Biochemical and Molecular Studies on Enzymes Related to Rubber Biosynthesis in *Hevea brasiliensis*

Thesis submitted to

Mahatma Gandhi University

For the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

(Faculty of Science)

By

AMBILY P. K.

Under the supervision and guidance of

Dr. Molly Thomas

RUBBER RESEARCH INSTITUTE OF INDIA
KOTTAYAM, KERALA
INDIA-686 009

March 2016

Dedicated to My Parents...

DECLARATION

I hereby declare that the thesis entitled **"Biochemical and Molecular Studies on Enzymes Related to Rubber Biosynthesis in *Hevea brasiliensis*"** is an authentic record of original research carried out by me under the supervision and guidance of Dr. Molly Thomas, Principal Scientist, Crop physiology division, Rubber Research Institute of India, Kottayam-9 in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY of the Mahatma Gandhi University and no part of this work has been presented for any degree, diploma or any other similar titles of any university.

Ambily.

RRII

23-03-2016.

Ambily P. K.

THE RUBBER RESEARCH INSTITUTE OF INDIA
RUBBER BOARD, KOTTAYAM- 686 009
(Ministry of Commerce, Government of India)

CERTIFICATE

This is to certify that the thesis entitled **"Biochemical and Molecular Studies on Enzymes Related to Rubber Biosynthesis in *Hevea brasiliensis*"** is an authentic record of original research carried out by Mrs. Ambily P. K., at Rubber Research Institute of India, Kottayam-9, under my supervision and guidance for the award of the degree of **Doctor of Philosophy in Biochemistry**, under the faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been presented or submitted earlier for any degree, diploma or any other similar titles of any university.

RRII
23.03.2016

Dr. Molly Thomas
Principal Scientist
Crop Physiology Division
Rubber Research Institute of India
Kottayam-9

Phone : 91-481-2353311 (10 lines) Fax: 91-481-2353327

ACKNOWLEDGMENT

I wish to express my deep sense of gratitude and indebtedness to my guide ***Dr. Molly Thomas****, Principal Scientist, Crop Physiology Division, Rubber Research Institute of India, Kottayam, for helping me to successfully complete my work. Her valuable advice and splendid supervision improved the quality of my work at all stages and her constant encouragement enabled me to present the work in this format. I sincerely thank her for the patience, confidence and faith she had in me during the course of my doctoral thesis.*

I sincerely express my deep sense of gratitude to ***Dr. James Jacob****, Director of Research, Rubber Research Institute of India, Kottayam for allowing me to conduct this doctoral research at Rubber Research Institute of India, Kottayam and his encouragement and support throughout my work.*

I convey my thanks to ***Dr. K. Annamalainathan****, Joint Director, Crop Physiology Division, Rubber Research Institute of India, Kottayam, for his valuable and timely suggestions and providing all the facilities during the course of my studies.*

I am deeply indebted to ***Dr. R. Krishnakumar****, Joint Director, Climate Change and Ecosystem Studies, Rubber Research Institute of India, Kottayam, for the support and encouragement rendered during my studies. His valuable suggestions and critical comments were highly helpful in refining the work at various stages of this study.*

I wish to express my heartfelt sense of gratitude to ***Dr. K. V. Sumesh****, Scientist B, Crop Physiology Division, Rubber Research Institute of India, Kottayam, for his timely help and support for the preparation of the thesis.*

The timely support given by ***Dr. M.B. Mohammed Sathik****, Principal Scientist Botany,* ***Dr. C.P. Reghu****, Joint Director (Retired) and* ***Dr. A. Thulaseedharan****, Joint*

Director, Biotechnology Division, Rubber Research Institute of India, Kottayam, were invaluable on academic and personnel level for which I am grateful.

Special thanks to ***Dr. Prakashkumar B.****, Associate Professor, School of Biosciences, M. G. University, Kottayam, for his timely advice during my research.*

It is a great pleasure to express my deep sense of gratitude to ***Dr. S. Sreelatha, Dr. Jayasree Gopalakrishnan, Dr. D. Bhuvanendran Nair, Dr. Jayantha Sarkar, Dr. Sheela P. Simon and Mr. Harikumar*** *of Crop physiology Division, Rubber Research Institute of India, Kottayam.*

I owe my thanks to ***Rajan Mathew, P.M. Sebastian, M.B. Rajan*** *and all other members of Crop Physiology Division for their timely help in different stages of this work.*

I wish to express my gratitude to ***Dr. Shaji Philip*** *and* ***Dr. Bindu Roy*** *of Plant Pathology Division,* ***Dr. K. U. Thomas*** *of Genome Analysis,* ***Dr. Karunaichami*** *and* ***Dr. Rajagopal*** *of Latex Harvest Technology Division for their valuable advices and suggestions.*

My heartfelt thanks for the support, encouragement and constant companionship of Dr. Mrudula P Musthapha, Mrs. Lisha P. Luke, Mrs. Linu Kuruvila, Mrs. Smitha M. Xavier, Mrs. Anu Mary Joseph, Mrs. Rejitha Rajan, Dr. Satheesh P. R., Mr. Joby Joseph and Mr. S. Pramod during my fabulous days in RRII.

I convey my thanks to staff members of library, computer section and other staff members of RRII, Kottayam.

I duly acknowledge with full heart the personal sacrifices, timely permission, incessant encouragement and moral support of my husband Mr. Kitie Lal and my three little kids Meghna, Riya and Rithu, which enabled me to complete this venture successfully.

I bow down to my parents, my brother, my in-laws, and all my family members for their moral support, immense patience and loving care that made to successfully complete my thesis work and showing confidence in every moments of my life.

I am greatful to Sri. Madhusoodhanan, Principal Scientist, Rubber Technology Division, Rubber Research Institute of India, for the preparation of the cover page.

I sincerely thank M/s Minitek Computers, Opp. M.G. University, Kottayam for the neat documentation of the thesis.

Above all, I bow my head before the ***God Almighty*** *whose blessings were always with me enabling to undertake this endeavor successfully.*

Ambily P. K.

ABSTRACT

Hevea brasiliensis is the primary source of natural rubber, an industrial/ engineering raw material. The properties of natural rubber make it superior and competitive to synthetic rubber for use in several industrial applications. As a result of the recent intensive research and development in crop improvement programmes, several new high yielding varieties of *Hevea* have been evolved and widely cultivated in India. There are clonal variations in the productivity of rubber and it depends on the capability of each clone to synthesize latex which contains rubber.

Sucrose is the primary precursor of natural rubber biosynthesized in the laticiferous tissue *via* isoprenoid pathway. The quantity of latex obtained from different *Hevea* clones may be the result of variations in the activities of the enzymes involved in rubber biosynthesis. The enzymes directly involved in the biosynthesis of rubber particle would be of great importance to specify the rate limiting steps in the pathway. Determining the activity of such key enzymes in the pathway would help in identifying markers for yield potential in *Hevea*. Molecular markers related to latex yield are useful in the crop improvement research programmes and they can reduce the duration of the breeding cycle of *Hevea*. Information is rather scarce on key biomolecules closely related to rubber yield in *Hevea*. The clonal variations in the activity of rubber biosynthetic enzymes and expression of the corresponding genes are not investigated thoroughly. Hence, the present work on biochemical and molecular studies on enzymes related to rubber biosynthesis in *Hevea brasiliensis* was carried out in clones with varying yield potentials for developing biochemical / molecular markers which can be used as tools for early yield prediction in *Hevea*.

In the present study, expression of 14 genes associated with rubber synthesis was analyzed in high/ low yielding clones of *Hevea brasiliensis* (with and without stimulant application). The level of expression of *HbSUT3*, a sucrose transporter was found to be significantly higher in high yielding than low yielding clones. In *H. brasiliensis* sucrose is the primary substrate for rubber biosynthesis. The expression of genes corresponding to enzymes like hydroxymethyl glutaryl- CoA synthase (HMGS), HMGR and mevalonate diphosphate decarboxylase (MVD) was found to be directly related to yield potential of clones, i.e. high expression in high yielding and low expression in poor yielding clones. The up regulation of these genes might result in an increased supply of IPP, the isoprenoid monomer, for rubber biosynthesis. Expression of genes in the downstream biosynthetic pathway like *FPPS*, *RuT* and *REF2* was also found to be significantly higher in high yielding clones than low yielding clones. The results suggest that high rubber yield potential is associated with relatively high expression of these genes. These are useful markers for high yield potential of *Hevea* clones.

The expression of genes associated with rubber biosynthesis was compared between ethylene stimulated and unstimulated trees of different clones. The expression of *HbSUT3* was significantly higher in stimulated than unstimulated trees of both high and low yielding clones. The results suggest that *HbSUT3* plays an important role in the sucrose loading of the laticifer tissue and thus in rubber production in stimulated trees. Productivity of rubber depends on sufficient recouping of the expelled latex before the next harvesting, which requires an efficient and continuous sucrose supply. The expressions of *hmgr*1 and *MVD* were induced by ethylene treatment (stimulant application) and the results suggest that the enzymes they encode are involved in enhanced IPP supply for rubber biosynthesis under ethylene stimulation.

The studies on activity of HMGR in different *Hevea* clones showed that high yielding clones had a correspondingly higher HMGR activity than the low yielding clones. HMGR activity showed a positive correlation with rubber yield. However, it is interesting to note that HMGR activity was not induced by ethylene stimulation. The study suggests that quantification of HMGR activity may be useful to score high yielding *Hevea* clones. Further, the cloning of *hmgr*1 gene in expression vector and the *in vitro* synthesis of HMGR protein was also successfully carried out. The resultant protein would be useful as a marker for screening yield potential of *Hevea* clones.

Keywords: Clonal variation, Enzyme assay, Gene expression analysis, *Hevea brasiliensis,* Rubber biosynthesis, Yield potential.

PREFACE

Hevea brasiliensis is an important crop under commercial cultivation in the world for natural rubber production, a vital industrial raw material. Natural rubber (*cis*-1, 4-polyisoprene) is synthesized from the precursor sucrose *via* the isoprenoid pathway in plants with laticiferous tissue. The processes involved in the biosynthesis are controlled by essential metabolic steps that involve the loading of sucrose in the laticiferous vessels and the regulation /activation of certain enzymes leading to rubber biosynthesis. Several high yielding clones of *Hevea* have been evolved as a result of successful breeding programmes. The productivity of a clone depends on its potential to synthesize latex and is associated with the rate of activities of enzymes involved in the isoprenoid pathway leading to the synthesis of rubber.

A lot remains unknown about the biochemical and molecular aspects of enzymes /factors involved in rubber biosynthesis in different clones of *Hevea brasiliensis*. Definite information is lacking correlating various biochemical factors with the yield of rubber which can be used as markers for high yield potential of *Hevea* clones. Hence, the present work on biochemical and molecular studies on enzymes related to rubber biosynthesis in *Hevea brasiliensis* was conducted using high and low yielding clones for developing markers for yield potential in *Hevea* clones.

In the present work, biochemical and molecular aspects of a few key enzymes associated with rubber biosynthesis in *Hevea brasiliensis* were studied in selected clones with different yield potentials. Gene expression analysis of some important rubber biosynthetic enzymes was carried out in different *Hevea* clones with and without yield stimulation. Hydroxy-methtyl-glutaryl-CoA reductase (HMGR), a rate limiting enzyme of rubber biosynthetic pathway, was quantified and HMGR activity was correlated

with total rubber yield. Molecular cloning and expression analysis of *hmgr*1 was done and *in vitro* synthesis of HMGR protein was successfully carried out. The general concept of the research topic, its significance and objectives of the study, review of literature, results and conclusions are described in six different chapters of the thesis.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

^{14}C	-	Carbon 14
BLAST	-	Basic Local Alignment Search Tool
BSA	-	Bovine serum albumin
CPT	-	cis-1, 4-prenyl transferase
Ct	-	Cycle threshold
CTAB	-	Cetyl trimethyl ammonium bromide – (hexadecyle trimethylammonium bromide)
DEPC	-	Diethyl pyrocarbonate
DMAPP	-	Dimethylallyl pyrophosphate
DOXP	-	1-deoxy-D-xylulose-5-phosphate
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetra acetic acid
eIF-5A	-	Eukaryotic translation initiation factor
EST	-	Expressed Sequence Tag
FPP	-	Farnesyl pyrophosphate
FPPS	-	Farnesyl pyrophosphate synthase
GGPP	-	Geranylgeranyl pyrophosphate
GGPS	-	Geranylgeranyl pyrophosphate synthase
GPP	-	Geranyl pyrophosphate
SUT	-	Sucrose transporter
HMG-CoA	-	Hydroxy-methyl glutaryl- CoA
HMGR	-	Hydroxy-methyl glutaryl reductase

HMGS	-	Hydroxy-methylglutaryl-CoA synthase
IPP	-	Isopentenyl pyrophosphate
IPTG	-	Isopropyl -β-D-1-thiogalactopyranoside
kDa	-	kilo Dalton
LB	-	Luria Burtany
MEP	-	2-C-methyl-D-erythritol-4-phosphate
MnSOD	-	Manganese superoxide dismutase
MOPS	-	3-Morpholinopropane-1-sulfonic acid
MVA	-	Mevalonate
MVD	-	Mevalonate decarboxylase
MVDP	-	Mevalonate diphosphate
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NCBI	-	National Center for Biotechnology Information
Oligo DT	-	Deoxy-thymine nucleotides
PAGE	-	Polyacrylamide gel electrophoresis
PB	-	Prang Besar, Malaysia
PPMVA	-	Pyrophosphomevalonate
PSD	-	Particle Size Dispersion
PVP	-	Poly vinyl pyrrolidone
RBIP	-	Rubber biosynthesis inhibitor protein
RBSP	-	Rubber biosynthesis stimulator protein
REF	-	Rubber Elongation Factor
Rf	-	Retardation factor

RRIM	-	Rubber Research Institute of Malaysia
RuT	-	Rubber transferase
SDS	-	Sodium dodecyl sulfate
SOB	-	Super Optimal Broth
TEMED	-	Tetra methyl ethylene diamine
Tjir	-	Tjirandji, Indonesia
TLC	-	Thin layer chromatography
TPD	-	Tapping panel dryness
Tris- HCl	-	Tris(hydroxymethyl)aminomethane hydrochloride
X-Gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

General Introduction

1.1. The rubber tree (*Hevea brasiliensis*)

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important perennial crop that produces natural rubber (NR), the indispensable industrial/engineering raw material for manufacturing several products that are in everyday use. NR is synthesized by more than 2000 plant species confined to 300 genera of seven families *viz.*, Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae, but most of them make short chain rubber molecules which are inferior in quality for commercial use (Archer *et al.*, 1963; Backhaus, 1985; Cornish, 1993). *Hevea brasiliensis* belongs to the genus *Hevea* of the Euphorbiaceae family. There are ten species under the genus *Hevea* viz, *H. benthamiana, H. brasiliensis, H. camargoana, H. camporum, H. guianensis, H. microphylla, H. nitida, H. pauciflora, H. rigidifolia* and *H. spruceana* (Schultes, 1990; Wycherley, 1992). Among the various species of the genus, *H. brasiliensis* (Fig. 1.1) is the major commercially grown species for the source of NR because of its high yield and superior rubber quality (Cornish, 2001; Asawatreratanakul *et al.*, 2003).

Rubber is water resistant, does not conduct electricity and is highly elastic. These properties are due to the large and complex molecular structure of rubber (*cis*-1, 4 polyisoprene). There are at least 40,000 different products made with natural rubber and over 400 medical devices (Cornish *et al.*, 1993; Mooibroek and Cornish, 2000). Due to the molecular structure and high molecular weight (greater than 1 million Daltons), this strategic molecule has high performance properties that cannot easily be mimicked by artificially

produced polymers like synthetic rubber (SR) (Sekhar, 1989; Venkatachalam *et al.*, 2006; Clement-Demange *et al.*, 2007; de Fay *et al.*, 2010). NR has the added advantage of being a renewable resource with environmental benefits. Production of NR results in sequestration of large amounts of atmospheric carbondioxide (CO_2) but the SR produced from petroleum stock result in the emission of green house gases in to the atmosphere.

The major producers of natural rubber are Thailand, Indonesia, Vietnam, China, Malaysia and India. In India, more than 92% of the rubber production is from the traditional rubber growing tracts of Kerala, some parts of Tamil Nadu and Karnataka. The remaining NR production is from the non-traditional regions like Maharashtra, parts of Andhra Pradesh and Northeastern states of India. Rubber cultivation in the non-traditional area has been expanding at a fast pace due to saturation of rubber plantations in the traditional area.

Rubber is the major constituent of latex which is produced and stored in specialized cells called laticifers or latex vessels, located in the phloem in the bark of the *Hevea* trees (Gomez and Moir, 1979). These latex vessels are derived from the cambium and are arranged as concentric rings in the bark, and between the vessels in each ring, there are anastomoses that allow withdrawal of latex from a large area of bark by means of controlled wounding called tapping (Gomez and Moir, 1979). Latex is present in almost all parts of the plant but laticifers of the trunk are commercially utilized for harvesting latex (Fig.1.1).

Fig.1.1. Rubber Plantation

1.2. Latex composition

Latex is a specialized form of cytoplasm containing a suspension of rubber and non rubber particles in an aqueous serum which contains 30-50% rubber (Dian *et al.*, 1995). Besides rubber particles and water, fresh latex contains carbohydrates, proteins, lipids and inorganic salts (Archer *et al.*, 1963). Quebrachitol, sucrose and glucose are the major soluble carbohydrates present in the latex (Low, 1978).

Latex can be separated into

1) a white upper layer of rubber particles,

2) an orange or yellow layer containing Frey-Wyssling particles,

3) an aqueous serum named C-serum and a bottom fraction containing grayish yellow gelatinous sediments by ultra centrifugation (Cook and Sekhar, 1953).

The serum contains most of the soluble substances including amino acids, protein, carbohydrates, organic acids, inorganic salts and nucleotide materials (Archer and Cockbain, 1969). The bottom fraction consists mainly of lutoid particles and also includes varying amount of Frey-Wyssling particles, mitochondria and other particulate components of plant cells having a density greater than that of serum.

Enzymes required for different metabolic pathways related to rubber biosynthesis are present in the C-serum of latex. Fats, waxes, sterols, sterol esters and phospholipids are the major lipids present in the latex. Lipids associated with the rubber and non-rubber particles in the latex play a vital role in the stability and colloidal properties of latex. While processing the latex, the rubber particles will coagulate and form a soft mass and float in the serum. The coagulated rubber is processed further for commercial use.

1.3. Rubber biosynthesis

Natural rubber is a huge linear biopolymer with isoprene as the monomeric subunit. Isopentenyl pyrophosphate (IPP) is produced *via* two

biosynthetic pathways in higher plants. In the cytosol the well described mevalonate (MVA) pathway synthesizes IPP from acetyl-CoA (Spurgeon and Porter, 1981). Another metabolic pathway for IPP synthesis is called 1-deoxy-D-xylulose-5-phosphate/2-*C*-methyl-D-erythritol-4-phosphate (DOXP/ MEP) pathway, which is located in the plastids (Rohmer *et al.*, 1996; Lichtenthaler *et al.*, 1997). Natural rubber biosynthesis is a side-branch of the ubiquitous isoprenoid pathway (Fig.1.2).

