DEVELOPMENT OF TRANSGENIC HEVEA BRASILIENSIS FOR THE OVER EXPRESSION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1 (hmgr1) GENE

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled "**Development of transgenic *Hevea brasiliensis* for the over expression of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase 1 (*hmgr* 1) gene**" is an authentic record of the original research work carried out by Smt Jayashree. R, at The Rubber Research Institute of India, Kottayam-9, under our joint supervision and guidance for the award of the Degree of **Doctor of Philosophy in Biotechnology**, under the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in the thesis has not been submitted earlier for any other degree, diploma or any other similar titles of any university.

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I hereby declare that the thesis entitled "**Development of transgenic *Hevea brasiliensis* for the over expression of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase1 (*hmgr*1) gene**", submitted to the M. G. University, Kottayam-9, for the award of the degree of Doctor of Philosophy in Biotechnology, is an authentic record of the research work carried out by me at the Biotechnology Division of the Rubber Research Institute of India, Kottayam, under the joint guidance of Dr. P. A. Nazeem, Professor and Co-Ordinator (DIC), CPBMB, College of Horticulture, K.A.U and Dr. P. Venkatachalam, Associate Professor, Department of Biotechnology, Periyar University. The work presented in this thesis has not been submitted earlier for any other degree or diploma at any university.

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PREFACE

Transgenic technology is a powerful tool in the hand of breeders for improving the crop where conventional methods of genetic improvement are laborious and time-consuming. Recombinant DNA and transformation techniques allow the use of genes from any source as tools for crop improvement. Transgenic methods enable the insertion of well characterized genes for specific traits into *Hevea* genome producing highly specific change only in the trait of interest, where only small variations in the genetic makeup is expected. In contrast, many of the unknown genes are introduced into the genome of *Hevea* in conventional methods while attempting the transfer of a desired gene. *Hevea* breeding programmes mainly aim at improving the latex yield of the plant combined with abiotic/biotic tolerance, resistance to tapping panel dryness and development of latex timber clones. The latex biosynthetic pathway starting from sucrose to polyisoprene is controlled by many enzymes among which the activity of one of the upstream enzymes catalyzing the conversion of 3-Hydroxy 3-Methylglutaryl Coenzyme A to mevalonate, an irreversible step, was reported to be lower. Several studies supported this view and linked this gene to the latex yield of *Hevea.* Thus the present study was undertaken with an objective of developing transgenic plants overexpressing laticifer specific *hmgr*1 gene. For achieving this goal, suitable *Agrobacterium* strain and the callus type giving better transformation efficiency were optimized. The transgenic plants were validated for transgene integration and expression using different molecular and biochemical methods.

The thesis contains six chapters; the first chapter briefly introduces the crop, describes the limiting factors of latex yield and the importance of this gene for transgenic work. The second part details relevant studies carried out by other researchers in this direction. This part reviews the work done in the isoprenoid pathway in general; *Agrobacterium* mediated genetic transformation and

transgenic plant development. The experimental details followed are dealt in the third section of the thesis. The fourth chapter critically scrutinizes the results under different experiments and these results are discussed in the fifth part of the thesis with probable explanations. In the final part of the thesis, various results are summarized with a road map on the future prospects of the work.

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ABSTRACT

The booming demand for natural rubber along with the inconsistency related to the production and consumption necessitates the development of superior clones with high yield potential in *Hevea*. Crop improvement through traditional methods is a long run process due to the lengthy breeding cycle and heterozygous nature of the crop. Hence the release of a new clone through conventional method is time consuming. In *Hevea*, yield improvement through transgenic technology attempts the transfer of key regulatory genes involved in the rubber biosynthesis pathway. *In vitro* plant regeneration pathway through somatic embryogenesis is an essential pre requisite for achieving this task. The biosynthetic pathway genes were well characterized by many researchers. It has been documented that among the enzymes catalyzing rubber biosynthesis, the one responsible for the irreversible conversion of 3-Hydroxy 3-Methylglutaryl Coenzyme A (HMG CoA) to mevalonate was said to be rate limiting, since its activity was lower compared to others up to isopentenyl pyrophosphate (IPP). Clonal variations in the 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) activity were established and a significant correlation has been worked out between the enzyme activity and yield of *Hevea*. Based on these observations an attempt was made to regenerate transgenic plants that overexpress laticifer specific *hmgr*1 gene in *Hevea brasiliensis* through *Agrobacterium* mediated genetic transformation. Different strains and callus types were experimented to ascertain the suitable strain and the tissue type giving excellent transformation frequency in *Hevea*. Experimental results proved EHA 105 as the best strain giving highest frequency of transformation. Amidst the callus types experimented, the embryogenic calli derived from the zygotic embryos gave maximum cell lines at an efficiency of 67%. Embryogenic suspensions from the anther tissue proved to the best among the clonal explants used, producing cell lines at a frequency of 27%. Influence of compounds and culture conditions beneficial in improving the efficiency of transformation were also assessed among which the incubation temperature during

co-cultivation had a definite role on accelerating the frequency of transformation. Optimum culture condition for *Agrobacterium* mediated transformation was a three day co-culture at 20°C in presence of acetosyringone where an incremental variation in the efficiency of transformation was noticed (32%). The PCR positive cell lines were proliferated and cultured for somatic embryogenesis. Components influencing somatic embryogenesis and plant regeneration from the transgenic cell lines were analyzed. Transgenic embryos were produced from the cell lines at an efficiency of 72%, where the basal salts, organic nitrogen sources, organic supplements, growth regulators, polyamines and carbohydrates had a well defined role. Half strength MS medium with an increase in KNO_3 concentration (3.0 g l^{-1}), omitting NH_4NO_3, resulted in embryo maturation. The impact of stress inducing compounds on somatic embryo maturation was studied. Polyethylene glycol (PEG), abscisic acid (ABA) combinations mediated maturation of *hmgr*1 transgenic embryos. Partial desiccation of the embryos improved the germination capacity and a growth regulator combination of GA_3, BA, IAA triggered plant regeneration. The regenerated plantlets were successfully hardened and transferred to net house conditions. Transgene integration was confirmed using PCR analysis and the pattern of integration of the transgene was assessed using Southern hybridization. Gene expression analysis was performed by ELISA technique and northern blotting. This is the first report on the development of transgenic plants in *Hevea* integrated with the laticifer specific *hmgr*1 gene. Forty five plantlets were successfully hardened and maintained in the containment facility. Selected transgenic plants were multiplied by bud grafting to study the yield pattern of the plants. Once proved to be positive, *hmgr*1 gene can be used to increase latex production of low yielding disease tolerant clones of *Hevea brasiliensis*.

Keywords: Agrobacterium mediated genetic transformation, Enzyme-linked immunosorbant assay, 3-hydroxy-3-methylglutaryl CoA reductase, Southern hybridization, Northern blotting Polymerase Chain Reaction, Polyethylene glycol

ABBREVIATIONS

%	:	Percent
°C	:	Degree centigrade
µg	:	Micro gram
µm	:	Micro mole
µl	:	Micro litre
2, 4-D	:	2, 4 Dichloro pheonoxyacetic acid
AACT	:	Acetyl CoA acetyl transferase
ABA	:	Abscissic acid
BA	:	6-Benzyladenine
cDNA	:	Complementary Deoxyribo nucleic acid
dCTP	:	Deoxycytidine tri phosphate
DEPC	:	Di ethyl pyrocarbonate
DNA	:	Deoxyribo nucleic acid
dNTPs	:	Deoxy nucleotide triphosphate
DXP	:	1-deoxy-D-xylulose 5-phosphate
EDTA	:	Ethylenediamine tetra acetic acid
EtBr	:	Ethidium Bromide
$g\ l^{-1}$	:	Grams per litre
G3P	:	Glyceraldehyde 3-phosphate
GA_3	:	Gibberellic acid
HMGR	:	3-hydroxy-3-methylglutaryl CoA reductase
Hrs	:	Hours
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
IPP	:	Isopentenyl diphosphate
IPTG	:	Isopropylthio-β- D-galactopyranoside
Kb	:	Kilobase
kDa	:	Kilodalton

Kin	:	Kinetin
mg l^{-1}	:	Milligram per litre
ml	:	Millilitre
mM	:	Millimolar
MVD	:	Mevalonate diphosphate decarboxylase
NAA	:	Naphthalene acetic acid
NCBI	:	National Centre for Biotechnology Information
nm	:	Nanometer
nM	:	Nanomolar
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
PMK	:	Phospho mevalonate kinase
PVP	:	Polyvinyl pyrrolidone
RNA	:	Ribo nucleic acid
rpm	:	Rotations per minute
SDS	:	Sodium dodecyl sulphate
sec	:	second(s)
TBE	:	Tris Borate EDTA
TDZ	:	Thidiazuron
TE	:	Tris-ethylenediamine tetra acetic acid
T_m	:	Melting temperature
T-Pilus	:	Transfer Pilus
w/v	:	Weight per volume
WPM	:	Woody Plant Medium

INTRODUCTION

Chapter 1

INTRODUCTION

Hevea brasiliensis Muell. Arg is the major commercially cultivated species of natural rubber. Natural rubber is produced in the form of latex in about 2000 plant species belonging to 311 genera. In spite of this diversity, *Hevea brasiliensis* serve as the prime source of commercial rubber. Natural rubber is one of the most important linear biopolymer of high economic importance with incomparable properties such as resilience, elasticity, abrasion resistance, efficient heat dispersion and impact resistance that cannot be mimicked by artificially produced polymers. This high molecular weight polymer ($>10^6$ Da) is formed from isopentenyl di phosphate (IPP) where the isoprene units were linked in cis- configuration producing cis-1, 4-polyisoprene. The high strength, low heat buildup and better wear resistance made rubber a congenial raw material for the automobile industry. It also serves as a strategic raw material in more than 40,000 consumer products including 400 medical devices (Mooibroek and Cornish 2000).

Hevea brasiliensis belonging to the family Euphorbiaceae is a sturdy perennial tree with orthotropic rhythmic growth attaining a height of 25-30 metres (Fig.1). The tree was introduced to South East Asia through the Kew Gardens during 1876 using a limited number of seeds collected from the Raio Tapajo's region of Brazil by Sir Henry Wickam (Dijkman, 1951). It is grown in the tropics having evenly distributed rainfall of 2000 to 3000 mm (125 to 150 rainy days per annum), maximum temperature of 29-34°C, 80 % atmospheric humidity and a bright sunshine of about 2000 h per annum (Watson, 1989; Rao and Vijayakumar, 1992). The genus *Hevea* include ten species all originated from the Amazon region and are strongly cross pollinated and monoecious (Schultes, 1990).

The tree is deciduous with regular, annual leaf shedding called wintering. The wintering starts during December and extends up to February in the traditional region. Refoliation occurs after wintering followed by flowering. Rubber tree is monoecious with declinous flowers which are short stalked and fragrant. *Hevea* appears to be insect pollinated and cross fertilized (Simmonds, 1982). After pollination the ovary develops into a three lobed regma. The tree has attracted attention as a substitute for the tropical rain forest as a wood resource since rubber wood can be used for making furniture and floor materials. Natural rubber also has the added advantage of being a renewable resource with environmental benefits. It plays an important role in lowering the environmental impact of vehicle based transport and contributes to the preservation of global environment as an efficient carbon sequester (Rahaman and Sivakumaran, 1998).

The global area under rubber cultivation comes to around 9.6 million hectares. The major rubber producing countries (Thialand, Indonesia, Malaysia, India, Vietnam, China, Ivory Coast and Sri Lanka) accounted for 83% of the natural rubber produced globally. Certain factors related to economical and political instability in the rubber producing countries accounted for the diminishing acreage of rubber plantations. However the steadily growing demand for natural rubber paved way in establishing alternative sources of domestic crops or engineering alternative substitutes (Cornish, 2001a; Mooney, 2009).

Fig.1. A mature rubber plantation

The prime objective of rubber tree breeding is the yield improvement of the crop combined with other characters like biotic/abiotic stress tolerance and the development of latex-timber clones. The major limiting factors of latex yield in *Hevea* include the flow characteristics determining the quantity of latex and *in situ* regeneration of the latex between successive tappings. Here the flow characteristics which contribute on yield include the initial flow rate per unit length of tapping cut, length of the cut, percentage rubber content and plugging index (Sethuraj, 1981). Traditional breeding attempts the development of superior clones with specific traits through hybridization, selection and evaluation. As rubber is a perennial tree with a broad life span, conventional methods take a long time to release a clone with desirable characters. The major constraints in the conventional techniques include highly heterozygous nature of the crop, long gestation period (6 to 7 years before tapping), seasonal flowering, low fruit set, lack of early selection parameters for estimating traits such as latex yield, susceptibility to Tapping Panel Dryness, wind damage and G x E interactions. Biotechnological tools take hand at this juncture where genetic improvement can be made at an easy pace through transgenics. In *Hevea*, yield improvement through transgenics attempts the transfer of key regulatory genes associated with yield. A plant regeneration pathway through somatic embryogenesis is an essential pre requisite for attaining this task. Plant regeneration through somatic embryogenesis was perfected in *Hevea* (clone RRII 105) using different explants *viz.*, immature anthers, inflorescence and leaf tissues (Kumari Jayasree *et al.*,1999; Sushamakumari *et al.*, 2000b; Kala *et al.*, 2005). Genetic engineering can be used as a breeding strategy where the transfer of large blocks of genetic material between the parents is avoided. *In vitro* plant regeneration techniques coupled with the developments in recombinant DNA technology helped in improving the clones by incorporating specific traits by gene transfer. Although the change observed by the

introduction of a single gene is rather small, these minor changes may be additive and have a substantial effect on the yield of the crop. The transfer of limited number of traits also caused least disruption to the existing genome of the plant.

Rubber biosynthesis occurs on the surface of the rubber particle suspended in the latex, mainly by the Mevalonate pathway (MVA). The latex biosynthetic pathway starting from simple sugar involves at least seventeen steps, each being controlled by a particular enzyme or an enzyme system. One of the key regulatory points in the isoprenoid pathway is the irreversible conversion of HMG-CoA to mevalonate catalyzed by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGR). Quantification work carried out by Lynen (1969) revealed a lower HMGR activity in *Hevea* latex (0.078 nmol MVA/ ml of latex), compared to other enzymes in the pathway up to IPP (isopentenyl pyrophosphate) strongly suggesting the rate limiting role of this enzyme in the rubber biosynthetic pathway. Clonal variations in the HMGR activity were noticed earlier and have been related to yield in *Hevea* (Wititsuwannakul and Sukonrat, 1984). Studies done by Wititsuwannakul (1986) in continuation with the above work also proved the association between the *hmgr*1 enzyme activity and rubber biosynthesis in *Hevea*. These findings led to the selection of this gene for the present work. HMGR is encoded by a group of three genes namely *hmgr*1, *hmgr*2 and *hmgr*3 which form a small gene family. It has been reported that *hmgr*1 is involved in rubber biosynthesis, *hmgr*2 with the defence responses induced by pathogens and wounding whereas *hmgr*3 is of a housekeeping nature (Chye *et al.*, 1992). In order to increase the latex yield in *Hevea brasiliensis* over expression of the *hmgr*1 gene was attempted through *Agrobacterium* mediated genetic transformation. The present study was focused with the following objectives.

Objectives

- Identification of a suitable bacterial strain and target tissue for *Agrobacterium* mediated genetic transformation with *hmgr*1 gene in *Hevea brasiliensis*.
- Development of transgenic *Hevea* plants over expressing *hmgr*1 gene.
- Validation of the transgene integration and expression using molecular and biochemical tools.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

Natural rubber (*Hevea brasiliensis*, Willd.ex Adr.deJuss.Muell.Arg) is a perennial out-breeder with a long life span, which makes crop improvement laborious and time consuming. During 1876, nearly 2000 seedlings raised from the seeds collected by Sir Henry Wickham were transported from the Kew gardens to Ceylon which formed the basis for rubber cultivation. The plants were first propagated from cuttings, where twigs collected from 2 to 3 year old trees were used.

Rubber tree is a sturdy perennial attaining a height of about 30 metres. It is deciduous with partial or complete wintering depending upon the clone, age of the plant or location. Refoliation and flowering follow wintering. Occasional off season flowering is also observed during September and October. *Hevea* is monoecious where the male flowers are smaller in size and more in number than the female flowers. Pistillate flowers are seen at the tip of the panicle and its branchlets. The gynoecium is tricarpellary and syncarpous with an ovule in each locule. Pollination is predominantly insect-mediated. The latex vessels in *Hevea* are articulated and anastomosing. They arise from the cells produced by the vascular cambium and are arranged in concentric rings around the cambial layer. The latex vessel characters like the number of rows, density of the vessels per row, diameter of latex vessels and the intensity of anastomosing are significant clonal characters which influence latex yield. The number of latex vessel rows is positively correlated with the yield and can be used as an early selection criterion in breeding programmes. Rubber tree is primarily grown in the traditional belt in the South Western region of peninsular India which was later extended to the non-traditional regions.

In *Hevea*, conventional breeding began during 1918 in Malaysia with the prime objective of releasing newer clones by shortening the breeding cycle. *Hevea* breeding started in India during 1954 when the first set of hybrids was formed. The clone RRII 105 (obtained by crossing Tjir and GL1), which was one among these hybrids, revolutionized rubber production in India through its high yield potential. The main breeding objectives in *Hevea* include high latex yield, improvement in the technological properties of natural rubber, timber yield and quality and disease tolerance. The first step in conventional breeding is the introduction of the planting material, which enriches the genetic diversity of the species and accelerates the process of genetic improvement. They were multiplied and then evaluated in field trials with the popular clone as the check. From these trials, the trees with outstanding yield were selected and cloned. They were utilized in the hybridization programmes based on hybridization objectives, *viz*, yield improvement, disease tolerance, drought and cold tolerance, improved vigor and timber properties. The hybrid seedlings were later evaluated in the nursery and selected.

The conventional breeding strategies for natural rubber improvement are considered as a long drawn process as the breeding cycle is too lengthy. The steadily increasing demand for natural rubber compelled researchers to utilize *in vitro* techniques to improve rubber production as well as productivity. Development of a high yielding clone is the need of the hour to achieve this goal of increasing productivity. Development of transgenic plants over expressing a key latex biosynthetic gene using *Agrobacterium* mediated transformation can be a solution for this need. Biotechnological approaches have a major role in improving the accuracy of the selection procedure and in developing clones integrated with specific traits. Through conventional breeding, a set of genes are transferred to a recipient plant from domestic crops or related genera. But biotechnological approaches attempt the transfer of

defined genes from any organism resulting in an increase in the availability of the gene pool for crop improvement. The aim of gene transfer techniques is the production of improved varieties through the incorporation of important genes into existing cultivars (Singh *et al.*, 2004). Together with the traditional plant breeding practices, biotechnological interventions helped in the development of novel methods to genetically alter and control plant development, its performance and the products. The developments of transgenic plants in any crop species require a perfect somatic embryogenesis pathway for plant regeneration.

Somatic embryogenesis can be used as an alternative to budding technique for clonal propagation in *Hevea*. This protocol comprises of induction, proliferation, histodifferentiation, maturation and germination phases. Developmental stages in somatic embryogenesis are similar to zygotic embryogenesis, where embryos were produced without any vascular connection to the parental tissue. Somatic embryogenesis, defined as a non-sexual developmental process, produces bipolar embryos usually from juvenile somatic tissues, which is a major limitation for the propagation of woody species. Carron and Enjalric (1982) induced calli and embryoids from the anther wall. Later, Wang and Chen (1995) developed plantlets from stamen cultures. Somatic embryogenesis was induced from the inner integument of immature *Hevea* seeds at Cirad (Carron and Enjalric. 1982). The influence of various factors on somatic embryogenesis was studied in detail by EI Hadrami *et al.* (1989, 1991), (EI Hadrami and d'Auzac 1992), Michaux-Ferrier and Carron (1989), Auboiron *et al.* (1990) and Etienne *et al* (1991). Etienne *et al.* (1993a, 1993b) described that a slow desiccation with 351 mM sucrose supplemented with 1mM ABA strongly improved germinability and plant conversion. Embryo development in the presence of ABA was also reported by Veisseire *et al.* (1994a, 1994b). Somatic embryogenesis was also promoted by the

incorporation of higher levels of sucrose (351 mol m^{-3}) and calcium (12 mM) (Montoro *et al.*, 1993, 1995). Temporary immersion that generates a stressful condition for the explants, enhanced somatic embryo production (Martre *et al.*, 2001). According to Blanc *et al.* (1999), carbohydrates have a crucial role on somatic embryo formation. A comparative study between somatic and zygotic embryos was conducted by Lardet *et al.* (1999). Although plant regeneration via somatic embryogenesis was successful in *Hevea*, the regeneration system is still confronting problems due to the low germination percentage and plant conversion as in many other tree species (Cailloux *et al.*, 1996; Linossier *et al.*, 1997). According to many reporters, individual genotypes within a given species vary greatly in their embryogenic capacity during somatic embryogenesis (Ammirato, 1983; Attree and Fowke, 1993; Dudits *et al.*, 1995; Merkle *et al.*, 1995). Reliable embryo formation is limited to a few *Hevea* genotypes like PB 260, PR 107, PB 235, RRII 105 and RRIM 600 where the embryo formation is momentary (Carron *et al.*, 1989; Kumari Jayasree *et al.*, 1999). Since 1990, research has been initiated for *in vitro* plant regeneration via somatic embryogenesis for the Indian *Hevea* clones at the Rubber Research Institute of India (RRII), kottayam. Both immature anthers (Kumari Jayasree *et al.* 1999) and inflorescence explants (Sushamakumari *et al.* 2000b) were found ideal for somatic embryogenesis and plant regeneration for the high yielding Indian *Hevea* clone RRII 105. The role of sucrose and ABA on callus development, embryo induction and plant regeneration was studied by Sushamakumari *et al.* (2000b) and the somatic embryo-derived plants were established in RRII experimental field. Recently attempts were also made to induce somatic embryos from leaf and root explants of *Hevea* (Kala *et al.* 2005, 2006; Sushamakumari *et al.* 2006). Although large quantities of somatic embryos were produced, normal plant development was difficult due to the

asynchronous maturation of the embryos and subsequent low germination and conversion rates.

In *Hevea*, optimization of plant regeneration techniques through somatic embryogenesis enabled us to apply genetic transformation techniques for breeding. Through genetic engineering, tree improvement can be made by the introduction of alien genes for desirable agronomic traits. This would help reduce the time required for *Hevea* genetic improvement and became an important part of *Hevea* breeding programmes. Genetic engineering has additional significance, since it allows the introduction of a desired gene in a single step for precision breeding. It offers a potential tool for crop improvement and opened new vista for the production of crop plants and forest trees with increased resistance to salinity stress, high temperature, diseases, insect pest, drought and other environmental conditions. In the last 15 years, amazing progress has been made in the production of transgenic plants in about 120 crop species, where plants with improved traits such as resistance to herbicides, insects, pests, diseases, microbial pathogens and viruses, physiological stresses and other quality traits have been produced. It is now technically possible to transfer genes across all taxonomic boundaries into plants- ie., from other plants, animals and microbes or even to introduce totally artificial genes.

2.1 *Agrobacterium*-mediated transformation in *Hevea brasiliensis*

The era of plant genetic engineering of crop plants began with the discovery of the ability of *Agrobacterium*, a soil bacterium to transfer a portion of its DNA (T-DNA) into the plant cells naturally. *Agrobacterium* is a gram negative soil dwelling bacterium that causes crown gall and hairy root disease in most dicots, gymnosperms and some monocots by a natural genetic engineering event in which part of the bacterial Ti (Tumor inducing) or Ri (Root inducing)

plasmid DNA (T-DNA) is transferred into the nuclear genome of plant cells (Nester *et al.*, 1984; Binns and Thomashaw, 1988). The T-DNA is integrated into the plant genome via illegitimate recombination. Genes in the T-DNA encode enzymes involved in the production of auxin and cytokinin compounds essential for plant growth and development. In addition, wild type T-DNA also carries genes that direct the synthesis of particular amino acids and sugar derivatives that are not normally found in plant cells. These plant tumor specific compounds are generally referred to as opines (Ellis and Murphy, 1981). Based on the type of opines encoded by the corresponding Ti plasmid, *Agrobacterium* can be classified as octopine, nopaline, succinopine and chrysopine strains (Dessaux *et al.*, 1993). In fact, only the T-DNA ends which form the border repeats of 24 base pairs are essential as recognition signals for the transfer system. By using such disarmed *Agrobacterium* strains, it is possible to obtain normally appearing, fertile transgenic plants (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). In addition to its applications in crop improvement, *Agrobacterium* mediated transformation offers a powerful research tool for studying plant biology especially the control mechanisms in gene expression. The availability of a reliable method for the regeneration of a complete plant from a transformed cell is considered as an essential pre requisite for virtually all systems of genetic manipulation. In *Hevea*, *Agrobacterium* mediated genetic transformation is the most successful method till date. It is widely used because of its efficiency and convenience. It has remarkable advantages over direct transformation methods including preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Czernilofsky *et al.*, 1986; Koncz *et al.*, 1989; Le *et al.*, 2001; Olhoft *et al.*, 2004) with the exclusion of vector DNA (Hiei *et al.*, 1997; Fang *et al.*, 2002), unlinked integration of co-transformed T-DNA etc (McKnight *et al.*, 1987; Komari *et al.*, 1996; Hamilton, 1997; Olhoft *et al.*, 2004). The transgenic plants are generally fertile and the

foreign genes are transmitted to progeny in a Mendelian manner (Rhodora and Thomas, 1996). Other advantages include low copy number and little rearrangement of the DNA insert with efficient plant regeneration capacity.

The transfer of the T-DNA and its integration into the plant genome is also influenced by several factors which include plant genotype, target tissue, plasmid vectors, bacterial strain, addition of synthetic phenolic compounds inducing *"vir"* genes, culture media composition, tissue damage, suppression and elimination of *Agrobacterium* after co-cultivation etc (Alt Morbe *et al.*, 1989; Bidney *et al.*, 1992; Hoekema *et al.*, 1993; Hiei *et al.*, 1994; Komari *et al.*, 1996; Nauerby *et al.*, 1997; Klee, 2000). The type and texture of explant, antibiotic resistance gene used in the gene construct, and nature of the promoter driving the transgene expression are other important factors, which determine the efficiency of transformation and regeneration of transgenic tissues.

One of the significant advancement in *Hevea* transformation was the stable introduction of the foreign genes into the nuclear genome of plants. Its possibility was first explored by Arokiaraj and Wan (1991) using *Agrobacterium* mediated transformation. Later the first transgenic *Hevea* plant was developed by the particle bombardment method using anther calli derived from the clone GL1 (Arokiaraj *et al.*, 1994). Subsequently, transgenic *Hevea* plants were developed by *Agrobacterium* mediated transformation using anther calli as the target tissue (Arokiaraj *et al.*, 1996, 1998). Montoro *et al.* (2003) used the callus from inner integument tissue of immature fruits (clone PB260) for genetic transformation. Recently, Blanc *et al.* (2006) developed transgenic plants of *Hevea* (clone PB 260) via *Agrobacterium*-mediated transformation. Transgenic plants integrated with the *SOD* gene, a functional gene under the control of CaMV 35S and FMV 34S promoter were produced at RRII (Jayashree *et al.*, 2003; Sobha *et al.*, 2003a). To ensure the stable supply of natural rubber, production of improved *Hevea* tree with increased productivity

is needed. Recent progress in the molecular biology techniques helped us in scrutinizing the mechanism of rubber biosynthesis.

2.2 Rubber biosynthesis in plants

Natural rubber is a secondary metabolite present as rubber particles in the latex, the fluid cytoplasm of laticifers in *Hevea brasiliensis*. Plants synthesize over 22,000 known isoprenoid compounds which are involved in many essential metabolic and regulatory activities (Connolly and Hill, 1992). Rubber is composed of isoprene units linked together to form a polymer which differs from the majority of isoprenoid compounds in two respects. It has a high and a variable molecular weight ranging from 100,000 to several million and the geometrical configuration of the double bond is exclusively "*cis*", whereas the other isoprenoid compounds have "trans" configuration with a fixed molecular weight ranging from 100 to 1000 units. Because of its molecular structure (high *cis*-bond about 99.5%) and high molecular weight ($> 10^6$ Da), natural rubber has many physical properties which makes it superior to synthetic rubber and therefore serve as an important raw material for rubber products. Generally, isoprenoids can be classified into primary and secondary metabolites. This multibranched pathway is one of the most complicated biosynthetic pathways in plants leading to diverse compounds such as photosynthetic pigments, growth regulators like abscisic acid and gibberellins, phytoalexins, antibiotics, sterols, plastiquinone, ubiquinone, isopentenyl-tRNA and prenylated proteins (Bach, 1987; 1995). It has been well documented that isoprenoids including natural rubber, sesquiterpenes, triterpenes, sterols and brassinosteroids are biosynthesized via the MVA pathway (Newman and Chappell, 1999), whereas gibberellins, abscisic acid, carotenoids, and chlorophyll side chains are biosynthesized via the MEP pathway (Lichtenthaler, 1999). All these compounds are produced from acetyl-CoA via mevalonate to a central intermediate isopentenyl diphosphate (IPP) (McGarvey and Croteau, 1995).

IPP is the basic building block from which these compounds are derived. These compounds are necessary for a broad range of functions, including cell growth, reproduction, disease resistance, respiration and photosynthesis. Evidence supporting this cytosolic pathway for rubber formation was derived from a high-level incorporation of radiolabelled pathway intermediates such as mevalonate (Skilleter and Kekwick, 1971) and HMG CoA (Hepper and Audley, 1969) into rubber. The initial steps of the isoprenoid pathway involve the fusion of three molecules of acetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then reduced to yield mevalonic acid in a NADPH-dependent double reduction. This step is catalyzed by mevalonate: NADP oxido reductase, CoA acylating; 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) (Rogers *et al.*, 1983). Only in the recent years has the plastidic 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (MEP) pathway been considered as a possible alternative route for rubber biosynthesis. The analysis of redundancy–reduced EST's and transcription-derived fragments revealed the existence of this alternate pathway (DXP/MEP) in addition to the well-known Mevalonate pathway for the synthesis of IPP (Ko *et al.*, 2003). This knowledge came from the discovery of 1-deoxy D-xylulose 5 phosphate, which was highly up regulated in the latex compared to the leaf tissue. They concluded that the plants have two different pathways for the synthesis of IPP. The mevalonate pathway starting from the condensation of Acetyl CoA and the second one, the 1-deoxy D-xylulose 5 phosphate/2-C methyl-D-erythritol 4 phosphate (DXP/MEP) pathway, where pyruvate and D glyceraldehyde-3-phosphate is the precursor of IPP (Rohmer *et al.*, 1993; Eisenreich *et al.*, 1998) (Fig. 2). Addition of inhibitors like mevinolin to intact protoplasts decreased sterol formation but had little or no effect on β-carotene, plastoquinone and fatty acid formation, indicating a selective inhibition of cytosolic HMGR (Schulze-Siebert and Schultz, 1987).

