations. The putatively transgenic cell lines were subcultured in the culture medium with frequent subculture duration of 20 days. The results were scored after two subcultures.

3.7.4 Influence of growth regulators on induction of somatic embryos

Different concentrations and combinations of growth regulators (Kin, NAA, GA$_3$ and zeatin) were inducted in the embryo induction medium for improving the rate of embryogenesis. The interactive effect of zeatin with NAA (0.5-2.5 mg l$^{-1}$) was compared with Kin (0.3 - 1.0 mg l$^{-1}$) and NAA (0.25 - 1.25 mg l$^{-1}$). Both the media commonly contained 0.5 mg l$^{-1}$ GA$_3$. The pH of the medium was adjusted to 5.7 with 1 N KOH and sterilized at 121$^\circ$C for 15 minutes by autoclaving. The cultures were incubated in the dark at 28 ±1°C, and periodically subcultured into fresh medium at 4 weeks interval. The percentage embryogenesis was assessed after 50 days of culture in the embryo induction medium.
3.7.5 Effect of spermidine and sucrose on somatic embryogenesis

Higher polyamine content has been reported in the embryogenic tissues by many researchers and hence exogenous application of spermidine was given along with different concentrations of sucrose. Higher levels of sucrose also positively influenced somatic embryogenesis. When different concentrations of spermidine were tried at a fixed sucrose level of 50 g l\(^{-1}\), promising results on somatic embryogenesis was noticed. Hence sucrose concentration of the embryo induction medium (AG\(_1\)) were varied from 50 - 90 g l\(^{-1}\) and used along with different concentration of spermidine (0-2.5 mg l\(^{-1}\)). The combined effect of spermidine and sucrose on somatic embryogenesis was evaluated and scored after 6 weeks of culture.

3.8 Maturation of somatic embryos

The globular embryos obtained in the embryo induction medium were separated and cultured for maturation in half strength MS, modified MS (MSO) and WPM. The medium was supplemented with casein hydrolysate (500 mg l\(^{-1}\)), CW (5 %), sucrose (30 g l\(^{-1}\)) along with growth regulators (BA and GA\(_3\) 0.5 mg l\(^{-1}\)). In this experiment the influence of major salts on somatic embryo maturation was studied. Later the effect of different stress inducing compounds on embryo maturation was monitored. Response of the somatic embryos on exposure to desiccation was also assessed.

3.8.1 Effect of major salts and amino acids on somatic embryo maturation

The nitrogen sources as well as the ratio of NO\(_3^-\) / NH\(_4^+\) play a significant role on cell growth. The importance of the nitrogen sources on somatic embryo maturation was studied by culturing the globular embryos in the medium containing higher levels of KNO\(_3\). The concentration of NH\(_4\)NO\(_3\) was either lowered or omitted from the basal medium. To study the effect of organic nitrogen sources on somatic embryo maturation, the medium was
fortified with amino acids like L-glutamine (100 - 1000 mg l\(^{-1}\)) and L-Proline (50-250mg l\(^{-1}\)). The maturation of the somatic embryos was assessed after 60 days of culture.

**3.8.2 Effect of stress inducing compounds on somatic embryo maturation**

The role of stress inducing compounds ABA (Abscisic acid), PEG (polyethylene glycol) and sorbitol on maturation of somatic embryos were studied by incorporating them in the basal medium (MSO). The medium contained varying concentration of these compounds (ABA 0.2 - 0.8 mg l\(^{-1}\); sorbitol 1.5 - 4 %; PEG 5 - 14 %) both individually and in combinations. The response was assessed after 40 days of culture. The pH of the medium was adjusted to 5.7 and solidified using 0.35% phytagel. The medium was autoclaved at 121°C for 15 min. Abscisic acid was filter sterilized using 0.2 µm millipore filter and added to the basal medium. The embryogenic calli containing globular embryos were subcultured in the medium with regular subculture to fresh medium at 4-weeks interval and the plates were kept at 28 ±1°C in dark. The observations were recorded after 9-weeks of culture.