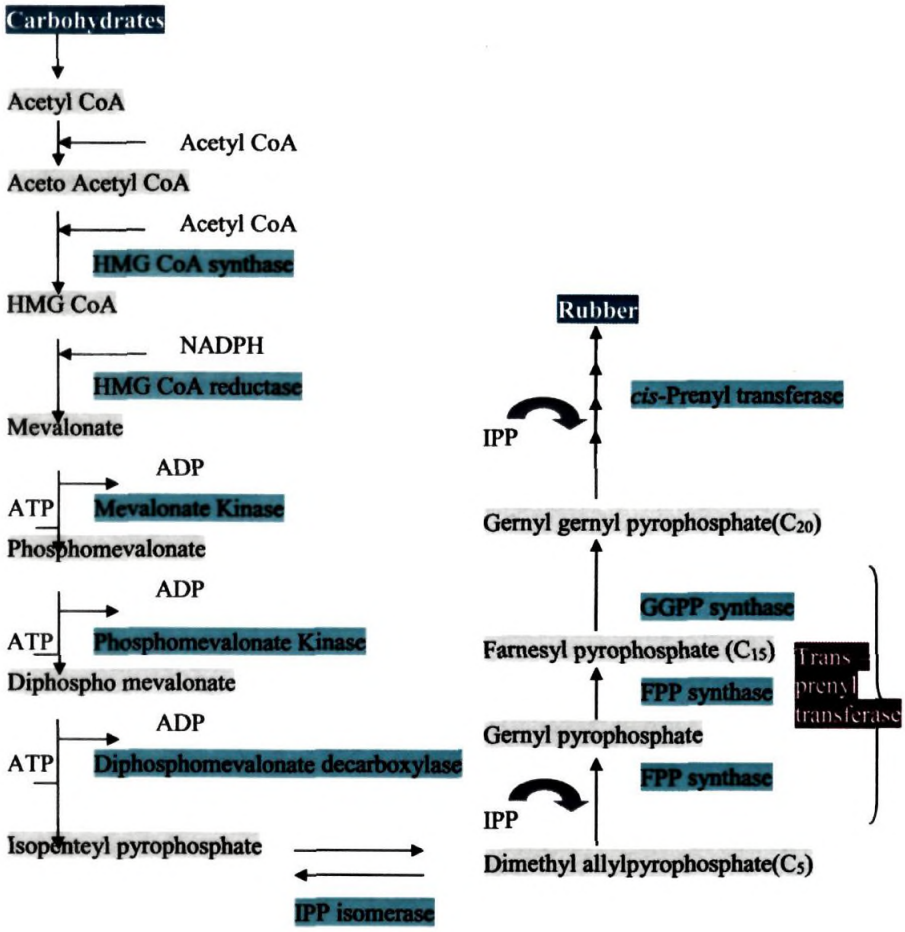


Fig. 1.2. Schematic Representation of rubber biosynthetic pathway in *Hevea*

The mevalonate (MVA) pathway leading to natural rubber biosynthesis can be divided into four steps:

1. The formation of isopentenyl pyrophosphate (IPP).
2. The initiation of rubber biosynthesis by the formation of other initiator molecules such as geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP).
3. The polymer chain elongation of rubber biosynthesis by the successive addition of IPP to initiator molecules with the help of an enzyme prenyl transferase.
4. The termination of rubber biosynthesis; the release of rubber molecule from the enzyme, rubber transferase.

The formation of IPP is by the condensation of three molecules of acetyl-CoA followed by reduction, phosphorylation and decarboxylation (Kush, 1994; Stermer *et al.*, 1994). The enzymes required for the conversion of acetate and mevalonate to rubber have been detected in latex (Lynen, 1969). In *Hevea*, the acetate molecule is produced mainly by the catabolism of sugar (Jacob, 1970; Tupy, 1973). Acetyl-CoA is generated from pyruvate *via* its decarboxylation and dehydrogenation, catalyzed by pyruvate dehydrogenase complex localized in the mitochondria and the transport of the acetyl-CoA *via* citrate to the cytoplasm.

Two molecules of acetyl-CoA condense to form acetoacetyl-CoA and the acetoacetyl-CoA condenses with another molecule of acetyl-CoA resulting the production of β-hydroxymethyl glutaryl-CoA (HMG-CoA) with

the help of an enzyme, HMG-CoA synthase. Mevalonic acid derived from HMG-CoA in a nicotinamide adenine dinuceotide phosphate-linked reduction (NADPH) also occurs in *Hevea* latex (Archer and Audley, 1967). The activity of the HMG-CoA-reductase enzyme in the latex is suspected to be the limiting factor for rubber biosynthesis (Lynen, 1969). Three genes *hmgr*1, *hmgr*2 and *hmgr*3 have been identified in rubber tree of which *hmgr*1 seems to be responsible for rubber biosynthesis (Kush *et al.*, 1990). The characterization of HMG-CoA reductase from different plant species revealed developmental and organ specific expression of HMG-CoA reductase isoforms (Rodwell *et al.*, 2000). This enzyme has been reported to occur in the serum fraction of preserved *Hevea* latex (Lynen, 1967). Hepper and Audley (1969) reported its occurrence in the bottom fraction of centrifuged latex.

In the isoprene pathway, the mevalonate is activated as phosphomevalonate by a cytosolic mevalonate kinase (Skilleter *et al.*, 1966). The next stage consists of activation of phosphomevalonate into pyrophosphomevalonate (PPMVA) by a phosphomevalonate kinase (Williamson and Kekwick, 1965). Rubber monomer, IPP is produced from PPMVA by means of a decarboxylase and is accompanied by dehydration of the molecule (Chesterton and Kekwick, 1968).

The formation of natural rubber from IPP requires the isomerisation of IPP to dimethylallyl pyrophosphate (DMAPP) and then a series of prenyl transfer occurs. The interconversion of IPP to its highly electrophilic isomer, DMAPP is catalyzed by the enzyme isopentenyl pyrophosphate isomerase

(IPP isomerase) (Oh *et al.*, 2000a). Initial condensation of IPP occurs only in the presence of "appropriate structured molecules" (Lynen and Henning, 1960). The polyisoprene is then formed by repeated addition of the precursor, IPP to the elongating molecules (Lynen and Henning, 1960). In *Hevea* latex, IPP isomerase appears to be distributed between the serum and the surface of rubber particles. It has also been found associated with the washed rubber particles (Lynen, 1969).

Sequential condensation of IPP with DMAPP results in the formation of 10-carbon intermediate called GPP which condensed with another IPP molecule yielding FPP, a 15-carbon intermediate. Farnesyl pyrophosphate synthase (FPPS) mediates the production of FPP from DMAPP or GPP (Poulter and Rilling, 1981). Further condensation of GPP with initiator molecules results in the formation of a C_{20} molecule, GGPP, thus the primary isoprenic unit is built up to stage C_{20}. The chain is lengthened by the terminal addition of IPP units by means of a transferase. There exist two different types of prenyl transferases in the plant system; *cis*-and *trans*-prenyl transferases. They have separate roles in the biosynthesis of *cis*-1,4-polyisoprene. The soluble *trans*-prenyl transferase from the latex of *Hevea* functions solely as FPP synthase and plays no direct role in *cis*-1,4-polyisoprene elongation (Cornish, 1993). The main function of this enzyme is to make initiator molecules, FPP, for further IPP condensation. The *cis*-1, 4-prenyl transferase or rubber transferase (CPT/RuT), which is firmly associated with the rubber particles, is responsible for elongating the rubber molecules.

The mechanism of polymerization of IPP has been elucidated in relation to terpene biosynthesis (Lynen *et al.*, 1959). Two steps involved in this process are,

1) isomerization of IPP to DMAPP by a shift of the double bond by IPP isomerase.

2) condensation of DMAPP with IPP by *cis*-polyprenyl transferase (Archer *et al.*, 1963; McMullen and McSweeney, 1966; Archer and Audley, 1967; Archer and Cockbain, 1969; Madhavan and Benedict, 1984), to give a molecule each of pyrophosphate and GPP.

This C_{10} molecule has an allelic structure and repeats the condensation, with another molecule of IPP. The propagation, repeated several times, results in the formation of natural rubber (*cis*-polyisoprene) with high molecular weight. The enzymes, IPP isomerase, trans-prenyl transferases (GPP, FPP and GGPP synthases) and *cis*-prenyl transferase (rubber transferase) have important roles in rubber biosynthesis.

The termination of rubber biosynthesis is the release of rubber molecule from the enzyme, rubber transferase. In *Hevea,* the chain termination process must have been quite consistent as the event yield polyisoprene with molecular weight distribution of about 100 to 1000 kDa (Subramanian, 1980). Mechanisms controlling molecular weight of rubber must exist because molecular weight of rubber in different rubber synthesizing plant species varies considerably (Archer and Audlely, 1973). Many plant species produce

natural rubber, but only a few produce the high molecular weight polymer that is required for high quality commercial products.

1.4. Genes associated with rubber biosynthesis

Genes expressed in the latex of *Hevea* can be divided into three groups based on the proteins they encode:

1) rubber biosynthesis-related proteins such as rubber elongation factor (REF), hydroxyl methyl glutaryl-CoA reductase (HMGR), hydroxyl methyl glutaryl-CoA synthase (HMGS), *cis*-prenyltransferase (CPT), geranylgeranyl pyrophosphate (GGPP) synthase, small rubber particle protein (SRPP), isopentenyl pyrophosphate (IPP) isomerase.

2) defense/stress-related proteins such as MnSOD, hevein, chitinase, β-1, 3-glucanase.

3) latex allergen proteins such as Hev.b.3, Hev.b.4, Hev.b.5, Hev.b.7, *etc* (Han *et al.*, 2000).

Kush *et al.*, (1990) have shown differential expression of several rubber biosynthesis-related genes in the latex. REF, an enzyme involved in rubber biosynthesis (Dennis and Light 1989), is highly expressed in laticifers (Goyvaerts *et al.*, 1991). Other rubber synthesis-related genes, such as HMG-CoA reductase (Chye *et al.*, 1992) and SRPP (Oh *et al.*, 1999), are also highly expressed in the latex. Tupy (1988a) reported the presence of ribosomes and polysomes in laticifers. About 200 distinct polypeptides are present in the latex (Posch *et al.*, 1997). Subsequently, cytosolic proteins

identified were the rubber biosynthesis stimulator protein which corresponds to eIF-5A (Yusuf *et al.*, 2000; Chow *et al.*, 2003) and a patatin like inhibitor (Yusof *et al.*, 1998).

It was observed that significantly higher level of expression of REF gene in the latex of high yielding rubber clones than in low yielding clones (Priya *et al.*, 2007). The expression of sucrose transporter gene (*HbSUT*3) was reported to be positively correlated with latex yield in *Hevea* (Tang *et al.*, 2010). The mRNA levels of *Hevea* HMG-CoA synthase (*hmgs*1) and HMG-CoA reductase (*hmgr*1) showed positive correlation with rubber yield (Sumanmanee *et al.*, 2013). Although there are few reports on the expressions of genes involved in rubber biosynthesis (Kush *et al.*, 1990; Priya *et al.*, 2007; Sumanmanee *et al.*, 2013), the genes associated with high yield potential are lacking. The expression of genes involved in rubber biosynthesis, therefore, needs to be studied in detail in different *Hevea* clones with varying yield potential to understand the molecular mechanisms involved in yield potential.

1.5. Rationale of the study

In India, major improvement in the productivity of NR has been made during the last half century. Due to the increasing demand for rubber in the domestic market an increase in latex yield from rubber plantations is very important and challenging. Exogenous factors such as eco-climatic factor, mechanical wounding, exploitation, diseases *etc.* as well as endogenous factors like cultivar, genetic stability, maturity, physiological disorders and

root stock-scion interaction *etc.* are known to affect the latex yield potential in rubber trees. Improvements in rubber yield have been made through breeding and selection of improved rubber clones, adopting better harvesting methods and improved agronomic and horticultural practices.

As a result of the research and development programmes in crop improvement, several high yielding clones of *Hevea* have been started cultivating in recent past. There are clonal variations in the productivity of rubber latex. Hence, this study aims to understand the variations in the enzyme activities related to the rubber biosynthetic pathway in different rubber clones with varying yield potential. Molecular markers related to latex yield or rubber biosynthesis may be used to reduce the duration of breeding cycle of the crop. The latex gene profiling performed by various workers (Ko *et al.*, 2003; Chow *et al.*, 2007) reported a significant proportion of encoded proteins related to rubber biosynthesis and stress or defense response. The biochemical and molecular nature of enzymes and its gene expression in different clones of *Hevea brasiliensis* are not studied well. To ensure the stable supply of natural rubber, genetically improved varieties of *Hevea* clones with high crop production potential is needed. It is important to study the molecular mechanisms involving with high yield potential. Hence, the present work on biochemical and molecular studies on enzymes related to rubber biosynthesis in *H. brasiliensis* was conducted with the following major objectives.

1.6. Objectives:

1. Gene expression of some important enzymes involved in rubber biosynthesis in *Hevea* clones with varying yield potential.
2. Quantification of HMG-CoA reductase activity in different *Hevea* clones.
3. Molecular cloning, characterization and expression of *hmgr*1 gene from *Hevea brasiliensis*.
4. Identification of biochemical/ molecular markers associated with yield potential in *Hevea*.

Chapter 2

Review of literature

2.1. *Hevea brasiliensis*

Natural rubber (NR) is an important raw material used for making more than 40,000 products due to its unique properties such as resilience, elasticity, abrasion resistance, efficient heat dispersion and malleability at cold temperatures (Cataldo, 2000; Cornish, 2001). *Hevea brasiliensis* is the only tree species cultivated commercially for harvesting NR (Clement-Demange *et al.*, 2007; Venkatachalam *et al.,* 2013). *Hevea* is a perennial tropical tree grown in the tropical rainforests of the Great Amazon Basin of South America. It is one of the most recently domesticated species in the world and is now being cultivated on large scale plantations in Southeast Asian and African countries.

The *Hevea* tree may grow to 30 m (100 ft) or more in the wild, although plantation trees generally reach heights of around 17 m (60 ft). The bark is the most important part of the tree because it contains the tissues that produce the latex containing rubber. Latex vessels are found in the tree's soft bark. They are modified sieve tubes running anti-clock wise in concentric cylinders at an angle of approximately 30° to the vertical axis of the stem. Natural rubber is obtained from *Hevea* by collecting and processing exuded latex from notches made on the tree bark of 6-30 year old trees. Latex is harvested from *Hevea* trees by making controlled wounding of the bark called tapping. When severed during tapping, high turgor pressure inside the laticifers expels latex which contains 30-50 % (w/w) of *cis*-1, 4-polyisoprene.

Latex consists of four main fractions,

1) rubber particles (25-40% of total latex volume),

2) lutoids (10-20%),

3) Frey- Wyssling particles (5%),

4) other elements like protein, resins, sugars, glycosides, tannins, alkaloids, mineral salts and secondary metabolites (Delabarre and Serrier, 2000).

Latex has two major functions: making the plant less attractive to pests and protecting the plant by sealing of the wounds, so that, no aggressors can penetrate the tree. Natural rubber is a secondary metabolite present as rubber particles in the latex, the fluid cytoplasm of laticifers in *H. brasiliensis*. Plant isoprenoids comprise around 23,000 compounds and are the most diverse class of natural compounds including substances such as gibberellins, carotenoids, chlorophyll side chains, plastoquinone side chains, sesquiterpenes, sterols, brassinosteroids, dolichol and mitochondrial ubiquinone side chains (Lichtenthaler *et al.*, 1997a; 1997b; Kasahara *et al.*, 2002; Nagata *et al.*, 2002). Natural rubber is composed of isoprene units, linked together to form a polymer which differs from the majority of the isoprenoid compound in two aspects. 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 to1000 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 many rubber products used globally in the day to day life.

2.2. Rubber biosynthesis

In *Hevea*, the latex yield is limited by two main intrinsic factors in the latex-producing tissues. The first is the latex flow rate and its duration, which are influenced by the turgour pressure of the inner bark tissues (d'Auzac *et al.*, 1989), the latex viscosity, which depends on the percentage of dry matter (dry rubber content (DRC) of latex) (Van Gils, 1951; Cornish and Brichta, 2002) and latex coagulation efficiency. The second intrinsic limiting factor is the ability of the latex cells to regenerate their lost cytoplasm, including rubber, between two consecutive harvesting, which mainly depends on their metabolic orientation and activity (Jacob *et al.*, 1989).

Isopentenyl pyrophosphate (IPP) is the basic building block, from which isoprenoids are derived. IPP is produced *via* two biosynthetic pathways in higher plants. In the cytosol the well described mevalonate (MVA) pathway synthesizes IPP from acetyl-CoA (Spurgeon and Porter, 1981; Kush, 1994). Another metabolic pathway for IPP synthesis is called 1-deoxy-D-xylulose-5-phosphate/2-*C*-methyl-D-erythritol-4-phosphate (DOXP/MEP) pathway, which is located in the plastids (Rohmer *et al.*, 1996; Lichtenthaler *et al.*, 1997b). 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).

2.3. MVA/MEP pathway

The mevalonate pathway consists of six steps that transform acetyl CoA to IPP, followed by an IPP isomerase that maintains a balance between IPP and dimethyl allyl pyrophosphate (DMAPP). The MEP pathway consists of seven enzymes that transform glyceraldehyde 3-phosphate and pyruvic acid to IPP and DMAPP in a ratio of 5:1 (Tholl and Lee, 2011; Sando *et al.*, 2008b). Evidence supporting the cytosolic mevalonate pathway for rubber formation was derived from a high level incorporation of radiolabeled pathway intermediates such as mevalonate (Skilleter and Kekwick, 1971) and 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) (Hepper and Audlely, 1969) into rubber. The initial step of the isoprenoid pathway involves the fusion of three molecules of acetyl CoA to produce HMG-CoA. The HMG-CoA is then reduced to mevalonic acid in a NADPH dependent double reduction. This step is catalyzed by 3-hydroxy-3-methylgultaryl coenzyme A reductase (HMGR; EC 1.1.1.34) (Rogers *et al.*, 1983). The plastidic MEP pathway been considered as a possible alternate route for rubber biosynthesis. The analysis of redundancy-reduced ESTs and transcription derived fragments revealed the existence of this alternate pathway (MEP) in

addition to the well known mevalonate pathway for the synthesis of IPP (Ko *et al.*, 2003).

Sucrose is the precursor molecule for the synthesis of IPP and rubber biosynthesis in *Hevea*. The individual steps in the synthesis of rubber from sucrose are well established (Lynen, 1969). The biosynthesis of rubber from sucrose involves more than 20 enzymatic reactions (Sando *et al.*, 2008a). Several proteins other than enzymes are reported to be involved in the biosynthetic process (Kang *et al.*, 2000). The expressions of genes corresponding to enzymes involved in rubber biosynthesis were reported in a few *Hevea brasiliensis* clones (Priya *et al.*, 2007; Chow *et al.*, 2012; Towaranonte *et al.*, 2010; Venketachalam *et al.*, 2009a, b).

2.4. Major enzymes in the MVA pathway

2.4.1. HMG-CoA synthase

3-Hydroxy-3-methylglutaryl coenzyme A synthase (HMGS, EC 2.3.3.10) catalyzes the condensation of acetyl CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). In mammals two forms of HMGS have been reported, one in the mitochondria and the other in the cytoplasm (Clinkenbeard *et al.*, 1975a, b). HMGS enzyme is also present in bacteria (Sutherlin *et al.*, 2002) and in plants (Lynen 1969; Alam *et al.*, 1991; Bach 1995; Alex *et al.*, 2000; Nagegowda *et al.*, 2005). HMGS activity has been demonstrated in *H. brasiliensis* latex by Lynen (1969). HMGS activity was found mainly in C-serum, which represents the cytosolic fraction of laticiferous cells (Suvachittanont and Wititsuwannakul, 1995).

HMGS activity is positively correlated with rubber yield in *Hevea*, suggesting a regulatory role of this enzyme in the diurnal variation of rubber biosynthesis (Suvachittanont and Wititsuwannakul 1995). *Hbhmgs*1 mRNA was more abundant in the latex, stem and petiole than in leaves of rubber seedlings and mature trees. The expression of the *hmgs*1 gene was shown to correlate with the presence of more laticiferous cells in the corresponding tissues (Suwanmanee, 2013).

Suwanmanee *et al.* (2002) cloned *hmgs* gene from *H. brasiliensis* and found that the levels of mRNA and activity of HMGS was closely related to accumulation of rubber in the plants. Sirinupong *et al.* (2005) isolated another *hmgs* gene, *hmgs*2, from *H. brasiliensis*. The homology of nucleotide and amino acid sequences between *hmgs*1 and *hmgs*2 was found to be 92% and 94%, respectively. RT-PCR analysis showed that their expressions in latex-producing cells and petioles were significantly higher than that in leaves, which suggests that *hmgs*1 and *hmgs*2 were two critical genes in the synthetic pathway of rubber. HMGS also plays an important role in the development and defense mechanism of plants.

2.4.2. HMG-CoA reductase

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyse the synthesis of mevalonate from HMG-CoA. Mevalonate is converted to IPP, which acts as precursor to a wide range of isoprenoid compounds including natural rubber (Stermer *et al.*, 1994; Newman and Chappell, 1999). HMGR catalyzes the initial step of the MVA pathway and

is understood to be the key enzyme in the pathway (Bach 1987; Goldstein and Brown, 1990). HMGR usually occurs in small gene families in plants and the number of genes encoding HMGR varies depending on the species (Gertler *et al.*, 1988). In *Arabidopsis* two genes encode HMGR (Enjuto *et al.*, 1994; 1995), three genes in potatoes (Korth *et al.*, 1997) and four genes in tomatoes (Daraselia *et al.*, 1996). Although the proteins they encode may share high sequence identity, the expression patterns of individual family members are generally distinct (Stermer *et al.*, 1994). Differences in gene expression pattern between *hmgr*1 and *hmgr*2 in *Arabidopsis* have been reported and *hmgr*1 is expressed throughout in all plant tissues and *hmgr*2 is expressed only in floral and meristematic tissues (Enjuto *et al.*, 1994).