The chloroplasts isolated from guayule (*Parthenium argentatum* Gray), incorporated labeled carbon compounds from [^{14}C] bicarbonate, [2-^{14}C] pyruvate, and [U-^{14}C]-3-phosphoglycerate into isopentenyl diphosphate (IPP) indicating the ability to utilize Calvin cycle intermediates for IPP synthesis via acetyl CoA and mevalonate (Reddy and Das, 1987). Location of plastidic enzymes for IPP synthesis (Wong *et al.*, 1982; Arebalo and Mitchell, 1984) in *Pisum sativum* and *Nepeta cataria* support the concept of separate pathways for IPP synthesis and disagree with the central IPP-pool hypothesis.

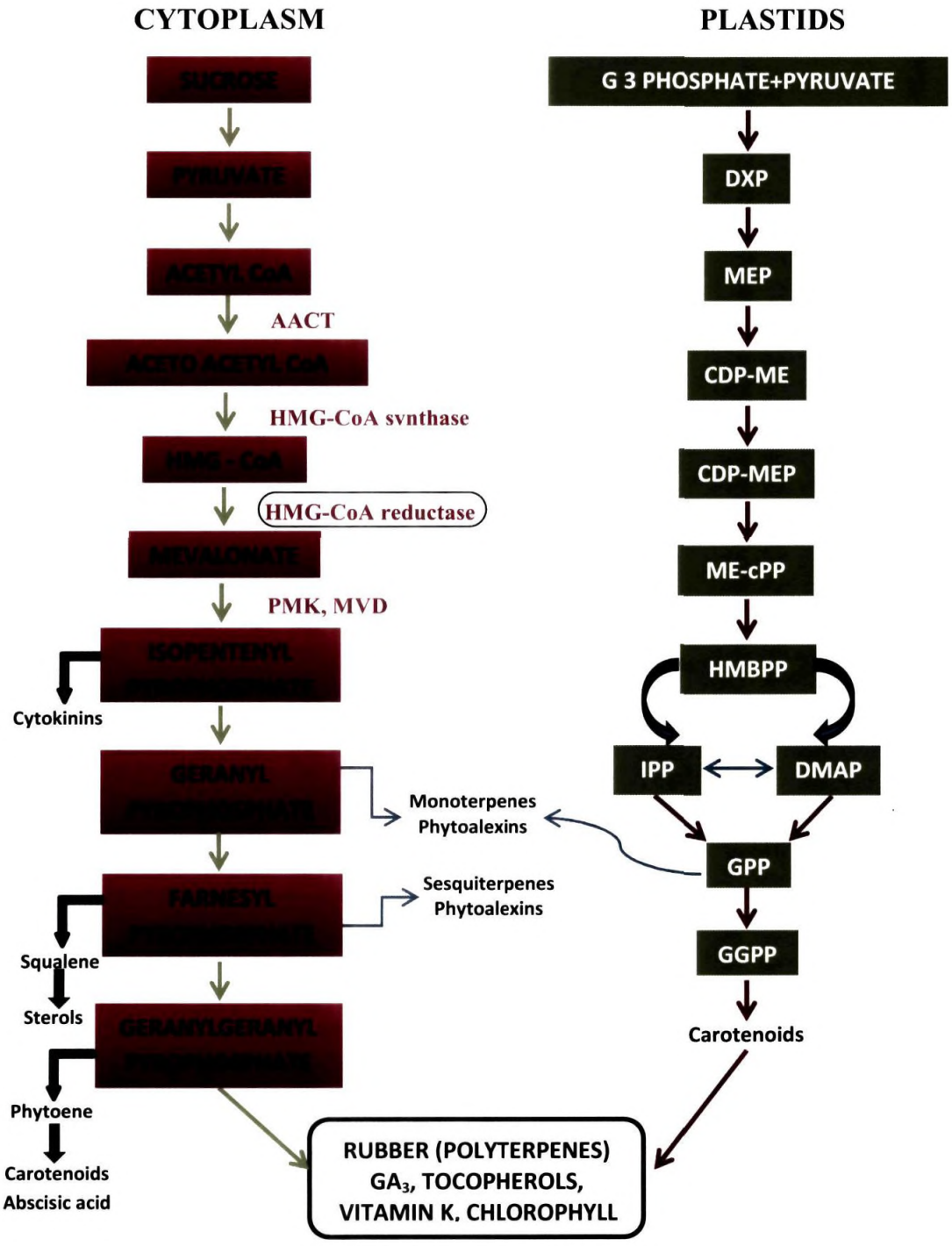


Fig.2. Mevalonate and MEP pathway for isoprenoid biosynthesis

Evidence for the existence of a novel pathway for the early steps of isoprenoid biosynthesis leading to IPP formation was also detected in eubacteria (Rohmer *et al.*, 1993). The expression of DXPS (1-deoxy-D-xylulose 5-phosphate synthase) in the *Hevea* latex and leaves suggests that the MEP pathway exists in laticifer and provide an alternative means of generating IDP for *cis*- polyisoprene synthesis (Ko *et al.*, 2003).

The existence of HMGR activities in various cellular compartments within the plant cell raises the possibility of multiple forms of the enzyme. This suggests that multiple HMGR genes might exist or that a single gene encodes for different forms through alternative mRNA processing or post-translational modifications. Discovery of the sub cellular localization of HMGR may be important in understanding the regulation of isoprenoid biosynthesis. There are multiple forms of HMGR in plants, which are distinctly regulated at the gene and protein level (Brooker and Russell, 1975 a, b; 1979; Choi *et al.*, 1992; Chye *et al.*, 1992; Learned, 1996; Chappell and Nable, 1987; Vogeli and Chappell, 1991; Ji *et al.*, 1992). In all plant species studied to date, HMGR is encoded by a small gene family of two or more members that are differentially expressed during development or in response to stress. Plants regulate HMGR activity at the level of mRNA, by differential induction of specific gene family members. Unlike mammalian HMGR, there is clear transcriptional activation as well as suppression of specific plant HMGRs. Genes encoding HMGRs have been isolated and characterized from many plant species including *Hevea* (Chye *et al.*, 1991, 1992), *Arabidopsis* (Enjuto *et al.*, 1994, 1995), tomato (Park *et al.*, 1992), potato (Korth *et al.*, 1997) and mulberry (Jain *et al.*, 2000). Unlike animals, which have a single-copy *hmgr* gene, plant *hmgr* occurs in small gene families and the number of genes encoding HMGR in plants varies depending on the species (Gertler *et al.*, 1988). Although the proteins they encode share high sequence identity, the expression patterns of individual family members

are generally distinct (Stermer *et al.*, 1994). HMGR is encoded by at least two and/or even larger multigene families in plants. The presence of multiple genes is consistent with the hypothesis that different isoforms of HMGR are involved in separate sub cellular pathways for isoprenoid biosynthesis. In the rubber plant, *Hevea brasiliensis*, HMGR is encoded by a small gene family of three genes, *hmgr*1, *hmgr*2, and *hmgr*3. During rubber (*cis*-polyisoprene) biosynthesis, transcriptional activation of specific HMGRs was observed. The expression of *hmgr*1 mRNA is induced by ethylene, but the activity of HMGR is not influenced by ethylene. The *hmgr*1 is highly expressed in laticifers where rubber biosynthesis occurs, while *hmgr*3 is not affected.

The *hmgr*3 isoform is probably involved in isoprenoid biosynthesis of a "housekeeping" nature, which infers that it is constitutively expressed in the plant. However, *hmgr*2 could be associated with the defense responses induced by pathogens and wounding (Chye *et al.*, 1992).

The study conducted by Chye *et al.* (1992) consistently showed the possible existence of families of isozymes each dedicated to the production of distinct classes of isoprenoids, which are independently regulated. Like mammalian HMGR, specific plant HMGRs is also subject to feedback regulation by the end products of the pathway. More recently, HMGR mRNA has been shown to accumulate in dark-grown *Arabidopsis*, since *hmgr*1 was suppressed in response to light (Learned, 1996). Using promoter/reporter gene fusions it was demonstrated that the suppression of *hmg*1 mRNA in response to light was mediated by cis-acting elements within the promoter.

2.3 Structure of HMGR

In general, the structure of HMGR is similar among plants. The structure has three distinct regions which include an N terminal trans membrane domain, linker region, and C-terminal domain. The catalytic domain located in the

C-terminal portion of the protein, is highly conserved across all plant species (74 to 98%). The most divergent among the plant HMGRs with respect to the size and the sequence is the linker region. The N-terminal domain (containing the putative membrane-spanning region) in plants also show striking variations in the size. The highly conserved nature of the transmembrane spans of plant *hmgr* can be considered as a reflection of functional importance. This conservation in the membrane domain region in plants, helped in anchoring the enzyme to specific membrane or in the regulation of the enzyme to different environmental or physiological stimuli (Denbow, 1997).

2.4 Yield variability in *Hevea brasiliensis*

Latex yield is a polygenic, complex trait influenced by different factors. The major yield components like initial flow rate per unit length of tapping cut, length of the cut, percentage rubber content and plugging index play an important role in regulating latex yield in *Hevea*. Here the duration of the latex flow is mainly controlled by the coagulation processes. Studies on the molecular basis of latex coagulation showed that Hevein induced the latex coagulation by bringing the rubber particles together. This hevein-rubber particle bridging is mediated by N-acetyl-D-glucosamine and a receptor on the surface of the rubber particle. This bridging is inhibited by the action of chitinases and N-acetyl-D-glucosaminidases. Therefore the expression level of these genes, namely hevein and chitinases in the latex which is a clonal character can be used as molecular markers for higher yield in *Hevea* (Chrestin *et al.*, 1997).

Maintenance of higher growth rate is also essential for higher latex yield (Sethuraj, 1981). Seasonal as well as clonal variations in the latex yield were also noticed in rubber trees. During the peak season, the trees experienced some kind of stimulation which increased the laticifer activity. During this period, a higher sucrose loading caused the excess sucrose to be drained out with the

latex serum. The number of laticifers is another important factor influencing rubber yield in *Hevea* (Gomez, 1982). It was demonstrated that the laticifer differentiation in *Hevea* was affected by the jasmonic acid. Introduction of genes encoding enzymes involved in the jasmonic acid synthesis can bring about latex vessel differentiation in *Hevea*. A key enzyme in this pathway, allene oxide synthase (AOS) has been cloned and work has been initiated in this direction (Arokiaraj *et al.*, 2002).

A positive influence of low temperature and wintering on the latex yield has been reported by Vinod (2001). Studies conducted in guayule (*Parthenium argentatum*) supported this statement showing a rapid increase in the rubber formation from 589 mg to 4438.0 mg per plant during the winter season. The HMGR activity during June, measured as 21.1 nmol mevalonic acid (MVA) $h^{-1}g^{-1}$ was increased to 29.9 nmol mevalonic acid (MVA) $h^{-1}g^{-1}$ fresh weight during winter. A similar rise was also noticed in the rubber transferase activity. The increase in the activities of these enzymes coincided with the acceleration in the rubber formation of the plant. But the activities of MVA kinase and IPP isomerase were almost unchanged in the fall and winter (Ji *et al.*, 1993). The influence of *hmgs* and *hmgr*1 mRNA levels on the dry rubber content in *Hevea* was investigated by Suwanmanee *et al.* (2007). The *hmgs* and *hmgr* 1 mRNAs showed a positive correlation with dried rubber content in rubber latex from 3 tapping's of ten trees with coefficients of 0.77 ($p<0.01$) and 0.71 ($p<0.01$), respectively. A positive correlation coefficient of 0.74 ($p<0.01$) was obtained in the expression of *hmgs* and *hmgr*1 gene. The duplex RT-PCR was used to determine mRNA levels of *hmgs* and *hmgr*1 from high and low yielding rubber trees. Both genes clearly showed higher expression in the high yielding clones than the low yielding ones. These results showed that rubber biosynthesis in *Hevea brasiliensis* was coordinately regulated by *hmgs* and *hmgr*1 genes. Therefore the expression of these genes could be used as early selection markers

in *Hevea* breeding programmes. Similarly northern blot analysis in *Hevea* also showed a diurnal variation in the *hmgr*1 mRNA level. The levels of *hmgr*1 mRNA varied with the tapping times and the peak was at 2.00 a.m. The relationship between *hmgr*1 mRNA and dry rubber content was again investigated in this study, where a positive correlation was observed between the dry rubber content and *hmgr*1 mRNA in the Malaysian clone RRIM 600 and PB 235. These results strongly prove the regulatory role of *hmgr*1 in rubber biosynthesis (Nuntanuwat *et al.*, 2006).

2.5 HMGR and Rubber biosynthesis

The commercial exploitation of *Hevea* tree for latex commenced by the end of 19th century (Carron *et al.* 1989). Rubber content accounts for about 2% of the dry weight of the tree. The generation of genetically modified rubber tree needs a proper understanding of plant physiology. A deep knowledge on the potential limiting factors of latex yield helped us to spot out some definite enzymes or proteins, a modification in the expression of their corresponding gene could augment yield in *Hevea.* At this context, the latex yield can be enhanced either by treatment or by the over expression of key rubber biosynthetic genes in transgenic plants. Many studies have proved that the three enzymes namely HMGR, FDP and RuT were very closely related to yield and quality of rubber. Richards and Hendrickson (1964) proved that acetate was incorporated into a large number of complex organic molecules. HMGR has been rigorously studied in animals and yeast due to its importance in catalyzing the rate-limiting reaction in cholesterol biosynthesis (Goldstein and Brown, 1990), and is very highly regulated in these systems (Hampton *et al.*, 1994). However, the rate limiting nature of HMGR in plants and its regulation remain controversial (Chappell, 1995; Stermer, 1994). Evidence for the rate-limiting action of HMGR in isoprenoid biosynthesis came from studies of several investigators (Chappell and Nable, 1987; Stermer and Bostock, 1987) where a

concomitant rise in the isoprenoid biosynthesis was noticed with an increase in the HMGR activity. However, it has also been demonstrated using pulse-labeling studies that other downstream enzymes in the pathway are also highly regulated and may be key control points (Threlfall and Whitehead, 1988; Vogeli and Chappell, 1988; Chappell *et al.*, 1991; Chappell *et al.*, 1995). To directly evaluate the role of HMGR in regulating carbon flow into plant isoprenoids, Chappell *et al.* (1995) constitutively expressed the hamster HMGR cDNA in tobacco plants (*Nicotiana tabacum* L.) resulting in an increase in total HMGR enzyme activity (3-6 fold) and an overall sterol accumulation (3-10 fold). However, only some classes of isoprenoids (eg, sterols) were affected while carotenoids, phytoalexins and the phytol chain of chlorophyll remained unchanged. Additional studies supported the role of specific isoforms of HMGR in mediating pathway flux especially during defense responses. HMGRs are highly regulated by a variety of developmental and environmental signals such as light, wounding, infection, hormones, herbicides, and sterols (Bach, 1995). The subcellular localization of plant HMGR is controversial, but enzyme activity has been associated with mitochondria (Bach *et al.*, 1986), chloroplasts (Brooker and Russell, 1975a; Arebalo and Mitchell, 1984) as well as the ER (Kondo and Oba, 1986; Enjuto *et al.*, 1994). Studies on plastid and cytoplasmic HMGR activites in pea revealed distinctive kinetic and regulatory properties (Brooker and Russell, 1975b; Brooker and Russell, 1979; Wong, *et al.*, 1982). HMGR activites are higher in rapidly growing parts of the plant such as apical buds and roots, but lower in more mature tissues. This is more evident in tomato where the activity is highest during the early stages of fruit development which later declined during fruit ripening (Gillaspy *et al.*, 1993). Similarly treatment with mevinolin (a competitive inhibitor of HMGR) can slow or inhibit plant growth and development (Gray, 1987; Narita and Gruissem, 1989).

Mevalonic acid is derived from β-hydroxy β methyl glutaryl CoA in a TPNH– linked reduction. *Hevea* latex is a colloidal suspension of rubber and other particles in an aqueous medium. Freshly tapped latex on centrifugation separated into three distinct fractions, a light rubbery phase of creamy appearance, middle aqueous C serum and the sediment bottom fraction (BF) of membrane–bound organelles. The BF is composed of the membrane bound organelles, the lutoids and Frey-Wyssling particles. The fresh latex is thus a colloidal mixture of these particles together with the cell soluble substances in an aqueous suspension. HMGR presumably one of the rate-limiting enzymes in the RB (rubber biosynthesis) pathway was purified from the washed BF membrane (Wititsuwannakul *et al.*, 1990; Benedict, 1983). It was shown that a Ca^{2+} binding protein in the C-serum functions as an activator of HMGR enzyme (Wititsuwannakul *et al.,* 1990). Rubber biosynthesis mainly occur on the bottom fraction particle surface (BF) or the washed bottom fraction membrane (WBM). Again the rubber biosynthesis activity of WBM (washed bottom fraction membrane) was much higher than the rubber particles (RP) surface (Wititsuwannakul *et al.*, 2004).

Lynen (1969) pointed out that the relative enzymatic activities of various enzymes in the pathway up to IPP were of comparable magnitude with the exception of HMG CoA reductase. This enzyme activity was much lower and is conservable that the constitutional level of this enzyme may be a limiting factor in rubber biosynthesis. HMGR in *Hevea* latex has been characterized and the activity appears to be exceptionally low (0.078nmol MVA/ ml of latex), compared to other enzymes. Thus, the level of this enzyme may be a limiting factor in the biosynthesis of natural rubber. Similar results were observed by Hepper and Audley, (1969) where mevalonic acid was utilized by the latex at a much faster rate than HMG CoA. Wititsuwannakul (1986) reported that the specific activity of HMGR was high in high–yielding *Hevea* clones and that the

diurnal variations observed in the rubber content of the latex coincided with the variations in the HMGR activity. It may therefore be possible to increase the rubber yield by promoting the transcription and translation of latex-specific HMGR.

According to Sando *et al.* (2008), the natural rubber biosynthesis by the mevalonate pathway consisted of six steps catalyzed by corresponding enzymes. The enzymes involved in the early steps of rubber biosynthesis (HMGS and HMGR), functioned jointly when the substrate is available (Suwanmancea *et al.*, 2002). Of the three members of the HMGR family, the laticifer specific *hmgr*1 was induced by ethylene and not influenced by ethylene (Wititsuwannakul *et al.*, 1986). The direct role of yield stimulants on the rate of rubber formation following tapping has not been proved so far. The *hmgr*2 could be associated with the defense reactions against wounding and pathogens and *hmgr*3 was expressed constitutively (Chye *et al.*, 1992; Wititsuwannakul *et al.*, 1986). Venkatachalam *et al.* (2009) reported molecular cloning and characterization of a full-length cDNA as well as genomic DNA of *hmgr*1 gene from *Hevea brasiliensis* (clone RRII 105). The Northern blot results suggested a high level expression of *hmgr*1 in the laticifers than in the leaves, emphasizing its involvement in rubber biosynthesis. The nucleotide sequence of the genomic DNA contained 4 exons and 3 introns giving a total length of 2440 bp. The *hmgr*1 cDNA contained an open reading frame of 1838 bp coding for 575 amino acids with a theoretical pI value of 6.6 and the calculated protein M_W of 61.6 kDa. The amino acid sequence of *hmgr*1 revealed highly conserved motifs that are common to other plant species. These conserved motifs play a vital role in the structural and catalytic properties of this enzyme. The existence of the three isoforms of *Hevea hmgr* was also confirmed by the genomic Southern blot analysis. The northern analysis further showed a higher *hmgr*1 mRNA transcript level in the latex cells compared to mature/immature leaves and seedlings.

Alignment analysis and phylogenetic studies conducted by Sando *et al.* (2008) revealed the existence of four HMGR genes in *Hevea brasiliensis* and that the MVA pathway genes were highly expressed in the latex. They coined different expression patterns for the multiple copies of HMGR and HMGS. Furthermore this report strongly supported the earlier findings that *hmgr*1 is highly expressed in the latex, showing its importance in the natural rubber biosynthesis in *Hevea.* If HMGR activity is shown to be correlated with the yield, there is every possibility to utilize this relationship to increase rubber biosynthesis by increased transcription and translation of HMGR and maximizing the specific activity of the enzyme *in situ.* Genetic transformation was carried out in *Hevea* using *hmgr*1 gene where *Hevea* anther callus was used as the explant. HMGR activity in the transformed callus ranged from 70-410% and the corresponding activity in the somatic embryos were from 250-300% compared to the control (Arokiaraj, 1995). Unfortunately, the transgenic embryos failed to germinate. Here the elevated activity of the *hmgr*1 in the transformed tissues could be explained in part by the level of mRNA. Studies by Berlin *et al.* (1993) proved that the over production of an intermediate in a biosynthetic pathway could lead to increased levels of the end product as evidenced in transgenic *Peganum harmala.* These observations showed that the activity of a key enzyme in the rubber biosynthetic pathway could be elevated through genetic transformation and this can lead to an increase in the end product. Hence, experiments were initiated at RRII to enhance rubber biosynthesis by developing transgenic plants integrated with the genes coding for *hmgr*1 and some of the downstream enzymes of the biosynthetic pathway.

It has been well established that the transformation efficiency depends on many factors including plant genotype, plasmid vectors, '*vir*' gene inducing compounds, medium composition, *Agrobacterium* strains etc. The choice of *Agrobacterium* strain is one of the key parameter determining the efficiency of

transformation. Arokiaraj *et al.* (1998) used *Agrobacterium* strain GV2260 (p35SGUSINT) that harboured the β-glucuronidase (*gus*) and neomycin phosphotransferase (*npt*II) genes for genetic transformation in *Hevea.* Subsequently, Montoro *et al.* (2000) investigated the influence of $CaCl_2$ on *Agrobacterium* gene transfer in *Hevea* friable calli using five different *Agrobacterium* strains (C58pMP90, C58pGV2260, AGL1, LBA4404 and EHA105) and two binary vectors, where the efficiency was higher using the strain EHA 105. Later Montoro *et al.* (2003) developed transgenic calli from *Agrobacterium* infected inner integument tissues of immature fruits (clone PB260) using the strain EHA105 harboring the binary vector pCAMBIA 2301. Blanc *et al.* (2006) produced many transgenic plants of *Hevea* (clone PB 260) via *Agrbacterium*-mediated transformation using pCAMBIA2301 vector combined with the strain EHA105. Most of the *Agrobacterium* strains appeared to have a wide host range but their rate of infection depended on the genotype within a single species. This may be due to the stress response of plants, which involves the secretion of phenolic compounds for switching the "*vir*" genes directing *Agrobacterium* to the infected area. Hence, *Agrobacterium* strain as well as the binary vectors played an important role in giving an efficient transformation in *Hevea brasiliensis.* Arokiaraj *et al.* (2009) proved this statement by transforming *Hevea* anther callus with two different strains of *Agrobacterium* (GV 2260 and GV 3850) harboring the super virulent plasmid p ToK47. It was shown that both the *Agrobacterium* strains were benefited by the super virulent binary vector giving a higher frequency of transformation resulting in the production of callus, embryoids and plantlets, proving the involvement of binary vector also in determining the transformation efficiency.

The importance of components other than the *Agrobacterium* strain and the binary vector which can influence the transformation efficacy has been tried by many researchers. The addition of $AgNO_3$ and the inclusion of thiol

compounds in the co-cultivation medium, desiccation of the explants, pretreatment of explants with the anti-necrotic mixture etc. were reported to have an influence on improving the efficiency of transformation in many crops mainly by suppressing the bacterial over growth, thereby facilitating plant cell recovery. Cheng *et al.* (2003) showed that the explant desiccation enhanced the T-DNA delivery and plant tissue recovery, resulting in an increased stable transformation in wheat and maize. According to Cheng and Fry (2000), desiccation treatment was effective in accelerating the efficacy of transformation not only in monocot species but also in recalcitrant dicots such as soybean. Pretreatment of the explants with the anti-necrotic mixture helped in reducing the oxidative burst. In sugarcane, transgenic cell lines were observed only when the explants were treated with the anti-necrotic mixtures (Enriquez-Obregon *et al.*, 1998). Antibiotic selection combined with the inclusion of thiol compounds in the co-cultivation medium, improved efficiency of transformation in maize (16.4%) (Olhoft *et al.*, 2003). Inclusion of silver nitrate in the co-culture medium enhanced stable transformation in maize (Armstrong and Rout, 2001; Zhao *et al.*, 2001). Studies on the effect of different temperature regimes and the explant type on the frequency of transformation has been reported by many workers. Salas *et al.* (2001) proved that the optimal temperature for stable transformation varied with each type of explant and also with the strain of *Agrobacterium*. Lower incubation temperature (19°C) was effective in improving the transformation of cotton using embryogenic callus (Jin *et al.*, 2005). In tobacco, a temperature of 22°C was optimum for the T-DNA delivery (Dillen *et al.*, 1997) whereas in wheat T-DNA delivery and stable transformation was obtained at 23-25°C (Rout *et al.*, 1996). Likewise, the use of suspension cultures improved the frequency of transformation in many crops. Infection of the 3-day-old suspension cultures with *Agrobacterium* on a filter paper moistened with N_6 medium resulted in a very high frequency

transformation in rice. In this experiment the transformation efficiency was 60-times higher than that obtained from calli co-cultured in N_6 solid media alone (Ozawa and Takaiwa. 2010). A transformation system was also developed in *Arabidopsis* using suspension cultures and stably transformed cells were recovered (Forreiter *et al.*, 1997). In Hybrid poplar, suspension cultures were mainly used to compare the efficiencies between the different strains of *Agrobacterium* (Howe *et al.*, 1994). Embryogenic cell suspension cultures established from unfertilized ovules were used as the target material for *Agrobacterium* mediated transformation in Citrus (Dutt and Grosser, 2010).

2.6 HMGR and cell growth

In any rubber producing plant, the fundamental building blocks are IPP and DMAPP. Therefore, efficient and sufficient supply of IPP is a major factor in rubber biosynthesis (Cornish, 2001a). The HMGS and HMGR which have a regulatory role in the isoprenoid biosynthesis are also essential for cell growth and division (Hepper and Audley, 1969; Bach, 1987 and 1995). Kaneko *et al.* (1978) postulated that HMGR has a role to play in the cell growth and the cells require small amounts of active HMGR to synthesize mevalonate-derived substance other than sterols for their growth. The enzyme HMG Co A reductase catalyzes the irreversible conversion of HMG CoA to mevalonate. Due to the irreversible nature of this reaction, this step was likely to be a regulatory point in sterol biosynthesis (Goldstein and Brown, 1990). Kush *et al.* (1990) have reported the differential expression of several rubber biosynthesis related genes in the latex of *Hevea*. He also demonstrated that laticifer is enriched with the transcripts coding the enzymes involved in the rubber biosynthesis and plant defense. Among these the most important ones were REF (Dennis and Light, 1989; Goyvaerts *et al.*, 1991), HMG CoA reductase (Chye *et al.*, 1991) and small rubber particle protein (SRPP) (Oh *et al.*, 1999). Whether these enzymes play a similar rate-limiting role in controlling plant isoprenoid biosynthesis

remains unresolved (Bach, 1986; Narita and Gruissem, 1989; Choi *et al.*, 1992). According to Venkatachalam *et al.* (2009) the *Arabidopsis* transgenic plants overexpressing *Hevea hmgr*1 cDNA were morphologically distinguishable from the control wild type plants.

MATERIALS AND METHODS

Chapter 3

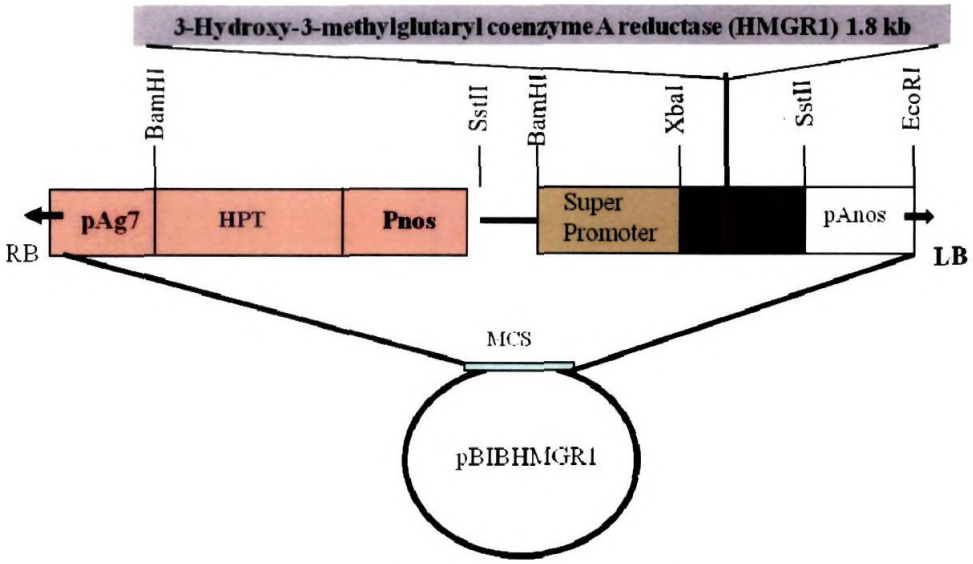
MATERIALS AND METHODS

Attempts were made to develop transgenic plants overexpressing laticifer specific *hmgr*1 gene in *Hevea brasiliensis*. Experiments were carried out to identify the most efficient *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 *hmgr*1 developed by Venkatachalam *et al.* (2009). The binary vector contained hygromycin phosphotransferase gene (*hpt*) as the plant selectable marker and *hmgr*1 gene from *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 *hmgr*1 cDNA was isolated from *Hevea* tree by a PCR based approach using the primers designed based on the published sequences of *hmgr*1 from the Malaysian clone RRIM 600 (Chye *et al.*, 1991; 1992). The *hmgr*1 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 *hmgr*1 cDNA isolated from *Hevea brasiliensis* (clone RRII105) was placed between the super promoter and nos terminator elements. The resulting plasmid was designated as pBIB *hmgr*1 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.