**3.8.3 Combined effect of ABA with sugar alcohols (sorbitol and PEG) on maturation of somatic embryos**

Primarily the individual effect of sorbitol and PEG\(_{6000}\) on somatic embryo maturation was assessed and finally used in combination with ABA to accelerate the maturation frequency. The combined effect of ABA (0 – 0.8 mg l\(^{-1}\)) and PEG (0 - 14%) on maturation of somatic embryos was assessed. Similarly the effect of different concentrations of sorbitol (1.5- 4.0 %) with varying levels of ABA (0.2-0.8 mg l\(^{-1}\)) was also experimented. The cultures were incubated in the dark at 28 ± 1°C with frequent subculture at monthly intervals.
3.9 Desiccation of somatic embryos

The matured embryos were desiccated to facilitate easy germination. The acquisition of desiccation tolerance is a signal of potential autonomy of the somatic embryos. The early cotyledonary stage embryos obtained in the maturation medium (MSO in Appendix B), were partially desiccated by placing them on an empty, sealed sterile plate, in a laminar flow chamber for 12-48 hrs or desiccated rapidly by placing in a sealed desiccator containing saturated Ca(NO$_3$)$_2$.4 H$_2$O for 2 to 6 hrs. The desiccation of the embryos was continued in darkness at 25°C. After the desiccation period, the embryos from the respective treatments were transferred to the germination medium.

3.10 Regeneration of transgenic plants

Germination experiments to study the effect of amino acids (L-glutamine 100-1000 mg l$^{-1}$, L-asparagine, 50-400 mg l$^{-1}$, L-proline 50 - 250 mg l$^{-1}$) were carried out using the somatic embryos derived from the zygotic material. The torpedo and the cotyledon shaped somatic embryos in the maturation medium were separated and cultured individually on to the plant regeneration medium. The basal medium for plant regeneration was $\frac{1}{4}$th MS. The medium contained additional amounts of KNO$_3$ and K$_2$SO$_4$. The effect of different growth regulators on plant germination was also studied. The morphogenic competence of the embryos was drastically improved by the nitrogen composition of the regeneration medium. The amount of organic nitrogen and the ratio of the organic to inorganic nitrogen were altered in the modified medium. Organic supplements (casein enzymatic hydrolysate 500 mg l$^{-1}$, yeast extract 100 mg l$^{-1}$) along with sucrose (30 g l$^{-1}$) were incorporated in the germination medium (MS$_4$) (Appendix B). The germination medium also contained growth hormones BA (0.5-1.0 mg l$^{-1}$), GA$_3$ (0.3-1.0 mg l$^{-1}$) and IAA (0.1-0.3 mg l$^{-1}$).
The cultures were maintained in the dark for one week and then transferred to light (85 µmol m\(^{-2}\) s\(^{-1}\)) at 25 ± 1 °C for 15 days. The green embryos showing shoot meristems were further subcultured into fresh media (MS\(_{3}\)), with lower levels of sucrose (20 g l\(^{-1}\)). The pH of the medium was adjusted to 5.8 with 1N KOH. The medium was solidified using phytagel (0.2%) and contained activated charcoal (0.2%).

### 3.11 Acclimatization of the transgenic plants

The plantlets were washed thoroughly with tap water to remove any traces of phytagel and transferred to earthenware pots. The pots were filled with potting mixture, which was previously sterilized by autoclaving at 121 °C, 15 lb for 20 min. The potting mixture was sand: soilrite: soil in the ratio 1:1:1. The potting medium was soaked with the fungicide, bavistin (0.1%) one week before transplantation. Plantlets were rinsed with water to remove the adhering agar of the medium. They were then dipped in bavistin solution for 5 min and then transferred to the earthenware pots filled with the potting medium and maintained in the growth chamber under controlled conditions. The relative humidity (RH) of the growth chamber was adjusted to 85 percent and the temperature was maintained at 27°C. The plantlets were watered on alternate days. Two weeks after transplantation, the plantlets were moistened with ½ x Hoagland’s solution at weekly intervals. After 2-3 weeks, the relative humidity of the growth chamber was reduced to 80 percent with a subsequent rise in the temperature (28 °C). Plantlets after two to three weeks were transferred to big polybags filled with soil: sand: cow dung in the ratio 2:1:1. They were placed in the growth chamber for one more week and then transferred to the shade house. Plants were watered on alternate days and given NPK Mg mixture (20:20:0:15) at monthly intervals.
3.12 Molecular characterization of the transgenic plants