In plants, the mevalonate is the general precursor of many identified isoprenoid compounds and many of which are vital for plant growth, development, and variety of other normal physiological activities. Harker *et al.* (2003) showed that HMGR is a key enzyme controlling overall flux in the sterol biosynthesis pathway in seed tissue by over expression of *Hevea hmgr*1 gene in tobacco. Suzuki *et al.* (2004) reported that in *Arabidopsis,* loss of function of *hmgr*1 lead to dwarfing, early senescence, male sterility and reduced sterol levels and suggested that *hmgr*1 plays a critical role in triterpene biosynthesis. HMGRs in plants determine the flux to isoprenoid pathway and HMGRs are regulated by a variety of developmental and environmental signals such as light, wound, infection, hormones, herbicides and sterols (Bach, 1995).

In *Hevea brasiliensis*, HMGR is encoded by a small gene family comprised of 3 members, *hmgr*1, *hmgr* 2 and *hmgr*3 (Chye *et al.*, 1992). It was reported that *hmgr*1 is involved in rubber biosynthesis and *hmgr*3 is of housekeeping nature in *Hevea* (Chye *et al.*, 1991). The northern blot results suggested that *hmgr*1 was expressed more in laticifers than in leaves, emphasizing that this gene is specifically involved in rubber biosynthesis. Ji *et al.* (1993) demonstrated that *hmgr*1 activity was positively correlated with rubber biosynthesis as well as latex yield. Sando *et al.* (2008a) cloned *hmgr*4 and *hmgr*5 and reported that both were specifically expressed at high levels in mature leaves and xylem respectively.

The sub cellular localization of plant HMGR is reported and the enzyme activity has been associated with mitochondria (Bach *et al.*, 1986), chloroplasts (Brooker and Russell, 1975; Arebalo and Mitchell, 1984) as well as endoplasmic reticulum (Kondo and Obab, 1986; Enjuto *et al.*, 1994). Like mammalian HMGR, plant HMGRs are also subjected to feedback regulation by the end products of the pathway. Studies on plastid and cytoplasmic HMGR activities in pea revealed distinctive kinetic and regulatory properties (Brooker and Russell, 1975; 1979; Wong, *et al.*, 1982). HMGR activities are higher in rapidly growing part of the plant such as apical buds and roots but lower in more mature tissues. In tomato, HMGR activity was highest during the early stages of fruit development which later declined during fruit ripening (Gillapsy *et al.*, 1993).

Treatment with mevinolin (a competitive inhibitor of HMGR) can slow or inhibit plant growth and development (Gray, 1987; Narita and

Gruissem, 1989). HMGR was purified from the washed bottom fraction membrane of *Hevea* latex (Wititsuwannakul *et al.*, 1990; Benedict, 1983). It has been shown that a Ca^{2+} binding protein in the C-serum functions as an activator of HMGR enzyme (Wititsuwannakul *et al.,* 1990). The relative enzymatic activities of various enzymes in the rubber biosynthesis pathway up to IPP were of comparable magnitude with the exception of HMGR (Lynen, 1969). The activity of this enzyme was much lower and is considered to be a limiting factor in rubber biosynthesis. 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 clones and the diurnal variations observed in the rubber content of the latex coincided with variation in HMGR activity. It may therefore be possible to increase the rubber yield by promoting the transcription and translation of latex specific HMGR. The *hmgr*1 and dry rubber content showed a positive correlation in studies conducted in *Hevea* clones RRIM 600 and PB 235 suggesting the regulatory role of *hmgr*1 in rubber biosynthesis (Nuntanuwat, 2006). Liu *et al.,* (2015) reported that the expression of most genes related to latex biosynthesis was inhibited in tapping panel dryness (TPD) and *hmgr*1 in the MVA pathway was concurrently suppressed.

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

2.4.3. Mevalonate decarboxylase

Mevalonate decarboxylase (MVD) (EC 4.1.1.33) catalyses the conversion of mevalonate diphosphate (MVDP) to IPP. This is an irreversible reaction and is dependent upon ATP and Mg^{2+} for its activity (Pang *et al.*, 2006). The homologues of MVD have been reported from plants such as *Hevea brasiliensis, Ricinus communis, Arabidopsis thaliana, Nicotiana langsdorffii* x *Nicotiana sanderae*, *Solanum lycopersicum*, *Ginkgo biloba* and *Panax ginseng*. In *Arabidopsis thaliana*, MVD was presumed to be transcribed at a low level in whole plant or in a tissue specific pattern (Cordier *et al.*, 1999) except for *Ginkgo biloba*, where MVD was reported to be equally expressed in root and leaf (Pang *et al.*, 2006). *HbMVD* which is highly expressed in latex, play an important role in rubber biosynthesis in *H. brasiliensis* (Sando *et al.*, 2008a).

2.4.4. Farnesyl pyrophosphate synthase

Farnesyl pyrophosphate synthase (FPPS) (EC 2.5.1.1) plays the central role in both eukaryotic and prokaryotic isoprenoid biosynthetic pathways (Poulter and Rilling, 1981). It catalyzes the consecutive condensations of DMAPP or geranyl pyrophosphate (GPP) with IPP to produce farnesyl pyrophosphate (FPP) as a final product. In plants, FPPS enzymes have been located in different cellular compartments, namely the cytosol (Hugueney *et al.,* 1996), mitochondria (Cunillera *et al.,* 1997), plastids (Sanmiya *et al.,* 1999) and peroxisomes in animals (Biardi and Krisans, 1996). FPP is situated at the branching point of the isoprenoid biosynthetic pathway for several isoprenoids, such as steroids, cholesterol, prenylated proteins, sesquiterpenes, heme A, and vitamin K2 (Ogura *et al.,* 1997). FPP is the allylic pyrophosphate initiator for successive condensations of IPP in the *trans-* or *cis*-configuration to produce *trans*-polyisoprene (chicle, gutta percha) or *cis*-polyisoprene (natural rubber).

FPPS synthesizes FPP in two separate steps (Chappell, 1995). FPP produced in the cytosol is dedicated to the biosynthesis of sterols (Biardi and Krisans, 1996), although it is also used for farnesylation and sesquiterpenoid biosynthesis (Chappell, 1995). In *Arabidopsis,* FPPSs are expressed in all organs throughout plant development, though at different levels. *FPPS*1 is widely expressed in all tissues throughout plant development, whereas expression of *FPPS*2 is mainly concentrated in floral organs, seeds and the early stages of seedling development (Cunillera *et al.,* 2000; Closa *et al.,* 2010). In the rubber tree, *HbFPPS*1 is expressed predominantly in the

laticifers and is likely to encode the enzyme involved in rubber biosynthesis (Adiwilaga and Kush, 1996). The expression of *HbFPPS*2 and *HbFPPS*3 is not cell-type specific. The involvement of *HbFPPS*2 and *HbFPPS*3 in isoprenoid biosynthesis is of a house-keeping nature (Guo *et al.*, 2015). Two copies of the FPPS gene have been isolated from guayule (Pan *et al.*, 1996). Immunoblot analysis of FPPS in the tissues of the *Hevea* indicated that some part of the FPPS is localized in laticifers and involved in rubber biosynthesis, while the other part of the FPPS is in epidermal cells and participates in the biosynthesis of other isoprenoid compounds. The FPP synthase in *Hevea* latex has a major role to produce the starter substrate FPP for the prenyl chain elongation in rubber biosynthesis (Takaya *et al.*, 2003).

2.4.5. Geranylgeranyl pyrophosphate synthase

Geranylgeranyl pyrophosphate synthase (GGPS, EC: 2.5.1.29), is a homodimeric, short chain *trans*-prenyltransferase responsible for the synthesis of GGPP and occurs ubiquitously in plants, animals and bacteria (Ogura and Koyama, 1998). It is a branch point enzyme, which regulates coordination with the other prenyltransferases (GDP and FDP synthase respectively) of the precursor flux towards mono-, sesqui- and diterpenoids production (Hefner *et al.*, 1998; Laskaris *et al.*, 2000). The activity of GGPS was first detected in plants, such as pumpkin (*Cucurbita moschata*) (Ogura *et al.*, 1972). GGPS catalyzes the condensation of IPP with an allylic prenyl pyrophosphate FPP to give GGPP, which is an essential precursor in the biosynthesis of several isoprenoids necessary for plant growth and development such as carotenoids, gibberellins, prenyl quinones, chlorophylls

and formation of geranylgeranylated proteins (Okada *et al.,* 2000). GGPS was detected in all *Hevea* tissues examined, though the expression levels were different from each other. Expression of the GGPS gene in leaf, flower and young leaf were higher than those in latex and petiole and GGPS in *Hevea* latex may be engaged in the biosynthesis of β-carotene, which is responsible for the characteristic colour of Frey – Wyssling particles (Takaya *et al.,* 2003).

GGPS cDNAs have been cloned and characterized from plant species, such as *Lycoperiscon esculentum* (Ament *et al.,* 2006), *Antirrhinum majus* (Tholl *et al.,* 2004), *Adonis aestivalis* (Cunningham and Gantt, 2007), *Hevea brasiliensis* (Takaya *et al.,* 2003), *Chrysanthemum morifolium* (Kishimoto and Ohmiya, 2006), *Catharanthus roseus* (Bantignies *et al.,* 1996) and *Sinapis alba* (Kloer *et al.,* 2006). In spite of the extensive studies of the GPPS genes in many plant species, only a few of the isolated cDNAs have been functionally characterized. Previous studies indicated that GGPS is an important branch point enzyme in terpenoid biosynthesis and GPPS gene is involved in the biosynthesis of diterpenes as well as in protein prenylation.

2.4.6. Rubber transferase

Rubber transferase (RuT; EC 2.5.1.20) is the *cis*-prenyltransferase that catalyses the polymerisation of IPP into *cis*-rubber (Archer and Audley, 1967; Archer and Cockbain, 1969; Archer and Audley, 1987; Light and Dennies, 1989). It was suggested earlier that the rubber biosynthesis in *H.*

brasiliensis is mediated by *trans*-prenyltransferase associated with a rubber elongation factor, a protein of molecular weight 14.6 kDa, tightly bound to the rubber particles in the laticifers (Dennis and Light, 1989). Later it was demonstrated that the *trans*-prenyltransferase functions as farnesyl pyrophosphate synthase and it plays no direct role in the *cis*-1,4-polyisoprene elongation (Cornish, 1993). The successive addition of IPP was found to take place only on the surface of pre-existing rubber particles implying that rubber transferase was located on the surface of the rubber particles (Archer *et al.*, 1963; McMullen and McSweeney, 1966).

Rubber transferase requires divalent cations, such as Mg^{2+} or Mn^{2+} for activity, but rubber transferase, IPP and Mg^{2+} together will not result in rubber biosynthesis; a second substrate, an allylic pyrophosphate (APP) is needed to initiate the polymerisation process (Archer and Audley, 1987; Madhavan *et al.*, 1989; Cornish and Backhaus, 1990; da Costa *et al.* 2005, 2006; Scott *et al.*, 2003). Rubber transferase accepts a broad range of initiator molecules, with FPP (C15) being preferred (Tanaka *et al.*, 1996; Castillon and Cornish, 1999; Mau *et al.*, 2003). The length of the polymer produced depends on concentrations of the substrate IPP, the ratio of concentrations of IPP and an initiator, allylic pyrophosphate (APP) and the size of the initiator (Castillon and Cornish, 1999). The activity of rubber transferase enzyme increased with an increase in the concentration of washed rubber particle (WRP) (Archer *et al.*, 1963). The presence of rubber transferase activity in crude extract of *Parthenium argentatum* was demonstrated by Madhavan and Benedict, (1984).

RuT was first cloned from *Arabidopsis* (Oh *et al.,* 2000b) and in *Arabidopsis* RuT catalyzes the formation of long-chain dolichols (C120). Two rubber transferase homologues (*RuT*1 and *RuT*2) were cloned from the *H. brasiliensis* latex (Asawatreratanakul *et al.,* 2003; Koyama, 2003). The activities of these enzymes were found in the bottom fraction and the supernatant cytosol (C-serum) of centrifuged fresh *Hevea* latex (Tangpakdee *et al.,* 1997). *RuT*1 and *RuT*2 require additional cofactors from the eukaryotic cells to produce distinct RuT activity.

2.5. Proteins involved in rubber biosynthesis

Besides the enzymes of rubber biosynthesis pathway, other ancillary proteins contribute to rubber formation. These include sucrose transporters (d'Auzac *et al.,* 1997), the major membrane protein of rubber particle, rubber elongation factor (REF) (Dennies and light, 1989), the small rubber particle protein (Oh *et al.,* 1999), a patatin-like inhibitor (Yusof *et al.,* 1996; 1998) and a rubber biosynthesis stimulator protein (Yusof, 1996; Yusof *et al.*, 2000; Chow *et al.,* 2003).

2.5.1. Sucrose transporter

The laticifers in the trunk bark of regularly tapped rubber tree represent a strong sucrose sink (Tupy, 1989). The mechanism of sucrose transport and metabolism in the laticifers is of fundamental importance for improving *Hevea* productivity (d'Auzac *et al.,* 1997). Sucrose transporters are responsible for loading sucrose in to cells and play a central role in sucrose portioning between its site of synthesis and its numerous sink tissues

(Braun and Slewinski, 2009). In *Hevea,* six sucrose transporters have been cloned in which *HbSUT*3 has been identified as the key member responsible for sucrose loading in to lacticifers (Tang *et al.,* 2010; Dusotoit *et al.,* 2010). The *Arabidopsis AtSUC*5, specifically expressed in the endosperm, was required for the supply of sucrose to seeds during early stages of development (Baud *et al.,* 2005). In regularly tapped *Hevea* trees of a metabolically active clone, *HbSUT*3 was revealed to be the dominant SUT member expressed in the latex as compared with other SUT genes. The gene expression of *HbSUT*3 in latex correlated positively with yields of rubber tree with in the same clone (Tang *et al.,* 2010). Correlation between transcript accumulation of sugar transporters and their transport activity has been reported (Sakr *et al.,* 1997; Lemoine *et al.,* 1999; Rosche *et al.,* 2002; Decourteix *et al.,* 2008; Hayes *et al.,* 2007; Zhou *et al.,* 2009).

2.5.2. Rubber elongation factor

Rubber elongation factor (REF) is present in large amounts, at the surface of the large rubber particles in all laticifer layers (Sando *et al.,* 2009). It plays an important role in the final polymerization step of rubber biosynthesis. It has a molecular mass of 14,600 Da and is tightly bound to rubber particles (Dennis and Light, 1989). It is also found as a noncovalent homotetramer of ~58 kDa (Czuppon *et al.,* 1993; Goyvaerts *et al.,* 1991). REF is not made as a preprotein, synthesized on free polysomes in the laticifer cytoplasm and the assembly of the rubber particles is likely to occur in the cytosol (Goyvaerts *et al.,* 1991). REF, also known as Hev b1, is one of the major allergens in *Hevea* latex (Czuppon *et al.,* 1993). There are at

least two loci for REF in the *Hevea* genome (Sookmark *et al.*, 2002). The amount of REF in whole latex is proportional to the rubber content. Quantitative analysis of REF and rubber content in whole latex revealed a ratio of one molecule of REF to one molecule of *cis*-1,4-polyisoprene (Dennis and Light, 1989). *Hevea* genes encoding for REF has been cloned (Oh *et al.*, 1999; Goyvaerts *et al.*, 1991) and various isoforms are identified (Priya *et al.*, 2006). REF proteins from *Hevea* latex are acidic, which are believed to be not post-translationally modified (Yeang *et al.*, 1996). REF gene expression is higher in latex than in leaf tissues of *Hevea* (Han *et al.*, 2000; Ko *et al.*, 2003 ; Priya *et al.*, 2007) and is markedly higher in high latex yielding clones than in low yielding ones (Priya *et al.*, 2007). All these results point to an association of REF with rubber production. In *Hevea*, three isoforms of REF (REF1, REF2, and REF3) have been identified. Ruderman *et al.*, (2012) reported that REF3 isoform was found to be higher in low yielding than high yielding *Hevea* clones.

2.5.3. Rubber biosynthesis stimulator /inhibitor protein

A rubber biosynthesis stimulator protein (RBSP) was purified from *H. brasiliensis* latex and identified as eIF-5A by amino acid sequencing (Yusof, 1996; Yusof *et al.*, 2000). A large family of nine RBSP genes, representing seven unique isoforms of the protein was reported (Chow *et al.*, 2003). Three genes in tobacco, (Chamot and Kuhlemeier, 1992) and four genes in tomato (Wang *et al.*, 2001) of eIF-5A were reported. Plant eIF-5A isoforms have been reported to be involved in other functions such as photosynthesis (Chamot *et al.*, 1992) and organ senescence (Wang *et al.*,

2003; 2005). Chow *et al.* (2006) reported a differential stimulatory effect of RBSP isoforms on rubber biosynthesis in *H. brasiliensis*. The translation initiation factor 5A (eIF5A) was reduced in tapping panel dryness (TPD) affected trees (Li *et al.*, 2010) and eIF5A negatively regulated programmed cell death (Hopkins *et al.*, 2008). Archer and Audley (1987) detected an inhibitor of rubber biosynthesis in the C-serum of the *H. brasiliensis* latex. Yusof *et al.* (1998) had purified the patatin like inhibitor protein (RBIP) with a molecular weight of 43.7 kDa.

2.6. Ethylene stimulation and rubber biosynthesis

Biosynthesis of natural rubber, like other secondary metabolites is affected by various plant hormones. Ethylene was the only plant hormones identified to stimulate the latex production in *Hevea.* The use of ethylene has led to a technological revolution in natural rubber production (Coupe and Chrestin, 1989). The treatment of rubber tree bark with ethylene releasing compounds such as ethephon, induces fresh latex production and stimulates latex regeneration between the latex harvesting periods (d'Auzac *et al.*, 1995; Coupe and Chrestin, 1989). Ethephon (2-chloro ethyl phosphonic acid) treatment in the bark tissues of the tapping panel prolongs the duration of latex flow and thus increases the total volume of latex during tapping (Ho and Paardekooper, 1965) and such treatment is now widely practiced in rubber plantations to increase the latex yield. Bark treatment with ethephon is known to increase the latex yield by 1.5 to 2 fold in rubber trees (Pujadae-Renaud *et al.*, 1994). Ethephon up on hydrolysis on the bark of the tree releases ethylene and will alter the composition of the latex. The expressions

of most genes involved in rubber biosynthesis are inhibited or unchanged upon ethylene stimulation (Zhu and Zhang, 2009; Adiwilaga and Kush, 1996). Stimulation is associated with obvious changes in both the physiology and metabolism of laticifers (Coupe and Chrestin, 1989). The changes in latex cells activities associated with ethephon includes, increase in RNA, invertase activity, sucrose content, latex stability and a decrease in starch content (Chong, 1981; Tupy, 1988a,b).

Ethylene could up regulate the activity of glutamine synthetase (GS), a key enzyme of nitrogen metabolism and its transcript levels in *H. brasiliensis* latex cells, suggesting the involvement of GS in stimulation of rubber production with ethylene (Pujade-Renaud *et al.,* 1994). Miao and Gaynor, (1993) reported that higher expression of MnSOD on ethephon application, prevented lutoid disruption by super oxide radicals leading to increased rate of latex flow. Kush *et al.* (1990) reported that laticiferic genes were induced by ethylene in *Hevea.* Ethylene increased invertase activity, which resulted in glycolysis acceleration and finally increased the supply of carbon sources for rubber biosynthesis (Alessandro *et al.,* 2006). Ethylene has little direct effect on rubber biosynthesis and have suggested that increases in latex yield could be attributed to a prolongation of latex flow (Zhu and Zhang, 2009; Tungngoen, 2009). During ethylene stimulation, the enzymes involved in carbohydrate metabolism were activated and the gene expression of several sucrose transporters in *Hevea* bark tissues and latex-producing cells were induced (Tang *et al.,* 2013). The transcripts of *HbSUT*1A and *HbSUT*2A, whose basal levels were low in the latex cells,

were enhanced by ethylene treatment in virgin trees and the transcript level of *HbSUT*1B was considerably increased by ethylene in the latex cells of exploited trees while it decreased in virgin trees (Dusotoit-Coucaud *et al.*, 2009).