RB –Right border, LB-Left border, HPT-Hygromycin, MCS-Multiple cloning site, nos-Nopaline synthase

Fig 3. Plasmid vector pBIB *hmgr*1 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^r) (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 $MgCl_2$ in a final volume of 20 µl. The reaction mixture was incubated in a thermal cycler under the following conditions.

Step 1	Initial denaturation	- 94°C for 10 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 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

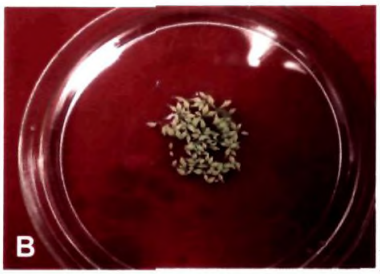
Individual colonies of *Agrobacterium* were grown in liquid LB medium with the respective antibiotics and proliferated on a gyratory shaker, at 250 rpm, overnight at 28°C. The OD of the bacterial culture was measured at A_{420} nm of 0.5 by taking 200µl bacterial suspension from the overnight grown culture 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 10^8 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.

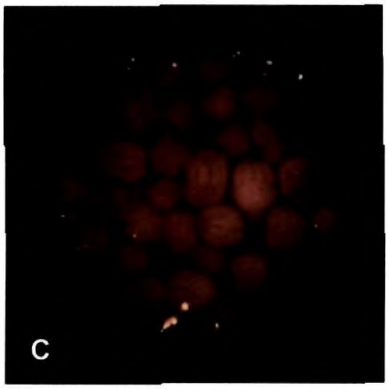
3.4 Source material for genetic transformation

Agrobacterium mediated genetic transformation with the *hmgr*1 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.

A- Inflorescence



B- Immature flower buds at the diploid stage



C- Immature anthers used for callus induction

Plate 1. (A-C) Explant for culture initiation

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 - 1.5 mg l^{-1}) and BA (0.25 – 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 % phytagel. 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 GA_3 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 NH_4NO_3 (500 mg l^{-1}) supplemented with phenolic compounds [acetosyringone (20 mg l^{-1}),

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° C. The phenolic compounds along with the growth regulators were filter sterilized using a 0.2 µm 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 ± 2°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°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 $AgNO_3$ 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 $AgNO_3$ (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

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 $AgNO_3$ 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 $AgNO_3$ (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 *hmgr*1 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'- CGGAATGCGCGTGACGCTCC-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'- CGTGCACAGGGTGTCACG 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

2X CTAB
2% CTAB
0.1 M Tris HCl (pH 8.0)
20m M EDTA (pH 8.0)
1.4 M NaCl
0.1% β-mercaptoethanol
1% polyvinyl polypyrrolidone

- 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 O.D at 260 nm = 50 ng /µl

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)

Glucose	50 mM
Tris HCl (pH 8.0)	25 mM
EDTA	10 mM (pH 8.0)
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)

NaOH	0.2 M (freshly diluted from 10M stock)
SDS	1%

- 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

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml
Autoclaved at 121°C, 15 lb before use	

- 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 *hmgr*1 gene. PCR analysis was carried out using promoter specific forward and *hmgr* specific reverse primers designed to amplify the *hmgr*1 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 *hmgr*1 gene were 5'- CGGAATGCGCGTGACGCTCC -3' and 5'-GACATATCTTTGCTGGATCTGT-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

Component	Volume	Final concentration
Template	2.0 µl	50 ng
Reaction buffer	2.0 µl	1 X
dNTP mix	2.0 µl	100 µM of each dNTPs
Forward primer	1.0 µl	10 pmol
Reverse primer	1.0 µl	10 pmol
Taq DNA polymerase	0.15 µl	0.75 U
Sterile D.W	12.85 µl	
Total volume	**20 µl**	

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	- 35cycles
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

(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 I D 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 AG_1 and WPM (Lloyd and McCown, 1980) (Appendix B). AG_1 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 GA_3 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 (AG_1) 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 sub cultures.

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°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_4NO_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 (50-250mgl^{-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_2O 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 $¼^{th}$ MS. The medium contained additional amounts of KNO_3 and K_2SO_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^{-2}) at 25 ± 1°C for 15 days. The green embryos showing shoot meristems were further subcultured into fresh media (MS_4), 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 *hmgr*1 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 *hmgr*1 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 *hmgr*1 specific primers was 640 bp. The PCR reactions were carried out as in Table 1. The reaction conditions for *hpt* and *hmgr*1 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 *hmgr*1 cDNA sequence in NCBI.

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.

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 over hang 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_4 DNA ligase	1.0 µl
H_2O	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 *hmgr*1 gene

3.12.3a Sequencing and sequence analysis

The sequencing of *hmgr*1 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 *hmgr*1 (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 *hmgr*1 cDNA obtained from the transgenic plant was compared with that of *Hevea hmgr*1 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, *EcoR*I and *Xba*I (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 *hmgr*1 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 *EcoR*I (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-^{32}P labeled *hpt* probe. The reaction mixture was prepared as described below.

DNA	-	20 µl (10 µg)
Enzyme buffer	-	7.5 µl
Res. Enzyme	-	15 µl
BSA	-	2 µl
D.D H_2O	-	5.5 µl
Total reaction	-	50 µl

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.

20x SSC
3M NaCl
0.3 M sodium citrate
pH adjusted to 7.0

- 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/cm^2 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-^{32}P 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 [NaCl - 0.1 M, Tris-HCl- 10 mM (pH 8.0), EDTA- 1mM (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 micro centrifuge 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.

1. Pre-hybridization solution

6x SSC

5X Denhardt's reagent

0.5 % SDS

2. Denhardts 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

Washing solution I.
2x SSC
0.1% SDS.

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

Washing solution II.
0.1x SSC
0.5% SDS.

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).

Formaldehyde gel running buffer (5x)
(MOPS buffer)
0.1M MOPS (pH 7.0)
40m M sodium acetate
5 mM EDTA (pH 8.0)

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.

Pre-hybridization solution
5 x SSC, 5x Denhardt's reagent, 1.0 % SDS, 50% formamide

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.

Solution I
2x SSC, 0.1% SDS.

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

Solution II
0.1x SSC, 0.5% SDS.

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_2PO_4, and 1.15 g of Na_2HPO_4. $2H_2O$ 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_2O_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/H_2O_2) was added to each well and incubated for 30 minutes. The reaction was stopped by adding 50µl of 1N H_2SO_4.

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.

PROTOCOL

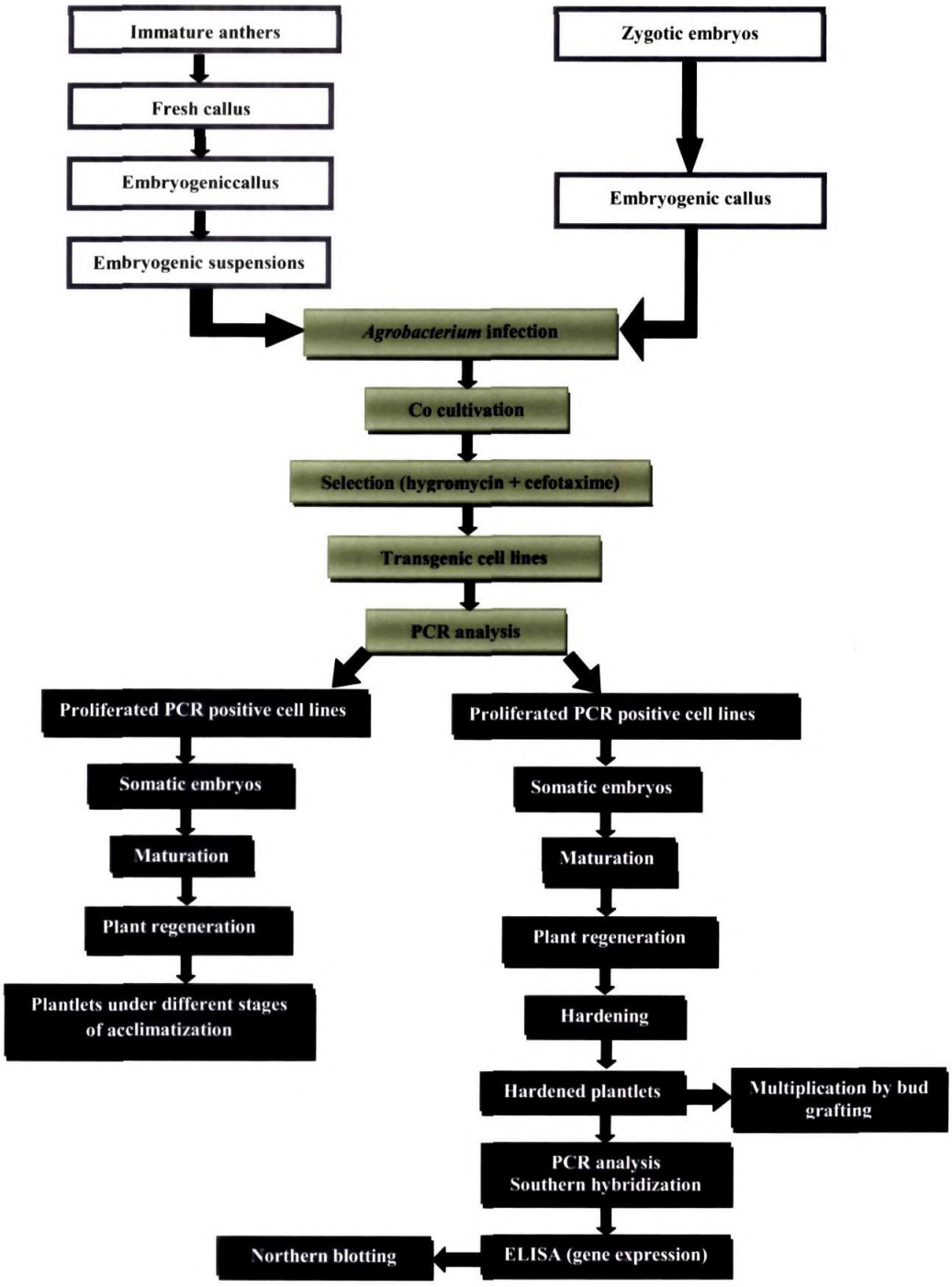


Fig. 4 The protocol followed in the transformation work

RESULTS

Chapter 4

RESULTS

A reproducible regeneration pathway for producing transgenic plants overexpressing *hmgr*1 gene was developed in *Hevea*. The most important factor determining the efficiency of transformation is the choice of a suitable target tissue. So the first part details the generation of the target tissue for transformation experiments. Thereafter the response of different callus types, both clonal and of zygotic origin on transformation frequency was evaluated. The most efficient *Agrobacterium* strain giving higher rate of transformation was identified by experimenting three different bacterial strains for genetic transformation. Plant selection was carried out using hygromycin, the second widely used negative selection system. Improvement in the efficiency of transformation was attempted by inducting compounds which are beneficial in accelerating the transformation frequency and also by altering the culture conditions. Factors influencing embryo induction, maturation and transgenic plant regeneration were critically analyzed. The transgene integration and expression were validated. The experimental results obtained under different aspects are given below.

4.1 Plasmid vector and bacterial strains

The glycerol stocks maintained at -80°C were revived once in a year. The *Agrobacterium* cultures were taken out of the deep freezer, and grown in solid LB plates containing 50 mg l^{-1} Kan and 20 mg l^{-1} Rif. The culture plates were dark incubated at 28°C. The bacterial colonies of the different *Agrobacterium* strains were screened for the presence of the insert by restriction enzyme digestion and stocks were prepared from fresh cultures.

4.2 Antibiotic sensitivity

The concentration of antibiotic (hygromycin) required for the selection of the transformed cell lines was determined from the kill curve experiment (Fig.5). Response of different callus to varying levels of the selection antibiotic hygromycin was different. Frequent sub culture at an interval of 15 to 20 days to fresh antibiotic media was essential for efficient selection. Antibiotic medium containing 10 mg l^{-1} hygromycin inhibited 20 percent of the callus growth. The callus texture remained unchanged in the case of embryogenic callus whereas the primary calli obtained from immature anthers turned brown. Subsequent increase in the concentration of the antibiotic decreased the survival percentage of both the callus.

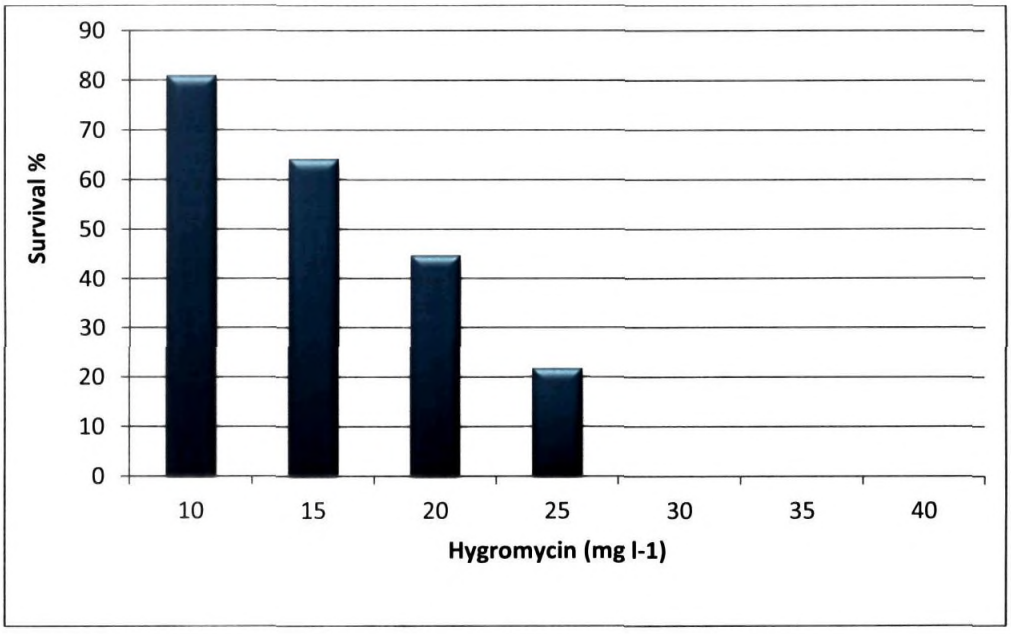


Fig. 5. Sensitivity of *Hevea* callus at different levels of hygromycin

Inclusion of 20 mg l^{-1} hygromycin in the selection medium retarded the growth of about 50 percent of the cells. The survival rate of the callus was determined as 20% when the concentration of the antibiotic was increased to 25 mg l^{-1}. When the control calli was subjected to a concentration of 30 mg l^{-1}, the primary callus turned black whereas the embryogenic tissues became white in colour indicating tissue death / inhibition of the callus growth (Plate 2 A, B.) Therefore the minimum concentration of hygromycin suitable for the selection was 30 mg l^{-1} where effective selection of the transformants was possible preventing any escapes.

4.3 *Agrobacterium* culture initiation

Individual colonies were formed from the three *Agrobacterium* strains carrying the binary vector in LB medium with the respective antibiotics (Kan^{50} and Rif^{20}). The presence of the insert in the colonies was confirmed through colony PCR. PCR analysis using the promoter specific forward and the *hmgr* specific reverse primers amplified a fragment of length 1.9 kb in the plasmid DNA (positive control) and in the bacterial colonies containing the insert (Fig.6). Individual colonies containing the binary vector were grown in liquid LB medium containing the respective antibiotics at 28°C with shaking at 250 rpm. The O.D of the overnight grown cultures was adjusted to 0.5 at A_{420} and pelleted. The pellet grown in induction medium attained good growth after 4 hrs which was used for infecting the target tissues.

4.4 Generation of source material for genetic transformation

4.4.1 Callus induction from anther tissue

Contamination free cultures could be initiated from immature anthers and callus induction was obtained in modified MS medium supplemented with 2,4-D,NAAand BA after forty days. Table 4.2 describes the combined effect of 2,4-D and BA in presence of NAA (0.5 mg l^{-1}) on callus induction from the n the cultured anthers. The basal medium containing lower levels of 2, 4-D (0.5 mg l^{-1})

and BA (0.25 mg l^{-1}) resulted in a low callusing efficiency (18%). An increase in the 2, 4-D concentration (0.5 - 1.25 mg l^{-1}), slightly improved the frequency, even though the level of BA remain unchanged. But this callus appeared to be watery in nature. An increase in the concentration of BA from 0.25 to 0.5 mg l^{-1} changed the texture of the callus. Compact calli was obtained in modified MS medium supplemented with 2, 4-D (1.0 mg l^{-1}), BA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}) (Plate 3A). Maximum callus induction (35%) was achieved in this combination. When the 2,4-D concentration was raised to 1.25 mg l^{-1} in presence of BA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}), the callusing efficiency decreased to 29%. Further rise in the concentration of 2, 4-D and BA impaired callus growth, reducing the efficiency of callus induction. Proliferation of the primary calli was attempted in the same medium with reduced levels of growth hormones 2, 4-D (0.5 mg l^{-1}), BA (0.5 mg l^{-1}) and NAA (0.2 mg l^{-1}).

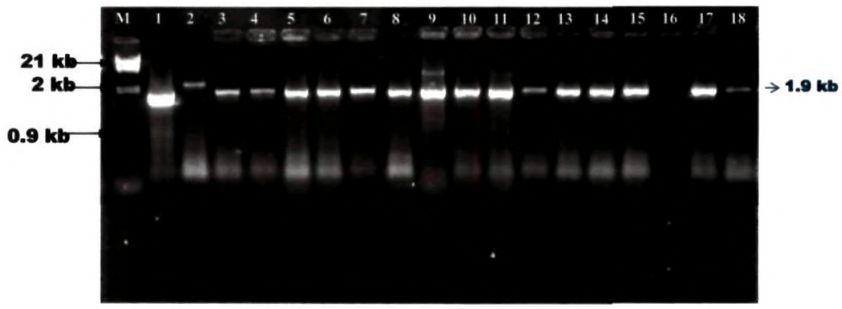


Fig 6. Colony PCR

M-λ Marker, 1-Positive control, 2-18 bacterial colonies

Plate 2 A-B Response of the callus upon exposure to hygromycin

A. Primary calli exposed to hygromycin (30 mgl^{-1})

B. Embryogenic callus on exposure to hygromycin (30 mgl^{-1})

4.4.2 Embryogenic callus formation

The proliferated primary calli cultured in half strength MS medium turned black after two weeks of culture. After six to seven months of culture in the same basal medium (with regular subculture to fresh media combinations) the cells which were competent in inducing embryogenic response produced embryogenic callus. The conversion of the primary callus to the embryogenic callus was noticed in the medium containing TDZ and NAA (Table 4.3) (Plate 3B). The basal medium containing lower concentration of TDZ (0.2 mg l^{-1}) and NAA (0.5 mg l^{-1}), produced friable callus. An increase in the NAA concentration slightly improved the friability of the callus. But the TDZ concentration of the media influenced embryogenic callus formation. Here the conversion of the primary callus to embryogenic calli was TDZ dependent. Further rise in the concentration of TDZ (0.6 mg l^{-1}) significantly enhanced the emergence of friable, embryogenic callus in presence of NAA. When the TDZ concentration was increased beyond 0.6 mg l^{-1}, no further improvement in the embryogenic callus formation was noticed. An increase in the level of NAA beyond 1.5 mg l^{-1} reduced the emergence of embryogenic callus, where the optimum concentration was 1.0 mg l^{-1}. Results proved that embryogenic callus formation was achieved in half strength MS basal medium containing TDZ (0.6 mg l^{-1}) and NAA (1.0 mg l^{-1}) in presence of BA (0.3 mg l^{-1}) at a frequency of 46%.

Table 4.2. Frequency of callus induction from immature anther explants

Conc. of BA (mg l^{-1})	Conc. of 2,4-D (mg l^{-1})				
	0.5	**0.75**	**1.0**	**1.25**	**1.5**
0.25	18±0.836	21±1.48	22±1.3	23±1.30	19±1.22
0.5	24±1.14	29±1.51	**35±1.14**	29±0.71	22±1.58
0.75	21±1.30	24±0.70	26±1.30	23±0.84	17±1.14
1.0	19±1.64	21±1.48	23±1.30	15±0.836	12±1.303

The callus induction frequency was represented as mean ± SD. Twenty anthers were inoculated per treatment with five replications

Table 4.3. Effect of NAA and TDZ on the conversion of primary callus to embryogenic callus (%)

Conc. of TDZ (mg l^{-1})	Conc. of NAA(mg l^{-1})				
	0.5	1.0	1.5	2.0	2.5
0.2	4.0 (7.56)	10.0(16.45)	14.0 (21.70)	4.0(7.58)	--(0.33)
0.4	20.0(26.28)	22.0(27.61)	16.0 (21.03)	6.0(11.2)	--(0.33
0.6	30.0(32.98)	**46.0(42.66)**	24.0 (29.24)	8.0 (12.83)	--(0.33)
0.8	10.0(16.45)	16.0(23.33)	10.0 (16.45)	-- (0.33)	--(0.33)
1.0	6.0(11.2)	10.0(16.45)	-- (0.33)	-- (0.33)	--(0.33)

CD (5%) = 11.81

The data was analyzed using Arc sine transformation and the values given in parenthesis are the transformed ones. The basal medium used was ½ MS with BA (0.3 mg l^{-1}).

4.4.3 Cell suspension culture

The embryogenic cell suspensions were established by culturing the embryogenic callus in ½ x MS liquid medium. Frequent replacement of the liquid medium at weekly intervals was essential for the continuous growth of the suspensions. Liquid medium containing higher levels of sucrose (60 g l^{-1}) and growth regulators like NAA (0.5 mg l^{-1}) and TDZ (0.3 mg l^{-1}) produced actively dividing cell suspensions (Plate 3C). Cell suspensions with a regeneration frequency of 20% were noticed in the cultures maintained in liquid medium for 2 weeks. The regeneration frequency improved to 33%, when the cell aggregates were cultured for 4 weeks. Suspension cultures with good regeneration potential was obtained at 5 weeks of culture in the liquid ½ x MS medium (67%) (Fig.7). The cell aggregates maintained beyond 5 weeks, showed a lower regeneration potential and therefore 5 week old cultures were selected for *Agrobacterium* infection. These suspensions showing good regeneration capacity were further sub cultured to fresh medium for 3 days and used as the target tissue for transformation.

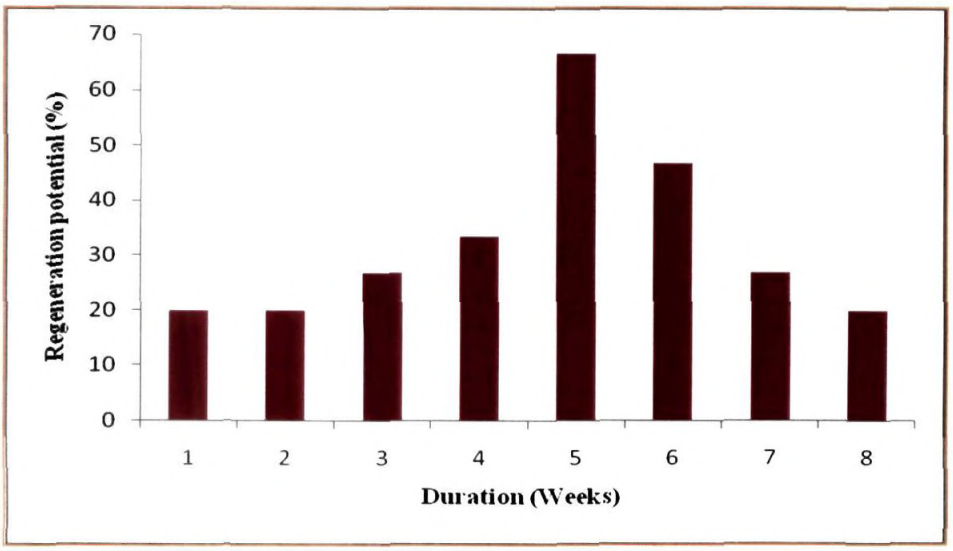


Fig.7. Regeneration potential of embryogenic suspensions at different time intervals

4.4.4 Embryogenic callus of zygotic origin

The embryogenic callus was obtained in Nitsch basal medium along with the developing embryos in a hormonal combination of zeatin (0.3 mg l^{-1}), kinetin (3.0 mg l^{-1}) and GA_3 (2.0 mg l^{-1}). The embryogenic callus obtained along with the developing zygotic embryos was separated and proliferated in half strength MS medium. The proliferation of the callus was obtained in a phytohormone combination of 2,4-D (0.3 mg l^{-1}), Kin (0.5 mg l^{-1}), NAA (0.3 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). The proliferated embryogenic callus of zygotic origin was used as the target material for genetic transformation studies (Plate. 3D).

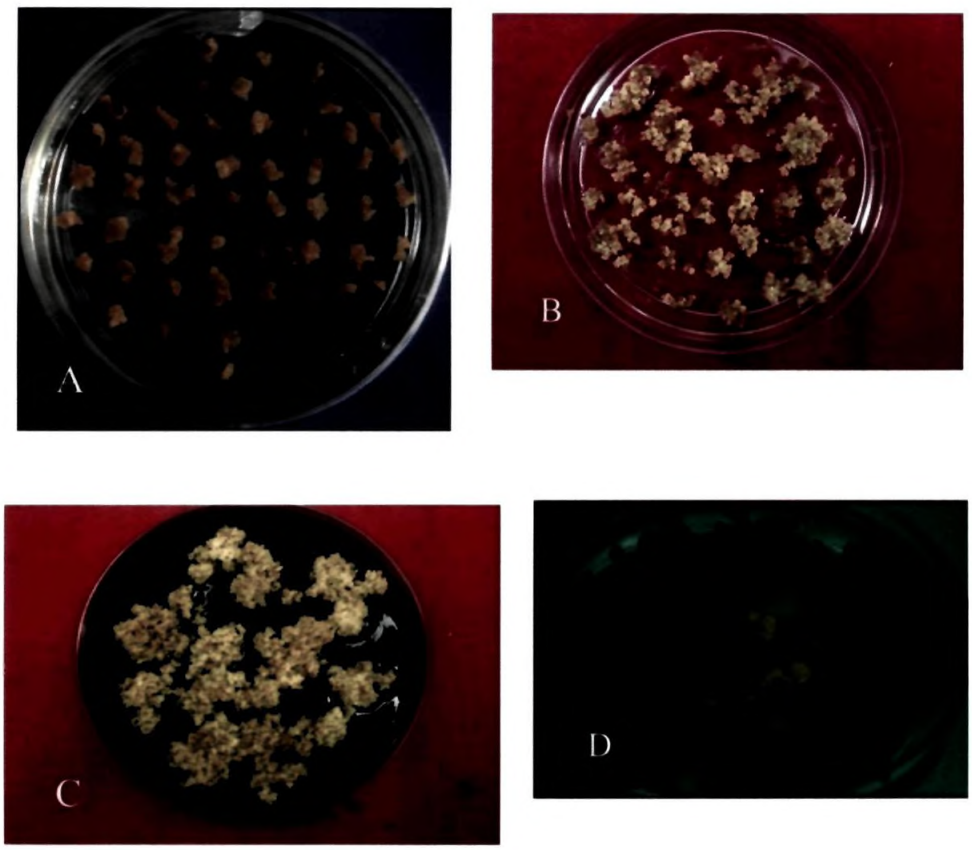


Plate 3 (A-D). Target tissues used for genetic transformation

A Primary callus

B Embryogenic callus

C Embryogenic suspensions

D Embryogenic callus of zygotic origin

4.5 Plant transformation protocol

4.5.1 Development of transgenic cell lines

The target tissues after *Agrobacterium* infection and a 3-day co-cultivation were transferred to the selection medium containing 500 mg l^{-1} cefotaxime and hygromycin 30 mg l^{-1}. Overgrowth-free cultures were obtained and these tissues were subcultured to fresh selection medium at monthly intervals. Transgenic cell emergence was observed after 50 days of culture.

4.5.1.1 Transformation efficiency- influence of the *Agrobacterium* strain and the target explant

The efficiency of transformation varied between the tested *Agrobacterium* strains where the succinamopine type of strain, EHA 105, gave the highest transformation efficiency with each kind of explant used. The results are presented in table 4.4. The observations showed that the strain EHA 105 gave a frequency of 9% (2.99) with the primary callus as the initial explant (Plate 4.A-B). The efficiency of transformation was increased to 15% (3.88) and 27% (5.19) using embryogenic callus (Plate 4.C-D) and embryogenic suspension cultures respectively (Plate 4. E-F). Genetic transformation using the embryogenic callus derived along with the zygotic embryos resulted in a very high frequency of transformation (67%) (8.18) (Plate 4.G). The experimental results showed that, irrespective of the target tissues used, highest frequency of transformation was obtained using EHA 105, which was the best strain.

Compared to EHA 105, the efficiency of transformation using the *Agrobacterium* strain pGV 1301 was lower with all the explants tried. Even with the most juvenile and responsive tissue (embryogenic callus of zygotic origin) the frequency of transformation recorded was 22% (4.67). Genetic transformation using the primary callus derived from anthers produced cell lines with a frequency of 7% (2.55) which was increased to 8% (2.78) on using

embryogenic callus of the anther. When the embryogenic suspensions were used as the target material for transformation, the frequency was further raised to 16% (3.99). Depending on the friability of the explant used for genetic transformation, the frequency varied, the most friable one producing the highest number of transformants. The third strain, LBA4404 responded poorly towards transformation and no transgenic cell line emerged after *Agrobacterium* infection. Hence this strain was considered as the least virulent one.