3.12.1 Confirmation of the transgene integration by PCR

The acclimatized transgenic plantlets were screened for the presence of hpt and hmgr1 gene sequences by PCR analysis using specific primers (section 3.8.1). Leaf samples were collected from the transgenic plants maintained in the growth chamber. The young, uninfected leaves were washed thoroughly in tap water and then rinsed with sterile water. After rinsing, the leaves were wiped with alcohol. The genomic DNA from the young leaves of the regenerated plantlets (transformed as well as untransformed control) was extracted according to the standard procedure (Doyle and Doyle, 1990) mentioned in section 3.6.2. The forward and the reverse primers corresponding to the hpt coding region were used for detecting hpt gene. The presence of hmgr1 transgene was detected using the gene specific forward and reverse primers. Plasmid DNA was used as the positive control, and the DNA extracted from the untransformed plant served as the negative control. The expected size of the amplified product using hpt primers was approximately 602 bp and using the hmgr1 specific primers was 640 bp. The PCR reactions were carried out as in Table 1. The reaction conditions for hpt and hmgr1 gene amplifications were mentioned in section 3.6.4. The PCR products were visualized on a 1.2 % agarose gel stained with ethidium bromide using TBE (0.5 x) as the running buffer (Sambrook et al., 1989). The gel images were captured using the Electrophoresis documentation and analysis system Kodak, USA (M/S Fotodyne, Kodak EDAS 290).

3.12.2 Cloning of the PCR product

In order to confirm the integration of T-DNA region into the plant nuclear genome, cloning of the PCR product was carried out. PCR was carried out using primers (super promoter forward and hmgr reverse primers)
mentioned in section 3.6.1. The product was eluted out of the gel, cloned into a vector (pGEM-T) and sequenced in order to compare the sequence information with the already reported \textit{hmgr1} cDNA sequence in NCBI.

\textbf{3.12.2a. Elution of the amplified product from the agarose gel}

The PCR product amplified from the transgenic plants were electrophoretically separated on a 1.0 \% low melting agarose gel. After viewing quickly under the UV light, the DNA bands were cut from the lane so as to avoid nicks. The gel slices were taken in a 1.5 ml microcentrifuge tube and kept at 65°C for 10 to 15 minutes to melt the agarose completely. To the melted agarose, 1/10 vol of 5M NaCl was added, mixed thoroughly and incubated at 65°C for 10 minutes. Equal volume of Tris- saturated phenol and chloroform was added, mixed gently and centrifuged at 8000 rpm for 10 min. The aqueous layer was removed. The DNA was precipitated using 0.1 volume of 3M sodium acetate and twice the volume of cold absolute alcohol. The precipitation was continued at -20°C for 30 minutes and was pelleted by centrifuging at 8000 rpm for 10 minutes at 4°C. The DNA was washed in 70\% ethanol and air-dried. The pellet was re suspended in TE buffer.

\textbf{3.12.2b. Ligation reaction}

The PCR products were cloned using the pGEM-T easy vector system (M/S Promega, USA) following the manufacturer’s instructions. The vector used was linearised with a single 3’ terminal thymidine at both ends. The ‘T’ overhangs at the insertion site greatly improve the efficiency of ligation of the PCR products by preventing the recirculation of the vector, providing a compatible overhang for the PCR products generated by certain thermostable polymerases. The polymerases added a single deoxyadenosine in a template – independent manner to the 3’ ends of the amplified fragments. The vector to the insert ratio was 1:3.
The ligation reaction was prepared as follows

- **2X Rapid ligation buffer**: 2.0 µl
- **p GEM-T easy vector**: 1.0 µl (50 ng)
- **PCR product**: 1.0 µl
- **T<sub>4</sub> DNA ligase**: 1.0 µl
- **H<sub>2</sub>O**: 2.0 µl
- **Total volume**: 10.0 µl

The reaction mixture was incubated overnight at 4°C.

### 3.12.2c. Transformation of *E.coli*

The competent cells of *E.coli* JM 109 supplied along with the pGEM–T easy vector system was used for transformation. One vial of the competent *E.coli* cells was removed from -80°C and placed in an ice bath until just thawed. The cells were mixed gently by flicking the tube. 50 µl of the cells were transferred to a centrifuge tube containing 2.0 µl of the ligation mixture. The reaction mixture was mixed gently and incubated in ice for 20 minutes. The cells were then subjected to heat shock for 45 seconds in a water bath at 42°C and immediately transferred to ice and incubated further for two minutes. SOC medium (950 µl) (Appendix C) was added to the vial and incubated at 37°C for 1.5 hours with shaking (220 rpm). This allows the bacteria to express the β-lactamase gene in the plasmid conferring resistance to the antibiotic ampicillin.