The key enzymes involved in rubber biosynthesis initiation, like FPPs and RuT were not significantly affected by ethylene. In the case of HMGR, *hmgr*1 gene was induced by ethylene but it does not influence HMGR activity (Adiwilaga and Kush, 1996; Zhu and Zhang, 2009, Chye *et al.*, 1992). HMGS gene expression and enzyme activity were significantly enhanced upon the addition of ethylene (Sirinupong, *et al.*, 2005). Wang *et al.* (2015) reported that the regulation of rubber latex production by ethylene stimulation might occur not solely at the gene level but also at the protein level, with post-translational modifications. Several aquaporins were also up regulated upon ethylene stimulation through the regulation of water exchange between the inner liber and latex cells (Tungngoen, *et al.*, 2009). Ethylene altered membrane permeability, prolonged latex flowing time, and as a feedback, finally regenerated the basic metabolism of rubber latex (Coupe and Chrestin, 1989). REF and SRPP were not changed at the gene or protein level upon ethylene treatment (Wang *et al.*, 2015). Wang *et al.* (2015) reported ethylene stimulation in *Hevea* could not directly induce most of the enzymes in the MVA pathway at either the gene or protein expression level.

2.7. Markers for yield potential in *Hevea*

As a result of the recent research and development in crop improvement programmes, several new high yielding varieties of *Hevea* clones have been developed and some are widely cultivated in plantation sector. However there are clonal variations in the productivity of rubber. The quantity of latex obtained from different *Hevea* clones may be associated with the variations in the activities of the enzymes involved in rubber biosynthesis. The enzymes directly involved in the biosynthesis of rubber would be of great importance to specify the rate limiting steps in the rubber synthesis pathway. Identifying the key enzymes in the pathway would be important for developing markers for yield potential in *Hevea*.

Other than biochemical markers, molecular markers related to latex yield or rubber biosynthesis are useful in the crop improvement programmes aiming to reduce the duration of the breeding cycle. Over the past two decades, there has been an exponential increase in data acquisition pertaining to the rubber tree, including genomic microsatellite markers (Le *et al.*, 2011; Mantello *et al.*, 2012), expressed sequence tag-simples sequence repeats (EST-SSRs) (Feng *et al.*, 2008; Triwitayakorn *et al.*, 2011; Li *et al.*, 2012), linkage maps (Lespinasse *et al.*, 2000; Souza *et al.*, 2013) and gene expression profiles (Chow *et al.*, 2007; 2012). More recently, a draft genome of the rubber tree was published (Rahman *et al.*, 2013). High-throughput genomic techniques are associated with innovative bioinformatics tools that can be important to rubber tree breeding and facilitate the development of

superior clones that are suited to different agroclimatic conditions (Saha and Priyadarshan, 2012).

Although earlier workers have identified and characterized few genes involved in rubber biosynthesis in *H. brasiliensis* (Ruderman *et al.,* 2012; Priya *et al.,* 2007; Towaranonte *et al.,* 2010; Venketachalam *et al.,* 2009a), the genes associated with high yield potential are not studied well. Thus, it is imperative to study the molecular mechanisms of high yield potential in *Hevea*. Hence, the biochemical and molecular studies on enzymes related to rubber biosynthesis in *H. brasiliensis* was conducted. In the present study, gene expressions analyses of certain enzymes involved in rubber biosynthesis in *Hevea* clones with varying yield potential were carried out. The activity of HMGR, the key enzyme identified in the rubber biosynthesis pathway, was quantified in different *Hevea* clones. Molecular cloning and expression of *hmgr*1 gene from *H. brasiliensis* was also performed.

Chapter 3

Expression of genes involved in rubber biosynthesis

3.1. Introduction

Rubber is synthesized in the cytoplasm of highly specialized cells called laticifers arranged in rings that are differentiated from the cambium (d'Auzac *et al.*, 1997; Hao and Wu, 2000). After maturation process, the laticifers in each ring anastomose together in to a reticulate laticiferous network. During latex harvesting through wounding the bark tissues, this ring structure facilitates the exudation of large amount of latex in single tapping (Tang *et al.*, 2010). Rubber biosynthesis is the primary metabolic activity of laticifers. Rubber usually constitutes 30-50% of the weight of fresh latex and over 90% of its dry weight (Jacob and Prevot, 1992). In *Hevea brasiliensis*, *Taraxacum koksaghyz* (Russian dandelion) and *Ficus elastica* (rubber fig) the rubber producing cells are laticifers (Gomez, 1982; Ko *et al.*, 2003; Sando *et al.*, 2009; Polhamus, 1962; Cornish *et al.*, 1993; van Beilen and Poirier, 2007), whereas in guayule, they are the parenchyma cells in the bark of stems and roots (Whitworth and Whitehead, 1991; Benedict *et al.*, 2008).

The latex yield of rubber tree is limited by two main intrinsic factors in the latex-producing tissues. The first is the latex flow rate and its duration, which are influenced by the turgor pressure of the inner bark tissues (d'Auzac *et al.*, 1989) and by the latex viscosity. The viscosity of the latex depends on the percentage of dry matter content (DRC) of latex (Van Gils, 1951; Cornish and Brichta, 2002), the rubber particle size, particle size dispersion (PSD) (Greenwood *et al.*, 1995; Blackley, 1997; Chu *et al.*, 1998; Cornish and Brichta, 2002) and the latex coagulation efficiency (Brzozowska-

Hanower *et al.*, 1978; Gidrol *et al.*, 1994; Kongsawadworakul and Chrestin, 2003; Wititsuwannakul *et al.*, 2008). The second intrinsic limiting factor is the ability of the latex cells to regenerate lost cytoplasm, including rubber, between two consecutive tappings, which mainly depends on their metabolic orientation and activity (Jacob *et al.*, 1989).

Using chemical stimulants for harvesting more latex from trees is a common practice in rubber estates. Biosynthesis of natural rubber, like other secondary metabolites, is affected by various plant hormones. Among the plant hormones, ethylene was identified to stimulate the latex production in *Hevea*, which is applied as ethephon (an ethylene releaser). Bark application of ethephon is known to increase the latex yield by 1.5–2 folds in rubber trees by extending latex flow duration during harvesting (Pujadae *et al.*, 1994).

Ethephon decomposes to ethylene, phosphate and chloride ion in aqueous solutions above pH 4-5. A significant increase in turgor pressure, initial flow rate and decrease in plugging index are the factors leading to prolonged latex flow and increase in the latex volume in *Hevea* due to ethephon treatment (Abraham *et al.*, 1971; Thomas *et al.*, 1999). Treatment with ethylene induces increased latex yield which can be perceptible as soon as 12 hours after the treatment and is maximum during 48-72 hours. The intensity and the duration of latex yield in response to ethylene are clonal characteristic. The mechanism of yield stimulation is generally related to the plugging of latex vessel terminals after severing them at tapping (Boatman, 1966; Buttery and Boatman, 1967). Plugging of vessel terminals, usually

measured as the plugging index (PI), vary with clone and with the length of the tapping cut (Milford *et al.*, 1961; Paardekooper and Samosorn, 1969).

Stimulation of trees increases the general latex metabolism including over expression of some genes in the laticiferous tissue (Pujade-Renaud *et al.*, 1997; Kush *et al.*, 1990). Hevin, a lectin like protein involved in the coagulation of latex, was mediated by ethylene (Broekaert *et al.*, 1990; Sivasubramaniam *et al.*, 1995; Gidrol *et al.*, 1994). Ethylene could up regulate activity of glutamine synthetase (GS), a key enzyme of nitrogen metabolism and its transcript levels in *H. brasiliensis* latex cells, suggesting the involvement of GS in the ethylene stimulation and latex production in *Hevea* (Pujadae-Renaud *et al.*, 1994). Higher expression of MnSOD, regulated by ethephon application, prevented lutoid disruption by superoxide radical (Miao and Gaynor, 1993) leading to increased rate of latex flow.

The precursor for the synthesis of rubber in the laticiferous tissue is sucrose (Sando *et al.*, 2008a) available from photosynthesis which is actively transported in to laticiferous cells through the plasmalemmic membrane. In *Hevea,* the symplasmic isolated nature of the laticifer from its neighboring cells (Hebant, 1981) suggests that sucrose transporters (SUTs) might play an active role in the transmembrane uptake of sucrose by the laticifers. It is then hydrolyzed in to glucose and fructose by invertase. These sugars are converted to acetyl-CoA through glycolysis. Three molecules of acetyl-CoA are condensed into mevalonic acid which is converted to isopentenyl pyrophosphate (IPP). Polymerization of thousands of IPP molecules assisted by the action of the enzyme rubber transferase (RuT) in association with

rubber elongation factor (REF), a molecule fixed on the rubber particle membrane, leads to the formation of high molecular weight rubber.

Hybridization coupled with vegetative propagation and clonal selection is the most important method of breeding of *Hevea brasiliensis*. This resulted in yield increase from 250 kg/ha/annum to more than 3000 kg/ha/annum (Licy *et al.*, 2003; Mydin, 2014). In contrast to the progress made in rubber tree breeding, the molecular mechanisms underlying high yield potential of a clone is not well understood. Molecular biological characterization of genes involved in rubber biosynthesis has not been adequately studied. Although earlier workers have identified and characterized few genes involved in rubber biosynthesis, the genes associated with high yield potential are not studied well. Hence, a study was conducted to determine the expression of genes corresponding to key enzymes involved in rubber biosynthesis in relation to latex yield trait. Genes related to rubber yield may eventually be used as specific molecular markers of yield potential in crop improvement programmes.

3.2. Materials and methods

3.2.1. Gene expression pattern under regular harvesting of latex

3.2.1.1. Plant material

Healthy *Hevea* trees of high yielding varieties (RRII 105, RRIM 600 and PB 217) and low yielding varieties (Tjir 1, RRII 33 and RRII 38) planted at Central Experiment Station, Chethackal, Pathanamthitta district, Kerala of Rubber Research Institute of India were selected for the study. These trees

were regularly harvested for latex in every three days under the S/2, d3 6d/7 system of tapping and the trees were in the 2nd year of tapping.

3.2.1.2. Latex yield and dry rubber content

Total latex yield in each tree was recorded by measuring the total latex volume on every tapping day. DRC in the latex was determined by gravimetric method. About 4-5 gram of latex was mixed well with an equal amount of distilled water and it was coagulated with 3% acetic acid. A thin film of rubber was prepared 3 or 4 minutes after coagulation. The coagulum was washed in running water and oven dried at 80°C for 72 hours. The oven dried material was used for calculating the DRC using the following formula.

$$DRC = \frac{Weight\ of\ oven\ dried\ rubber}{Weight\ of\ fresh\ latex} \times 100$$

The rubber yield was calculated as per the formula shown below and represented as g/tree/tap.

$$Yield = \frac{Total\ volume\ of\ latex \times DRC}{100}\ g\ /\ tree\ /\ tap$$

3.2.1.3. Latex sample collection for RNA extraction

Fresh latex was collected from the selected trees after discarding first few drops of latex after tapping. Latex (10 ml) was collected in 10 ml extraction buffer (1:1 v/v). All these samples were brought to the laboratory in ice and stored at -80°C until the extraction of total RNA.

3.2.2. Gene expression pattern in response to ethylene stimulation

3.2.2.1. Plant material

The study was carried out in mature *H. brasiliensis* trees planted at Rubber Research Institute of India farm, Kottayam. Two high yielding clones (RRII 105 and PB 217) and two low yielding clones (RRII 38 and Tjir 1) were selected for the study. The trees were tapped regularly for harvesting latex every three days under the S/2, d3 6d/7 tapping system and the trees were in the 13th year of tapping.

3.2.2.2. Ethephon stimulation

Ethylene treatment was administered by applying commercially available Ethephon (2-chloro ethyl phosphonic acid) diluted to 5% (v/v) with palm oil. The tapping panel of the trees were scraped well (1 inch below the tapping cut) in order to remove the dead tissues and rubber particles present on it. The compound was applied on the bark of the tapping panel 1 inch below the tapping cut using a brush. For each treatment, three trees were used along with control trees. Tapping panel of control trees was also scraped and treated with palm oil without ethephon. The dry rubber yield was measured from stimulated and control trees.

3.2.2.3. Latex sample collection

Fresh latex was collected from *Hevea* clones before stimulation and three days after stimulation (72 hrs). Latex from trees was collected in extraction buffer. Initial few drops of latex were discarded and fresh latex (10 ml) was collected in 10 ml extraction buffer (1:1 v/v). All these samples

were brought to the laboratory in ice and stored at -80°C until the extraction of total RNA.

3.2.3. Total RNA isolation

Latex RNA was isolated according to Chang *et al.* (1993).

Reagents:

- **RNA extraction buffer:** 2% CTAB (hexadecyl trimethylammonium bromide); 2% PVP (polyvinyl pyrrolidone K 30); 100 mM Tris-HCl (pH 8.0); 25 mM EDTA; 2.0 M NaCl; 0.05% spermidine; 2% v/v β-mercaptoethanol (added just prior to use).
- Chloroform: Isoamyl alchol (24:1)
- 8 M Lithidium chloride
- **SSTE:** 1.0 M NaCl; 0.5% SDS; 10 mM Tris-HCl (pH 8.0); 1.0 mM EDTA

The latex samples were centrifuged at 7000 rpm at 4^0C for 20 min. The middle serum layer was collected and mixed with equal volume of chloroform and isoamyl alchohol. The tubes were spun at 7000 rpm for 10 min at room temperature. The top aqueous phase was transferred into fresh centrifuge tubes and the extraction repeated using chloroform:isoamyl alcohol mixture. The top aqueous phase was again transferred to another tube and mixed with 0.3 volume of 8 M lithium chloride and kept the tubes at 4^0C over night. The tubes were spun at 7000 rpm for 20 min at 4°C. The pellet was washed with 2 ml of 2M lithium chloride. The tubes were spun at 7000 rpm for 20 min at 4°C. The pellet was suspended in 500 µl SSTE and mixed with equal volume of chloroform: isoamyl alcohol (24:1). The tubes were

spun at 10,000rpm for 10min at 4°C. The upper phase was transferred to another tube and mixed with double volume of absolute ethanol. The tubes were kept at -20°C overnight. The tubes were spun at 10,000 rpm for 20 min to pellet the RNA. The pellet was then dried using vacuum and dissolved in autoclaved DEPC treated water.

3.2.4. DNase treatment

The RNA samples were subjected to DNase treatment by using Sigma Deoxyribonuclease I Amplification Grade (AMPD 1) in order to eliminate the DNA from RNA preparations according to manufacturer's instructions. Concentration of RNA was measured using NanoDrop (ND-1000 Spectrophotometer, USA).The quality of the total RNA was checked on a formaldehyde denaturing gel.

3.2.5. Formaldehyde gel electrophoresis

Formaldehyde gel (1.4%) was prepared by adding required amount of agarose in 10x MOPS buffer and DEPC water. It was melted to homogeneity and allowed to cool down to about 50^{0}C. After adding required quantity of formaldehyde and proper mixing, it was poured in to casting tray with appropriate sized comb. After about 30 min, the gel was run in an electrophoretic tank containing 1x MOPS buffer after loading RNA sample plus loading dye along with suitable marker. Electrophoretic separation was carried out at 80V till the dye front reached the end of the gel. RNA was visualized under a UV transilluminator.

3.2.6. cDNA synthesis

cDNA was synthesized from total RNA using Superscript™ III First strand synthesis kit from Invitrogen, RNA mix (10 µl) after incubation at 65^{0}C for 5 min followed by incubation in ice for a minute was mixed with cDNA mix (10 µl). After a brief spin, the mixture was incubated at 50^{0}C for 50 min (oligo dT) and at 85^{0}C for 5 min. After a brief incubation in ice, 1 µl of RNase H was added to each tube and incubated for 20 min at 37^{0}C. The cDNA was quantified and stored at -20^{0}C.

3.2.7. Quantitative PCR (qPCR)

The primers (Table 3.1) were designed and synthesized by Eurogentec, Belgium. Quantitative PCR was performed in triplicate, using specific primers and generated cDNA as template in real- time PCR machine (Light cycler 480 II, Roche, Germany). qPCR was performed in a 20 µl reaction mixture containing 1 µl of 1/10 dilution of first-strand cDNA reaction, 125 nM of each primer and 10 µl of Light cycler 480 SYBR Green I Master (Roche Diagnostics Gmbh, Germany). qPCR was performed by incubating the mix at 95° C for 7 min, followed by 40 cycles of 95 °C for 20 seconds and 60 °C for 30 seconds. This was followed by a melt curve analysis (95 °C for 20 seconds, 60 °C for one minute, 95 °C for 5 minutes). Each experiment was repeated two to three times and each PCR reaction was performed in triplicate with no template controls (NTC). Reaction efficiency of both the target genes and the endogenous control was calculated based on the formula, Efficiency = $10^{(-1/slope)}$-1). The slope values of the primers were between -3.2 and -3.5. The Relative Quantification (RQ) values were analyzed (using the software of Light Cycler 480; release1.5.0) and the

expression rate of genes is represented as fold change. The $2^{-\Delta\Delta ct}$ method was adopted to analyse the relative changes in gene expression from qPCR experiments (Livak and Schmittgen, 2001). 18S rRNA was used as endogenous control for qPCR analysis.

3.2.8. Data analyses

For each treatment, three biological replications were included in the qPCR analysis. Statistical analysis was performed with the relative quantification data using ANOVA. The ratio with a P value ≤ 0.05 was adopted as significant.

Table. 3.1. List of genes and corresponding primers used for qPCR analysis.

Sl. No.	Gene	Forward primer (5'-3')	Reverse primer (5'-3')
1	*hmgr*1	CTGTTCTTCTCGGTGGGCGTATTAC	AGAGGCAATGAGGGAGACAATAGC
2	*hmgr*2	GCGAGGCAATTATCAAGGAAGAGG	GCACCAGCAACAGCGGAAC
3	*hmgr*3	GGCGTGGGAAGTCTGTTGTATG	AGAGAACCTGCTACGGCTGAG
4	*hmgs*	ACACTGACATTGAAGGCGTTGAC	CACTACAAGTCCATAGCGTCCATC
5	*MVD*	CTGCTGCTGGATTGGCTTCTTC	GCACTGCCTGAACCTTGTCTTG
6	*FPPS*	CGGCACGAGTGATTTCAGAGTTTC	CCAGGCACATTGTAGTCCAACATC
7	*GGPS*	GCTGCCATTCCACTCCAAGAACC	TCATTCCCACCAACAAGCTCACAC
8	*RuT*	CAGTCAAGACCGCAGCAGATAAG	AGCATACAGCAATGAGAAGCACAC
9	*REF*1	AGTTTATGCCAGGGCTTCTTTCTC	AGTATTGACGCCAGGCTTGAATG
10	*REF*2	AGATGCGTCCTTGACAATTGG	GCCCGGTTGTTGCATCTG
11	*REF*3	GTTTGTAGACAGCACGGTTGTTG	GAAGAAGCCAGAGAACGAGCAG
12	*HbSUT*3	CACCACAACCACCATCAC	GTGGAAGAGGTTCAGAAGAG
13	*RBSP*	ACACCAAGGATGATCTCAGGCTTC	AGACATGACGGTCACCACAAGG
14	*RBIP*	TGCTGCGACCAATCCTACACTAC	CACTAGCCTGCTCTTGCTCTCC
15	18S *rRNA*	CAACCATAAACGATGCCGACCAG	TTCAGCCTTGCGACCATACTCC

3.3. Results

3.3.1. Gene expression under regular harvesting of latex

Genes associated with yield potential were studied in six clones of *Hevea* with varying total rubber yield (Fig. 3.1.a).