Table 4.4 Influence of *Agrobacterium* strain and explant type on transformation efficiency in *Hevea brasiliensis*

Target tissue used	Transformation frequency (%)		
	EHA 105	**pGV 1301**	**LBA 4404**
Primary callus of anther	9.0(2.99)	7.0 (2.55)	--
Embryogenic callus of anther	15.2(3.88)	8.0 (2.78)	--
Embryogenic suspensions of anther callus	27 (5.19)	16 (3.99)	--
Embryogenic callus of zygotic origin	67(8.18)	22 (4.67)	--
CD (5%)	**0.58**	**0.81**	

Twenty-five callus clumps were used per treatment replicated four times. The values given in parenthesis are the transformed values

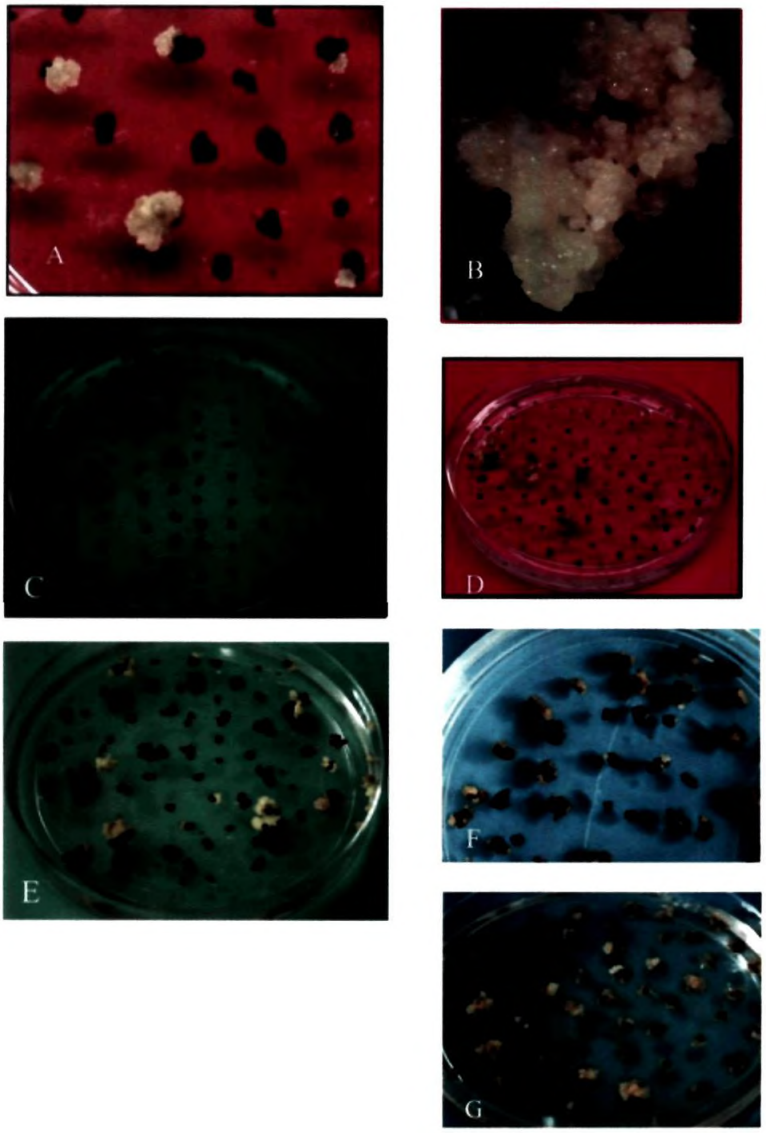


Plate.4 (A-G) Emerging transgenic cell lines from the infected callus of *Hevea*

A-B. Transgenic cell lines from primary callus

C-D. Transformed cell lines from embryogenic callus

E-F. Transgenic cell lines from suspension cultures

G. Transgenic cell lines from embryogenic callus of zygotic origin

4.5.1.2. Factors improving the frequency of transformation

Initial experiments proved *Agrobacterium* strain EHA 105 as the best strain and embryogenic suspension cultures derived from immature anther cultures as the most suitable clonal explants for transformation. Hence using this strain and explant, experiments were conducted to assess the impact of compounds which are beneficial in improving the transformation frequency.

4.5.1.2a Pre-culture of explant

Agrobacterium infected tissues which were exposed to the anti-necrotic mixture for 1 to 10 hrs failed to improve the transformation frequency. Compared to the control cultures, transgenic cell emergence in the treated cultures was at a frequency of 4%. When the exposure time of the anti-necrotic treatment was increased from 2 to 10 hrs and infected with *Agrobacterium*, the calli turned white within a few days of culture in the selection medium which eventually dried up (Table. 4.5). Thus it can be concluded that the anti-necrotic pretreatment failed to improve the efficiency of transformation in *Hevea* with the *hmgr*1 gene.

4.5.1.2b Explant desiccation

Desiccation of the explants after *Agrobacterium* infection proved to be ineffective in accelerating the frequency of transformation with the *hmgr*1 gene in *Hevea brasiliensis*. The desiccation of the infected tissues from 1 to 5 hrs, followed by a 3-day co-culture, resulted in tissue blackening though they remained viable and healthy. Transgenic cell emergence was minimum in these cultures upon transfer to the selection medium (Table.4.5). Prolonged desiccation of the callus (beyond 6 hrs) led to callus drying. Transgenic cell emergence was absent from these cultures indicating the inefficiency of these treatments on elevating the transformation frequency.

4.5.1.2c Influence of thiols and $AgNO_3$

The influence of the thiol compound, L-cysteine and $AgNO_3$ on ameliorating transformation frequency was assessed. The tissues had a fresh appearance during the initial weeks of culture when treated with lower levels of L-cysteine and with $AgNO_3$. But after the initial vigor, they slowly turned black. A further increase in the concentration of L-cysteine resulted in drying of the infected tissues in the selection medium. Similar effect was observed with silver nitrate also. Transgenic cell emergence was minimum in the cultures exposed to this modified medium (Table. 4.5).

4.5.1.2d Effect of incubation temperature

Table 4.5 describes the combined effect of incubation temperature and acetosyringone (AS) on transformation frequency. The *Agrobacterium* infected tissues were co cultured for three days in the co cultivation medium at different temperature regimes (4°C, 20°C and 28°C) and transferred to selection medium. Among the temperatures tried, incubation at 20°C was more appropriate for the emergence of transgenic cell lines in presence of acetosyringone (20 mg l^{-1}). Co-cultivation of the infected callus at 28°C in acetosyringone containing medium resulted in the formation of transgenic cell lines at a frequency of 27%, which was the control treatment. Exposure of the infected tissues to a lower incubation temperature (20°C) enhanced the efficiency of transformation from 27 to 32%, in presence of acetosyringone. But in the absence of acetosyringone, a drastic reduction in the frequency of transformation was observed in the cultures incubated at 20°C (6%) and 28°C (7%). Thus in general, low temperature induced increase in the transformation efficiency was depended on the presence of acetosyringone in the medium. Incubation at 4°C resulted in overgrowth free cultures but the infected calli became white in color after one week of culture indicating the death of the tissue. Considering these parameters, it can be stated that genetic transformation using the *hmgr*1 gene was more

effective at a lower incubation temperature (20°C) culminating in the production of new transformed cell lines.

4.5.2 Multiplication of the cell lines

Callus proliferation rate of the putatively transgenic lines varied with the explant used for transformation. The transformed cell lines obtained from the embryogenic suspension cultures and from the embryogenic callus of zygotic origin showed good proliferation in CCM_2 (Appendix B) fortified with growth regulators 2,4-D (0.5 mg l^{-1}), NAA (0.2 mg l^{-1}) and Kin (0.5 mg l^{-1}). The proliferated callus obtained from embryogenic suspensions and the embryogenic callus of zygotic origin had a friable texture (Plate 5A, B).

Table 4.5. Effect of different treatments on transformation efficiency of the *Agrobacterium* strain EHA 105 (%)

Sl. No	Treatments	Transformation efficiency (%)
1.	Control (28°C)	**27**
2.	Anti-necrotic mixture	4.8 (12.52)
3.	Desiccation	4.0 (4.81)
4.	L-cysteine and silver nitrate	5.6 (13.49)
5.	Incubation temperature (4°C) (with acetosyringone)	- (0.33)
6.	Incubation temperature (4°C) (without acetosyringone)	- (0.33)
7.	Incubation temperature (20°C) (with acetosyringone)	32 (34.43)
8.	Incubation temperature (20°C) (without acetosyringone)	5.6 (13.49)

CD (5%) = 3.79

Analysis was carried out using Arc sine transformation and the transformed values were given in parenthesis

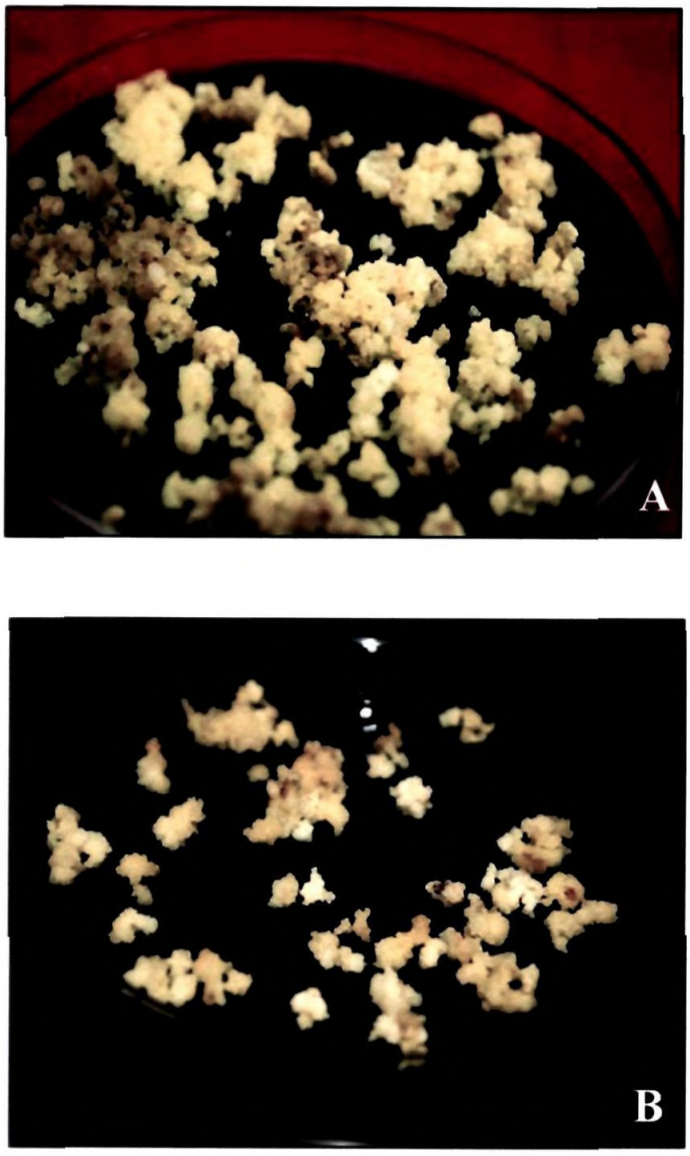


Plate 5 (A-B). Proliferated transgenic callus

A- Transgenic calli of zygotic origin

B- Transgenic calli derived from suspension cultures

4.6 Molecular characterization of the transgenic callus

4.6.1 Primers used for the amplification of the transgenic tissues

Three sets of primers were designed, the promoter specific, gene specific and marker specific for amplifying the transgene in the transformed tissues. The promoter specific primer helped to rule out the endogenous *hmgr*1 amplification. The size was expected to be approximately 1.9 kb. The gene specific primers were designed to amplify a smaller fragment of size approximately 640 bp in the transgenic tissues. Marker specific primers were used to amplify a fragment of length 602 bp in the transgenic tissues.

4.6.2 & 3 Genomic and plasmid DNA isolation

Good quality DNA without RNA contamination could be extracted from the transformed as well as untransformed cell lines using the CTAB method. Agarose gel electrophoresis confirmed the quality of the DNA and the concentration was calculated to be around 1µg /µl. Plasmid DNA isolated from the *Agrobacterium* culture was used as the positive control in the subsequent experiments.

4.6.4. Determination of transformants by PCR

PCR analysis using the promoter specific forward and HMGR specific reverse primers amplified a fragment of approximately 1.9 kb in the transformed cell lines and also in the positive control (Fig. 8A). The corresponding band was absent in the non-transformed callus. The use of the primers from the promoter region rules out the possibility of endogenous HMGR amplification. The presence of the integrated *hmgr*1 transgene was confirmed in all the transgenic cell lines tested and the positive control (plasmid DNA), where a fragment of length 1.9 kb was amplified. PCR analysis using the marker specific primers (*hpt*), amplified a fragment of approximately 602 bp in size in the transgenic cell lines and in the plasmid DNA (Fig.8B).

The corresponding band was absent in the non-transformed cell lines. positive amplification in the transgenic cell lines indicated the presence of *hmgr*1 transgene in the genome of the *Hevea* transformed tissues.

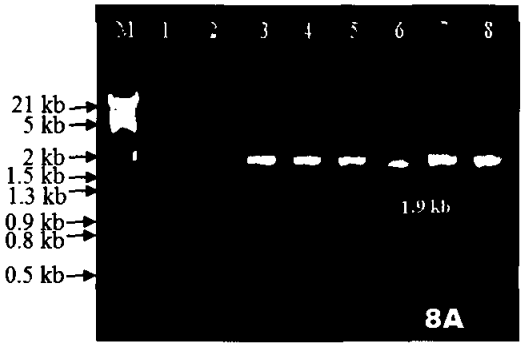
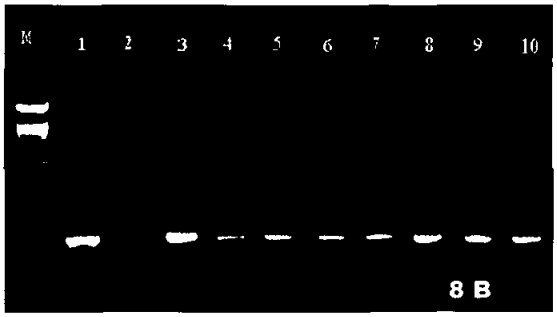


Fig. 8. PCR amplification of transformed callus

A. Using promoter specific forward and gene specific reverse primer

M - Marker (λ-Hind III/BamH1)
Lane 1 - Positive control
Lane 2 - Negative control
Lane 3-8 - Transgenic cell lines



B. Amplification using *hpt* specific primers

M - Marker (λ-Hind III/BamH1)
Lane 1 - Positive control
Lane 2 - Negative control
Lane 3-10 - Transgenic cell lines

4.7 Somatic embryogenesis from the transformed cell lines

The PCR positive friable embryogenic callus from the suspension cultures and from the zygotic material was subcultured for embryo induction.

4.7. 1 Effect of basal salts on somatic embryogenesis

The active role of the basal salts on somatic embryogenesis was evaluated in the present experiment. The rate of embryogenesis from the proliferated cell lines (Plate 5 A-B) in different media combinations were summarized in Table 4.6. The basal medium AG_1, a modified version of MS was optimal for the induction of somatic embryos. When MS basal medium was used for somatic embryogenesis, the frequency of embryogenesis was low (15%). A reduction in the NH_4NO_3 concentration of MS medium from 1650 mg l^{-1} to 200 mg l^{-1}, $MgSO_4$ concentration from 370 mg l^{-1} to 90mg l^{-1}, with a subsequent rise in the $KN0_3$ concentration from 1.9 g l^{-1} to 2.0 g l^{-1} improved the embryo induction frequency to 39% in modified MS medium, denoted as AG_1. The frequency of embryogenesis in the Woody Plant Medium was 29%.

4.7.2 Polyamines and somatic embryogenesis

The exogenous application of different polyamines clearly indicated the promotive effect of spermidine on somatic embryogenesis. The individual effect of polyamines was compared in Table 4.7 where putrescine, spermine and spermidine at concentrations 1.0 - 5.0 mg l^{-1} were included in the embryo induction medium. Addition of putrescine failed to show a positive response on somatic embryogenesis. Inclusion of spermine and spermidine accelerated somatic embryogenesis. But compared to the control treatment, spermidine was the most effective polyamine triggering somatic embryogenesis. Experimental results showed maximum embryo induction of 41% in the medium fortified with 2.0 mg l^{-1}spermidine. Higher levels of spermidine (greater than 2.0 mg l^{-1}), failed to improve somatic embryogenesis. Spermine at 2.0 mg l^{-1} induced

somatic embryogenesis at frequency of 27%. Further increase in the spermine concentration reduced the frequency of embryogenesis. Thus among the three polyamines tried, spermidine was efficacious in accelerating somatic embryogenesis.

4.7.3 Organic nitrogen sources on somatic embryogenesis.

The effect of six amino acids on somatic embryogenesis has been summarized in Table 4.8. Among the varying concentrations of amino acids experimented, maximum embryo induction was observed with L-alanine, followed by L-asparagine, L-glutamine and L-serine. Inclusion of L-arginine and L-proline also promoted somatic embryogenesis. Addition of L-alanine induced somatic embryogenesis at a frequency of 40.96%, followed by L-asparagine (39.22%), L-proline (38%), L-glutamine (37.44%) and L-serine (27.94 %). The incorporation of amino acids [L-alanine 100 mg l^{-1}, L-glutamine 400 mg l^{-1}L-asparagine 300 mg $l^{-1}$and L proline 100 mg l^{-1}] in the embryo induction medium improved the efficiency of embryogenesis in the transgenic cell lines.

Table 4.6. Influence of basal salts on somatic embryogenesis from the transgenic callus of *Hevea brasiliensis*.

Treatments	* Embryo induction frequency (%)
MS	15±0.447
AG_1 (Modified MS)	**39.3±0.836**
Woody Plant Medium	29±1.303

**The embryo induction frequency is represented as mean ± standard deviation of the percentage obtained from the five replications. Each treatment contained 30 callus clumps*

Table 4.7. The influence of polyamines on somatic embryo induction from the transgenic cell lines of *Hevea brasiliensis* (%)

Treatment	Conc. of polyamines (mg l^{-1})				
	1.0	**2.0**	**3.0**	**4.0**	**5.0**
Putrescine	3.0 (9.92)	6.4 (14.61)	1.25 (5.14)	-- (0.33)	-- (0.33)
Spermine	22.6(28.37)	27.4 (31.56)	20.4(26.84)	10.6(18.97)	3.2(10.23)
Spermidine	36.4(37.10)	**41.4(40.05)**	28.0(31.95)	10.4(18.79)	2.4 (8.7)

CD (P<0.05) =1.68

The basal medium was AG_1 and each treatment contained twenty five callus clumps replicated five times. Analysis was carried out using Arc sine transformation and the transformed values were given in parenthesis

4.7.4 Role of phytohormones on somatic embryogenesis

The influence of growth regulators on somatic embryogenesis has been depicted in Table 4.9 and 4.10, where modified MS basal medium (AG_1) was used along with the best combination of amino acids (L-alanine 100 mg l^{-1}, L-asparagine 300 mg l^{-1}, L-glutamine 400 mg l^{-1} and L-proline 100 mg l^{-1}). Experiments carried out using different levels of zeatin (0.5-2.5 mg l^{-1}) and NAA recorded an embryo induction frequency of 60% with 1.0 mg l^{-1}zeatin and 2.0 mg l^{-1} NAA (Table 4.9). NAA concentration of the medium strongly influenced the embryo induction efficiency. The highest embryo induction frequency was observed when 2.0 mg l^{-1}NAA was used in combination with zeatin (1.0 mg l^{-1}). Lower levels of zeatin induced embryogenesis from the embryogenic cell masses at a very low frequency. Addition of NAA to the medium containing zeatin gradually improved the frequency of embryogenesis, maximum level being observed at 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} zeatin. Further increase in the concentration of both the phytohormones reduced the efficiency of embryogenesis.

Table 4.8. Efficacy of amino acids on somatic embryo induction in *Hevea brasiliensis*.

	Aminoacids	Concentration (mg l^{-1})	Embryo induction[1](%)
Control	Nil	Nil	39 %
1.	L-Asparagine	50	24.30^{d}±0.93
		100	34.48^{b}±1.57
		200	38.02^{a}±1.52
		300	39.22^{a}±0.93
		400	29.96^{c}±1.05
2.	L-Arginine	100	28.63^{a}±0.84
		200	32.49^{a}±1.60
		300	28.51^{a}±1.78
		400	23.43^{b}±1.51
		500	21.05^{b}±1.07
3.	L-Alanine	50	34.40^{b}±1.25
		100	40.96^{a}± 1.15
		150	35.58^{b}±2.01
		200	29.96^{c}±1.05
4.	L-glutamine	100	30.60^{bc}±1.24
		200	33.13^{ab}±1.69
		400	37.44^{a}±1.20
		600	33.13^{ab}±1.69
		800	27.87^{cd}±1.43
		1000	24.11^{d}±1.95
5.	L-serine	10	27.18^{b}±1.34
		20	33.18^{a}±0.99
		30	27.94^{b}±0.84
		40	22.67^{c}±1.29
		50	21.05^{c}±1.07
6.	L-proline	50	30.60^{bc}±1.24
		100	38.02^{a}±1.52
		150	33.74^{ab}±1.80
		200	26.50^{cd}±1.14
		250	24.87^{d}±1.97

Means followed by a common letter are not significantly different at $p<0.05$ by DMRT. The data represent transformed values (Arc sine transformation) ± *SE.*

[1]Twenty embryogenic groups were cultured per treatment and each experiment was replicated five times

Table 4.9. Effect of NAA and zeatin on induction of somatic embryos from the putatively transgenic callus of *Hevea brasiliensis* (%)

Conc.of Zeatin(mg l^{-1})	Conc. of NAA(mg l^{-1})					
	0.5	**0.75**	**1.0**	**1.5**	**2.0**	**2.5**
0.5	13(3.74)	16(4.12)	18(4.35)	23(4.90)	33(5.83)	24(5.00)
0.75	14(3.86)	17(4.23)	25(5.10)	38(6.24)	46(6.85)	15(4.00)
1.0	16(4.12)	18(4.35)	35(6.00)	44(6.71)	**60(7.81)**	12(3.59)
1.5	13(3.74)	15(3.98)	18(4.35)	14(3.86)	32(5.74)	--(1.00)
2.0	6(2.62)	13(3.74)	11(3.44)	14(3.86)	27(5.29)	--(1.00)
2.5	--	--	--	--	--	--

CD (5%) = 0.63

The data was analyzed using square root transformation and the transformed values were given in parenthesis. Basal medium was AG_1 +Alanine+glutamine +Asparagine+proline

Table 4.10. Influence of Kinetin and NAA on somatic embryogenesis from the transformed cell lines (%).

Conc.of NAA (mg l^{-1})	Conc. of Kinetin (mg l^{-1})				
	0.3	**0.5**	**0.7**	**0.9**	**1.0**
0.25	8.0(16.2)	12.0(20.15)	15.0(22.68)	19.0(25.8)	12.0(20.15)
0.5	11.0(19.3)	22.0(27.87)	27.0(31.3)	29.0(32.56)	20.0(26.52)
0.75	31.0(33.8)	33.0(35.05)	45.0(42.14)	51.0(45.6)	32.0(34.45)
1.0	48.0(43.87)	55.0(47.9)	**62.0(52.04)**	48.0(43.87)	25.0(29.9)
1.25	17.0(24.25)	20.0(26.52)	23.0(28.62)	12.0(20.15)	6.0(13.99)

CD (5%) = 5.25

The data was analyzed using Arc sine transformation and the transformed values were given in parenthesis

The interactive effect of kinetin with NAA was found to be better than zeatin, NAA combinations in improving the frequency of embryogenesis. When kinetin was used along with NAA, the embryo induction frequency improved further. Even lower levels of kinetin stimulated embryo induction in the transgenic cultures when used along with higher concentration of NAA. The embryo induction medium containing reduced levels of kinetin and NAA produced somatic embryos at a lower frequency. But when the concentration of NAA was increased, a gradual rise in the embryo induction efficiency was noticed. The embryo induction medium containing kinetin (0.5mg l^{-1}) along with 1.0 mg l^{-1} NAA produced somatic embryos at a frequency of 55%. A further rise in the kinetin concentration from 0.5 to 0.7 mg l^{-1}augmented embryo induction frequency up to 62 % (Table 4.10). Thus modified MS basal medium containing kinetin (0.7 mg l^{-1}) and NAA (1.0 mg l^{-1}) produced somatic embryos at a frequency of 62% and therefore this growth regulator combination were used in subsequent experiments.

4.7.5 Effect of spermidine and sucrose on somatic embryogenesis

Table 4.11 describes the combined effect of sucrose (50-90 g l^{-1}) and spermidine (0 - 2.5mg l^{-1}) on somatic embryogenesis. An increase in the sucrose concentration from 50 g l^{-1} to 80 g l^{-1} tremendously augmented the embryo induction percentage. Although individual application of spermidine (2.0 mg l^{-1}) induced globular embryos (41%), the frequency of embryogenesis was increased reaching a maximum of 72% when 1.5 mg l^{-1}spermidine was used along with higher concentration of sucrose (80 g l^{-1}). Here spermidine induced somatic embryogenesis at a lower concentration of 1.5 mg l^{-1} from the proembryogenic masses cultured (Plate 6 A-F) (Table 4.11). Further increase in the sucrose concentration (90 g l^{-1}) resulted in callus proliferation thereby decreasing the embryo induction efficiency (63%).

Table 4.11. The influence of spermidine and sucrose on embryo induction (%)

Conc. of Sucrose ($g\ l^{-1}$)	Conc. of Spermidine ($mg\ l^{-1}$)					
	0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
50	32(5.65)	40(6.32)	55(7.39)	67(8.24)	51(7.11)	32(5.65)
60	37(6.10)	43(6.52)	60(7.74)	68 (8.24)	59(7.65)	40(6.30)
70	39(6.20)	49(7.01)	57(7.56)	61(7.83)	49(7.01)	28(5.28)
80	31(5.54)	36 (5.99)	60(7.74)	**72(8.48)**	48(6.92)	21(4.61)
90	29(5.40)	31(5.52)	47(6.81)	63(7.92)	28(5.28)	16(3.98)

CD (5%) = 0.64

The experiment contained 25 callus/ treatment with four replications. Analysis was carried out using square root transformation and those given in parenthesis are the transformed values.
The basal medium was AG_1+alanine+glutamine+asparagine+proline +Kin+NAA.

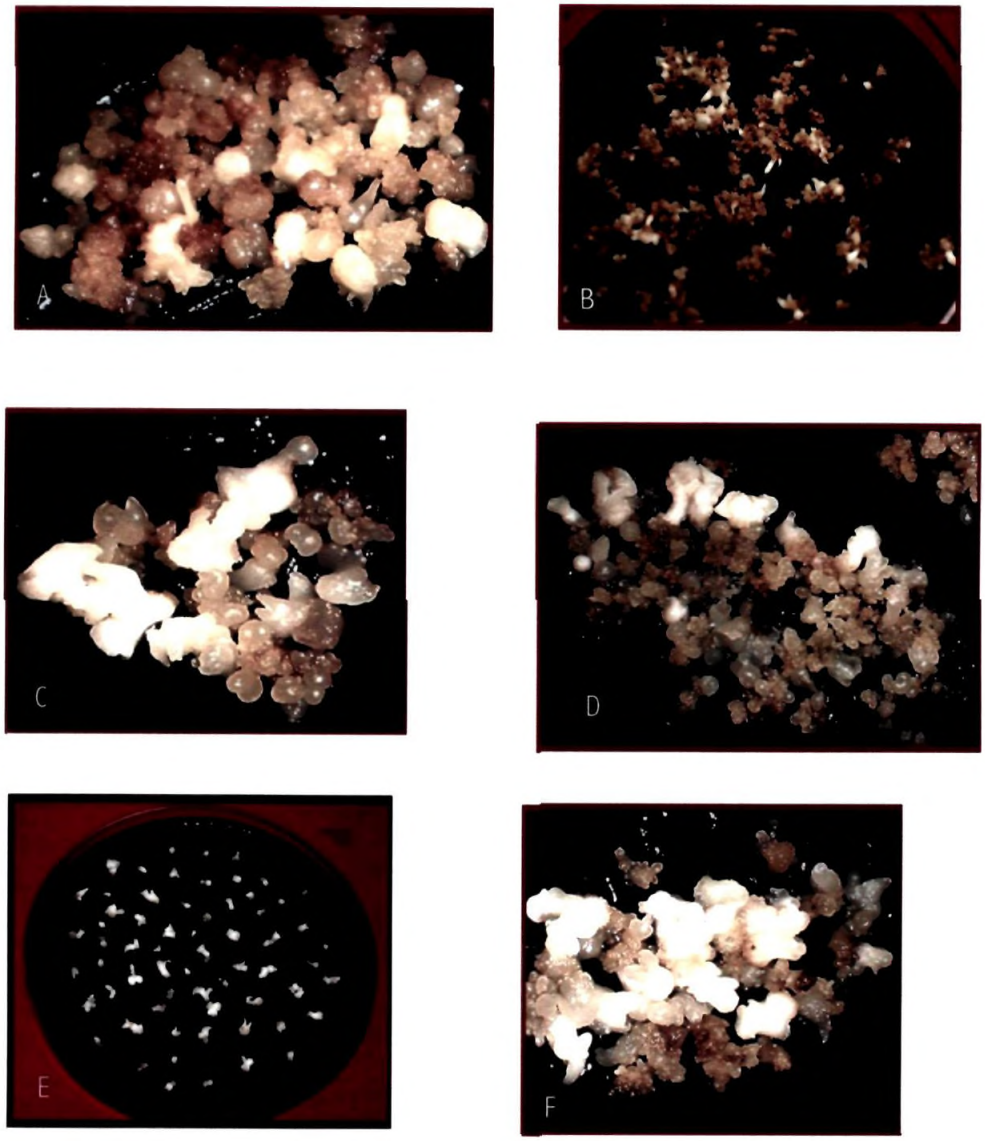


Plate 6 (A-F). Somatic embryogenesis from the transgenic cell lines

4.8. Maturation of somatic embryos

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

Table 4.12 illustrates the effect of oxidized/reduced nitrogen on somatic embryo maturation. The omission of NH_4NO_3 from the maturation medium (MSO), with a subsequent rise in the KNO_3 concentration (3.0 g l^{-1}) accelerated maturation of somatic embryos. When half strength MS medium was used for somatic embryo maturation, the efficiency of maturation was 19%. When the KNO_3 concentration of MS medium was increased to 3.0 g l^{-1} with the simultaneous omission of NH_4NO_3, embryo maturation could be increased to 25%. Using Woody Plant Medium (WPM), maturation of the embryos was obtained at a frequency of 13%.