### 3.12.2d. Screening of the transformed colonies

LB medium was prepared in plates with the selection antibiotic ampicillin (50 µg/ml) (M/S Sigma-Aldrich, USA). The surface of the LB-ampicillin plate was coated with 100 µl of IPTG (100 mM) and 20 µl (50 mg/ml) X-gal (5-Bromo, 4-Chloro, 3-indolyl β-D-galactoside in dimethyl
formamide) (Appendix C) and incubated at 37°C for 30 minutes for absorption. The transformed cell suspension (50-100 µl) was spread over the pre-warmed plates and incubated at 37°C for 16 hours. The transformed colonies were selected visually by blue-white screening. Colony PCR of the white colonies were carried out for further confirmation of transformation. Colony PCR was carried out as in section 3.3.1. The amplified PCR products were visualized on a 1.2% agarose gel stained with ethidium bromide using 0.5x TBE (Appendix C) as the running buffer (Sambrook et al., 1989). The transformed white colonies were selected and inoculated in liquid LB medium, overnight at 37°C for plasmid isolation.

3.12.2e. Plasmid DNA isolation and purification

The plasmid isolation from the recombinant bacteria was carried out according to the alkaline lysis method described in section 3.6.3.

3.12.2f. PEG purification of the plasmid DNA

The plasmid DNA isolated through alkaline lysis method was purified by PEG precipitation for sequencing.

- The pelleted plasmid DNA was resuspended in 32 µl water, 8 µl of 4M NaCl and 40 µl of 13 percent PEG and mixed thoroughly.
- The mixture was incubated in ice for 20 minutes and centrifuged at 10,000 rpm at 4°C for 15 minutes.
- The supernatant was discarded and pellet was rinsed with 70 percent ethanol.
- The pellet was air-dried and resuspended in 20 µl of sterile double distilled water and stored at -20°C.
3.12.3. *In silico* analysis of *hmgr1* gene

3.12.3a Sequencing and sequence analysis

The sequencing of *hmgr1* gene insert in pGEM–T easy vector was carried out at M/S Macrogen, Korea using pUC/M13 forward and reverse primers. The nucleotide sequence of *hmgr1* (3-hydroxy 3-methyl glutaryl CoA reductase) obtained was edited to discard the vector sequences at either ends and compared with the already reported cDNA sequence published in NCBI database using the BLASTn programme (www.ncbi.nih.gov/BLAST/Altshul et al., 1990).

The deduced amino acid sequence of *hmgr1* cDNA obtained from the transgenic plant was compared with that of *Hevea hmgr1* gene and with the corresponding sequence from various taxa and a dendrogram was created to determine the phylogenetic relationship. The Multiple Sequence alignment and comparison of the sequences was carried out using Clustal Omega (Sievers et al., 2011).

3.12.4 Southern blotting

The integration of the T-DNA into the nuclear genome of the transgenic plants and the insertion pattern of the transgene was determined using genomic Southern blot hybridization. The genomic DNA was restricted using restriction enzymes and probed using *hpt* gene probe to detect fragments of the integrated transgene.

3.12.4a. Restriction digestion of the genomic DNA

The integration of the T-DNA into the nuclear genome of the transgenic plants has to be confirmed by genomic southern blot hybridization. Four PCR positive transgenic plants and one untransformed control plant were selected. Genomic DNA was extracted from these plants and restricted using restriction
enzymes. Three restriction enzymes namely Bam HI, EcoRI and XbaI (M/S Promega) were used for digesting the DNA (10 µg) in separate reactions. The digested products were probed with radioactively labeled hpt gene probe (since hmgr1 gene was present in Hevea) to detect the integrated gene fragments.

The genomic DNA from two transgenic plants and the plasmid DNA were double digested with Bam HI and Xba I to release the hpt transgene. The restricted products were transferred to the nylon membrane and probed with the radiolabeled hpt gene probe.

In order to determine the number of independent insertions of the transgene in the nuclear genome of the transgenic plants, the genomic DNA from two transgenic plants was restricted using Bam HI (M/S Promega) and the DNA from the other two transgenic plants was digested with EcoRI (M/S Promega) in separate reactions. The genomic DNA from the untransformed plant was digested with Bam HI and used as the control. The restriction enzyme Bam HI was having a unique site on either side of the marker gene. The digested products were then transferred to the nylon membrane and probed with α-32P labeled hpt probe. The reaction mixture was prepared as described below.