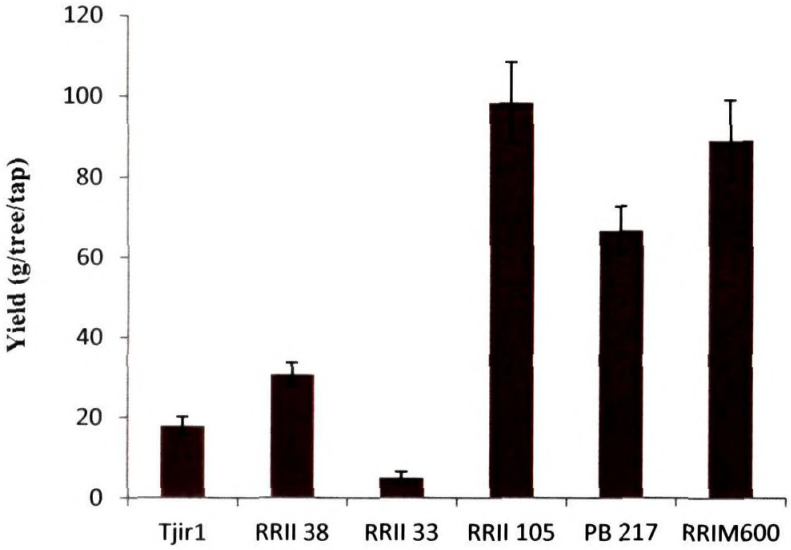


Fig. 3.1.a. Dry rubber yield (g/tree/tap) of *Hevea* clones

The latex was collected from high yielding (RRII 105, PB 217 and RRIM 600) and low yielding clones (Tjir 1, RRII 38 and RRII 33) for isolating total RNA. The optical density ratio A_{260}/A_{280} of the RNA isolated ranged from 1.9 to 2.1 indicating the good quality of RNA. The presence of two narrow bands of 28S and 18S RNA in the gel indicated that the RNA was intact and of good quality (Fig. 3.1.b).

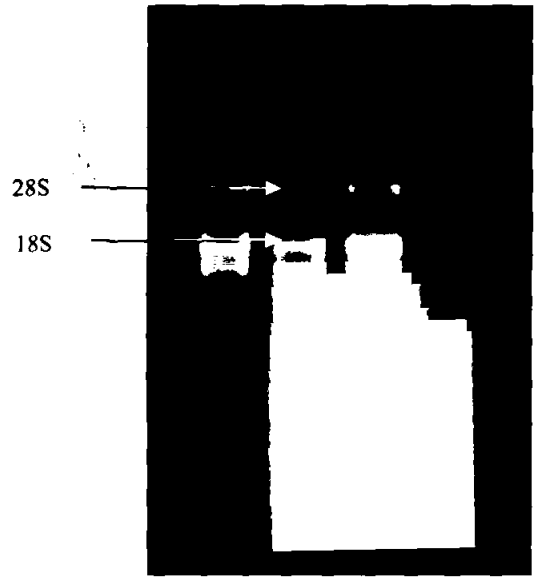


Fig. 3.1.b. Visualisation of total RNA (2 μg) from *Hevea* latex on 1.4% denatruring formaldehyde agarose gel.

These RNA samples were used for cDNA synthesis and as template after proper dilution for the PCR and qPCR analyses. The melting peaks (Fig. 3.1.c.), slope value of the primer (Fig. 3.1.d.) and amplification curves with Ct values (Fig. 3.1.e.) confirmed the amplification efficiency in qPCR.

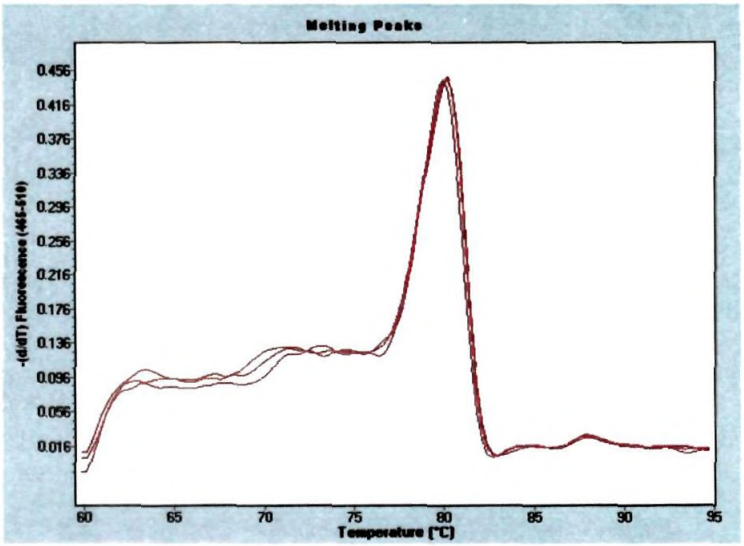


Fig. 3.1.c. Melting peak of 18S rRNA of *H. brasiliensis* in qPCR

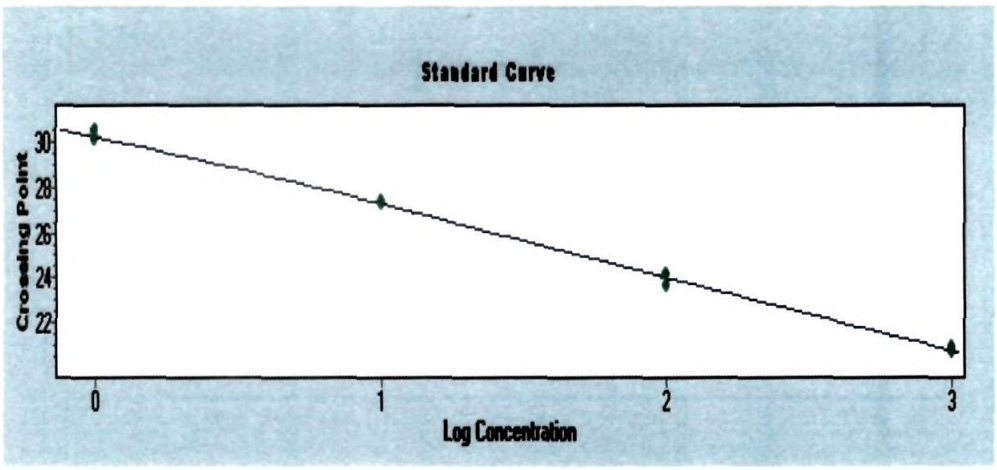


Fig. 3.1.d. Slope value of 18S rRNA of *H. brasiliensis* in qPCR

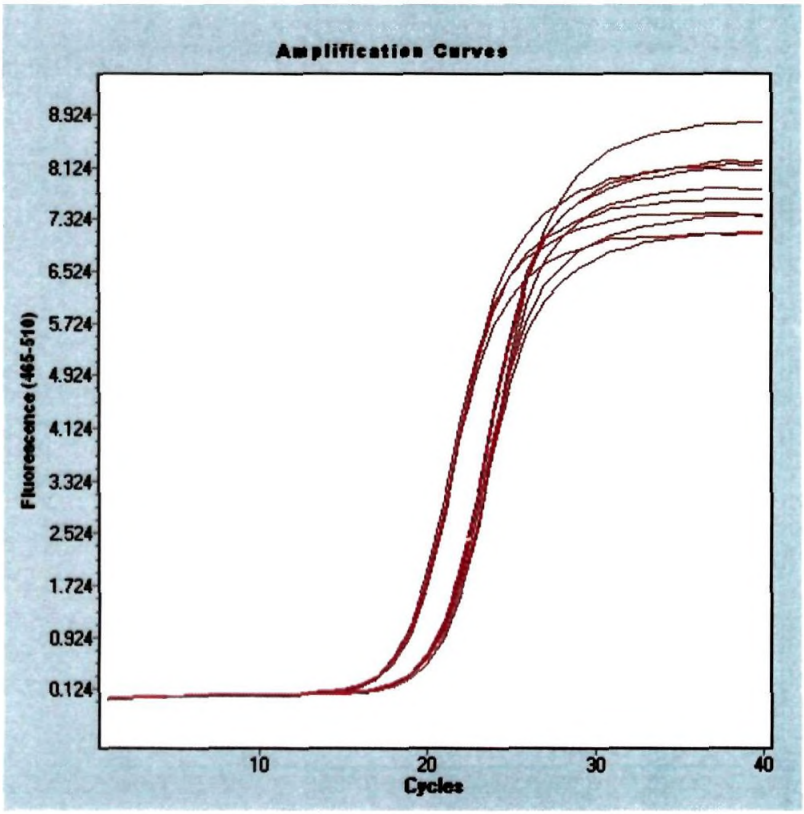


Fig. 3.1.e. Amplification curve of 18S rRNA and *hmgs* of *H. brasiliensis* in qPCR.

In order to determine the association of the selected genes to rubber biosynthesis and evaluate their differential expression in different *Hevea* clones with varying yield potential, qPCR analyses were performed. The low yielding *Hevea* clone (Tjir 1) was used as calibrator in this study and the results are given in Table 3.2. and Fig.3.1.f. to 3.1.i.

Table.3.2. Relative quantification (fold change) of genes expressed in *Hevea* clones with different yield potential

Genes	Calibrator (Tjir 1)	RRII 38	RRII 33	RRII 105	PB 217	RRIM 600	CD
hmgs	1	2.07	1.38	6.37*	6.77*	5.62*	2.13
*hmgr*1	1	1.68	1.94	5.51*	2.84*	2.87*	0.98
*hmgr*2	1	1.84	1.04	2.08	1.50	1.47	0.81
*hmgr*3	1	0.90	0.99	0.86	0.65	0.95	0.31
MVD	1	2.11	1.51	7.04*	8.01*	5.62*	2.09
FPPS	1	1.22	1.25	2.02*	2.59*	3.76*	0.37
GGPPS	1	1.26	0.91	1.45	1.67	0.96	3.82
RuT	1	2.14	2.07	5.17*	7.38*	10.80*	1.29
*HbSUT*3	1	2.30	2.57	6.21*	7.63*	6.95*	1.74
*REF*1	1	1.35	1.17	1.30	1.42	1.05	0.55
*REF*2	1	2.84	1.98	8.89*	13.35*	4.55*	2.18
*REF*3	1	2.37*	1.45	1.86*	2.85*	1.45	0.78
RBSP	1	1.37	1.46	1.65*	1.78*	1.35	0.55
RBIP	1	1.09	0.87	1.19	1.60	1.12	0.42

* indicates significance at $P \leq 0.05$

Hydroxy-3-methyl-glutaryl-CoA synthase (HMG-CoA synthase, HMGS) catalyze the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-CoA. The HMG-CoA produced by this enzyme in plants acts as a substrate for HMG-CoA reductase (HMGR) to generate mevalonate which is further converted to isoprenoid compounds. The relative gene expression of *hmgs* was found to be significantly higher in high yielding *Hevea* clones (RRII 105, RRIM 600 and PB 217) than the low yielders (Fig. 3.1.f.).

In *Hevea brasiliensis,* HMGR is encoded by a small gene family comprising of five members, *hmgr*1, *hmgr*2, *hmgr*3, *hmgr*4 and *hmgr*5. In this study, the expressions of *hmgr*1, *hmgr*2 and *hmgr*3 were analyzed. Expression of *hmgr*1 was found higher in high yielding clones than the low yielders. It was significantly high in RRII 105 followed by RRIM 600 and PB 217 (Fig. 3.1.f). Among the *Hevea* clones studied, *hmgr*2 expression was high in RRII 105 and RRII 38. Its expression was similar in *Hevea* clones PB 217 and RRIM 600. The expression pattern of *hmgr*3 was almost similar in all the *Hevea* clones studied (Fig. 3.1.g.).

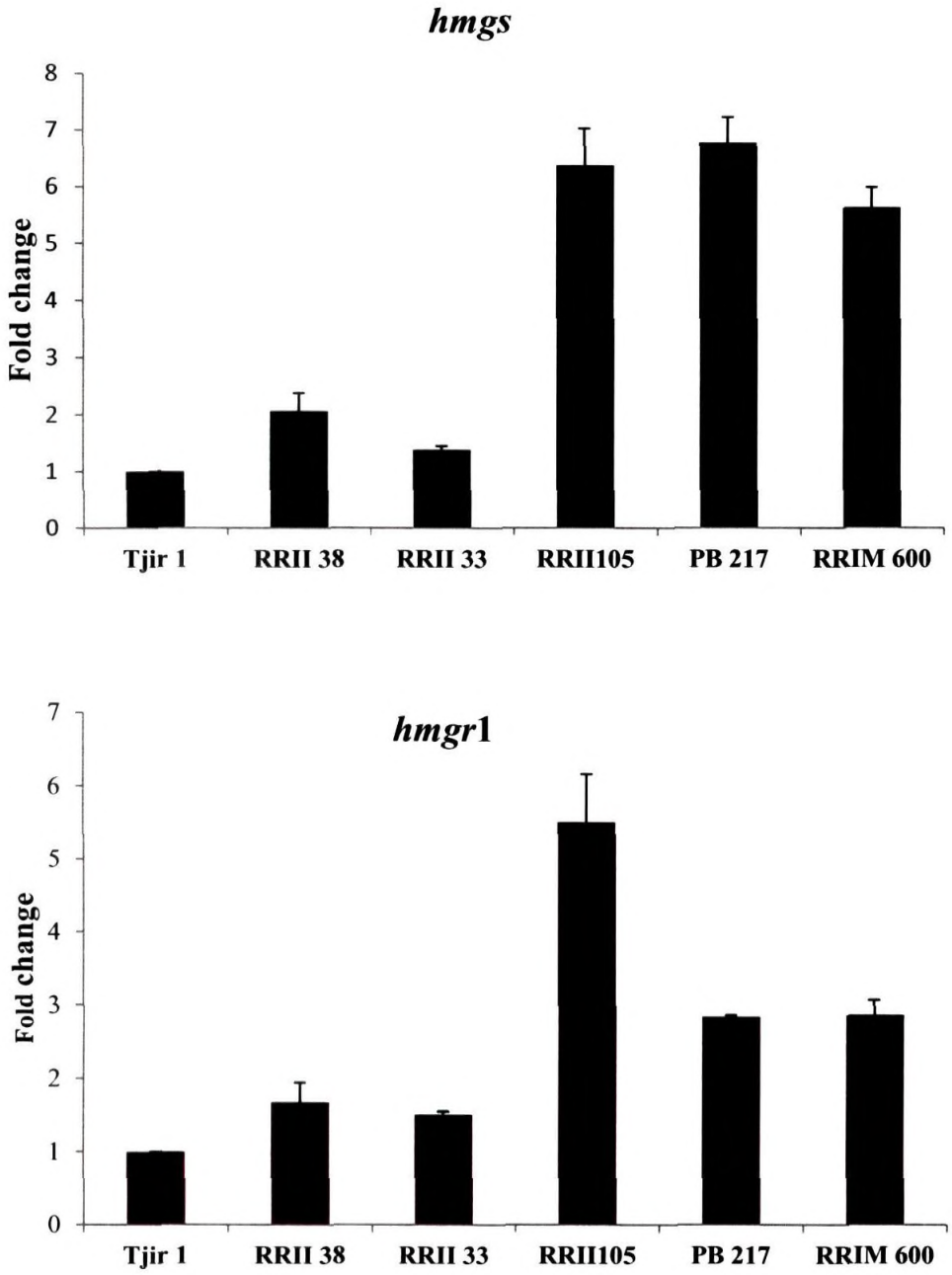


Fig. 3.1.f. Quantitative expression analysis of *hmgs* and *hmgr*1 in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.

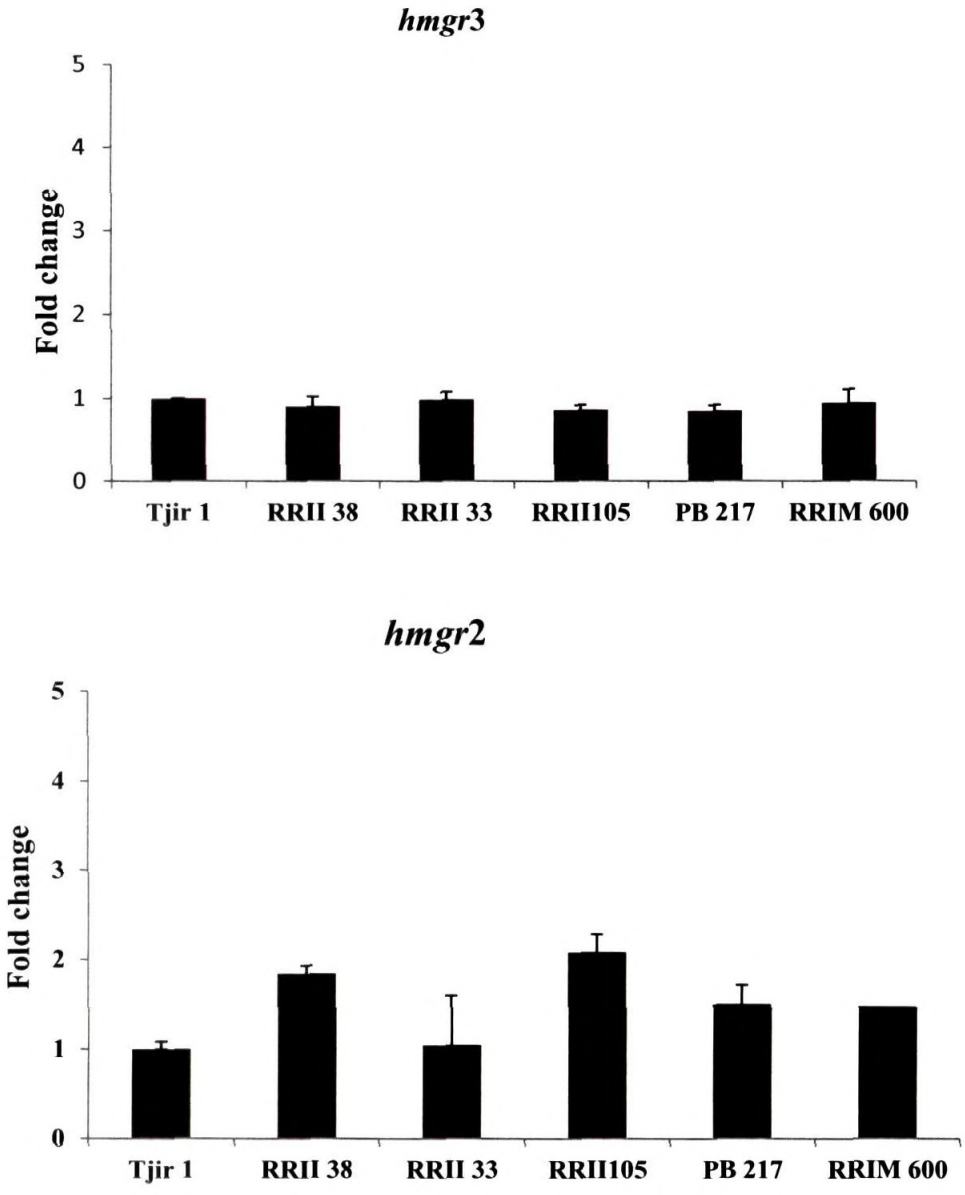


Fig. 3.1.g. Quantitative expression analysis of *hmgr*2 and *hmgr*3 in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.

The gene expression of Mevalonate diphosphate decarboxylase (MVD) which catalyses mevalonate formation was significantly higher in high yielding *Hevea* clones than low yielding clones (Fig. 3.1.h). Among the high yielding clones, RRII 105 showed higher *MVD* gene expression compared to PB 217 and RRIM 600. *FPP synthase* gene expression was significantly higher in high yielders compared to the low yielding clones (Fig. 3.1.h.).

GGPP synthase gene expression was not significantly different between the high and low yielders of *Hevea* clones. The expression of this gene was observed high in PB 217 followed by RRII 105 and RRII 38 (Fig. 3.1.i.). The gene expression of rubber transferase (*RuT2*) was also significantly higher in high yielding *Hevea* clones than the low yielding clones. Among the high yielders, RRIM 600 showed higher gene expression followed by PB 217 and RRII 105. The low yielding clones (Tjir 1, RRII 38 and RRII 33) displayed a low gene expression (Fig.3.1.i).