Organic nitrogen sources like glutamine and proline, triggered somatic embryo maturation (Table 4.13.). Among the different levels of glutamine (100 mg l^{-1} - 1.0g l^{-1}) and proline (50 – 250 mg l^{-1}) used, glutamine (1.0 g l^{-1}) and proline (200 mg l^{-1}) helped in the maturation of somatic embryos. Compared to the control treatment, the frequency of maturation was improved by the inclusion of L glutamine, reaching a maximum of 35%.

Table 4.12. Effect of basal medium on somatic embryo maturation in *Hevea brasiliensis*

Treatments	Maturation efficiency (%)
Half strength MS	19 (4.34)
MSO	**25 (4.99)**
WPM	13 (3.57)

CD (0.05) = 0.67

Twenty globular embryos were cultured in each treatment and the experiment was replicated five times. The analysis was done using square root transformation and the transformed values were given in parenthesis

Table 4.13. Effect of amino acids on somatic embryo maturation and germination in *Hevea brasiliensis*

Sl No.	Amino acids	Conc (mg l^{-1})	Embryo maturation[1](%)	Embryo germination[2](%)
Control	Nil	nil	25%	
1.	L-Asparagine	50		1.93^{c}± 0.57
		100		2.39^{bc}±0.57
		200		3.57^{ab}±0.25
		300		4.27^{a}±0.43
		400		1.93^{c}±0.57
2.	L-glutamine	100	11.51^{d}±2.99	1.93±0.57
		200	16.23^{d}±1.35	2.39±0.57
		400	22.56^{c}±1.82	3.11±0.58
		600	27.18^{bc}±1.34	3.36±0.65
		800	30.60^{ab}±1.24	4.08±0.31
		1000	35.05^{a}±0.75	3.11±0.58
3.	L-proline	50	17.10^{d}±1.88	2.39±0.57
		100	23.43^{bc}±1.51	1.93±0.57
		150	27.25^{ab}±0.69	2.64±0.71
		200	31.21^{ab}±1.65	1.93±0.57
		250	21.80^{c}±1.54	1.46±0.46

Means followed by a common letter are not significantly different at $p<0.05$ by DMRT. The data represent transformed values± SE.

[1] Twenty embryos were used for each treatment with five replications

[2] Ten matured embryos were cultured per treatment and the experiment was replicated five times.

4.8.2 Effect of stress-inducing compounds on maturation of somatic embryos

The effect of stress inducing compounds on somatic embryo maturation was shown in Table 4.14. Here the individual effect of sorbitol and PEG on somatic embryo maturation was compared. Among the different stress-inducing compounds used for maturation, PEG was optimal giving fairly good response followed by ABA (data not given). PEG as a non-penetrating (apoplastic) osmoticum enhanced the conversion of globular embryos to the torpedo staged ones. When used alone, PEG stimulated the formation of torpedo staged somatic embryos. A gradual increase in the PEG concentration enhanced the maturation efficiency of the embryos. Maturation frequency was maximum (28%) in presence of 12% PEG. Further increase in the PEG concentration reduced the maturation percentage of the embryos. Inclusion of sorbitol produced matured somatic embryos at a frequency of 19% but compared to the control treatment the frequency of maturation was less. Addition of sorbitol (3.0%) assisted in the conversion of the embryos from the globular to early torpedo stage, after which they failed to mature. Continuous exposure resulted in the senescence of the embryos. Thus among the two stress inducing agents tried on maturation, PEG was optimal in increasing frequency of maturation of the somatic embryos compared to sorbitol which showed a negative effect on embryo maturation.

Earlier studies with ABA proved its ameliorating effect on conversion of the embryos from globular to the late torpedo shaped ones. Though sorbitol alone did not have a promotive effect on embryo maturation, the joint effect of sorbitol with ABA was tried to see whether sorbitol in presence of ABA can improve maturation of the somatic embryos (Table 4.15). From the table it can be inferred that the combined use of sorbitol and ABA reduced the embryo maturation frequency to 10% from the earlier obtained efficiency of 19% when sorbitol alone was tried. The results proved the inefficiency of sorbitol on

maturation of somatic embryos when used alone or in combination with ABA. The combined effect of ABA with PEG was also assessed which indeed helped in the advancement of the embryos from the late torpedo to the cotyledonary stage.

Table 4.16 describes the joint effect of PEG (0, 6, 8, 10, 12 and 14%) and ABA (0 – 0.8 mg l^{-1}) (Plate 7A-C; Plate 8 A). When supplied alone, ABA (0.4 - 0.6 mg l^{-1}) stimulated somatic embryo maturation at a frequency of 24%. Addition of PEG to the ABA containing medium enhanced the embryo maturation efficiency to a maximum of 48%. The optimum concentration of ABA was 0.4 mg l^{-1} since this concentration accelerated maturation of embryos at all concentrations of PEG (6 -12%). Here the addition of ABA to the PEG medium doubled the maturation frequency from 28% (which was the frequency when PEG alone was used) to 48%. When the ABA concentration of the medium was increased further, a decrease in the maturation frequency was noticed. Thus the embodiment of the stress inducing agents helped in the conversion of the somatic embryos from the late torpedo to the cotyledon stage. The sucrose concentration of the maturation medium was 30 g l^{-1}. Though maturation frequency differences were not observed between the embryos of clonal and zygotic origin, visually the zygotic embryos were big and healthy with well-developed cotyledons.

Table 4.14. Effect of osmotic agents (sorbitol and polyethylene glycol) on maturation of somatic embryos

Conc. of Sorbitol (%)	Maturation (%)	Conc. of Polyethylene glycol (%)	Maturation (%)
1.5	6.67 (2.62)	6	10(3.29)
2.0	11.33(3.42)	8	18(4.11)
2.5	14.67(3.88)	10	24.4(4.94)
3.0	**19.3 (4.44)**	12	**28.4(5.33)**
3.5	8.00 (2.89)	14	5.00(2.21)
4.0	-- (0.71)		

CD (5%) = 0.66 **0.51**

Each experimental unit contained 25 embryos in MSO basal medium replicated six times. Data was analyzed using square root transformation and the transformed values were given in parenthesis.

Table 4.15. Influence of sorbitol and ABA on transgenic embryo maturation

Conc. of ABA(mg l^{-1})	Somatic embryo maturation (%)					
	Conc. of Sorbitol (%)					
	1.5	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**
0.2	--(1.00)	4.0(2.12)	9.0(3.15)	--(1.00)	--(1.00)	--(1.00)
0.4	4.0(2.12)	8.0(2.96)	**10.0 (3.3)**	--(1.00)	--(1.00)	--(1.00)
0.6	10.0(3.24)	6.0(2.62)	5.0(2.31)	--(1.00)	--(1.00)	--(1.00)
0.8	7.0(2.8)	--(1.00)	--(1.00)	--(1.00)	--(1.00)	--(1.00)

CD (5%) = 0.82

Data was analyzed using square root transformation and the transformed values are given in parenthesis

4.9. Effect of desiccation on somatic embryo germination

It was observed that partial drying of the embryos (24 hrs) on a laminar flow hood was beneficial for bipolar differentiation upon transfer to the germination medium. Here the rate of drying mainly depended on the stage of the treated embryos. Partial drying was optimum for the induction of shoot meristem when the embryos were at an early cotyledonary stage. The influence of partial desiccation was obvious in the case of the embryos obtained from the clonal material whereas the embryos of zygotic origin germinated even without the desiccation treatment. On the other hand desiccation of the embryos using saturated levels of Ca $(NO_3)_2.4H_2O$ (2 to 6 hrs), failed to germinate, and the embryos gradually senesced after the treatment.

Table 4.16. Effect of PEG and ABA on somatic embryo maturation (%)

Conc.of ABA (mg l^{-1})	Conc. of PEG (%)					
	0	6	8	10	12	14
0	6 (2.62)	13(3.71)	19(4.46)	22(4.78)	25(5.09)	11(3.45)
0.2	11(3.43)	25(5.09)	26(5.18)	33(5.82)	35(5.99)	21(4.68)
0.4	24(4.99)	32(5.74)	39(6.32)	41(6.47)	**48(7.00)**	22(4.79)
0.6	24(4.99)	28(5.38)	25(5.09)	22(4.79)	19(4.46)	14(3.83)
0.8	11(3.43)	14(3.86)	13(3.70)	--(1.00)	--(1.00)	--(1.00)

CD (5%) = 0.55

The experimental unit contained 25 embryos replicated four times. Embryos were cultured in MSO medium with glutamine + proline. Data was analyzed using square root transformation and the transformed values were given in parenthesis

4.10. Regeneration of transgenic plants

The germination of the partially desiccated somatic embryos was accomplished in ¼th MS basal medium containing double strength MS minor elements. Addition of KNO_3 (2.0 g l^{-1}) and K_2SO_4 (400 mg l^{-1}) in the basal medium promoted plant regeneration. Table 4.13. describes the beneficial effect of amino acids on embryo germination. The organic nitrogen supplied by the addition of L-asparagine (300 mg l^{-1}), L-glutamine (800 mg l^{-1}) and proline (150mg l^{-1}) favored plant regeneration. The germination commenced one month after culture in the respective medium, but the germinating plantlets were maintained for two months for the complete development of the leaves. Somatic embryos showing abnormal development was also visualized in the medium.

Table 4.17 depicts the influence of phytohormones on plant regeneration. Inclusion of phytohormones BA (1.0 mg l^{-1}) and GA_3 (0.8 mg l^{-1}) stimulated germination of the somatic embryos. Further addition of IAA (0.3 mg l^{-1}), increased the plant germination percentage reaching a maximum of 5% (Table 4.17) (Plate 7D). Inclusion of GA_3 in the germination medium induced rapid germination of the embryoids to plantlets. Although plantlet development was observed at 2% sucrose, healthy plantlets with normal leaf development were noticed in medium containing 3% sucrose. Plant regeneration from the somatic embryos derived from the callus of zygotic origin utilized slightly lower levels of growth hormones for germination. A very high regeneration efficiency (44%) was exhibited by these embryos where a combination of BA (0.75 mg l^{-1}), GA_3 (0.6 mg l^{-1}) and IAA (0.2 mg l^{-1}) favored plant regeneration (Table 4.17) (Plate 8 A-B). Plant regeneration efficiency was very high in the case of the embryos obtained from the zygotic material and the transgenic plants were very healthy and showed vigorous growth. Very good rooting system was seen in these embryos. But the clonal material showed a low plant regeneration efficiency and some of the plantlets were abnormal.

4.11. *Ex vitro* performance of the regenerants

The transgenic plantlets were transferred from the *in vitro* to *ex vitro* conditions, when the leaves of the plantlets matured. They were initially transferred to earthenware pots and kept under controlled conditions in a growth chamber (Plate 9.A). The plantlets in the earthenware pots were watered on alternate days and moistened with ½ x Hoagland mixtures (Hoagland and Arnon, 1950) at weekly intervals. The plantlets were maintained under controlled conditions for 3 to 4 weeks. The emergence of new flushes can be viewed as the sign of acclimatization. After the emergence and maturation of the newly formed leaves (2 to 3 whorls), the plantlets were transplanted to big poly bags (9 ½"x 22") filled with soil: sand: cow dung in the ratio 2:1:1 (Plate 9.B-C). One week after transplantation, the polybags containing the plantlets were transferred to the net house under ambient conditions (Plate 9.D).

Table 4.17. The effect of phytohormones (BA, GA_3 and IAA) on plant regeneration

Growth hormones (mg l^{-1})			Plant regeneration (%)	
BA	**GA_3**	**IAA**	**Anther derived embryos**	**Polyembryony derived embryos**
0.5	0.5	0.1	1.6 (1.21)	24 (4.94)
0.75	0.6	0.2	3.3(1.29)	**44 (6.66)**
1.0	0.8	0.3	**5 (1.57)**	33 (5.77)
1.5	1.0	0.4	2.4 (1.31)	9 (2.97)
2.0	1.25	0.5	--(1.00)	4 (2.08)
			CD (5%) 0.34	**0.58**

The data was analyzed using square root transformation and the transformed values are given in parenthesis

Plate -7 (A-G) Embryo maturation and plant regeneration from clonal explants

A-C Embryos at different stages of maturation

D-G Germinating embryos and regenerating plantlets

Plate-8 (A-B) Embryo maturation and plant regeneration from zygotic material

A-Stages of embryo maturation

B- Regenerating plantlets

4.11.1 Difficulties encountered during hardening of somatic plants of clonal origin

The influence of the initial explant on normal plant regeneration as well as hardening was evident from the experimental results. The percentage survival of the transgenic plants raised from the callus of zygotic origin was 45% whereas the survival rate of the anther derived plants were low. Significant changes during embryogenesis and maturation were not observed among the transgenic cell lines obtained from different explants. But the zygotic material derived embryos were larger with well-developed cotyledons. The plant regeneration frequency varied between the clonal and zygotic material and a very high plant conversion rate was observed in the zygotic embryo derived cultures (Plate 8B). These plants had a good rooting system with more number of lateral roots assisting hardening process. Forty five plantlets were successfully hardened and maintained under the shade net (Plate 9. D). The anther derived plants failed to survive the hardening phase and efforts are progressing to germinate normal plantlets so that they can be easily acclimatized.

Plate 9. A-D Hardening of transgenic plants

A Plants in the earthenware pots

B, C Transgenic plants in polybags

D Acclimatized plants in the containment facility

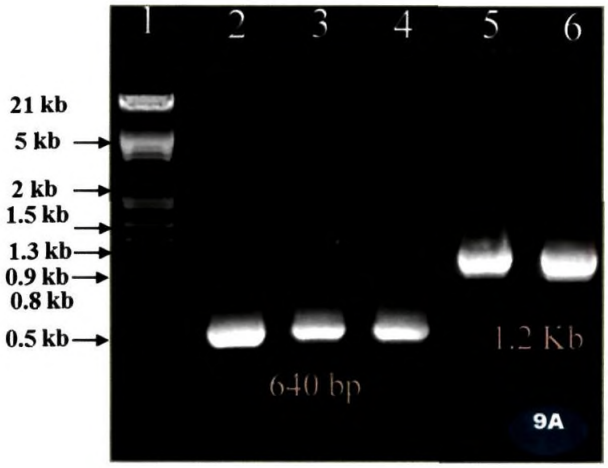
4.12. Molecular analysis of the transgenic plants

4.12.1. PCR analysis of the transgenic plants

PCR analysis performed using the gene specific primers amplified the expected DNA fragment of length 640 bp in the transgenic and positive control respectively. But a fragment of approximate length 1.2 kb was amplified in the untransformed control which included one intron in between and therefore a bigger fragment was amplified which was the endogenous HMGR amplification (Fig.9A). PCR analysis was also carried out using the marker specific primers (*hpt*) which amplified a fragment of length 602 bp in the transgenic plants and in the positive control (Fig. 9B). The respective band was absent in the non-transgenic plant. These results confirmed the presence of the two transgenes in the nuclear genome of *Hevea* transgenic plants.

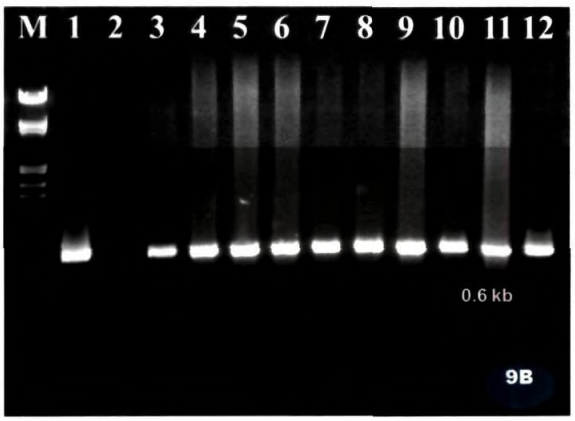
4.12.2. Cloning of the PCR product

The pGEM –T easy vector system is a simple, efficient method for cloning the PCR product. The vector insert ratio of 1:3 was optimum and ten colonies were formed in LB plate containing ampicillin. A blue/white ratio of 20% was observed in presence of X-gal and IPTG. PCR analysis was carried out using promoter specific forward and gene specific reverse primers and the plasmid isolation was performed from the selected colonies using alkaline lysis method. A fragment length of 1.9 kb was amplified from most of the white colonies, confirming the presence of the insert (Fig.10). The isolated recombinant plasmids were purified using PEG for sequencing.



A-Amplification using *hmgr* specific primers

Lane l-	Marker
Lane 2-	Positive control
Lane 3-4	Transgenic plants
Lane 5-6	Untransformed plants



B. PCR amplification using *hpt* specific primers

M -	Marker
Lane l -	Positive control
Lane 2 -	Untransformed plants
Lane 3 -12	Transgenic plants

Fig. 9. PCR amplification of the transgenic plants

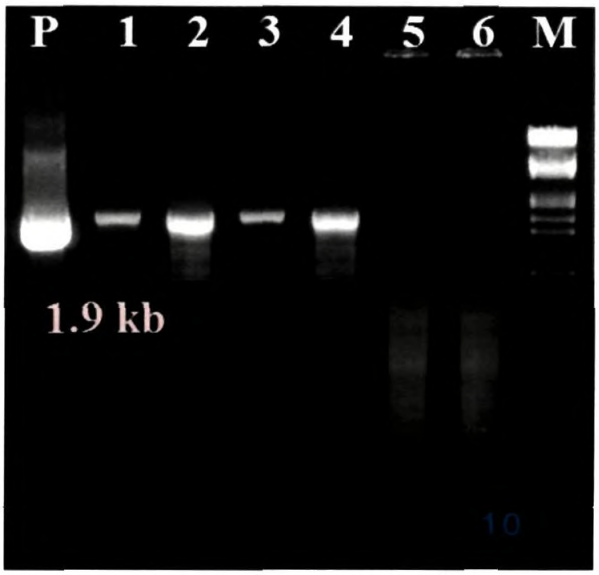


Fig 10. Colony PCR

M - Marker (λ – *Hind*III / *Eco*RI)

P - Positive control

1-6 - Bacterial colonies

4.12.3. *In silico* analysis of *hmgr*1 gene

4.12.3a Nucleotide sequencing and sequence analysis

When the purified recombinant plasmids containing the insert were sequenced using the pUC/M13 forward and reverse primers, a 1.9 kb cDNA fragment was obtained which aligned with the earlier reported sequence of *hmgr*1 gene in the Blastn programme. A maximum similarity of 99% was obtained with the already reported cDNA sequence of the gene (Venkatachalam *et al.*, 2009) (Fig. 11, 12).

The multiple sequence alignment of the deduced amino acid sequence of hmgr 1 cDNA from the transgenic plant was carried out with that of *Hevea hmgr*1, *Euphorbia pekinensis, Taraxacum koksaghyz, Solanum lycospersicom, Litchi chinensis, Medicago trunculata, Ricinus communis* and *Arabidopsis thaliana* (Fig. 13). The alignment showed 77% identity with *Arabidopsis*, 70 - 72% identity with those of *Taraxacum, Medicago* and *Ricinus*, 81% identity with *Litchi* and *Euphorbia* and 60% with that of *Solanum*. Phylogenetic relationship was drawn from this data (Fig. 14). The dendrogram showed sequence similarity between *Hevea*, *Litchi chinensis* and *Euphorbia pekinensis* which came in one cluster. The *hmgr* sequence of *Solanum* differed from that of *Hevea.*

CGGAATGCGCGTGACGCTCCCGGTGACGCCATTTCGCCTTTTCAGAAATGG
ATAAATAGCCTTGCTTCCTATTATATCTTCCCAAATTACCAATACATTACAC
TAGCATCTGAATTTCATAACCAATCTCGATACACCAAATCGACTCTAGACC
CTTTCTCTCTCCTGCGCCGGCATATTTTTACATGGACACCACCGGCCGGCTC
CACCACCGAAAGCATGCTACACCCGTTGAGGACCGTTCTCCGACCACTCCG
AAAGCGTCGGACGCGCTTCCGCTTCCCCTCTACCTGACCAACGCGGTTTTCT
TCACGCTGTTCTTCTCGGTGGCGTATTACCTCCTTCACCGGTGGCGCGACAA
GATCCGCAACTCCACTCCCCTTCATATCGTTACTCTCTCTGAAATTGTTGCT
ATTGTCTCCCTCATTGCCTCTTTCATTTACCTCCTAGGATTCTTCGGTATCGA
TTTTGTGCAGTCATTCATTGCACGCGCCTCCCATAACGTGTGGGACCTCGAA
GATACGGATCCCAACTACCTCATCGATGAAGATCACCGTCTCGTTACTTGC
CCTCCCGCTAATATATCTACTAAGACTACCATTATTGCCGCACCTACCAAAT
TGCCTACCTCGGAACCCTTAATTGCACCCTTAGTCTCGGAGGAAGACGAAA
TGATCGTCAACTCCGTCGTGGATGGGAAGATACCCTCTTATTCTCTGGAGTC
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AGAGGATGACAAGGAGGTCGCTGGAAAGGCTTGCCAGTAGAAAGGGTTCG
ATTACGAGTCGATTTTAGACAATGCTGTGAAATGCCAGTGGGATACGTGCA
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GCCGCGGAGTTGAAGTTCTTCTTGGAGGACCTGACAATTTTGATACTTTGGC
CGTAGTTTTTAACAAGTCCAGTAGATTTGCGAGGCTCCAAGGCATTAAATG
CTCAATTGCTGGTAAGAATCTTTATATAAGATTCAGCTACAGCACTGGCGA
TGCAATGGGGATGAACATGGTTTCTAAAGGGGTTCAAAACGTTCTTGAATT
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TCAGTTGTTTGTGAGGCAATTATCAAGGAAGAGGTGGTGAAGAAGGTGTTG
AAAACCAATGTGGCCTCCCTAGTGGAGCTAAACATGCTCAAGAATCTTGCT
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TCGTATCTGCAATCTTTATTGCCACTGGCCAGGATCCAGCACAGAATGTTG
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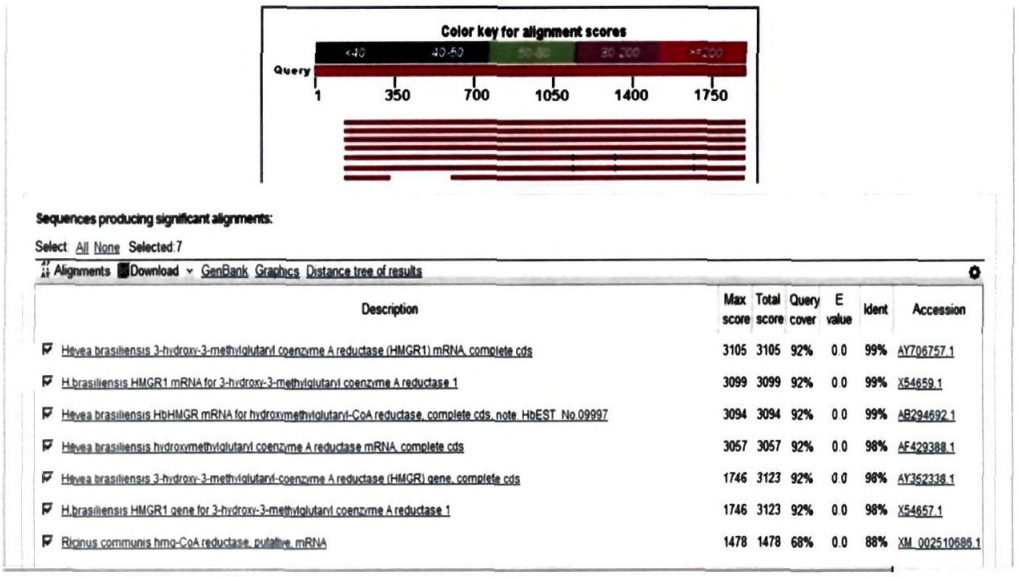
TGCAAACAAAGAGTTGCCAGGATCAAGCTCAAGGCTCCTTGCTGCCATCGT
AGCTGGTTCAGTTTTGGCTGGTGAGCTCTCCTTGATGTCTGCCATTGCAGCT
GGGCAGCTTGTCAAGAGTCACATGAAGTACGACAGATCCAGCAAAGATAT
GTC

SUPER PROMOTER FORWARD CGGAATGCGCGTGACGCTCC

HMG REVERSE GACATATCTTTGCTGGATCTGT

Fig.11 The cDNA sequence amplified from the transgenic plants



Sequences producing significant alignments:

Select All None Selected:7

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Hevea brasiliensis 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR1) mRNA, complete cds	3105	3105	92%	0.0	99%	AY706757.1
H.brasiliensis HMGR1 mRNA for 3-hydroxy-3-methylglutaryl coenzyme A reductase 1	3099	3099	92%	0.0	99%	X54659.1
Hevea brasiliensis HbHMGR mRNA for hydroxymethylglutaryl-CoA reductase, complete cds, note: HbEST No.09997	3094	3094	92%	0.0	99%	AB294692.1
Hevea brasiliensis hydroxymethylglutaryl coenzyme A reductase mRNA, complete cds	3057	3057	92%	0.0	98%	AF429388.1
Hevea brasiliensis 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) gene, complete cds	1746	3123	92%	0.0	98%	AY352338.1
H.brasiliensis HMGR1 gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase 1	1746	3123	92%	0.0	98%	X54657.1
Ricinus communis hmg-CoA reductase, putative, mRNA	1478	1478	68%	0.0	88%	XM_002510686.1

Fig.12. Blastn output

Clustal Omega

Tools > Multiple Sequence Alignment > Clustal Omega

Results for job clustalo-I20131007-143200-0532-2224145-oy

```
CLUSTAL O(1.2.0) multiple sequence alignment


Solanum          MDVRRR----PVKPLCTSKDASA--------------GEPLKQQQVSSPKASDALPLPLY
Taraxacum        MDVGRRSSLKPAATK-GKT-M-------------AEDQYDDQIFKADKKVVDAVVNPLPVG
Ricinus          MEASR----RPTN----ATAK---AVQPLK-----VK---MVDDETCAPKSSDALPVSLY
Medicago         MDARRRLKSLPPRSPAGGENLKTQKLKSLP---------------TTTTGENLNSQTVFLC
Arabidopsis      MDLR--R--RPPKPPVTNNNNSNGSFRSYQPRTSDDDHRRRATTIAPPPKASDALPLPLY
Litchi           MDVR--R--RPPK-----------------PPRVANDE---HRTTTPQSPKASDAVPLPLY
Transgenic       MDT--------TG-------------RLHHRKHAT-PVE---DR--SPTTPKASDALPLPLY
Hevea            MDT--------TG-------------RLHHRKHAT-PVE---DR--SPTTPKASDALPLPLY
Euphorbia        MDSTKSR--RPIR-------------HLHHQKRISEEVD---DHRCLSPPLKASDALPLPLY
                 *:                                                      : :

Solanum          LTNGLFFTMFFSVMYFLLVRWREKIRNSIPLHVVTLSELLAMVSLIASVIYLLGFFGIGF
Taraxacum        ISNGVFFTVFFSVVYFLLIRWREKIRSSTPLHVVTMSEMAAIFFFVASFIYLVGFFGMSF
Ricinus          ITNAICFTLFFSVVYFLLSRWREKIRTSTPLHVVTLSEIAAIFGFIASFIYLLGFFGFDF
Medicago         VTNAVFFGVFFSVAYFLLHRWREKIRTETPLHVVTVSETAAIVSLIASAVYLLGFFGIGS
Arabidopsis      LTNAVFFTLFFSVAYYLLHRWRDKIRYNTPLHVVTITELGAIIALIASFIYLLGFFGIDF
Litchi           LTNAIFFTLFFSVAYFLLHRWRDKIRSSTPLHVVTLSEIAAIVSLIASFIYLLGFFGIDF
Transgenic       LTNAVFFTLFFSVAYYLLHRWRDKIRNSTPLHIVTLSEIVAIVSLIASFIYLLGFFGIDF
Hevea            LTNAVFFTLFFSVAYYLLHRWRDKIRNSTPLHIVTLSEIVAIVSLIASFIYLLGFFGIDF
Euphorbia        LTNAVFFTLFFSVAYYLLHRWRDKIRNSTPLHVVTLSEIAAIVSLIASFIYLLGFFGIGF
                 ::*.:: * :**** *:** ***:*** . ***:**::*  *:. ::** :**:****:

Solanum          VQSFVSRSN--SDSWDIEDENAEQLIIEEDSRRGPCAAATTLGCVVPPPPVRKIAPMVPQ
Taraxacum        VQATPYDEE---EELEVD--E-TEIVRKEDTRVTPCGAALDCESDVVVKQ----------
Ricinus          VQSLILRPPTDDDVWEVEDA----LLKDDSRR-FSCGQALDCSLS---SP--------PQ
Medicago         RTSFPDDLS--DEEI---------LAKEDSRKPGPCPAALVD--TDVKPP--------PA
Arabidopsis      VQSFISRAS--GDAWDLADT----IDDDDHR-LVTCSPPTPI------VS--------VA
Litchi           VQSFISRAS--NDAWDLDDEAATVIAAR--------PPP--------TSM--------PK
Transgenic       VQSFIARAS--HDVWDLEDTDPNYLIDEDHR-LVTCPPANISTKTTIIAA--------PT
Hevea            VQSFIARAS--HDVWDLEDTDPNYLIDEDHR-LVTCPPANISTKTTIIAA--------PT
Euphorbia        VQSLIARPS--HDTWDLDDADRSYLIDGDHR-LVTCSPAKVAPVN--SPP--------PA
                   :      :          :

Solanum          QPAKAALSQTEKPAPIIMPALSEDDEEIIQSVVQGKTPSYSLESKLGDCMRAASIRKEAL
Taraxacum        -----VVKKELVFTPTDTTVVTEEDEEVIQSVVSGKTPSYSLESKLGDCKRAAFIRRVAL
Ricinus          PPKPKVVEEIPTAISSTTFDLGEEDEEIIRSVVDGKTPSYSLESKLGDCKRAAAIRRLAL
Medicago         TL-TPIVAPVKIYEVVAPVNLTPEDEEIAKSVVTGSIPSYSLESRLADCRKAAAIRRSAV
Arabidopsis      K--------LPNPEPIVTESLPEEDEEIVKSVIDGVIPSYSLESRLGDCKRAASIRREAL
Litchi           L--------AAPPEPIVSAFSSVEDEEIVKSVVDGSIPSYSLESKLGDCKRAALIRREAL
Transgenic       K--------LPTSEPLIAPLVSEEDEMIVNSVVDGKIPSYSLESKLGDCKRAAAIRREAL
Hevea            K--------LPTSEPLIAPLVSEEDEMIVNSVVDGKIPSYSLESKLGDCKRAAAIRREAL
Euphorbia        K--------MSVPEPIVSPLASEEDEEIVKSVVNGTIPSYSLESKLGDCKRAAEIRREAL
                                  :** : .**: *  ********:*.** :** **: *:

Solanum          QRITGKSLEGLPLEGFDYESILGQCCEMPVGYVQIPVGIAGPLLLDGREYSVPMATTEGC
Taraxacum        ERITGKSLDGLPLEGLDYESILGQCCEMPVGYVQIPVGVAGPMLLNGKEFSVPMATTEGC
Ricinus          ERITGKSLSGLPLEGFDYNSILGQCCEMPIGYVQIPVGIAGPLLLDGKEYSVPMATTEGC
Medicago         QTITGKSLEGLPLEGFDYDSILGQCCEMPIGFVQIPVGVAGPLLLDGVEYTVPMATTEGC
Arabidopsis      QRVTGRSIEGLPLDGFDYESILGQCCEMPVGYIQIPVGIAGPLLLDGYEYSVPMATTEGC
Litchi           QTVTGRSLQGLPLDGFDYESILGQCCEMPVGYVQIPVGIAGPLLLDGFEYWVPMATTEGC
Transgenic       QRMTRRSLEGLPVEGFDYESILGQCCEMPVGYVQIPVGIAGPLLLNGREYSVPMATTEGC
Hevea            QRMTRRSLEGLPVEGFDYESILGQCCEMPVGYVQIPVGIAGPLLLNGREYSVPMATTEGC
Euphorbia        QRMTGRSLEGLPVEGFDYESILGQCCEMPVGYVQIPVGIAGPLLLDGREYSVPMATTEGC
                 : :* :*:.***:::**:**:**********:*:*****:***:**:* *: *********
```

Fig. 13. Multiple sequence alignment (cont..)