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>20 µl (10 µg)</td>
</tr>
<tr>
<td>Enzyme buffer</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Res. Enzyme</td>
<td>15 µl</td>
</tr>
<tr>
<td>BSA</td>
<td>2 µl</td>
</tr>
<tr>
<td>D.D H2O</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Total reaction</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The restriction digestion was continued at 37°C overnight and the fragments was size fractionated in a 1.0% agarose gel (10 µg DNA per lane) containing 0.1% (w/v) ethidium bromide. The electrophoresis was continued at 50V until the dye front migrated three - fourth length of the gel. The gel was
viewed on a transilluminator and documented. The gel was marked at one corner with a slanting cut.

**3.12.4b. Blotting of the DNA**

The method was based on the standard protocol developed by Southern (1975). The gel was documented before blotting and processed after electrophoresis.

- The DNA in the gel was depurinated by soaking in a solution of 0.25 N HCl for 15 min. The gel was rinsed twice with distilled water briefly, followed by alkali. This shortens the DNA fragment by alkaline hydrolysis at the depurinated sites.

- The gel was treated in the denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 45 min with gentle shaking and rinsed with water.

- The gel was neutralized by soaking in the neutralization buffer (1M Tris-HCl (pH 7.4) and 1.5 M NaCl) for 45 min.

During all the treatments, the gel was completely immersed in the solutions. The DNA was then transferred from the gel to the nylon membrane (Hybond N+, Amersham, UK) by capillary blotting method (Sambrook et al., 1989). Procedure followed was as follows.

- After neutralization treatment, the gel was washed briefly in 10x SSC and kept ready.

<table>
<thead>
<tr>
<th>20x SSC</th>
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</thead>
<tbody>
<tr>
<td>3M NaCl</td>
</tr>
<tr>
<td>0.3 M sodium citrate</td>
</tr>
<tr>
<td>pH adjusted to 7.0</td>
</tr>
</tbody>
</table>
• A tray was filled with 10x SSC to a height of 5cm and a platform of dimension bigger than that of the gel was placed in the tray.

• The platform was covered with Whatman No.3 filter paper which was presoaked in the 10x SSC. The ends of the filter paper were kept immersed in the 10x SSC solution kept in the tray. Three sheets of Whatman No.3 filter paper, pre-soaked in the 10x SSC (dimension same as that of the gel) were placed above the platform. Any air bubbles were removed by rolling the surface with a glass rod.

• The gel was carefully placed over the filter paper, upside down and a hybond N+ nylon membrane, presoaked in 10x SSC was placed on the top of the gel. Air bubbles formed were removed by gently rolling a glass rod on the surface of the gel.

• Three sheets of Whatman No.3 filter paper presoaked in 10x SSC were placed over this assembly. Dry Whatman No.1 filter papers were stacked on it. Above this ordinary filter papers cut to the dimension of the gel were stacked to a height of 10 cm. A suitable weight of about 250 g was placed over this. The weight should be sufficient to keep the papers tight, but it should not crush the gel.

• The DNA transfer was allowed to proceed overnight for 12 to 16 hrs.

• After the transfer, the assembly was separated and the nylon membrane was washed briefly in 5x SSC and air dried.

• The gel was checked on a Transilluminator to confirm the transfer of the DNA.

• The nylon membrane was placed in a UV–cross linker (Hoefer, USA) at 12000J/cm² for fixing the DNA. The membrane was wrapped in saran wrap between ordinary filter papers and stored at -20°C until use.
3.12.4c. Preparation of labeled probes

The gene probe was radio labeled using the Multiprime DNA labeling system from Amersham (UK) following manufacturer’s protocol. Random hexanucleotides were utilized for priming. DNA synthesis occurs on denatured template DNA at numerous sites along its length. The labeling proceeds following manufacturer’s instructions.