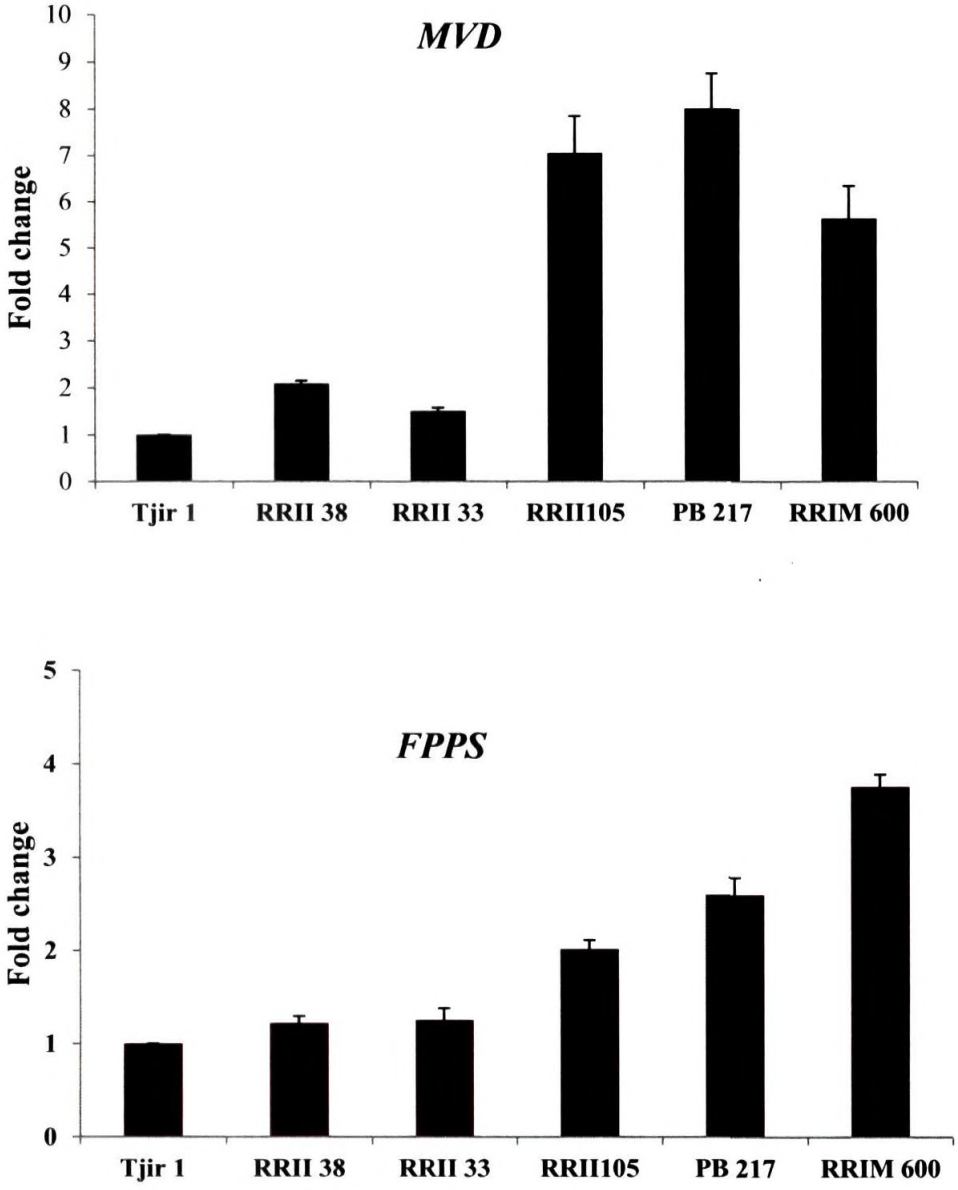
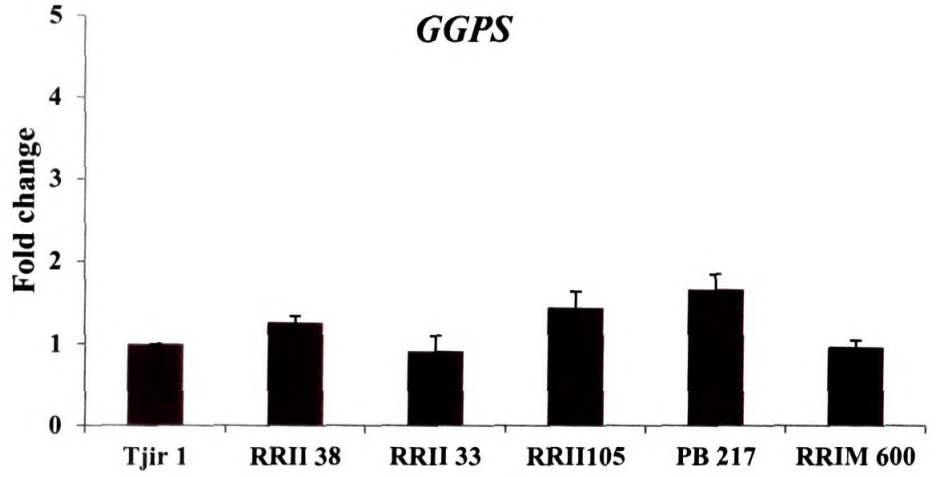


Fig. 3.1.h. Quantitative expression analysis of *MVD* and *FPPS* in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.



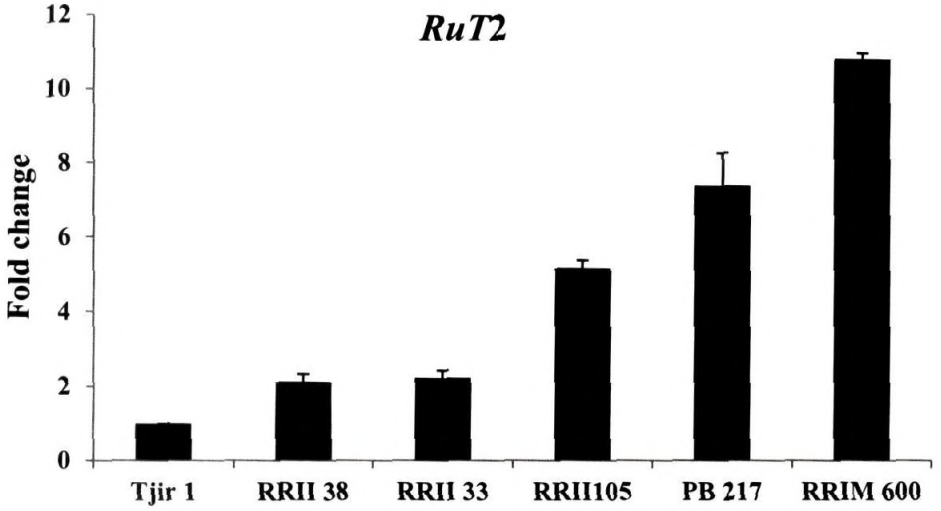


Fig. 3.1.i. Quantitative expression analysis of *GGPS* and *RuT* in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.

The gene expression of different isoforms of REF was also studied (Fig. 3.1.j.). *REF1* showed a low level of expression in all the selected *Hevea* clones. No significant difference was observed between high and low yielders. The gene expression of *REF2* was found up regulated in high yielding *Hevea* clones. Among them, PB217 showed a higher expression followed by RRII 105 and RRIM 600.

REF3 showed a different expression pattern, a low yielder (RRII 38) and a high yielder (PB 217) showed higher expression compared to other selected *Hevea* clones. *Hevea* sucrose transporter 3 (*HbSUT3*) was significantly up-regulated in high yielding *Hevea* clones (RRII 105, PB 217 and RRIM 600) than the low yielding clones (Tjir 1, RRII 33 and RRII 38) (Fig.3.1.k). Among the high yielders PB 217 showed a higher fold level change in gene expression (7.63) than other clones RRIM 600 (6.95) and RRII 105 (6.21) respectively. No significant difference was observed for the expression of rubber biosynthesis stimulator protein *(RBSP)* and rubber biosynthesis inhibitor protein *(RBIP)* between high and low yielding *Hevea* clones (Fig. 3.1.l.).

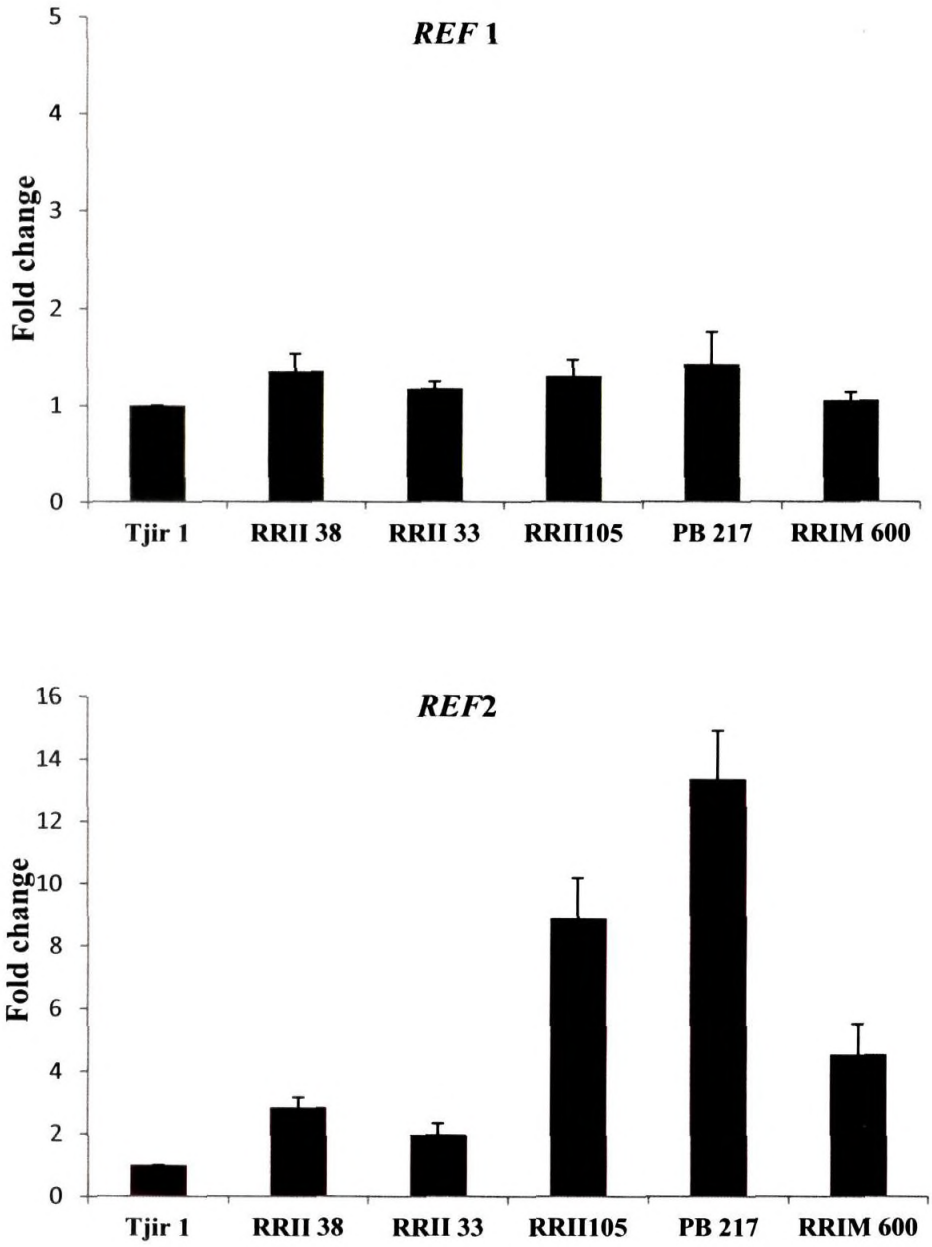


Fig. 3.1.j. Quantitative expression analysis of *REF*1 and *REF*2 in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.

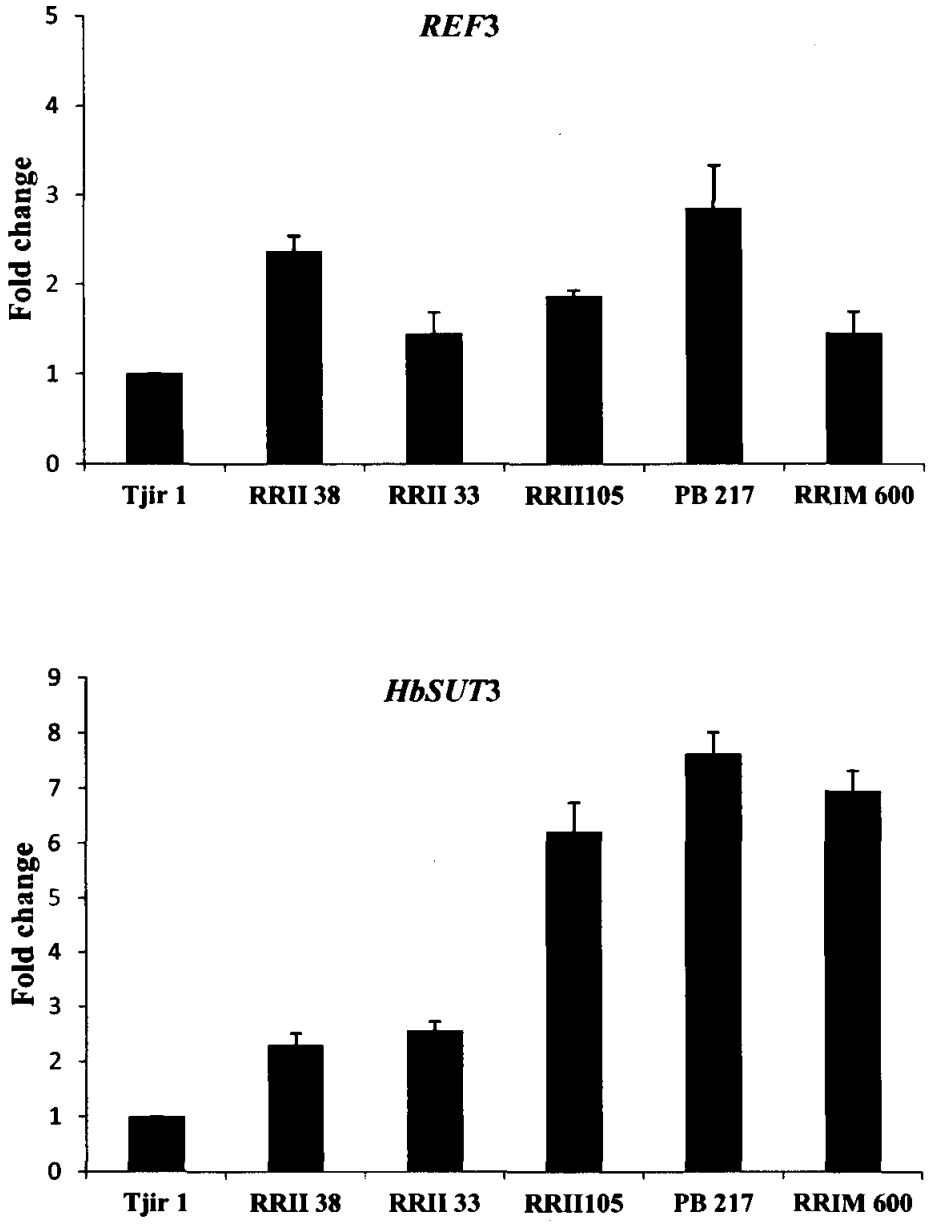


Fig. 3.1.k. Quantitative expression analysis of *REF*3 and *HbSUT*3 in *Hevea* clones with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. Error bars indicate standard error of three biological replicates.

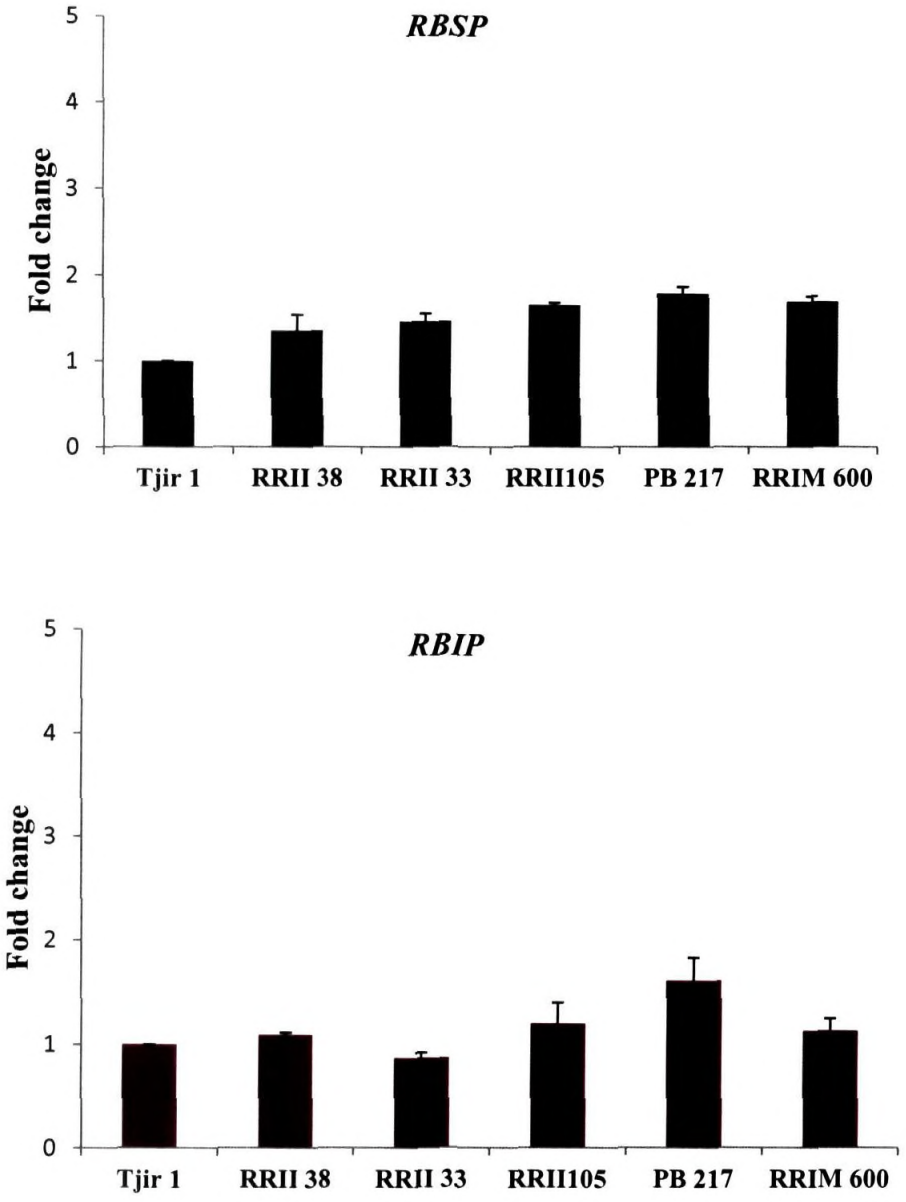


Fig. 3.1.1 Quantitative expression analysis of *RBSP and RBIP* in *Hevea clones* with different yield potential. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

3.3.2. Gene expression in response to ethylene stimulation

The effect of ethylene stimulation was confirmed by observing increased latex volume collected from stimulated trees and the rubber yield was found higher than the unstimulated control trees (Fig. 3.2.a).

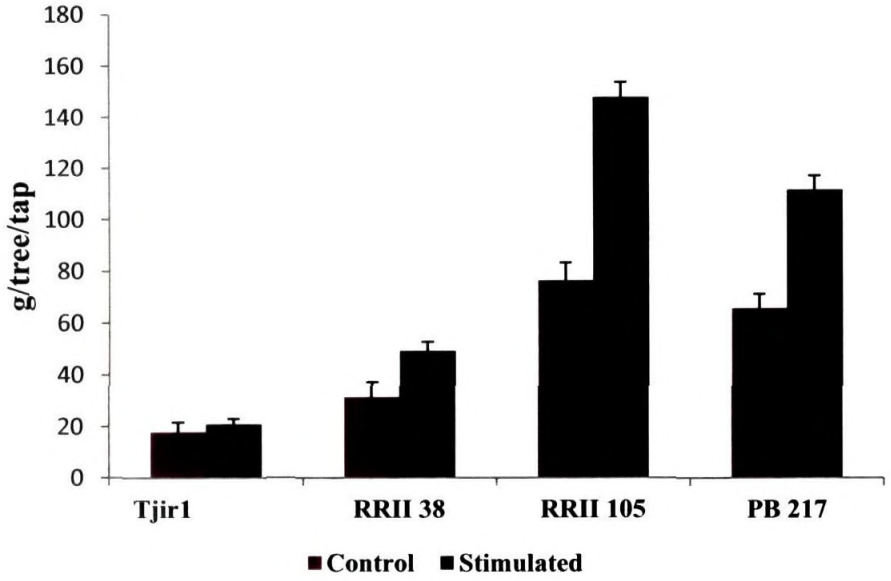


Fig. 3.2.a. Effect of stimulation on dry rubber yield (g/tree/tap) of *Hevea* clones.

The gene expression of above mentioned gene transcripts (14 nos.) was compared between ethylene treated and control trees of each clone. *Hevea* 18S rRNA was used as the endogenous control and the expression in control trees of each clone was used as the calibrator for gene expression analyses and the results are given in Table 3.3 and Fig. 3.2.a to 3.2.e).