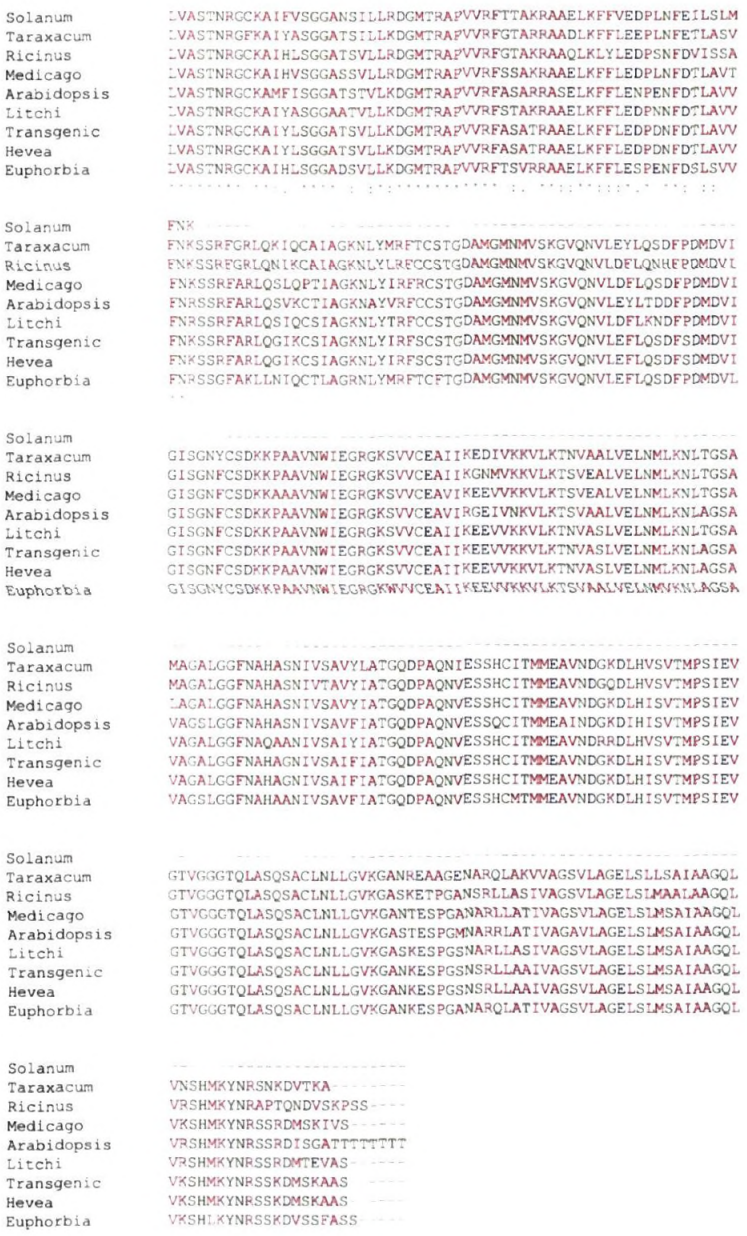


Fig. 13.Multiple sequence alignment

(*) denotes a match across all the sequences, (. and :) shows semi-conservative and conservative substitutions. The alignment was performed using Clustal Omega programme.

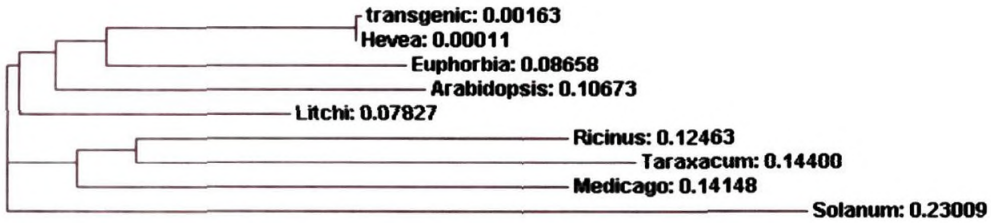


Fig. 14. Phylogenetic tree indicating the relationship of the *hmgr* amino acid sequences of transgenic plant with *Hevea hmgr* and other species. The alignment was generated using Clustal omega program and analyzed by Tree View program

4.12.4. Southern hybridization analysis

Putative transgenic plants derived from different transgenic events were chosen for genomic Southern blot analysis to confirm the integration of the T-DNA region into the nuclear genome of the transgenic plants. Good quality DNA was extracted from the leaves and digested using restriction enzymes, *Bam* HI, *EcoR*I and *Xba* I (M/S Promega) respectively (Fig.15A, Fig 15B.). The single enzyme digests were expected to give mostly larger fragments. These enzymes were selected since they completely digest *Hevea* genome and have no restriction sites inside the *hpt* gene sequences. The digestion of the DNA samples from two transgenic plants with *Bam* HI liberated the internal *hpt* gene cassette. Hybridization of the digested samples with α- [^{32} P]- d CTP labeled *hpt* gene probe produced a strong hybridization signal in the transgenic plants confirming the integration of the transgene in the genome of the plants. (Fig. 16. Lane 1,2).

When the digestion of genomic DNA was carried out using *EcoR*I which has a unique restriction site near the LB of the T- DNA, and hybridized with the gene probe, a single hybridization signal was obtained in one transgenic plant corresponding to the size of the integrated *hpt* transgene (Fig. 16; Lane 4) but the signal was absent in the other plant (Fig. 16; Lane 3). The absence of hybridization signal may be due to the low copy number of the transgene in the genome of the transgenic plants. The single hybridization signal obtained in one plant indicates a single copy insertion of the transgene in the genome of the transgenic plant. As expected the hybridization signal was absent in the non-transgenic control plant too (Fig.16; Lane 5).

The double digestion of the genomic DNA from the two transgenic plants using the restriction enzymes *Bam* HI and *Xba* I liberated the insert along with the promoter sequence. The product was separated and hybridized with the α- [^{32} P]-d CTP labelled*hpt* gene probe which produced strong hybridization

signal (Fig. 17; Lane 1, 2) in two transgenic plants and in the positive control (Lane 3) (Fig. 17), which once again confirmed the integration of the inserted transgene in the genome of the plants.

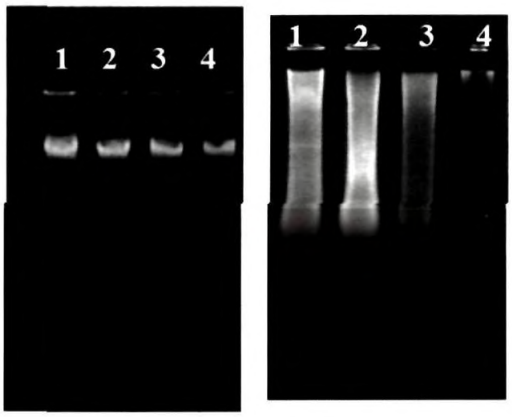


Fig. 15 (A -B) Extraction of Genomic DNA and digestion with restrictic enzymes

A- Isolated genomic DNA
B-Restriction enzyme digestion of the genomic DNA
Lane 1,2 - *Bam* HI
Lane 3 - *EcoR*I
Lane 4 - *Xba* I

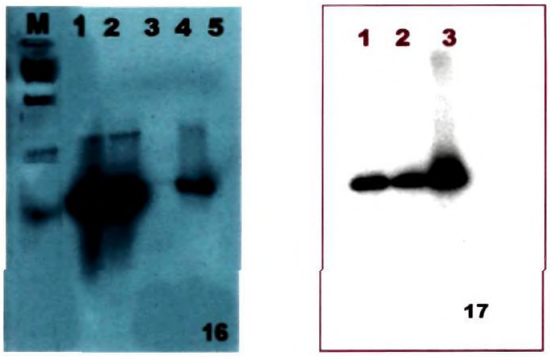


Fig. 16. Southern hybridization using ^{32}P labeled *hpt* gene probe

Lane 1,2 - Digestion of the two transgenic plants with *Bam* HI
Lane 3,4 - Genomic DNA digestion with *EcoR*I
Lane 5 - Digestion of the control plant with *Bam* HI

Fig. 17. Southern blot after insert release

Lane 1,2 - two transgenic plants
Lane 3 - Positive control

4.12.5 Expression analysis in the transgenic plants

4.12.5.1 Northern hybridization

Total RNA was extracted from the leaves of one transgenic and one control plant using the method described by Venkatachalam *et al.*, 1999. The concentration of RNA was observed in a 1.0% agarose gel (Fig.18A). Approximately 25 µg RNA without any DNA contamination was loaded in each well and the gel was run in 1 x MOPS buffer for 4 hours at 50 volt and 20 milli ampere, until the bromophenol blue migrated to 2/3 rd of the gel. The samples were duplicated in two more wells which were observed in the transilluminator after cutting that portion of the gel and staining with EtBr. The RNA was transferred from the gel to the nylon membrane, UV cross linked and probed with ^{32}P labeled *hmgr1* gene and exposed to the image plate of the phosphor image analyzer.

The results showed a hybridization signal in the transgenic plant indicating a higher transcript level in the leaves of the transgenic plant (Fig.18 B). But the hybridization signal was absent in the untransformed control. As we attempted the over expression of *hmgr*1 gene which is an endogenous gene, a hybridization band was expected in the untransformed control too. Lower mRNA level in the untransformed plant may be the reason for the absence of the hybridization signal in this plant. The experimental results thus confirmed the success of transgenic technique for the over expression of *hmgr*1 gene in *Hevea* where a constitutive level of gene expression was expected.

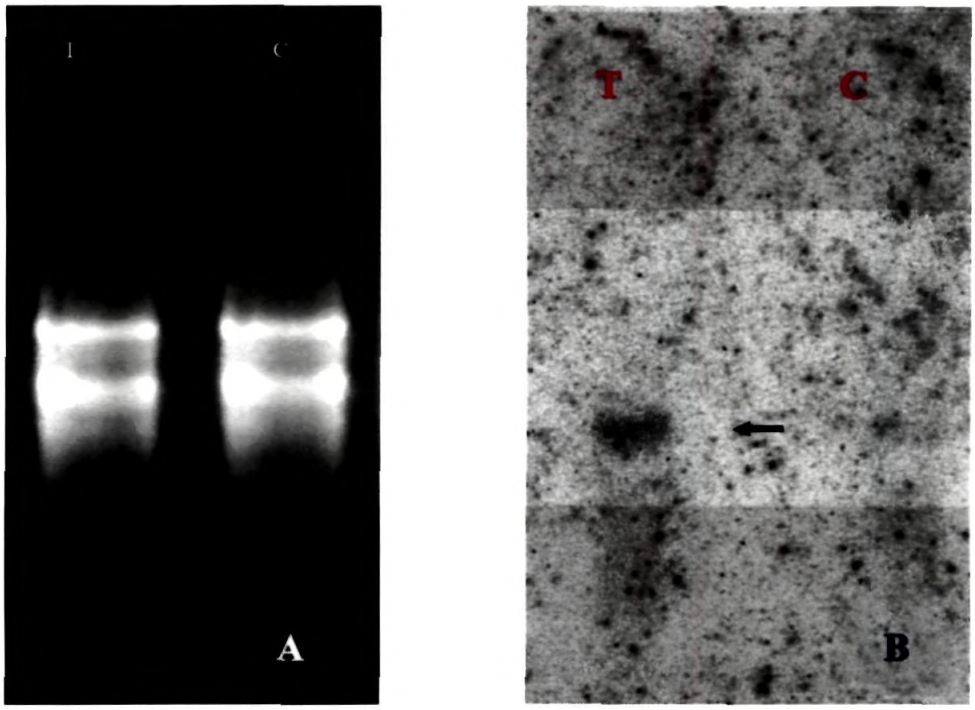


Fig 18. Northern analysis of the transgenic plant with the untransformed control plant

A. RNA isolated from the transgenic and control plant
B. Northern blot showing the transcript level in the transgenic plant

4.12.5.2. HMGR activity in the transgenic plants

HMGR activity was measured in the leaves of transgenic plants along with the control. The crude extract was collected from the leaves of the transgenic and the control plants and coated over the ELISA plate. The absorbance was recorded at 450 nm. The protein content of the extract obtained from the leaves was assessed using Bradford (1976) for calculating the specific activity. The specific activity = absorbance/protein content. The experimental results showed that the transgenic plants had a higher HMGR activity compared to the control plants. Among the four plants tested, three showed higher values than the control. The enzyme activity in one of the transgenic plant was comparable to the control plant. This lower HMGR activity possibly explains the absence of the hybridization signal in this plant during Southern hybridization. The values of the transgenic and the control plants are depicted in Table 4.18. From the table it was observed that the enzyme activity was very high (8 fold) in one of the transgenic plant compared to the three other transgenic tested and the control.

Table 4.18. HMGR enzyme activity in the control and transgenic *Hevea* plants (T_1-T_4)

Plants	Absorbance	Protein	Specific Activity (units/mg protein)
T_1	0.06183	0.014809	4.1755
T_2	0.09883	0.012657	7.8085
T_3	0.07266	0.03758	2.032
T_4	0.38033	0.02123	17.914
Control	0.082166	0.028463	2.886

DISCUSSION

Chapter 5

DISCUSSION

Genetic improvement programmes in *Hevea* are mainly aimed at accelerating the latex yield of the crop combined with biotic/abiotic stress tolerance and timber yield. Latex is the economic product of rubber tree contained in an anastomosing latex vessel system situated in the bark. Yield is a complex trait controlled by two intrinsic factors, the latex flow rate, its duration and the ability of the latex cells to regenerate their lost cytoplasm, including rubber between the two consecutive tappings (Jacob *et al.*, 1989). The latex flow rate (F) is determined by the turgor pressure inside the bark tissues, latex viscosity which depend on the dry rubber content and the rubber particle size and the latex coagulation efficiency (Ruderman *et al.*, 2012). Among these factors the rubber content (C_r) is mainly determined by rate of rubber biosynthesis and the intensity of exploitation. Clonal variations in the rubber biosynthesis are expected (Sethuraj, 1992).

The rubber biosynthetic pathway is controlled by different enzymes and among the genes regulating these enzymes, the irreversible step catalysed by HMGR is said to be a key regulatory point according to Lynen (1969). While quantifying the enzyme activity, Lynen noticed a lower HMGR activity in *Hevea* latex (0.078 nmol MVA/ ml of latex), compared to other enzymes in the pathway up to IPP (isopentenyl pyrophosphate) strongly suggesting its rate limiting role. Significant differences in the HMGR activity have also been reported in the bark of high and low yielding clones of *Hevea brasiliensis* (Nair and Kurup, 1990). Researchers published many articles proving clonal variations in the HMGR activity and a statistically significant interclonal and intraclonal correlation between the total HMGR activity and rubber yield in *Hevea* has been established (Wititsuwannakul and Sukonrat, 1984;

Wititsuwannakul *et al.*, 1988). The diurnal variations observed in the HMGR activity showed its close association with the rubber content in *Hevea* latex (Wititsuwannakul, 1986). These associations suggested the possible role of HMGR in controlling rubber biosynthesis in *Hevea*. In the absence of a reliable molecular tool for determining the yield associated genes in *Hevea*, transgenic approach was employed and laticifer specific *hmgr*1 gene was selected for generating transgenic plants, assuming it to be a probable candidate linked to yield in *Hevea*. The factors influencing *hmgr*1 transgenic plant regeneration and its validation in *Hevea brasiliensis* were discussed. After studying the yield pattern of these plants, the aim was to confirm the rate limiting nature of this enzyme so as to use as a biochemical marker in *Hevea* breeding programmes.

5.1 Binary vector

The glycerol stocks were revived every year after screening the insert in the bacterial colonies using restriction enzyme digestion. The plasmid vector (pBIB *hmgr*1) has been used earlier in *Arabidopsis* where the recombinant clones were screened for the presence of the insert by restriction enzyme digestion prior to the transformation work (Venkatachalam *et al.*, 2009).

5.2 Antibiotic sensitivity

The kill curve experiment conducted using the screening agent revealed hygromycin @ 30 mg l^{-1} as the lethal dose for selection of the transformed cell lines. The *E.coli* gene *aphIV* (*hph*, *hpt*), coding for hygromycin B phosphotransferase confers resistance to the plant cells by detoxifying hygromycin B *via* an ATP-dependent phosphorylation of a 7˝-hydroxyl group (Miki and McHugh, 2004). Hygromycin was successfully used for selection in diverse plant species including monocots, dicots and gymnosperms (Ortiz *et al.*, 1996; Tian *et al.*, 2000). High efficiency transformation was reported in rice (Hiei *et al.*, 1994), where the transformants were selected using a range of

hygromycin concentrations (30 to 50 mg l^{-1}) (Dong *et al.*, 1996; Rashid *et al.*, 1996; Aldemita and Hodges, 1996). In our study, a lower concentration of hygromycin (30 mg l^{-1}) was effective in selection, preventing escapes. The added advantage of this antibiotic is that in addition to effective selection, it also allows good growth and regeneration as evidenced in wheat (Juan pablo *et al.*, 1996). In oil palm, a higher concentration of the antibiotic (50 mg l^{-1}) was used for selecting the transformants (Sujatha and Sailaja, 2005). Efficient selection using hygromycin was possible in castor where the plant regeneration was better compared to the kanamycin selected cell lines (Parveez *et al.*, 2007).

5.3 Colony PCR

Genetic transformation studies generally start with the confirmation of the inserted transgene sequences in the bacterial colonies. Bacterial cultures were grown in plates and the PCR analysis of the bacterial colonies was performed using specific primers designed based on the inserted sequences. In this study, the presence of the inserted transgene in the plasmid was confirmed by colony PCR where a fragment of length 1.9 kb was amplified using promoter specific forward and *hmgr* specific reverse primers. The bacterial colonies containing the insert proved by colony PCR was grown in liquid culture medium with the respective antibiotics and used for infecting the target tissues. According to Wise *et al.* (2006) it is always advantageous to confirm the genetic material carried by the bacterial engineer before starting the transgenic work. The recombinant clones obtained were subjected to colony PCR as well as restriction enzyme digestion to confirm the cloned DNA insert, prior to the start of the transformation work using *Hevea hmgr*1 gene in *Arabidopsis* (Venkatachalam *et al.*, 2009). Similarly the presence of the insert was verified in the bacterial cultures by earlier workers before the transformation work in the small cereal tef (*Eragrostis tef*) (Gebre *et al.*, 2013).

5.4 Generation of callus types for genetic transformation

The first part details the generation of callus types for transformation experiment. Clonal as well as zygotic materials were used as the initial explant. Clonal explants experimented include three types of tissues established from the immature anthers of *Hevea*, *viz* primary callus, embryogenic callus and embryogenic suspension cultures. Immature anther derived callus was obtained in a phytohormone combination of 2, 4-D (1.0 mg l^{-1}), BA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}). The combined use of both the auxins in presence of the cytokinin improved the callus texture. In castor, a combination of BA along with NAA produced callus from the cotyledon explants at a very high frequency (Ganesh kumari *et al.*, 2008). According to Kala *et al.* (2009), a hormonal combination of 2, 4-D, BA and NAA favored callus formation from the leaf cultures of *Hevea*. Sushamakumari *et al.* (2000b) has also proved the positive response of these hormonal combinations (2, 4-D, Kin and NAA) on callus induction and proliferation from immature inflorescence of *Hevea*. Similiar observations were made in *Melia azedarach* and in many dicots, where a combination of high level auxins (2, 4-D or NAA) with low amounts of cytokinins (BA or Kin) helped in the initiation of organogenic callus (Vila *et al.*, 2003; Caboni *et al.*, 2000; Rugini and Muganu, 1998).

The conversion of primary callus to the embryogenic callus was obtained in half strength MS salts supplemented with TDZ (0.6 mg l^{-1}), NAA (1.0 mg l^{-1}) and BA (0.3 mg l^{-1}). Simultaneously liquid basal medium (½ MS) supplemented with NAA (0.5 mg l^{-1}) and TDZ (0.3 mg l^{-1}) at reduced levels helped in the establishment of embryogenic cell suspensions. The addition of TDZ caused alterations in the level of antioxidant enzymes especially peroxidases which promote embryogenic response in the tissues. These alterations in the enzyme kinetics might be the reason for the morphological changes in the tissues (Guo *et al.*, 2011). Literature proved the effect of TDZ in

giving a wide variety of responses when used along with other phytohormones rather than using alone. TDZ is a urea based cytokinin, nondegradable by cytokinin oxidase enzyme and therefore persistent in the tissues, transforming them from cytokinin dependance to cytokinin autonomy. Similiar results were observed in *Lilium longiflorum*, where embryogenic cells were established in medium containing TDZ (0.2 mg l^{-1}) and NAA (1.0 mg l^{-1}) (Nhut *et al.*, 2006). NAA/TDZ combination also accelerated embryogenic callus formation in *Cymbidium* orchid (Huan *et al.*, 2004). Beneficial role of TDZ alone on embryogenic callus initiation has been reported in certain crops. In triploid banana cultivars and paradise tree (*Melia azedarach*), TDZ induced embryogenic callus at a concentration of 2.0 mg l^{-1} and 4.54 µM respectively (Srangsam and Kanchanapoom, 2003; Silvia *et al.*, 2007). In the egyptian wheat cultivar, TDZ supported embryogenesis and also plant regeneration (Fahmy *et al.*, 2006). Yellowish–white embryogenic callus was obtained from the root segments of *Oncidium* in TDZ- 2,4-D combination (Chen and Chang, 2000b). Contrasting reports were also seen in crops where other phytohormones induced such embryogenic responses. A hormonal combination of 2,4-D and BA induced embryogenic callus in grape vine and suspension cultures were raised from this callus in liquid medium containing 2,4-D (Colova *et al.*, 2007).

The embryogenic callus obtained along with the developing zygotic embryos was also tried as the target material for obtaining *hmgr*1 transgenic cell lines. The proliferation of this embryogenic callus was obtained in the basal medium containing 2,4-D (0.3 mg l^{-1}), Kin (0.5 mg l^{-1}), NAA (0.3 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). This type of tissues was used earlier in the genetic transformation experiments due to its high regeneration potential. A similar hormonal combination was used in the induction of embryogenic callus from immature zygotic embryos of Royal Poinciana (*Delonix regia*) (Abdi and Hedayat, 2011). Zygotic embryo derived embryogenic callus was used as the

initial explant in wheat transformation due to its high frequency transformation and regeneration capacity (Raja *et al.*, 2010). Frame *et al.* (2011) also used the embryogenic callus from the scutellar cells of maize immature embryos for genetic transformation studies due to the amenability of the tissue towards transformation and regeneration.

5.5 Development of transgenic cell lines

5.5.1 Influence of callus type on transformation efficiency

The *Agrobacterium* strain and the type and texture of the explant used are the two main factors contributing towards transformation efficiency in *Hevea brasiliensis*. The use of an ideal target explant is an important parameter determining the transformation efficiency. In the present experiment highest frequency of transformation was observed using the embryogenic callus derived from the zygotic embryos. The friable, embryogenic nature of this material contributed towards higher rate of transformation and the zygotic origin made it more amenable towards plant regeneration than the clonal explants. Moreover the fine texture of this callus also contributed to the high frequency transformation. Here the explant juvenility and friability accounted for the higher rate of transformation (67%) (Fig.19). Similar results were observed in barley where the polyembryogenic cell masses obtained along with the immature embryos served as an excellent target tissue for genetic transformation studies (Nuutila *et al* ., 2000). Transgenic plant regeneration was successful from the embryogenic callus derived from immature zygotic embryos in wheat (Juan pablo *et al.*, 1996; Cheng *et al.*, 2003; Raja *et al.*, 2010). In grape vine, high frequency plant regeneration was obtained from the callus obtained from the immature zygotic embryos (Scorza *et al.*, 1995). Cervera *et al.* (2005) also pointed out the sensitivity of *Agrobacterium* cells towards juvenile tissues than the matured ones as evidenced in citrus.

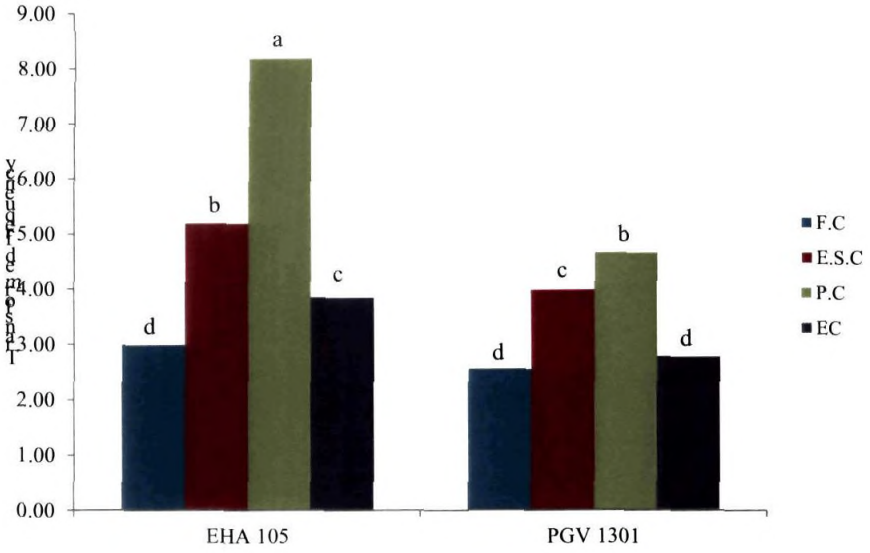
Considering the response of clonal explants on transformation, maximum efficiency (27%) was observed using embryogenic suspension cultures (Fig.19). The performance of the 3-day-old embryogenic suspensions was encouraging in *Hevea* giving a higher frequency of transformation. The importance of the type and texture of the target material on genetic transformation was once again proved in this study. Embryogenic suspensions represent synchronous embryogenic systems which facilitate high frequency embryogenesis and plant conversion in many crops (Colova *et al.*, 2007). Compared to the response of embryogenic callus, suspensions served as an attractive option since *Agrobacterium* require actively dividing cells for successful gene transfer (Okada *et al.*, 1986). The added advantage of suspensions is that large amount of uniform transgenic tissues are available from the infected cultures (Droste *et al.*, 2001). Moreover suspension cultures were commonly used in less responsive genotypes due to its beneficial effect on transformation. The suspensions raised from the somatic embryos were used for *Agrobacterium* infection in soya bean. Results of the rice transformation studies are also in line with our observations where a higher frequency of transformation was observed using 3-day-old suspension cultures (Ozawa and Takaiwa, 2010). According to Finer & McMullen, (1990) and Fromm *et al.* (1990) the embryogenic suspensions were generally more responsive to transformation. Transgenic cell lines were also developed from the embryogenic cell suspensions in cotton (*Gossypium hirsutum L.*) using particle bombardment where the selection was performed using hygromycin (Finer and McMullen, 1990). Use of suspension cultures for high frequency transformation has been emphasized by An (1985). This protocol was further elaborated and applied in *Arabidopsis* where stably transformed cultures were recovered (Forreiter *et al.*, 1997). The efficiency of transformation between different *Agrobacterium* strains was compared using embryogenic suspensions in poplar (Howe *et al.*,

1994). In recalcitrant varieties of sweet potato and *Citrus*, successful transformation and plant regeneration system were developed employing suspension cultures (Xing *et al.*, 2008; Dutt and Grosser, 2010).