- 2 µl of 25 ng of the template DNA (positive control) mixed with 5 µl of random primer and diluted with 18 µl of double distilled water.
- The sample was boiled for 5 min to denature.
- The samples were chilled immediately on ice for 5 min and then keep at room temperature.
- To the sample, 5 µl of the buffer was added, followed by 4 µl each of all d NTP’s except dCTP.
- 2.5 µl of nuclease free water was added to the sample.
- To this, 2.5 µl of α-32P labeled dCTP (sp. Activity-3000Ci/mMol or 10µCi/ µl) was added.
- Finally 3 µl of the enzyme (klenow fragment of DNA polymerase I) was added and mixed gently.
- The sample was spun for few seconds and incubated at 37°C for one hour.
- The reaction was stopped by adding 5 µl of 0.2 M EDTA.

The purification of the labeled probe was carried out by passing through a Sephadex G-50 column as follows.
1. Sephadex G-50 was prepared in STE buffer to form a slurry (10g of dry powder yields around 160 ml slurry).

2. At the bottom of 1ml column, glass wool was placed and 1 ml of the slurry was added without trapping any air bubbles.

3. Now the column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

4. The sephadex was tightly packed up to 1 ml by adding more slurry to the column.

5. Next the column was equilibrated with STE buffer \([\text{NaCl} - 0.1 \text{ M}, \text{Tris-HCl} - 10 \text{ mM (pH 8.0)}, \text{EDTA} - 1\text{mM (pH 8.0)}]\) and then with distilled water.

6. The labeled probe was then passed through the column and purified. The eluted fraction was collected in a 1.5 ml microcentrifuge tube.

7. The purified probe was then denatured by boiling at 100°C for 3 min and immediately chilled on ice. After denaturing the probe was stored in the freezer until use.

   This purification step helps in removing the small as well as unincorporated nucleotides to avoid background signals in the blot.

3.12.4d. Hybridization

   The hybridization of the labeled probe to the nylon membrane was performed according to Sambrook and Russell (2001). The following reagents were used for the purpose.
Materials and Methods

1. Pre-hybridization solution
   6x SSC
   5X Denhardt’s reagent
   0.5 % SDS

2. Denhardt’s solution (50x)
   BSA - 1.0 g
   PVP - 1.0 g
   Ficoll - 1.0 g
   made up to 100 ml with sterile double distilled water

3. Hybridization solution
   Pre-hybridization solution containing α-\(^{32}\)P labeled denatured probe DNA.

- The membrane was placed in the hybridization tube and an appropriate amount of pre-hybridization solution (0.2 ml/cm\(^2\) of the blot - 25 ml for 13×10 cm membrane) was added.

- The pre-hybridization was carried out at 65°C for 1 hr in a hybridization oven (Amersham-UK) at very low speed with rotary movements.

- The pre-hybridization solution was poured out and the hybridization solution was poured into the tube and incubated at 65°C. The incubation was carried out with slow rotation for 12 to 16 hrs.
3.12.4e. Washing of the blot and autoradiography

After hybridization, the membrane was washed twice at room temperature for 5 to 15 min with the wash solution I

<table>
<thead>
<tr>
<th>Washing solution I.</th>
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</thead>
<tbody>
<tr>
<td>2x SSC</td>
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<tr>
<td>0.1% SDS.</td>
</tr>
</tbody>
</table>

The blot was then subjected to high stringent washing at 65°C for 30 min with wash solution II. These washes were performed twice.

<table>
<thead>
<tr>
<th>Washing solution II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1x SSC</td>
</tr>
<tr>
<td>0.5% SDS.</td>
</tr>
</tbody>
</table>

The membrane was then floated briefly in 0.1x SSC at room temperature, air dried and then subjected to autoradiography. The membrane was wrapped in a cling film and exposed to the image plate BAS IP (MS 2025) of the phospho image analyzer (M/S Fujifilm, FLA 5000, Japan) and kept sealed in the BAS cassette for 4 hrs. After the exposure, the membrane was removed and the image plate was analyzed.

3.12.5 Gene expression analysis

3.12.5.1 Northern hybridization

3.12.5.1a RNA isolation

The RNA was extracted from the leaves according to the protocol developed by Venkatachalam et al. (1999). The reagents for the extraction were prepared in DEPC treated water. The steps involved is given below
• The leaves were collected in an ice bucket and first washed thoroughly in running water and then with autoclaved DEPC treated distilled water.

DEPC treated water
- Add 1 ml of DEPC to 1000 ml
- Stir overnight
- Autoclave to inactivate DEPC
- Cool at room temperature before use

• One gram of the collected leaf was then ground in liquid nitrogen to a fine powder.