Table.3.3. Relative quantification (fold change) of genes in response to ethylene stimulation. The control plants of respective clone as calibrator.

Genes	Calibrator	Tjir 1	RRII 38	RRII 105	PB 217	CD
*hmgr*1	1	1.450*	2.276*	1.743*	1.833*	0.165
*hmgr*2	1	0.303*	0.885	0.367*	0.585*	0.253
*hmgr*3	1	1.080	1.300*	1.145	1.130	0.198
hmgs	1	0.711*	1.328	0.277*	0.319*	0.125
MVD	1	1.689*	1.454*	1.982*	1.895*	0.385
FPPS	1	0.455*	0.441*	0.698*	0.750*	0.131
GGPS	1	0.680*	0.846	0.876	0.697*	0.241
RuT	1	0.711*	0.840	0.876	0.748	0.288
*HbSUT*3	1	5.477*	4.226*	7.787*	8.456*	0.611
*REF*1	1	0.928	0.869*	0.606*	0.404*	0.156
*REF*2	1	1.300	1.250	1.230	1.310	0.421
*REF*3	1	0.511*	0.680*	0.673*	0.667*	0.132
RBSP	1	1.060	1.120	0.930	1.112	0.387
RBIP	1	1.043	1.110	1.263	1.353	0.453

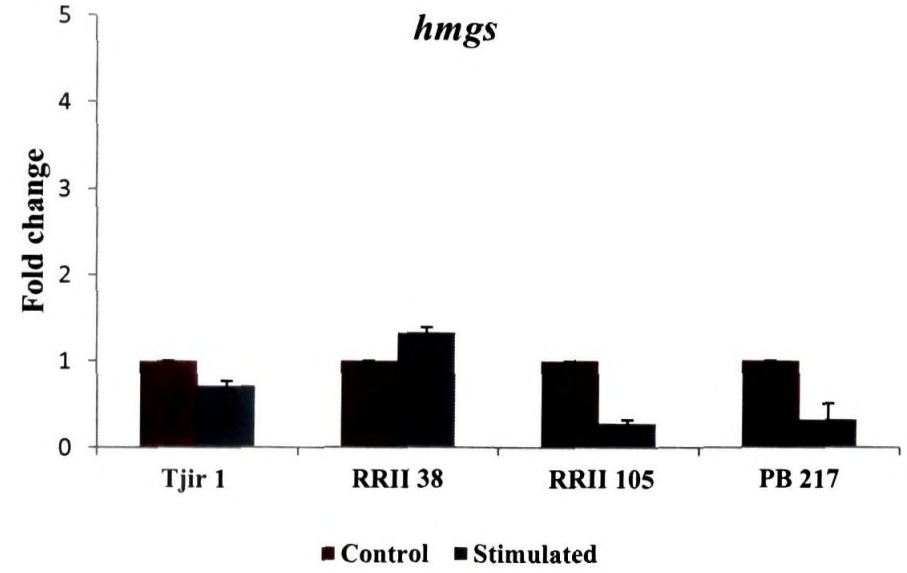
* indicates significance at $P \leq 0.05$

Irrespective of clones, the gene expression of *hmgs* was down regulated both in stimulated and unstimulated trees (Fig. 3.2.b). The effect of stimulation in the gene expression of *hmgr* gene family was studied. The expression of *hmgr*1 was significantly up regulated in all the stimulated

Hevea clones. The *hmgr*1 is the responsible gene for rubber biosynthesis and it was induced by ethylene (Fig. 3.2.b). The *hmgr*2 gene expression was down regulated in all the stimulated than the unstimulated trees of the corresponding clones. No significant difference was observed in the gene expression of *hmgr*3 between stimulated and control trees (Fig. 3.2.c).

MVD gene expression was up regulated in all *Hevea* clones irrespective of the yield potential. A significantly higher gene expression was observed in stimulated trees of high yielders (RRII 105 and PB217) and low yielders (Tjir 1 and RRII 38) than the unstimulated control trees (Fig. 3.2.d). The expressions of *FPP* synthase (Fig.3.2.d.), *GGPP* synthase and *RuT* (Fig. 3.2.e) were down regulated in all the selected *Hevea* clones after ethylene stimulation.

*HbSUT*3 expression was significantly up regulated in stimulated than the control trees (Fig. 3.2.f). The low and high yielding clones showed significantly higher level of expression for *HbSUT*3 in stimulated trees than in the control trees. In the case of *REF* gene families, *REF*1 (Fig.3.2.f) and *REF*3 (Fig.3.2.g) were also down regulated in all stimulated trees irrespective of the clones. The gene expression of *REF*2 (Fig.3.2.g) was induced with ethylene treatment compared to the control trees. There was no significant difference for the expressions of *RBSP* and *RBIP* between stimulated and unstimulated trees of different *Hevea* clones (Fig. 3.2.h).



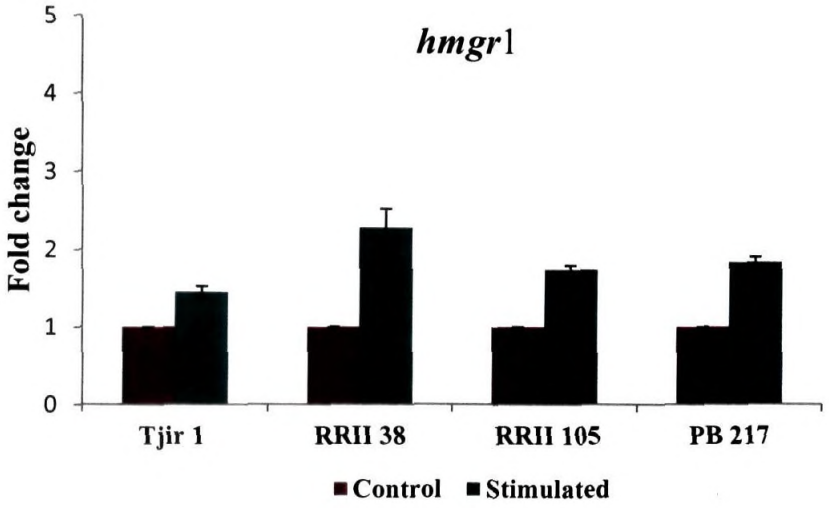
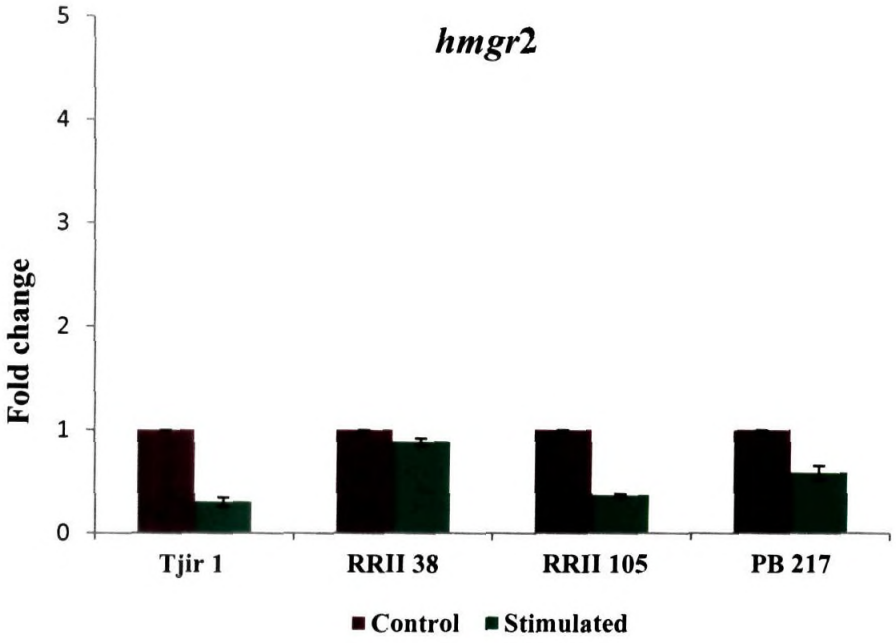


Fig. 3.2.b. Quantitative expression analysis of *hmgs and hmgr*1 in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.



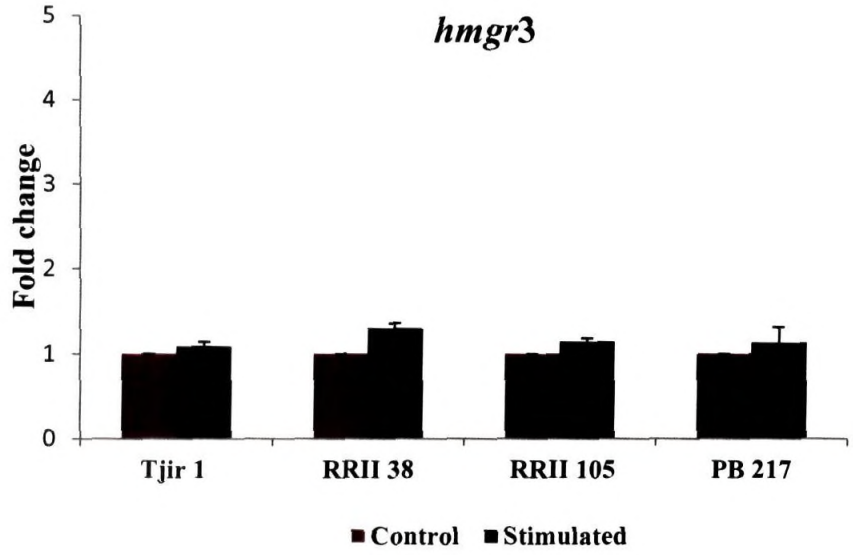


Fig. 3.2.c. Quantitative expression analysis of *hmgr2 and hmgr3* in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

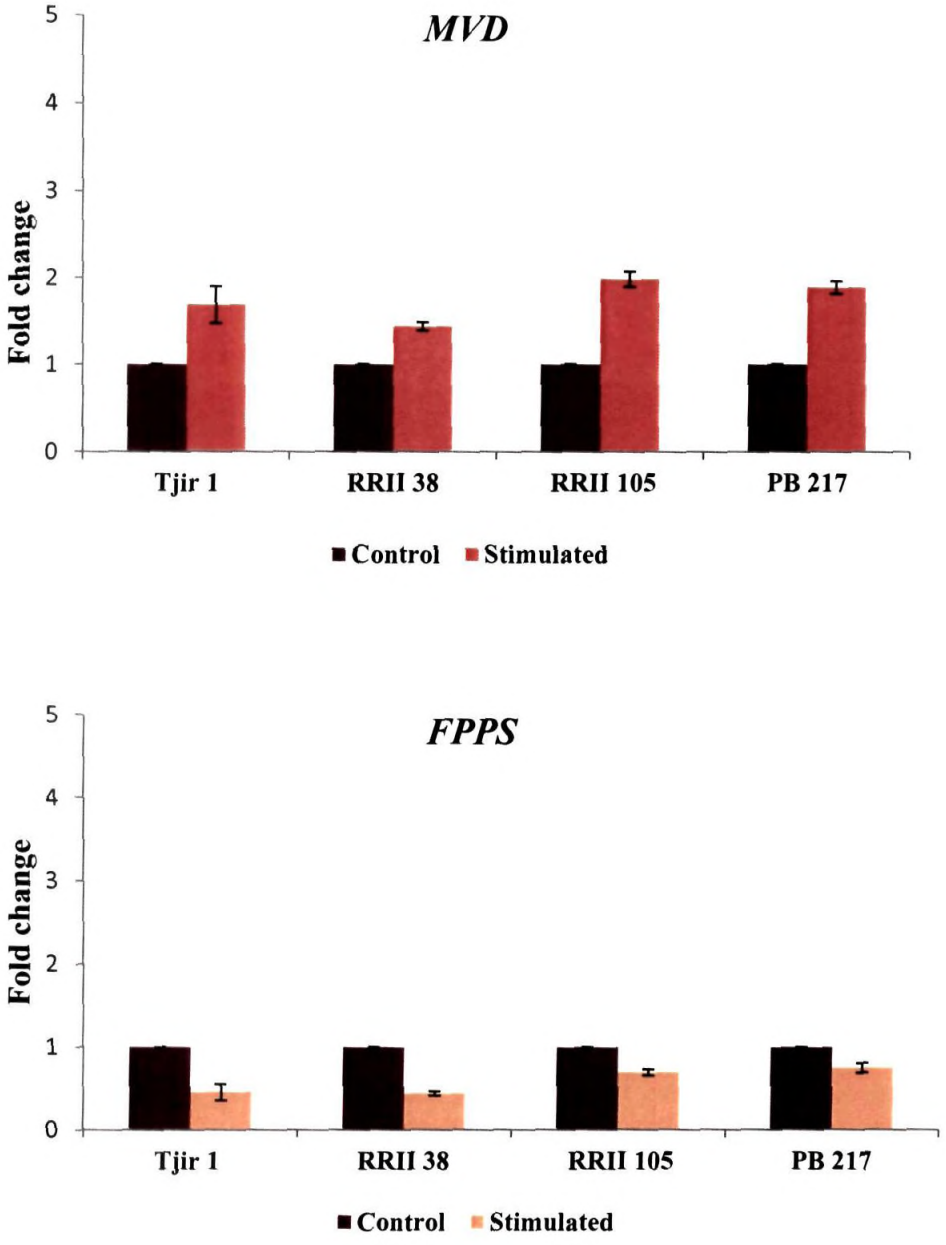


Fig. 3.2.d. Quantitative expression analysis of *MVD* and *FPPS* in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

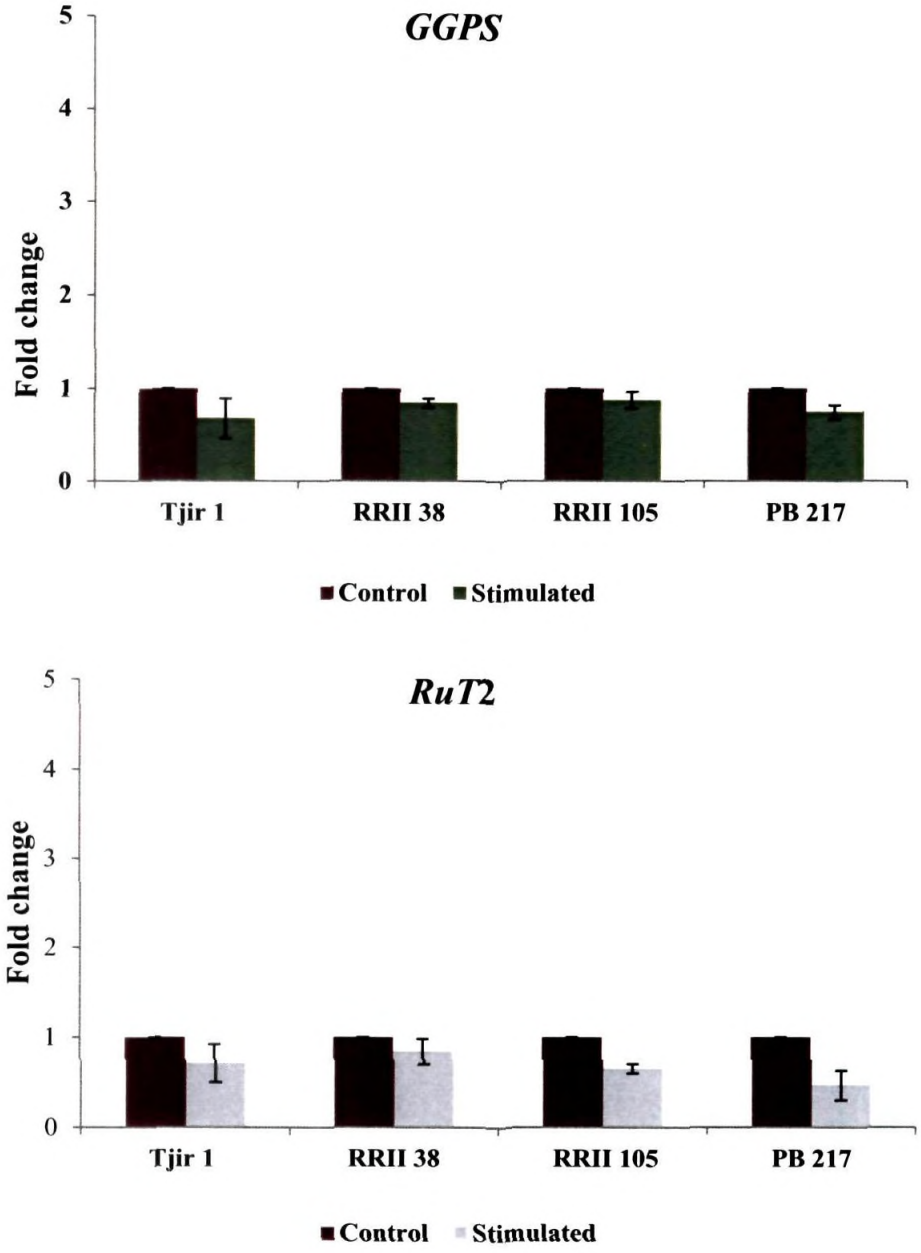
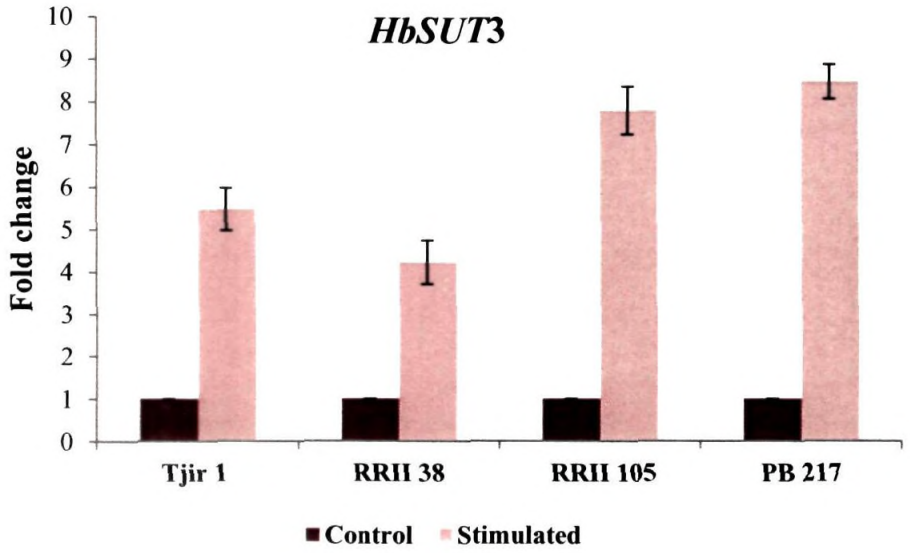


Fig. 3.2.e. Quantitative expression analysis of *GGPS* and *RuT2* in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.