Embryogenic callus is another attractive tissue for transgenic work because once established, the material is available throughout the year. Another advantage is that the use of embryogenic cultures reduces the time span for the transgenic plant regeneration, where the most difficult step, the conversion of the hard primary calli to the embryogenic callus is bypassed. In the present work, response of the embryogenic callus towards frequency of transformation was lower (15%) compared to the embryogenic suspension cultures (Fig.19). Contrasting reports were published earlier in *Hevea*, where infected embryogenic callus gave a very high frequency of transformation with a different *Agrobacterium* strain-binary vector combination (Rekha *et al.*, 2006; Kala *et al.*, 2006). Use of long term/established embryogenic calli for transformation studies were also reported in coffea (*Coffea arabica* L), where a frequency of 17% was observed (Ribas *et al.*, 2011). Earlier reports also revealed the development of a reliable transformation system using embryogenic callus in woody species like Prunus, grapevine, rubber and chestnut tree (Machado *et al.*, 1995; Dhekney *et al.*, 2009; Blanc *et al.*, 2006; Andrade *et al.*, 2009). An accelerated transformation and regeneration system was reported in *Citrus sinensis*, using embryogenic callus as the target tissue (Li *et al.*, 2003). In all these published reports, the rate of transformation and regeneration are mainly influenced by the type of the callus tissue experimented (Brisibe *et al.*, 2000).

Agrobacterium infection with the primary callus using the *hmgr*1 gene constructs resulted in a still lower frequency of transformation (9 %) (Fig.19). The reduction in the frequency of transformation may be due to the low phenol content and lack of callus friability. The hard callus might inhibit the easy entry

of *Agrobacterium* accounting for the low transformation frequency. Earlier reports also revealed a lower transformation frequency of 4% and 6% with the infected primary callus of *Hevea* (Jayashree *et al.*, 2003; Sobha *et al.*, 2003a). From these observations, it is obvious that the friable embryogenic callus and the embryogenic suspensions derived from them are the most preferred target material for transformation resulting in the development, proliferation and embryogenic growth rather than the use of compact, hard callus types. These differences in the efficiencies between the callus types, *ie*, primary calli, embryogenic and embryogenic suspensions might be due to the variations in the size of the cell aggregates.



Analysis of variance was performed. Vertical bars showing different letters are significantly different at p= 0.05 using Duncans Multiple Range Test (DMRT)

Fig. 19. Influence of the strain and the callus type on transformation frequency

5.5.2 Effect of *Agrobacterium* strain on transformation frequency

Among the three experimented *Agrobacterium* strains for genetic transformation, EHA 105 gave the highest transformation frequency irrespective of the tissue types used. The second best strain was pGV 1301, a nopaline one which has a cured Ti plasmid in the C58 chromosomal background (Fig. 19). The less virulent strain LBA4404 responded poorly with all the callus types. *Agrobacterium* strains generally differ in their ability to infect tissues and transfer its T-DNA (Suzuki *et al.*, 2001; Khanna *et al.*, 2004). The efficiency/ host range of a particular strain can be attributed to the interactions of the T_i plasmid with certain bacterial chromosomal background (Gelvin *et al.*, 2003). The differences in the "*vir*" region and the chromosomal background between the strains may also affect the susceptibility range of the plants (Hood *et al.*, 1993). Earlier Montoro *et al.* (2000) studied the influence of five different *Agrobacterium* strains (C58pMP90, C58pGV2260, AGL1, LBA4404 and EHA105) and two binary vectors on transformation of *Hevea* friable calli. He found that the GUS activity was higher in the cell lines generated using EHA 105, a succinamopine strain containing the disarmed pEHA 105 in the C58 chromosomal background. As in our study, the efficiency of EHA 105 strain has been proved by many researchers. Wang *et al.* (2006) confirmed higher transformation efficiency using EHA 105 over the three other strains experimented (GV3101, LBA 4404, A281) and a higher regeneration potential was also observed in the transgenic cell lines generated using EHA 105. The superiority of the virulent strain EHA 105 was again proved in *Vitis vinifera* and in pea (Torregrosa *et al.*, 2002; Nadolska and Waclaw, 2000). Similarly in *Phalaenopsis violaceae*, the hyper virulent strain EHA 105 performed well in terms of *UidA* gene expression (Subramanian and Rathinam, 2010).

In our case LBA 4404, which has a disarmed Ti plasmid p AL 4404 in the Ti Ach5 chromosomal background was considered as the least virulent

strain, failing to produce transgenic cell lines. Similar observation was made by Baloglu *et al.* (2007) in leaf disc transformation of sugar beet where GUS expression was absent in the LBA 4404 infected leaf discs. In contrary, the superiority of this strain over EHA 105 has been proved in potato varieties (Sarker and Mustafa, 2002) and in cases where a higher O.D was experimented (Islam *et al.*, 2010). In recalcitrant crops, the frequency of transformation is benefitted not only by the *Agrobacterium* strain but also by the binary vector chosen or by the strain-vector combinations used. The strain LBA 4404 in combination with the pCambia 2301 vector produced numerous transgenic cell lines in Onion (Mythili *et al.*, 2012). Similarly numerous transgenic plants were produced using EHA105 - pCAMBIA 2301 combinations in *Hevea* (Blanc *et al.*, 2006).

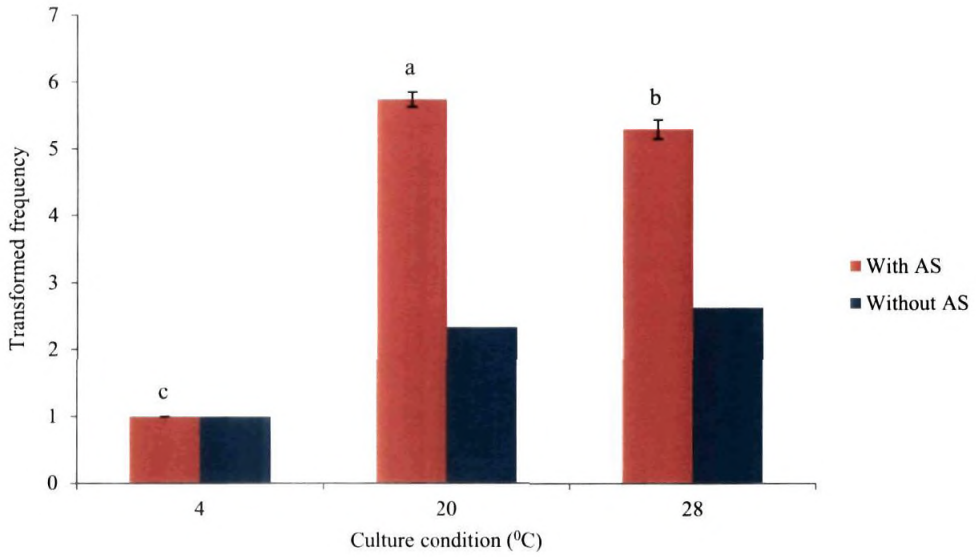
The virulence of any strain can also be improved by exchanging its T_i plasmid with another one or by including additional "*vir*" genes in the helper plasmid (Hood *et al.*, 1987). In wheat, LBA4404 strain was improved by adding *vir* genes from pT_i Bo542 (Jones *et al.*, 2005). According to Arokiaraj *et al.* (2009), the transformation frequency with the strain GV2260 can be improved using a super virulence plasmid containing additional "*vir*" genes. Thus the efficiency of transformation depends on so many factors where the bacterial strain is one among them. The influence of binary vector and the additional "*vir*" genes on transformation frequency should also be accounted.

5.5.3 Additional factors improving the frequency of transformation

In addition to the bacterial strain and binary vector, certain other factors also played a vital role on the frequency of transformation in *Hevea*. The frequency of transformation can be raised by enhancing the competency of the plant tissue and also by the '*vir*" gene induction (Chakrabarty *et al.*, 2002). Experiments using the *hmgr*1 gene construct proved that low temperature

incubation (20°C) of the infected tissues in the co-culture medium containing acetosyringone enhanced the frequency of transformation from 27% to 32% (Fig.20). During the period of co-culture, the phenolic compound acetosyringone (20 mg l^{-1}) worked along with the signaling factors like acidic pH, temperature shock etc. improving the expression of the virulence genes. This contributed towards increased transformation frequency. The strong influence of the medium pH, temperature shock and osmotic conditions on the induction and expression of the virulence genes was suggested by Fisk and Dandekar (1993). Here the phenolic compounds, temperature shock and the medium pH induced "*vir*" gene expression accelerated T-pili formation required for the attachment of *Agrobacterium* cells with the plant cells (Dillen *et al.*, 1997; Chakrabarty *et al.*, 2002; De Clercq *et al.*, 2002). The T-pilus assisted in the T-DNA transmembrane transport. According to Fullner and Nester (1996), mating between the bacteria took place at 19°C, at a medium pH of 5.3 in presence of acetosyringone. Fullner and Nester (1996) proved the role of acetosyringone on *vir* gene induction which was essential for the pilus assembly. According to them, lower incubation temperature brought tenfold increase in the pilated cells than those observed at 28°C. In the tobacco cultures, low temperature resulted in an efficient and stable GUS gene expression which was in accordance with our results (Su *et al.*, 2012). Thus the accelerated transformation frequency which was noticed in our cultures might be due to action of the T- pilus. Jin *et al.* (1993) studied the effect of temperature on VirA and Vir D proteins and found that high temperature negatively influenced the phosphorylation of VirG by VirA, which showed the temperature sensitivity of Vir A. The pilus of *Agrobacterium* strains was most stable at lower incubation temperatures (approximately 18 to 20°C) although "*vir*" gene induction was maximum at 25 to 27°C (Gelvin, 2003). Similiar reports were also available in maize, cotton etc. where a higher transformation frequency was

reported at an incubation temperature of 19 to 20°C (Jin *et al.*, 2005; Frame *et al.*, 2002). Therefore, co- culturing *Agrobacterium* cells with the plant tissues at lower temperatures during the initial days of transformation can improve the efficiency of transformation.



Vertical bars showing different letters are statistically significant at P=0.05 using Duncans Multiple Range test (Duncan, 1995).

Fig. 20. Effect of co-cultivation temperature and acetosyringone on transformation efficiency

In our experience, anti-necrotic treatment, explant desiccation and the use of L-cysteine and siver nitrate in the co culture medium failed in accelerating the transformation frequency in *Hevea* using *hmgr*1 gene construct. Tissue necrosis and cell death are the main reasons limiting transformation frequency in many crops during *Agrobacterium* mediated gene transfer (Pu and Goodman, 1992; Sangwan *et al.*, 1992). This type of *Agrobacterium* induced necrosis leads to oxidative burst in the target cells resulting in the generation of super oxide radicals. This resulted in plant cell death, necrosis and bacterial cell

death leading to the induction of PR proteins, phytoalexins etc. (Mehdy, 1994). The oxidative burst can be suppressed using anti-oxidants like cysteine or bactericidal agents like silver nitrate. They are capable of quenching the oxidative burst by scavenging the reactive oxygen species. The anti-necrotic treatment provided a congenial environment for the interaction of the *A. tumefaciens* with the plant cells. But in the transformation experiment using *hmgr*1 gene construct, no improvement in the frequency was noticed by the inclusion of anti-necrotic treatments which was in agreement with the findings of Baloğlu *et al.* (2007) where no significant improvement in the transformation frequency was observed with the use of L-cysteine in the co-culture medium. The reason might be the reduction in the tissue necrosis happening during *Agrobacterium* infection.

Contrasting results were observed in many crops where anti-necrotic mixture and thiol compounds had a beneficial role on transformation. Addition of silver nitrate and L-cysteine in the co-cultivation medium increased the transformation frequency in grape vine (Perl *et al.*, 1996) where *Agrobacterium* induced necrosis of the infected tissues has been reported earlier (Perl *et al.*, 1996; Das *et al.*, 2002). Enriquez-Obregon *et al.* (1998, 1999) reported an efficient transformation system in sugarcane and rice by the application of anti-necrotic mixture which reduced tissue necrosis. Addition of L-cysteine augmented the frequency of transformation in soya bean due to the inhibition of polyphenol oxidases and peroxidases (Somers *et al.*, 2003). Partial desiccation of the infected tissues also proved to be useful in rice transformation expediting the frequency by inhibiting the bacterial overgrowth (Yi *et al.*, 2001).

5.6 Confirming the transgene by PCR analysis

PCR analysis using the promoter specific and marker specific primers confirmed the presence of the transgene in the transgenic tissues (Fig. 8 A, B). The PCR positive cell lines were cultured for embryogenesis and transgenic plant regeneration. In the absence of a reporter gene, this approach seems to be valuable in regenerating transgenic plants preventing false positives.

5.7 Somatic embryogenesis from the transformed cell lines

A typical somatic embryogenesiss protocol for *Hevea brasiliensis* involves a series of consecutive stages starting from callus induction, embryogenic callus multiplication, somatic embryo induction, maturation and plant regeneration. The embryogenic capacity of the cells depend on several factors including the composition of the basal medium, supply of suitable osmoticum, growth regulators and the culture conditions. The complexity of the system depends on requirement of these ingredients during each step and the lengthy incubation period associated with each stage reaching up to one and a half years or more to develop complete plantlets.

5.7.a Impact of the media constituents on somatic embryogenesis

The media constituents for somatic embryogenesis from varying explant sources have been standardized in *Hevea.* Transformation process result in an alteration in the genetic makeup of the cell, hence the basal requirements for embryogenesis changes and therefore modifications in the media constituents are needed for successful embryogenesis and plant regeneration from transgenic tissues. The experimental results proved the value of this statement and modified MS medium with lower levels of NH_4NO_3 and $MgSO_4$ with an increase in the $KN0_3$ concentration promoted somatic embryogenesis in the transgenic cell lines. Nitrogen in the reduced form (NH_4^+) is essential for the onset of embryogenesis, especially during the induction phase (Rangaswamy,

1986). The NH_4^+ requirement (or of N in a reduced form) for embryogenic induction and differentiation was proved in different species and culturing systems (Halperin and Wetherell 1965, Wetherell and Dougall 1976, Kamada and Harada 1979, Walker and Sato 1981, Meijer and Brown 1987, He *et al.*, 1989). In many woody plants, full strength MS salts inhibited organized cell growth and this toxicity can be reduced by lowering the amount of ammonium or total nitrogen in the medium (Bonga and Von Aderkas, 1992). It was observed that the addition of NH_4NO_3 decreased cell growth and embryo induction percentage in *Juniperus excelsa* (Shanjani *et al.*, 2003). The reason being the inhibition of glutamine synthase activity resulting in the depletion of crucial amino acids and enzymes essential for cell growth and embryogenesis (Tachibana *et al.*, 1986). Therefore, dilution of the media sustained cell division, promoting growth of the calli, leading to direct differentiation according to Davidonis and Hamilton (1983). Work carried out by Merkle *et al.* (1995) proved that ammonium is required only for the initiation of embryogenesis in the cultured cells whereas nitrate alone is sufficient for further development of the pro-embryos or embryogenic cell clusters. Hence a reduction in the NH_4NO_3 concentration accounted for the increased cell growth and embryogenesis. This was also evident in cotton, where the rate of embryogenesis was higher in presence of $KN0_3$ (1.9 g l^{-1}), since K^+ play a key role in cell growth and embryogenesis in addition to NO_3^- (Ikram and Zafar, 2004; Kumria *et al.*, 2003). These arguments supported our findings where a reduction in NH_4NO_3 concentration and a rise in KNO_3 level induced somatic embryogenesis.

Amino acids in the basal medium played a crucial role during somatic embryogenesis which was obvious from the results. Among the amino acids experimented, maximum embryo induction was observed with L-alanine 40.96%, followed by L-asparagine (39.22%), L-proline (38%), L-glutamine

(37.44%) and L-serine (27.94 %). Kamada and Harada, (1979) and Gamborg *et al.* (1968) reported that NH_4^+ can be replaced either partially or fully with the organic nitrogen which has a pronounced effect on plant cell growth and differentiation. In this study, a reduction in the NH_4^+ concentration might be compensated by the organic nitrogen supply in the embryo induction medium. Addition of glutamine and L-alanine was crucial for the induction of somatic embryogenesis. Literature suggested accelerated cell division in presence of L alanine during early somatic embryogenesis. This may be due to the quick transformation of L-alanine to glutamic acid by alanine amino transferase which could be utilized as the nitrogen source (Kamada and Harada, 1979). Earlier reviews also proposed L-alanine as the most active amino acid when supplemented along with KNO_3 (20m M), where glutamine, asparagine, arginine and proline had a stimulatory effect. Our results are in consonance with this statement. Inclusion of glutamine and arginine resulted in enhanced protein accumulation in the somatic embryos supporting good growth and embryogenesis (Merkle *et al.*, 1995).

Phytohormones played a major role in stimulating somatic embryogenesis as apparent from the results. Combinations of growth regulators are highly essential for the synchronized growth of somatic embryos. NAA-zeatin combination induced embryogenesis at a frequency of 60%. The influence of zeatin in combination with GA_3 on somatic embryo induction was studied in the immature inflorescence culture in *Hevea*, where lower levels of NAA or 2,4-D was essential for triggering embryo induction (Sushamakumari *et al.*, 2000 b). When zeatin was replaced with kinetin, a modest increase in the embryo induction frequency (62%) was noticed. NAA-Kinetin combinations were optimum for somatic embryogenesis from the *hmgr*1 transgenic cell lines. In our experience lower levels of Kin also triggered somatic embryogenesis when experimented with NAA (2.0 mg l^{-1}). Similar observations were made in

Hevea anther culture where a combination of 0.7 mg l^{-1} Kin and 0.2 mg l^{-1} NAA favored somatic embryogenesis (Kumari Jayasree *et al.*, 1999). This study utilized lower concentrations of NAA which was in contrast to our report where, higher levels of NAA (1.0 mg l^{-1}) favored somatic embryogenesis. Repetitive embryogenesis was also induced in *Hevea* cultures in a growth regulator combination of 0.5 mg l^{-1} NAA, 2.0 mg l^{-1} Kin, 0.5 mg l^{-1} IAA and 4.0 mg l^{-1} 2,4-D (Asokan *et al.*, 2001). In *Gentiana cruciata* L, the cytokinin BA (0.5 mg l^{-1}), induced somatic embryogenesis along with NAA (Mikula *et al.*, 2005).

The experiment conducted using polyamines proved the beneficial effect of spermidine on somatic embryogenesis. Spermidine is a small aliphatic polyamine, ubiquitous, involved in developmental regulation of plants. Inclusion of spermidine in the embryo induction medium triggered embryogenesis at a frequency of 41%. But the requirement for polyamine is genotype dependant or specific during certain physiological conditions of the explants. They behave as a new class of growth substances or secondary hormonal messengers (Galston, 1983; 1987). Polyamines interact with negatively charged macromolecules like DNA, RNA, proteins, phospholipids etc activating and stabilizing them due to their polycationic nature at the physiological pH (Evans and Malmberg, 1989). In *Hevea*, the need for polyamines during the onset of embryogenesis has been reported earlier by El Hadrami (1989). Direct role of polyamines on somatic embryogenesis was confirmed by the high level expression of these compounds in the embryogenic cultures of *Hevea* maintained in an auxin free medium. According to Minocha *et al.* (1995), early stage somatic embryos showed significantly higher levels of spermidine and putrescine. This was obvious from the studies of Merkle *et al.* (1995), where the activity of arginine decarboxylase important in putrescine biosynthesis was elevated during somatic embryogenesis. Significance of

polyamines during somatic embryogenesis in angiosperms has been emphasised by researchers, the inhibition of which retarded embryogenesis (Meijer and Simmonds, 1988; Minocha, 1988; Galston and Flores, 1991). An exogenous supply of putrescine or arginine increased the embryogenic potential of the callus with a subsequent rise in the spermidine level which accounted for the enhanced embryogenic capability of the tissue (El Hadrami *et al.*, 1989; EI-Hadrami and D' Auzac, 1992; Carron *et al.*, 1995b). But during embryo maturation, the spermidine level declined which can be related to the ABA content in the maturation medium (Minocha *et al.*, 1993). The above observations strongly proved the role of spermidine during somatic embryogenesis and the importance of polyamine metabolism during somatic embryos development.

An increase in the sucrose concentration (80 g l^{-1}) of the embryo induction medium containing spermidine resulted in a frequency of 72% in the *hmgr*1 transgenic cell lines. The experimental results showed that the medium osmoticum and polyamine metabolism are important during somatic embryogenesis. Importance of sucrose concentration during different stages of somatic embryogenesis and plant regeneration has been proved earlier by Sushamakumari *et al.* (2000b). Higher levels of sucrose positively influenced somatic embryo induction from the immature inflorescence tissue of *Hevea.* Supportive data showing the capacity of higher sucrose concentrations on somatic embryogenesis has been reported in *Vietanamese ginseng* (Nhut *et al.*, 2012). Contradictory reports showed the influence of lower levels of sucrose (4%) in inducing nucellar embryogenesis in *mangifera indica* L, when supplied along with spermidine (Maneesh *et al.*, 2010).

5.8 Somatic embryo maturation

The maturation of *hmgr*1 somatic embryos progressed in ½ MS basal medium devoid of NH_4NO_3 with a subsequent rise in the level of KNO_3 (3.0 g l^{-1}). Further addition of glutamine (1.0 g l^{-1}) and proline (200 mg l^{-1}) stimulated maturation of the embryos. Enhanced maturation and germination of the somatic embryos was supported by KNO_3. In cotton, addition of KNO_3, which is an oxidized form of nitrogen, helped in the conversion of the embryogenic callus to embryos and plantlets (Trabelsi *et al.*, 2003). According to Ikram and Zafar (2004), somatic embryo maturation occurred in presence of KNO_3 while NH_4^+ affected anthocyanin production. Inclusion of nitrate and organic nitrogen in the culture medium strongly influenced embryo maturation and plant regeneration in the polyembryogenic cultures of barley (Nuutila *et al.*, 2000). The influence of nitrogen during somatic embryogenesis may be due to the pH variations occurring in the culture medium. Nitrate increased the pH of the medium while ammonia reduced it. The addition of casein hydrolysate promoted growth and maturation of somatic embryos. Casein hydrolysate had a buffering effect in the pH of the culture medium.

Experimental results showed that the addition of L-glutamine (1.0 g l^{-1}) and proline (200 mg l^{-1}) triggered maturation of *hmgr*1 transgenic embryos. The importance of glutamine and proline on transgenic embryo maturation were proved earlier in *Hevea* (Jayashree *et al.*, 2003). Similiar results were also observed in *Acacia mangium*, where the conversion of the globular somatic embryos to the torpedo and cotyledonary staged ones were stimulated by amino acids especially L-glutamine and L-proline in a low sucrose medium (3%) (Deyu *et al.*, 2004). Glutamine has a regulatory as well as nutritive role on embryo maturation. It gets converted to 5-oxo proline upon autoclaving. The addition of L-glutamine and potassium sulphate, an inorganic sulphur source facilitated storage protein accumulation in the somatic embryos accelerating

maturation and germination which was in accordance with our results. The supply of amino acids (organic nitrogen sources) enhanced embryogenesis and differentiation in barley, maize and carrot cell cultures (Nuutila *et al.*, 2000). In another study, the exogenous supply of L-glutamine and casein hydrolysate promoted somatic embryogenesis, when the nitrate assimilation was unoperative (Kirby *et al.*, 1987; Szczygiel and Kowalczyk, 2001; Trabelsi *et al.*, 2003). The beneficial role of L glutamine, an organic form of reduced nitrogen, accelerating somatic embryogenesis was again proved in Norway spruce (Verhagen *et al.*, 1989).

The promotive effect of PEG and ABA on *hmgr*1 transgenic embryo maturation was shown in the present experiment. Earlier reports in *Hevea* also proved the stimulatory effect of exogenous ABA (10^{-5} M) on globular embryo formation. Further, the conversion of the globular embryos to torpedo shaped ones was assisted by PEG (14%) and thus the effect of ABA was PEG dependant (Linossier *et al.*, 1997). Pretreatment with ABA or any stress was essential in triggering desiccation tolerance in the embryos, helping in normal maturation of the somatic embryos, preventing precocious germination. The role of ABA in the cotyledon development, shoot meristem induction and accumulation of protein reserves in the developing embryos was reported in *Hevea* (Etienne *et al.*, 1993b). Negative effect of ABA on somatic embryo maturation was also shown in the inflorescence culture of *Hevea* which was in contrast to our results (Sushamakumari *et al.*, 2000b). The beneficial role of PEG, ABA combinations on somatic embryo maturation was proved by many researchers. The positive influence of ABA on maturation in a water stressed medium is due to the accumulation of storage proteins. The accumulation of these necessary reserves helped in the germination of the embryos. In mangifera, the embryos attained desirable size in a low nitrogen medium containing PEG, resulting in synchronized growth and germinability

(Chaturvedi *et al.*, 2004). In *Araucaria angustifolia*, embryo maturation was attained with 1% PEG $_{8000}$ regardless of ABA (Astarita *et al.*, 1998). Maturation of the somatic embryos in *Algerian fir*, *Pinus armandii* etc. was observed in MS medium containing PEG (7.5 - 10%), ABA (10 mg l^{-1}) and maltose (4 - 5%) (Vookova and Kormutak, 2002; Maruyama *et al.*, 2007). Addition of PEG along with higher levels of ABA (20–40μM), resulted in the formation of early torpedo shaped embryos in *Abies alba* (Schuller *et al.*, 2000). In certain cases, maturation was influenced by water stress imparted by the gelling agent as seen in Avocado where 10% agar promoted maturation of the somatic embryos (Marquez-Martín *et al.*, 2011). Similar results were also seen in walnut where the embryos matured either in presence of gelrite (0.3%) or by the addition of 5 % PEG (Mohammad Ali *et al.*, 2010). The sucrose concentration of the maturation medium was further reduced to 30 g l^{-1}. This reduction in the sucrose concentration also accounted for the conversion of embryos to the cotyledonary stage.

5.9 Influence of desiccation on somatic embryo germination

Results showed that partial desiccation improved the germination capacity of the cotyledonary embryos, on transfer to the plant regeneration medium. The acquisition of desiccation tolerance is a signal of potential autonomy of the embryo. The improvement in the germinability may be due to the elicitation of the endogenous ABA levels accelerating the induction of shoot meristem in the embryos. Literature supported this view, where an increase in the endogenous ABA level in the desiccated somatic embryos accelerated the expression of specific genes involved in plant development (Oishi and Bewley, 1990, Skriver and Mundy, 1990). The rate of drying is determined by the stage of the embryos treated. If the embryos are immature, slow drying in a closed petri plate for one week may be optimal for germination. But if they are fully mature, rapid drying on a laminar flow hood is preferable (Senaratna *et al.*,

1989; 1990). In *Hevea*, slow desiccation of the somatic embryos or maturation on sucrose medium (351 mol m^{-3}) supplemented with ABA improved the germinability of the embryos and vigor of the developed plantlets (Etienne *et al.*, 1993a). Slow desiccation of the somatic embryos which was matured in an ABA-PEG medium accelerated the viability, shoot elongation and plant conversion in horse chestnut (Capuana and Debergh, 1997). Maturation drying terminates the developmental mode of the embryo, switching on to the germination mode (Kermode *et al.*, 1985; Attree *et al.*, 1991). The desiccated embryos showed higher germination (92%) and plant regeneration capacity (80%) as evidenced in cassava (Mathews *et al.*, 1993).

5.10 Transgenic plant regeneration

The organic nitrogen sources (L-glutamine, L-proline and L-asparagine) promoted plant regeneration in the *hmgr*1 transgenic embryos. The beneficial role of glutamine is its ability to supply amino nitrogen required for the synthesis of nitrogen containing molecules, seed storage proteins etc. In presence of glutamine and proline, the need for reduced nitrogen is met and the energetically expensive process of nitrate reduction is avoided (Guevin and Kirby, 1996). Importance of asparagine on improving the germination rate of the somatic embryos has been reported earlier which was also in accordance with our results. Asparagine increased the tap root length of the germinating embryos as observed in the case of white spruce (Robichaud *et al.*, 2004).

The induction of phytohormones BA, GA_3 and IAA in the plant regeneration medium accelerated germination of the *hmgr*1 transgenic embryos. Plantlets remained healthy and grew vigorously under *ex vitro* conditions. Earlier reports in *Hevea* also suggested the importance of growth regulators, GA_3, Kin and IBA on bipolar differentiation and transgenic plant regeneration (Jayashree *et al.*, 2003; Sobha *et al.*, 2003a). The inclusion of BA promoted

shoot development in the somatic embryos whereas the role of GA_3 might be associated with dormancy breakage allowing easy germination. The influence of GA_3 on somatic embryogenesis and plant regeneration was once again proved in the anther culture of *Hevea* (Kumari Jayasree and Thulaseedharan, 2001). Gibberellic acid (GA_3) also stimulated high frequency plant regeneration (83%) in *Kalopanax pictus* (Moon *et al.*, 2005). In the Siberian ginseng and coconut, the growth regulator GA_3 accelerated somatic embryo germination and plant conversion (Choi *et al.*, 1999a; Montero-Cortes *et al.*, 2010). Shoot regeneration from the nodal explants of cassava, was observed in solidified MS medium containing BA (2.2 μM) and GA_3 (1.6μM) (Bhagwat *et al.*, 1996). Indole acetic acid has a role in increasing the plant conversion rate of the somatic embryos where the plantlets grown in IAA medium accumulated sufficient quantity of ABA to support normal plant regeneration. Supportive evidences were given by Faure *et al.* (1998) where IAA assisted in the release of dormancy and helped in plant regeneration. Somatic embryo germination in the European spindle tree (*Euonymus europaeus* L.) was achieved in a combination of IAA and kinetin (Bonneau *et al.*, 1994). The reduced sucrose concentration (2 to 3%) of the medium also accounted for the plant conversion. Plant regeneration in presence of reduced sucrose concentrations were reported earlier in *Hevea* (Carron *et al.*, 1995b; Sushamakumari *et al.*, 2000 b; Kala *et al.*, 2009).