• The homogenate was transferred to polypropylene tube containing 1:1 volume of the RNA extraction buffer and extraction buffer saturated phenol. A pinch of PVPP (polyvinyl poly pyrrolidone) and β- mercaptoethanol (200µl) was freshly added to the mixture.

RNA extraction buffer
0.2 M NaCl, 0.1 M Tris-HCl (pH 8.5), 0.01 M EDTA and 1.5 % SDS.

• The samples were mixed thoroughly and centrifuged at 10000 rpm at RT for 30 min to separate the phases.

• The aqueous layer was treated with equal volume of chloroform and mixed well to remove carbohydrates, lipids and any traces of phenol.

• The sample was centrifuged at 10000 rpm for 20 minutes at RT. The organic layer was discarded.

• The aqueous layer was collected and the total RNA in this layer was precipitated by adding 1/3 volume of 8M LiCl. The precipitation was continued overnight at -20°C.
The RNA was pelleted by centrifugation at 10000 rpm for 20 min at 4°C.

The pellet was once again washed with 2M LiCl. The centrifugation was carried out at 10000 rpm for 15 min.

The pellet was washed with 70% ethanol to remove any soluble polysaccharides. Centrifugation was continued at 10000 rpm for 15 min at 4°C.

The pellet was air-dried and dissolved in 500µl of DEPC treated water.

The RNA was further purified and concentrated by precipitation with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute alcohol.

The sample was centrifuged at 10000 rpm, 10 minutes at 4°C for precipitation.

The pellet was washed in 70% ethanol, air-dried and re-suspended in sterile DEPC water.

The quantity of the RNA was checked using UV-spectrophotometer and the DNA contamination as well as the quality was visualized by running the sample on a 1% agarose gel.

The RNA isolated was used immediately or stored in 3 volumes of ethanol at -80°C.

3.12.5.1b. Electrophoresis of RNA

The agarose gel electrophoresis was carried out using the standard protocol described by Sambrook et al. (1989). The gel was prepared by initially melting the appropriate amount of the agarose. The melted agarose was cooled to 60°C and 5x formaldehyde gel running buffer and formaldehyde was added to get a final concentration of 1x and 2.2M respectively. The gel was casted in a
chemical hood and allowed to set for at least 30 min at RT (Lehrach et al., 1977; Miller, 1987).

<table>
<thead>
<tr>
<th>Formaldehyde gel running buffer (5x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MOPS buffer)</td>
</tr>
<tr>
<td>0.1M MOPS (pH 7.0)</td>
</tr>
<tr>
<td>40m M sodium acetate</td>
</tr>
<tr>
<td>5 mM EDTA (pH 8.0)</td>
</tr>
</tbody>
</table>

About 20 µg of RNA was incubated at 65°C for 15 min with formaldehyde gel running buffer (4.5 µl), formaldehyde (3.5 µl) and formamide (10 µl). After a brief spin, formaldehyde gel loading buffer (4µl) was added to the sample and loaded in the gel. Before loading the samples, the gel was pre run for 5 min at 5V/cm. The gel was run in 1x MOPS buffer at 50V for 3 to 4 hrs (until the bromophenol blue has migrated to ¾ th of the gel). The gel was viewed, photographed and transferred to the nylon membrane.

3.12.5.1c. Blotting of the RNA

Prior to transfer, the gel was washed thoroughly with DEPC treated water 3 to 4 times to remove the formaldehyde in the gel. The gel was then soaked in 20x SSC for 45 minutes. The nylon membrane (Hybond N⁺, Amersham, UK) which was cut into the size of the gel was presoaked in 10x SSC. Blotting was carried out as done in Southern blotting (section 3.12.4b). After the transfer, the membrane was air-dried and placed in a UV- Cross linker.

3.12.5.1d. Hybridization, washing and autoradiography

The nylon membrane was first placed in the hybridization tube and appropriate volume of the pre-hybridization buffer was added. It was incubated at 42°C for 3 hrs in a hybridization oven with rotary movement at a low speed.
Materials and Methods

The pre-hybridization solution was poured out and the hybridization solution was added into the tube. The preparation of the probe and its purification was as described in the Southern protocol (section 3.12.4c). The labeled probe was added to the tube and incubated at 42°C, overnight with slow rotation. The membrane was first washed with solution I for 5 minutes at room temperature.

After this wash, two stringent washes were given with solution II for 5 min each at room temperature.