5.11 *Ex vitro* performance of the transgenic plants

Acclimatization is considered as the process of adaptation to the changing environment. It is essential for the better survival and establishment of the plants. The *hmgr*1 plantlets with two to three whorls of matured leaves and with a well-developed root system were transferred to *ex vitro* conditions. A dip in bavistin solution (0.1%) protected the plantlets from the fungal attack. The potting medium used in our experiment was autoclaved sand: soilrite: soil in the ratio 1:1:1. Different types and combinations of substrates were tried for hardening *in vitro* raised plantlets by many workers (Deb and Imchan, 2010). The *in vitro* raised plantlets showed many abnormalities, both morphological and physiological as a result of the *in vitro* culture conditions. During *in vitro* culture, the plantlets were grown in a relatively air tight culture tubes, in sucrose medium with growth regulators under conditions of high humidity, controlled light and temperature. The growth of the plantlets in the closed culture tubes limited the inflow of CO_2 and the out flow of gaseous products from the plant. Culture in air tight containers also protected the plantlets from the microbial attack. The sugar concentration of the medium indeed reduced the water potential considerably. These conditions induced abnormalities in the *in vitro* raised plantlets (Kumar and Rao, 2012). Another probable reason for the mortality rate of the tissue culture plants is the sudden exposure, particularly the root system, to the microbial community of the soil. Thus upon transfer to *ex vitro* conditions, the plantlets suffered transplantation shocks and therefore a period of acclimatization is needed to correct these abnormalities. The use of growth chambers, which provide controlled conditions to the i*n vitro* raised plantlets, may be helpful in hardening the plantlets. The use of growth chambers helped in the acclimatization of *in vitro* raised plantlets by gradually lowering the humidity of the air (Pospíšilová *et al.*, 1999). During the hardening phase, the *hmgr*1 transgenic plants were watered on alternate days and moistened with

½ x Hoagland's solution, two weeks after transplantation for better survival. Deb and Imchan, (2010) used MS salt solution as the nutrient source for *in vitro* raised plants for one month after which it was replaced with tap water.

In the present study, maintenance of the cultures under controlled conditions in a growth chamber, adjusted to an RH of 85%, reduced the mortality rate of the plantlets and a fairly good response was seen in the case of transgenic plants obtained from the zygotic material. A survival rate of 45% was observed in the transgenic plants raised from the embryogenic callus of zygotic origin. Moreover the plants grew vigorously and they were morphologically similar to their non-transgenic counter parts.

However the survival rate of the plantlets derived from anther tissue was lower. Though tap root formation was noticed, the absence of lateral roots minimized the absorption of water and mineral salts from the potting medium, inhibiting plant growth. Hence hardening process was tedious. Here the main factor assisting zygotic embryogenesis and plant survival is the storage product accumulation so that the embryo gets its reserve during and after germination until the plantlet reaches its autotrophic potential. But in the case of somatic embryos, the lack of food reserve especially triglycerides affected the final stage of development and plant conversion. In addition, transplantation to the growth chamber conditions affected their survival. The impaired absorption of the roots combined with the fungal infections happening at the root shoot junctions, retarded the growth of the plantlets resulting in plant death.

The gene transfer mediated by *Agrobacterium* can bring about alterations in the genetic makeup of the cell. Integration of the transgene over a functional gene can result in the functional loss of that gene culminating in impaired developmental changes. So a workable solution may be the generation of plants from more number of events (pattern of integration may be different in

different events) so that appropriate selection is possible and normal plant regeneration is facilitated. The transgenic plants established even though meager in number, can be multiplied vegetatively by bud grafting. But the plants of zygotic origin were capable of withstanding the hardening process. These differences in the plant conversion between the zygotic and clonal materials under the same germination conditions have been reported in different crops (Sujatha, 2011). The probable reason is the expression of glutamine synthase implicated in the amino acid metabolism in the somatic embryos of clonal origin compared to their zygotic counterparts. The expression of glutamine synthase indicated lack of reserve proteins in these somatic embryos. Analysis of the total protein content between the zygotic and the somatic embryos by two dimensional electrophoresis identified proteins related to glycolysis, citrate cycle and ATP synthesis in the somatic embryos whereas storage and stress related proteins were abundantly seen in the zygotic embryos. The glycolytic, citrate cycle enzymes in the somatic tissues indicated more active energy metabolism and ATP demand which permitted these embryos to enter rapidly in to the germination phase without undergoing a dormancy phase. But in the zygotic derived embryos, the abundance of the protein groups helped in the accumulation of carbohydrates and storage proteins, which assisted in the desiccation of the embryos (Sghaler-Hammamia *et al.*, 2009).

5.12 Molecular characterization of the integrated transgene in *Hevea brasiliensis*

5.12.1. PCR analysis of the transgenic plants

The presence of the transgene was confirmed in the transgenic plants using gene specific and marker specific primers. PCR analysis using *hmgr* specific primers amplified a 640 bp fragment in the transgenic plants and in the positive control (Section 4.12.1, Fig. 9A). The cDNA sequence encoding *hmgr*1

gene was 1.8 kb. But as the primers were designed from the internal sequences of *hmgr*1, it could only amplify a product of 640 bp in the transgenic plants. This fragment was absent in the genome of the non-transgenic plant. The native *hmgr*1 gene was amplified in the non-transformed control where a bigger fragment of size 1.2 kb was amplified which included one intron sequence in between. The presence of the marker gene (*hpt*) was also confirmed in the transgenic and in the positive control where a fragment of length 602 bp was amplified (Section 4.12.1, Fig. 9B).

5.12.2 Cloning and sequencing the PCR product

The identity of the PCR product was confirmed by comparing the sequence information of the PCR product amplified from the transgenic plants with the already reported cDNA sequence published in NCBI.

5.12.3 *In silico* analysis of *hmgr*1 gene sequence

The sequenced PCR product on comparison proved to be 99% similar with the reported cDNA sequence of *hmgr*1 gene availabe in NCBI data base. The amino acid sequence of *hmgr*1 from the transgenic plants was aligned with that of *Hevea hmgr*1, *Euphorbia pekinensis, Taraxacum kok-saghyz, Solanum lycopersicum, Litchi chinensis, Medicago trunculata, Ricinus communis and Arabidopsis thaliana* and a dendrogram was created based on the gene sequences obtained from the GenBank. Here the *Neighbour-joining* method was used to build the tree. Considering the dendrogram created, it was observed that *Hevea* and *Euphorbia pekinensis* came in one cluster indicating their sequence similarity. The *hmgr* sequence of *Solanum* differed from that of *Hevea.* The protein sequence of *Taraxacum* and *Ricinus* were closely related and thus formed a single cluster along with *Medicago* (Section 4.12.3, Fig 14). Multiple sequence alignment is useful in comparing homologous sequences. It plays a crucial role in building phylogenetic relationship. The phylogenetic relationship

between the *hmgr* gene sequences of *Hevea* with other species was drawn by Venkatachalam *et al.* (2009) which showed five clades. He also observed a sequence similarity between *Hevea hmgr* and that of *Euphorbia* and *Morus*, placing them in one clade.

5.12.4 Southern blot analysis of the transgenic plants

Southern blot analysis confirmed the integration of the T-DNA into the nuclear genome of the transgenic plants. The restriction of the DNA from the transgenic plants using individual enzymes mostly yielded fragments of larger size. When the DNA was restricted using *Bam* HI, an internal cassette of *hpt* gene was released. Hybridization with the α- [^{32} P]- d CTP labeled *hpt* gene probe generated strong hybridization signal (Section 4.12.4, Fig. 16) with the *Bam* HI digested transgenic plants indicating insertion of the integrated gene. The *Xba* I and *Bam* HI double digested genomic DNA liberated the *hpt* transgene in the T-DNA and produced a strong hybridization signal in the transgenic plants and positive control on hybridization with the labeled *hpt* probe. The different hybridization patterns observed in the transgenic plants indicated the integration of the T-DNA in the genome of the transgenic plants. Other smaller bands could be detected due to the incomplete digestion of the genomic DNA of the transgenic plants. This type of insertion has been reported earlier in transgenic *Hevea* plants integrated with the *Hb* MnSOD gene (Jayashree *et al.*, 2003).

Digestion with *EcoR*I which has a site near the left border of the T-DNA produced a single hybridization band in one transgenic plant. This type of banding pattern confirmed single copy insertion of the transgene in the transgenic plant. However the signal was absent in the other plant. The absence of the signal may be due to the lower enzyme activity in the plant as observed in the ELISA test. The hybridization signal was absent in the non-transformed

control plant too. These results provide strong evidence that the *hpt* transgene was stably integrated in the genome of the transgenic plants through *Agrobacterium* mediated transformation.

5.12.5 Expression analysis in the transgenic plants

Transgene expression studies are valuable in examining the ultimate effect of transgene for which the study of enzymes and metabolites are essential. The transcript level in the transgenic plant can be quantified by northern analysis. Transgenic proteins can be analyzed by many techniques, one among them is ELISA.

5.12.5.1 Northern blotting

The transcript level of *hmgr*1 gene was quantified in the transgenic plant using northern blotting. The probe used to confirm the over expression was *hmgr*1cDNA from *Hevea brasiliensis*. The absence of the hybridization signal in the untransformed control plant may be due to the lower transcript of that particular isoform (*hmgr*1) in the leaves of the young plant analyzed. Moreover, high expression is expected in the laticifers which could be analyzed only at later stages. Strong hybridization signal was observed in the transgenic plant as we tried to overexpress this particular gene using a strong promoter so that expression is expected in all the tissues. The overexpression of *hmgr*1 gene using the constitutive promoter might have resulted in the accumulation of the transcript in all the tissues which produced strong hybridization signal in the sample.

5.12.5.2 Enzyme analysis in transgenic plants

Analysis of the HMGR activity in the leaves of the transgenic plants using indirect ELISA, demonstrated a higher activity in the transgenic plants compared to control plants. The specific activity varied between the tested plants. Among the four plants experimented, the specific activity of one tested

transgenic plant was comparable to the control plant, while others showed 2-8 fold increase in the specific activity (Fig. 21). Measurement of enzyme activity through ELISA has been reported in maize (Moore *et al.*, 2003). Integration of *Hevea hmgr*1 gene in tobacco resulted in an increased enzyme activity in three of the five tested transgenic plants (Schaller *et al.*, 1995). This may be due to the deregulation of the native gene caused by the active transcription of the transgene resulting in a modest increase in the enzyme activity. Similar observations were made by Re *et al.* (1995) in *Arabidopsis* where a higher mRNA level was noticed in the transgenic plants with only a marginal increase in the HMGR activity.

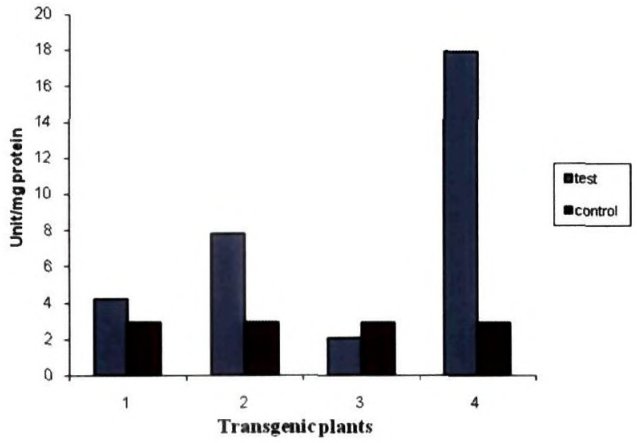


Fig.21. Specific activity of HMGCoA reductase in the leaf samples of transgenic and control plants of *Hevea*.

The plantlets were acclimatized and maintained in the net house. The contribution of the increased enzyme activity in the transgenic plants towards latex yield in *Hevea* can be ascertained only after test tapping the plants when they attain a tappable girth.

5.13 Biosafety issues related to the release of transgenics

After conducting the gene expression studies, the transgenic plants have to be evaluated for the yield trait. Plants showing better yield can be selected after tapping the trees when they reach a tappable girth. The promising ones can be picked and multiplied for initiating a small scale trial. During the transgenic work, the biosafety aspects were strictly followed and the progress of the transgenic work was evaluated at a regular basis by the Institutional Biosafety Committee. For conducting a small scale trial, permission has to be accorded by the Review Committee on Genetic Manipulation (RCGM). The trial location, biological details of the crop, method of pollination or the pollinating agents if any, details of its wild relatives, full details of the construct, including sequence information of the vector with all its genes (marker genes, reporter genes, promoter sequences) etc. have to be provided to RCGM with ample evidence proving the beneficial effect of the transgenics for obtaining the sanction for conducting the small scale trial. The Genetic Engineering Approval Committee (GEAC) considers the application and issues the orders for the conduct of the trial.

The general concern on the release of the transgenic plants includes environmental and agronomic impacts of transgene escape. The transgene escape from the ecological perspective depends on whether the crop has a wild relative and its ability to cross pollinate them. These issues can be sorted out by commending that *Hevea* is not a native plant of Asia and therefore no sexually compatible wild relatives of the crop is available to spread. *Hevea* being propagated vegetatively by bud grafting minimizes the risk further. The short and the long term effects caused on the environment by the release of the transgenic crops should be analyzed properly. For this a minimum isolation distance has to be observed during the initiation of the field trial. The risk of

pollen flow can be prevented further by planting tall barrier plants which physically prevent it.

Another vital issue is the depletion in the biodiversity. Policy makers should take a decision not to release the transgenics into its own centers of origin or to the delicate ecological zones or pockets rich in biodiversity. In our case, we are only attempting to overexpress an endogenous gene and therefore the release of such transgenic plants might not threaten the environment. The ill effects of the selectable marker genes or its products to mankind, insects etc. has to be studied thoroughly, since the transfer of these genes from the plants to the pathogenic organisms may lead to the evolution of antibiotic resistant pathogens. Above all, there is an urgent need to create public awareness on the benefits as well as the risk factors related to transgenics and also the importance of protecting the genetic diversity.

SUMMARY AND CONCLUSION

Chapter 6

SUMMARY AND CONCLUSION

The increasing global consumption of natural rubber resulted in a demand supply imbalance, which necessitated an increase in the production capacity of natural rubber. The production capacity can be improved by accelerating the productivity of rubber plantations and by expanding the crop to new and non-traditional areas. The productivity of the crop depends on the growth of the trunk both in the immature/ mature stages. Another important aspect is the land productivity improvement and optimum planting density. The land productivity can be achieved by cultivating high–yielding clones with desirable characteristics (resistance to abiotic/biotic stresses, wintering etc.) which aims at increasing the yield per tree. The yield potential of each tree in a plantation is now becoming an important parameter in order to improve the land use efficiency.

Development of superior clones with enhanced latex yield and stress tolerance (abiotic/biotic) is essential for the extension of the crop to the non-traditional belts so as to obtain a meaningful amount of yield under stressful conditions. The efforts made by the breeding and the latex harvesting group has improved rubber productivity, but a stage will be reached when the rubber biosynthetic rate of the tree itself become a limiting factor. At this juncture, yield enhancement can be made only by manipulating the factors influencing the rate of rubber biosynthesis. Biotechnological tools assisted in the transfer of genes for agronomically important traits, where specific changes can be made within a short time frame. The quantification of the pathway enzymes related to yield indicated a lower HMGR activity in *Hevea* latex compared to other downstream enzymes up to IPP. The diurnal variation in the HMGR enzyme activity, indicating its close correlation with the yield, forced us to select *hmgr*1

gene as a potent candidate for genetic transformation experiments to enhance the rubber yield. Hence transgenic approaches were utilized to over express laticifer specific *hmgr*1 gene in *Hevea brasiliensis* and to develop transgenic plants *via Agrobacterium* mediated transformation.

The target material for genetic transformation experiments were raised from immature anthers as well as from the zygotic embryos. Primary callus from the immature anthers was obtained in modified MS medium supplemented with 2,4-D (1.0 mg l^{-1}), BA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}). This callus was made embryogenic in half strength MS medium containing TDZ (0.6 mg l^{-1}) and NAA (1.0 mg l^{-1}) in presence of BA (0.3 mg l^{-1}). Actively dividing cell suspensions were raised from the embryogenic callus in ½ x MS liquid medium containing higher levels of sucrose (60 g l^{-1}) along with growth regulators NAA (0.5 mg l^{-1}) and TDZ (0.3 mg l^{-1}). These three callus types obtained from the immature anthers of *Hevea* were used as the clonal explants for genetic transformation. The embryogenic callus obtained along with the developing zygotic embryos in Nitsch basal medium were proliferated in a hormonal combination of 2,4-D (0.3 mg l^{-1}), Kin (0.5 mg l^{-1}), NAA (0.3 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). The embryogenic calli of zygotic origin were used as the initial explant for genetic transformation.

The importance of the *Agrobacterium* strain in determining the transformation efficiency was proved where EHA 105 was superior to the other two strains tried. LBA 4404 was the least virulent strain since transgenic cell emergence was absent from the LBA 4404 infected tissues. Embryogenic suspension cultures gave the highest transformation efficiency (27%) among the clonal explants used. Transformation frequency of 15% was obtained with the embryogenic callus followed by the primary callus (9%) derived from immature anthers of *Hevea*. Among the different tissues experimented, maximum transformation frequency (67%) was observed using the embryogenic callus of

zygotic origin, irrespective of the *Agrobacterium* strains used. The second best strain was pGV1301 producing transgenic cell lines with a lower frequency with all the tissue types experimented. Even with the most juvenile and responsive tissue (embryogenic callus of zygotic origin) the frequency of transformation recorded was 22%, followed by embryogenic cell suspensions producing transgenic cell lines at an efficiency of 16%. Using the primary callus, the frequency of transformation was 7% which was slightly improved with the embryogenic callus of the anther (8%). Pretreating the target tissues using the anti-necrotic mixture, desiccating the tissues and inclusion of L-cysteine and silver nitrate in the co-culture medium failed to improve the transformation frequency with the *hmgr*1 gene construct in *Hevea*. However co cultivation of the infected tissues at low incubation temperature (20°C) elicited the transformation efficiency (only in presence of acetosyringone) with this gene construct from 27% (control treatment) to 32%. The cell lines obtained in the selection medium were proliferated and the presence of the transgene was confirmed in the transformants by PCR analysis using gene specific and marker specific primers.

Plantlets were produced from the putatively transgenic cell lines obtained from the embryogenic cell suspensions of immature anthers and from the embryogenic callus of zygotic origin. The importance of the basal salts on somatic embryogenesis was confirmed where lower levels of ammonium nitrate along with an increase in the KNO_3 concentration improved the embryo induction efficiency. Addition of organic nitrogen sources also stimulated somatic embryogenesis from the transformed cell lines. Maximum embryogenesis was observed with the inclusion of L-alanine 40.96%, followed by L-asparagine (39.22%), L-proline (38%), L-glutamine (37.44%) and L-serine (27.94 %). The efficiency of embryogenesis varied with different growth regulators, maximum being observed using Kinetin (0.7 mg l^{-1}) and

NAA (1.0 mg l^{-1}) combinations. Polyamines regarded as the secondary hormonal messengers also influenced somatic embryogenesis. Inclusion of the polyamine, spermidine was better than spermine and putrescine, where somatic embryos were produced at a frequency of 41% with the addition of spermidine (2.0 mg l^{-1}) in the basal medium. The embryo induction medium containing spermidine (1.5 mg l^{-1}) along with higher levels of sucrose (80 g l^{-1}) triggered somatic embryogenesis at a very high frequency (72%). Maturation medium devoid of NH_4NO_3, supplemented with higher concentrations of KNO_3 helped in the maturation of somatic embryos (25 %). Organic nitrogen sources (glutamine 1.0 g l^{-1} and proline 200 mg l^{-1}) promoted the growth and maturation of the embryos. The conversion of the somatic embryos from the late torpedo to the cotyledon stage was accomplished in presence of ABA (0.4 mg l^{-1}) and PEG (12%). Partial desiccation of the embryos (24 hrs) on a laminar flow hood accelerated the induction of shoot meristem in the cotyledon staged embryos. Somatic embryo germination was achieved in one–fourth MS (¼ MS) medium with double strength minor elements additionally supplemented with KNO_3 (2.0 g l^{-1}) and K_2SO_4 (400 mg l^{-1}). Plant regeneration was favored in a hormonal combination of BA, GA_3 and IAA. A plant regeneration frequency of 5% was noticed from the suspension raised transgenic cell lines. Transgenic plant development was faster from the zygotic material where the plant regeneration efficiency was 44%. The germinated plantlets were initially maintained under controlled conditions in a growth chamber after which the acclimatized plantlets were transferred to big poly bags (9 ½"x 22") filled with soil: sand: cow dung in the ratio 2:1:1.

The presence of the transgene was confirmed in the transgenic plantlets using PCR analysis where gene specific primers amplified a 640 bp fragment in the transgenic and positive control whereas a fragment of 1.2 kb length was amplified in the untransformed control. Amplification using marker specific

primers amplified a fragment of 602 bp in the transgenic plants and in the positive control. The corresponding band was absent in the non-transgenic plants.

The integrity of the PCR product was further confirmed by cloning and sequencing the eluted PCR product from the transgenic plants amplified using the promoter specific forward and *hmgr*1 specific reverse primers. The sequence analysis showed a maximum similarity of 99% with the already reported c DNA sequence of *hmgr*1 gene. Multiple sequence alignment of the predicted amino acid sequence of *Hevea hmgr*1 gene with the corresponding genes from various taxa using Clustal omega showed 77% identity with the amino acid sequence of *Arabidopsis,* 70 - 72% similarity with those of *Taraxacum, Medicago* and *Ricinus,* 81% with *Euphorbia* and *Litchi* and a 60% similarity with *Solanum* respectively. From the dendrogram created, *Hevea, Euphorbia pekinensis* and *Litchi* came in one cluster indicating their sequence similarity. The amino acid sequence of *Solanum* was distinct and differed from that of *Hevea.* The protein sequence of *Taraxacum* and *Ricinus* were closely related, forming a single cluster, along with *Medicago.*

The integration of T DNA region into the nuclear genome of the transgenic plants was confirmed by genomic Southern blot analysis. The *Bam* HI, *Xba* I double digestion of the genomic DNA liberated the inserted *hpt* gene sequence from the two transgenic plants and the plasmid DNA. Subsequent hybridization with the labeled *hpt* gene probe resulted in a strong hybridization signal in the transgenic plants and in the positive control. Single enzyme digestion of the genomic DNA from the two transgenic plants with the *Bam* HI, followed by hybridization with the labeled *hpt* gene probe produced a strong positive signal in the transgenic plants indicating the insertion of the transgene in the nuclear genome of the transgenic plants. The genomic DNA digestion using *EcoR*I, having a restriction site near the LB of the T- DNA, followed by

hybridization resulted in a single hybridization band in one plant, whereas the signal was absent in the other one, may be due to the low copy number of the integrated gene in the plant. Further, the activity of the *hmgr*1 was semi-quantified in the transgenic plants using ELISA. The enzyme activity varied between the tested plants. Among the plants experimented, a 2-8 fold increase in the specific activity was noticed in three transgenic plants whereas the activity in one transgenic plant was comparable to the control plant. The expression of the transgene was further confirmed using northern analysis where the transcript level was observed in the transgenic plants; but absent in the untransformed control.

An *Agrobacterium* - mediated genetic transformation and plant regeneration system was developed in *Hevea* overexpressing *hmgr*1 gene. Parameters influencing genetic transformation, somatic embryogenesis and transgenic plant regeneration were analyzed. Transgenic plants integrated with the laticifer specific *hmgr*1 gene were developed in *Hevea* which is the first report of its kind. Forty five transgenic plants were successfully hardened and established in the net house under ambient conditions. PCR analysis and Southern confirmed the integration of the transgene in the plants. The transgenic plants were morphologically undistinguishable from the control plants. Selected PCR positive plants were multiplied through bud grafting to study the yield pattern in the plants. HMGR activity can be used an early selection tool if a positive correlation is established between enzyme activity and the latex yield. Thus the transgenic technology can be used for improving yield in low yielding *Hevea* plants tolerant to abiotic/biotic stresses. The present study utilized constitutive expression of the integrated transgene which may be replaced with laticifer specific promoters to get a tissue specific expression of the transgene which will be of great importance in future.

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APPENDICES

APPENDIX - A

Luria Bertani medium (LB)

Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	10 g l^{-1}
Bactoagar	15 g l^{-1}
pH	7.0

Culture medium for *Agrobacterium* growth and infection of the target tissues

Components	Induction medium (IM)	Co culture medium (CCM1)	Selection medium (SM)
Major *NH_4NO_3	MS (modified)* 500 mg l^{-1}	MS (modified)* 500 mg l^{-1}	MS (modified)* 500 mg l^{-1}
Minor	MS	MS	MS
FeNaEDTA	37.5 mg l^{-1}	37.5 mg l^{-1}	37.5 mg l^{-1}
Myoinositol	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}
Vitamins	B_5	B_5	B_5
Sucrose	20g l^{-1}	30g l^{-1}	30g l^{-1}
2,4-D	--	1.0 mg l^{-1}	0.2mg l^{-1}
Kinetin	--	--	0.5 mg l^{-1}
BA	--	0.5 mg l^{-1}	0.5 mg l^{-1}
NAA	--	--	0.4 mg l^{-1}
Acetosyringone	20 mg l^{-1}	20 mg l^{-1}	--
Glycine betaine hydrochloride	153.6 mg l^{-1}	153.6 mg l^{-1}	--
Proline	115.5 mg l^{-1}	115.5 mg l^{-1}	--
Cefotaxime	--	--	500 mg l^{-1}
Hygromycin	--	--	30 mg l^{-1}
Phytagel	--	0.2%	0.3%
pH	5.2 – 5.3	5.7 – 5.8	5.7 – 5.8

APPENDIX - B

Basal components of plant tissue culture medium

Components	Murashige & Skoog Medium (MS)	Nitsch & Nitsch medium (N_6)	WPM(Lloyd and McCown)
Major (mg l^{-1})			
NH_4NO_3	1650	--	400
$CaCl_2.2H_2O$	332.2	166	96
$Ca(NO_3)_2.4H_2O$	--	--	556
$MgSO_4.7H_2O$	370	185	370
KNO_3	1900	950	--
KH_2PO_4	170	68	170
K_2SO_4	--	--	990
Minor (mg l^{-1})			
H_3BO_3	6.2	10.0	6.2
$CoCL_2.6H_2O$	0.025	--	--
$CuSO_4.5H_2O$	0.025	0.025	0.25
$MnSO_4.H_2O$	16.9	18.9	22.3
KI	0.83	--	--
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$ZnSO_4.7H_2O$	8.6	10.0	8.6
Na_2EDTA	37.3	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8	27.8
Organics (mg l^{-1})			
Myoinositol	100	100	100
Glycine	2.0	2.0	2.0
Nicotinic acid	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	0.5	1.0
Biotin	--	0.2	--

Culture medium for somatic embryogenesis and plant regeneration from transgenic cell lines

Components	Proliferation medium (CCM_2)	Embryo induction medium (AG_1)	Maturation medium (MSO)	Plant regeneration medium (MS_4)
NH_4NO_3	--	200 mg l^{-1}	--	400 mg l^{-1}
KNO_3	--	2000 mg l^{-1}	3000 mg l^{-1}	2000 mg l^{-1}
$MgSO_4.H_2O$	--	90 mg l^{-1}	370 mg l^{-1}	90 mg l^{-1}
KH_2PO_4	--	170 mg l^{-1}	370 mg l^{-1}	40 mg l^{-1}
K_2SO_4	--	--	300 mg l^{-1}	400 mg l^{-1}
$CaCl_2.2H_2O$	--	333 mg l^{-1}	333 mg l^{-1}	80 mg l^{-1}
$Ca(NO_3)_2.4HO$	--	--	--	--
Minor	MS	MS	MS	MS
2,4-D	0.5 mg l^{-1}			
NAA	0.2 mg l^{-1}	1.0 mg l^{-1}	--	--
Kinetin	0.5 mg l^{-1}	0.7 mg l^{-1}	--	--
ABA	--	--	0.4 mg l^{-1}	--
BA	--	--	--	1.0 mg l^{-1}
GA_3	--	--	--	0.8 mg l^{-1}
IAA	--	--	--	0.3 mg l^{-1}
Hygromycin	30 mg l^{-1}	--	--	--
L-alanine	--	100 mg l^{-1}	1000 mg l^{-1}	--
L-glutamine	--	400 mg l^{-1}	--	800 mg l^{-1}
L-asparagine	--	300 mg l^{-1}	--	300 mg l^{-1}
Serine	--	--	--	--
L-arginine	--	--	--	--
Spermidine	--	1.5 mg l^{-1}	--	--
L-proline	--	100 mg l^{-1}	200 mg l^{-1}	150 mg l^{-1}
Casein hydrolysate	--	--	500 mg l^{-1}	300 mg l^{-1}
Yeast extract	--	--	--	100 mg l^{-1}
Sucrose	30 g l^{-1}	80 g l^{-1}	30 g l^{-1}	30 g l^{-1}
Coconut water	--	10%	10%	10%
PEG	--	--	12%	--
Charcoal	--	0.2%	0.2%	0.2%
Phytagel	0.3%	0.3%	0.3%	0.25%
pH	5.7 – 5.8	5.7 – 5.8	5.7 – 5.8	5.7 – 5.8

APPENDIX - C

Loading dye (6X buffer)

Bromophenol blue	- 0.025 g
Xylene cyanol	- 0.025 g
Glycerol	- 30 %
Milli Q water	- 10 ml

5X TBE

Tris- base	- 540 g
EDTA (2 mM)	- 46 g
(pH 8.0)	
Boric acid	- 276 g
Distilled water	- 10 litre
Stored at room temperature	

TE buffer

1M Tris HCl (p H 8.0)	- 1 ml
EDTA 0.5 M	- 0.2 ml
Mixed 1 ml of 1M Tris and 0.2 ml of 0.5 EDTA in an autoclaved bottle and made up to 100 ml by adding 98.8 ml of milli Q water. Autoclaved and stored	

RNase A

Pancreatic RNase A at a concentration of 10 mg /ml was dissolved in 0.01M sodium acetate (pH 5.2). Heat to 100°C for 15 minutes. Cool to room temperature. Adjust the pH by adding 0.1 volume of 1 M Tris-Cl (pH 7.4). Dispense into aliquots and store at -20°C.

IPTG

IPTG - 1.2g

Added water to get a final volume of 50 ml. Filter sterilized and stored at 4° C.

X-gal (2 ml)

5-Bromo,4-Chloro,3-indolyl β-D-galactoside - 100 mg

Dissolved in 2 ml N,N'-dimethyl-formamide.

Covered with aluminium foil and stored at -20° C.

SOC medium

Bacto –tryptone - 2.0 g

Bacto –yeast extract - 0.5 g

1M NaCl - 1.0 ml

1M KCl - 0.25 ml

2M $MgCl_2$ (filter sterilized) - 1.0 ml

2M glucose (filter sterilized) – 1.0 ml