This was followed by another stringent wash with pre-warmed solution II at 42°C for 15 to 20 min. Finally the membrane was rinsed with 2x SSC and then blotted dry with a blotting sheet to remove the excess liquid. The membrane was then wrapped in a UV transparent plastic wrap (saran) and exposed to image plate of the phospho image analyzer (M/S Fujifilm, FLA 5000, Japan) for 4 hrs and analyzed later (section 3.12.4e).

3.12.5.2 Enzyme assay in the transgenic plants using ELISA

ELISA is a complex technique where multiple layers of antibodies were used for boosting the signal. Among the different ELISA types, the indirect ELISA is highly sensitive since more than one labeled antibody is bound to the
primary antibody. The antigen coated to the multi well plate was detected in two stages. In the first stage an unlabelled primary antibody specific for the antigen was added. During second stage, an enzyme labeled secondary antibody was added which binds to the first one. This secondary antibody is an anti-species antibody which is polyclonal. Using indirect ELISA, the relative levels of the analyte in the assay samples can be compared since the intensity of the signal varies with the concentration of the antigen. The enzymatic label produces the distinguishable signal which can be directly equated to the binding of the antigen to the antibody. The assay signal can be measured using spectrophotometric or fluorescent plate reader.

For determining the HMGR protein in the leaf tissues of the transgenic as well as the control plantlets, indirect enzyme linked immunosorbant assay (ELISA) technique was used. Polyclonal antibodies raised in rabbit against the Arabidopsis HMGR protein was used as the primary antibody to study Hevea HMGR protein. The assay was carried out after the protocol described in the assay kit manual of Bangalore GENEI.

Reagents used for the assay included

1. **Washing buffer** - Phosphate buffer saline (PBS) with Tween-20; pH 7.4

   PBS was prepared by dissolving 8g NaCl, 0.2 g KCl, 0.2 g KH$_2$PO$_4$, and 1.15 g of Na$_2$HPO$_4$. 2H$_2$O in 1000 ml of distilled water.

2. **Casein** - 2%

   2 % casein was prepared in PBS (The pH was adjusted to 7.0 with 1N NaOH).

3. **Primary Antibody**

   Polyclonal antibody raised in rabbit for Arabidopsis HMGR protein, from Bangalore GENEI was used at a dilution of 1: 2000 (prepared in 2% casein).
4. Secondary antibody

Peroxidase labeled anti-rabbit IgG (Bangalore GENEI), at a dilution of 1:2000 (prepared in 2% casein) was used as the secondary antibody.

5. Substrate

Tetra-methylbenzidine/hydrogen peroxide (TMB/H\textsubscript{2}O\textsubscript{2}) was used as the enzyme substrate.

The ELISA was carried out using the procedure described below

The crude enzyme extract was prepared by grinding the leaf samples (250 mg) in liquid nitrogen and homogenizing in 4 ml 0.1 M Phosphate buffer saline (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and used as the crude protein extract. HMGR in the crude protein extract was determined by the following ELISA techniques.

1. 150µl of the primary antigen (crude protein extract) was coated on the 96 well ELISA plate and kept overnight at 4°C.

2. The wells were washed manually for three times with washing Buffer.

3. The unbound area of the wells was blocked with 250µl of 2% casein by keeping the plate for 1 hour at 37°C for blocking.

4. The wells were washed three times with washing buffer (Phosphate Buffer Saline with Tween 20).

5. 200µl of pre-diluted primary antibody was added to each well and the plate was incubated at 37°C for 1 hour for antigen-antibody reaction.

6. The contents were discarded and the wells were washed three times with washing Buffer.

7. 200 µl of pre-diluted second antibody (Goat anti-rabbit IgG-HRP) was added to each well and incubated at 37°C for 1 hr.
8. The contents were discarded and the wells were washed three times with the washing buffer and 200 µl of substrate (TMB/H₂O₂) was added to each well and incubated for 30 minutes. The reaction was stopped by adding 50µl of 1N H₂SO₄.

9. The colour developed in the wells was read at 450 nm wavelength using an ELISA reader (BioTek). Buffer coated wells in the ELISA plate was processed in the same way and was treated as blank.

10. The protein content of the crude protein extract was analyzed as per Bradford, (1976). The HMGR specific activity was expressed in units/mg protein.
Fig. 4 The protocol followed in the transformation work

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RESULTS