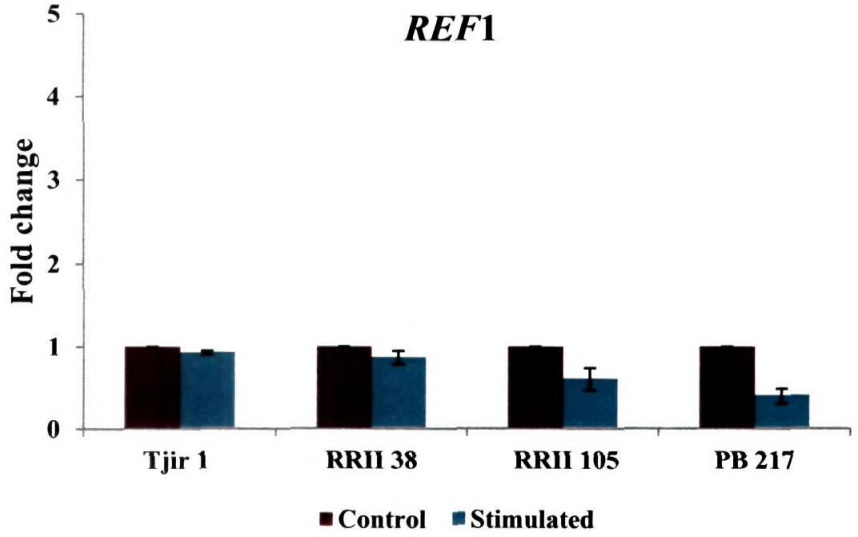


Fig. 3.2.f. Quantitative expression analysis of *HbSUT*3 and *REF*1 in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

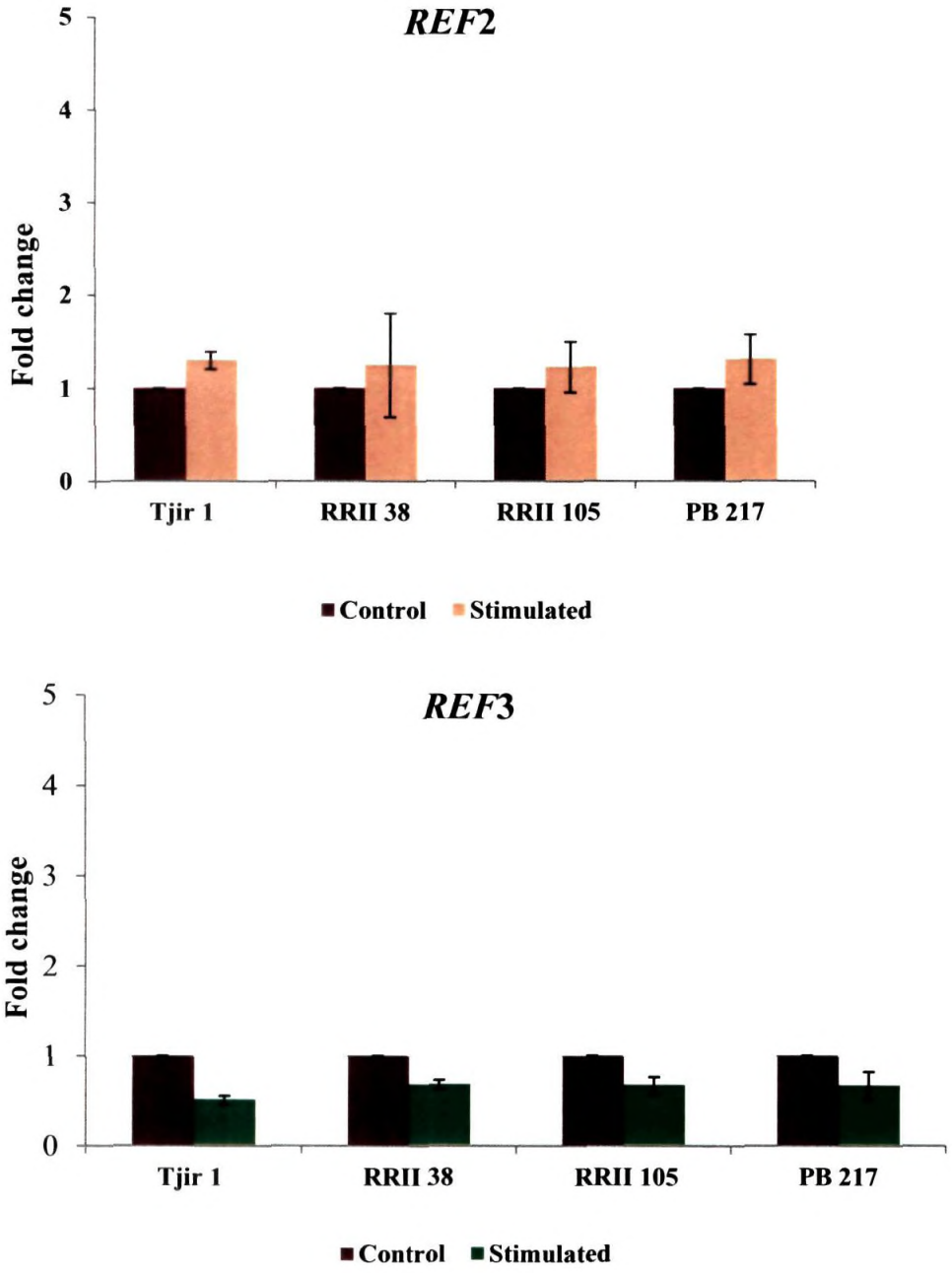


Fig. 3.2.g. Quantitative expression analysis of *REF*2 and *REF*3 in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

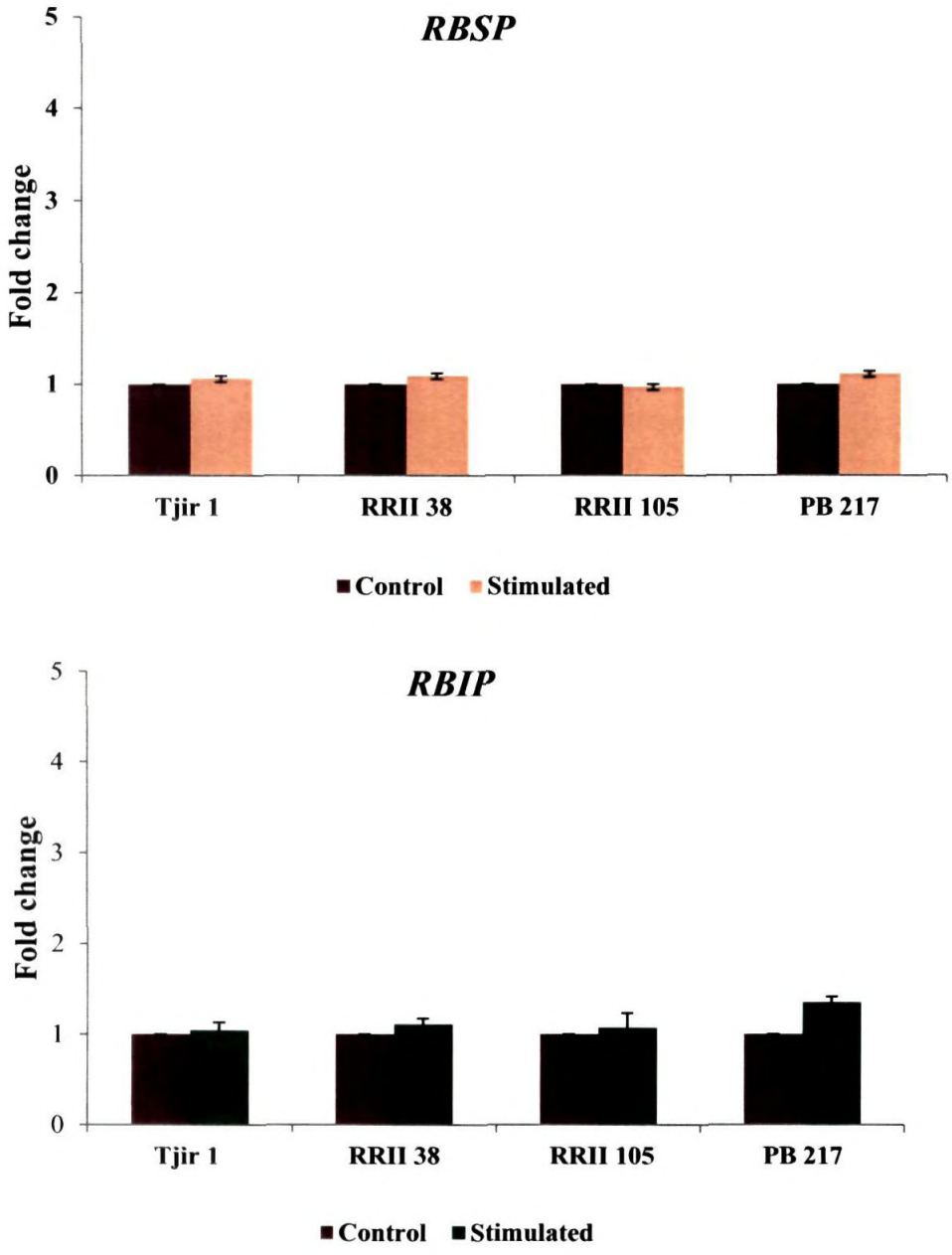


Fig. 3.2.h. Quantitative expression analysis of *RBSP* and *RBIP* in *Hevea* clones with different yield potential under ethylene stimulation. Clones are given in X axis and fold change (RQ values) in the Y axis. ± Error bars indicate standard error of three biological replicates.

3.4. Discussion

The biosynthesis of rubber begins with the precursor molecule, sucrose. The first isoprenoid molecule, IPP is formed from sucrose through more than 20 enzymatic reactions. The IPP serves as the fundamental building block for the formation of the final high molecular weight rubber molecule (Sando *et al.*, 2008a). Ethephon application is required to enhance latex flow and activate metabolism for latex regeneration between the successive harvesting time (Tungngoen *et al.,* 2009). Stimulation is associated with marked changes in the physiology and metabolism of latex cells, which induce a large but transient increase in latex production (Gidrol *et al.*, 1988). Stimulation is associated with obvious changes in both the physiology and metabolism of laticifers (Coupe *et al.,* 1989).

Both the HMGS and HMGR have shown to be involved in the initial stages of rubber biosynthesis (Hepper and Audley, 1969). The two enzymes possibly function in concert in the supply of substrates for biosynthesis of rubber similar to the synthesis of cholesterol in animals (Balasubramaniam *et al.*, 1977). HMGS activity has been positively correlated with rubber production in *Hevea brasiliensis*, suggesting that this enzyme has a regulatory role (Nagegowda *et al.*, 2004). Two members of HMGS (1.8 kb and 1.9 kb cDNAs) from *H. brasiliensis* (*hmgs*1 and *hmgs*2) were cloned and characterized (Suwanmane *et al.*, 2002; Sirinupong *et al.,* 2005). The expression of *hmgs*1 was found to be higher in laticiferous cells than in leaves whereas the abundance of *hmgs*2 transcripts were more in laticifer and petiole than in leaves. In plants, *hmgs* expression showed correlation with

rapid cell division and growth. Wounding and application of chemicals like methyl jasmonate, salicylic acid and ozone are known to induce *hmgs* expression suggesting that *hmgs* is involved in plant defence mechanisms (Alex *et al.*, 2000; Wegener, 1997). The expression of *hmgs*1 showed positive correlation with rubber yield (Li *et al.*, 2015). *hmgs* showed up regulation in tapping panel dryness (TPD, situation where partial or complete cessation of latex flow occurs) affected trees (Liu *et al.*, 2015). *hmgs* expression, HMGS activity and dry rubber content were reported to be increased after ethephon treatment (Suwanmanee *et al.*, 2004). However, in the present study, a down regulation of *hmgs* was observed in the latex samples collected 72 hrs after stimulation. Wang *et al.* (2015) reported that the gene and the protein level expressions of HMGS and HMGR were inhibited by ethylene treatment.

HMGR, a rate limiting enzyme in the rubber biosynthetic pathway which catalyses the NADP-dependent synthesis of mevalonate from HMG-CoA, is the most committed step of the MVA biosynthetic pathway (Caelles, *et al.*, 1989). In plants, mevalonate is the general precursor of many identified isoprenoid compounds, which are vital for normal growth, development and a variety of other normal physiological activities of plants. *hmgr*1 is expressed predominantly in laticifers, the cells specific to rubber biosynthesis. *hmgr*1 was more abundant in latex than in other tissues like leaf, flower and seedlings (Venkatachalam *et al.*, 2009). Schaller *et al.* (1995) reported that an increased expression level of *hmgr*1 was positively correlated with enzymatic activity of HMGR. Loss of function of *hmgr*1 in

Arabidopsis lead to dwarfing, early senescence and male sterility, and reduced sterol levels (Suzuki *et al.*, 2004). *hmgr*1 was significantly up regulated in CATAS8-79 (a high yielding clone) than PR 107 (a low yielding clone), suggesting that *hmgr*1 is critical for providing IPP (Chao *et al.*, 2015). It has been reported that *hmgr*3 expression is not cell specific and its role in isoprenoid biosynthesis is of a house keeping nature (Chye *et al.*, 1992). *hmgr*4 and *hmgr*5 were specifically expressed in high levels in mature leaves and xylem respectively (Sando *et al.*, 2008a). In the study, *hmgr*2 expression showed no significant difference between high and low yielding *Hevea* clones. Li *et al.* (2015) reported that the expression of *hmgr*1 was more in a high yielding clone compared to a low yielding clone. The expression of most of the genes related to rubber biosynthesis was severely inhibited in TPD affected trees and *hmgr*1 is one among them (Liu *et al.*, 2015). Chye *et al.* (1992) reported that *hmgr*1 was inducible by ethylene, while *hmgr3* gene was constitutively expressed. *hmgr*2 could be linked to the defence reactions against wounding and pathogens. HMGRs were reported to be involved not only in isoprenoid biosynthesis, but also in many other cell biosynthesis pathways such as sterols, membrane components, hormones *etc.*

The mevalonate diphosphate decarboxylase is an essential enzyme in mevalonate (MVA) pathway that catalyzes the irreversible decarboxylation of 6-carbon compound mevalonate-5-pyrophosphate (MVAPP) into 5-carbon IPP, the building block of sterol and isoprenoid biosynthesis. The biochemical pathway leading to rubber biosynthesis begins with the synthesis of IPP, the universal isoprene unit which then undergoes

polymerization to form polyisoprene. The up regulation of *MVD* gene might have resulted in an increased in IPP supply. In a recent proteomic study of the ethylene stimulation of natural rubber production MVD and mevalonate kinase were found to be inhibited by ethylene at both the gene and protein level (Wang *et al.,* 2015).

Cornish (1993) explained that there may exist two different types of prenyl transferases in the plant system; *cis* and *trans* prenyl tranfreases. A *trans* prenyl transferase that catalyses the formation of GDP and FDP in *Hevea brasiliensis* was demonstrated by Light and Dennies (1989). Although they claimed that the soluble enzyme behaved as rubber transferase in the presence of rubber particle and in the absence of rubber particle, it actually catalysed the formation of GDP and FDP from DMAPP. DMAPP primes the sequential head to tail condensations of isopentenyl pyrophosphate molecules by *trans* -prenyl transferase to form geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranyl geranyl pyrophosphate (GGPP) (van Beilen and Poirier, 2007). It is demonstrated that the soluble *trans* prenyl transferase from latex of *Hevea* functions solely as FPP synthase and plays no direct role in *cis*-1, 4-polyisoprene elongation (Cornish,1993). The main function of FPP synthase is to make initiator molecules for the synthesis of rubber.

Expression levels of *FPP synthase* and *GGPP synthase* genes were differentially expressed in various tissues of *H. brasiliensis*, which reflects their roles and specific functions in each tissue (Takaya *et al.,* 2003). Expression of *GGPP synthase* gene in leaf, flower and young leaf were

higher than those in latex and petioles. On the contrary, the expression of *FPP synthase* was high in latex as well as in flower. It is likely that the *GGPP synthase* has similar functions as that of *A. thaliana GGPS*, which is located in the chloroplast and is engaged in the biosynthesis of biologically important isoprenoids such as carotenoids, chlorophylls and gibberellins (Okada *et al.*, 2000). In TPD affected trees, the expression of GPP synthase (*GPPS*) and FPP synthase (*FPPS*) was dramatically down regulated (Liu *et al.*, 2015). The expression of *GPPS* and *FPPS* were reported to be significantly higher in a high yielding clone compared to a low yielding clone (Li *et al.*, 2015). FPP is a branch point and FPPS is considered as a regulatory enzyme in the pathway. In the present study, FPP synthase was down regulated after ethylene stimulation in all the selected *Hevea* clones. Adiwilaga and Kush (1996) reported that the expression of FPP synthase was not significantly affected by hormone treatment. GGP synthase gene expression was also found down regulated in the latex 72 hrs after the ethylene stimulation.

Rubber transferase (RuT), belonging to the *cis* prenyl transferase family catalyse the polymerization of IPP in to *cis*-rubber (Archer and Audley, 1967; Archer and Cockbain, 1969; Archer and Audley, 1987). The successive addition of IPP was found to take place only on the surface of pre-existing rubber particles (Archer *et al.*, 1963, McMullen and McSweeney, 1966) implying that rubber transferase was located on the surface of the rubber particles. Two RuT homologues, designated *RuT*1 and *RuT*2, were cloned from the *H. brasiliensis* latex (Asawatreratanakul *et al.*,

2003). The activities of these enzymes were found in both the bottom fraction and the cytosol (C-serum) of centrifuged fresh *Hevea* latex (Tangpakdee *et al.*, 1997). The activity of RuT is a reliable index for the latex yield prediction in *Hevea*. Cornish and Bartlett (1997) studied the stabilization capacity of the particle bound rubber transferase activity in stored and purified rubber particles. In TPD affected *Hevea,* RuT enzyme is fully active in *vitro* in the presence of adequate supply of their intermediate substrate (Krishnakumar *et al.*, 2001). Ethephon did not have any effect on the gene expression and activity of RuT (Luo *et al*, 2009; Zhu and Zhang 2009). In a recent study, *RuT*1 and *RuT*2 were reported to be repressed by ethylene stimulation (Wang *et al.*, 2015).

The sucrose transporter gene *HbSUT*3 plays an active role in sucrose loading to laticifer and rubber productivity in exploited trees of *H. brasiliensis* (Tang *et al.*, 2010). *HbSUT*3 was transcribed in all the tissues, suggesting a functional redundancy of this gene. The preferential expression of this gene in latex and female flowers suggests its major role in these two tissues (Tang *et al.*, 2010). Sucrose transporters belong to the major facilitative superfamily (MFS) and they have 12 typical transmembrane-spanning domains (Sauer, 2007). The expression of *HbSUT*3 in the present study was found to be significantly higher in high yielding clones than low yielding clones. The results showed the involvement of *HbSUT*3 in sucrose loading in to laticifers and in rubber productivity. The up regulation of *HbSUT*3 in high yielding clones suggests an enhanced sucrose loading to the laticifers of high yielding clones. It has long been believed that the efficiency

of sucrose transportation and metabolism and rubber biosynthesis are closely associated with the ability of latex regeneration between intervals of successive tappings (Tang *et al.*, 2010). Several unigenes associated with carbohydrate metabolism was found to be expressed at higher level in CATAS8-79 (a high yielding clone) than that in PR107 (a low yielding clone), suggesting a more efficient sucrose transportation and carbohydrate metabolism in CATAS8-79 (Chao *et al.*, 2015).

HbSUT3, showed an active role in rubber productivity in response to ethylene treatment also (Tang *et al.,* 2010). Its activity was reported to be finely modulated by exogenous (Decourteix *et al.*, 2006) as well as endogenous signals such as sucrose (Chiou and Bush, 1998) and hormones (Chincinska *et a*l., 2008). The significantly higher expression of *HbSUT3* in the latex than that of any other *SUT* genes, demonstrated that *HbSUT3* is the predominant SUT member involved in rubber biosynthesis (Tang *et al.,* 2010). The up regulation of *HbSUT3* after ethylene stimulation suggests an enhanced sucrose loading to the laticifers and demonstrates its active involvement in rubber biosynthesis and rubber productivity. Sucrose contents and its metabolism intensity were considered as limiting factor for rubber biosynthesis (Alessandro *et al.*, 2006). Acceleration of sucrose metabolism by ethylene action may be one of the main reasons for the stimulation of latex yield by ethylene releasing compounds (Zhu and Zhang, 2009).

REF present in large amounts at the surface of the rubber particles is involved in rubber biosynthesis (Sando *et al.*, 2009). REF gene is encoded by a small gene family consisting of three members and it plays an important

role in the final polymerization of rubber. REF expression showed positive correlation with *Hevea* latex yield (Priya *et al.*, 2007). REF was not obviously changed at the gene or protein level on ethylene treatment (Wang *et al.*, 2015). A rubber biosynthesis stimulator protein (*RBSP*) was purified from *H. brasiliensis* latex and identified as *eIF*-5A by amino acid sequencing (Yusof, 1996; Yusof *et al.*, 2000). Three genes in tobacco (Chamot *et al.*, 1992) and four genes in tomato (Wang *et al.*, 2001) of *eIF*-5A were reported. Plant *eIF*-5A isoforms have been reported to be involved in other functions such as photosynthesis (Chamot *et al.*, 1992) and organ senescence (Wang *et al.*, 2003; 2005). Chow *et al.* (2006) reported a differential stimulatory effect of RBSP isoforms on rubber biosynthesis in *H. brasiliensis*. The translation initiation factor 5A (*eIF* 5A) was reduced in TPD affected trees (Li *et al.*, 2010) and *eIF* 5A negatively regulated programmed cell death (Hopkins *et al.*, 2008). Archer and Audley (1987) detected an inhibitor of rubber biosynthesis in the C-serum of the *H. brasiliensis* latex. Yusof *et al.* (1998) had purified the patatin like inhibitor protein (*RBIP*) with a molecular weight of 43.7 kDa.

3.5. Summary and conclusions

In the present study, expression of genes involved in rubber biosynthesis were analyzed in different *Hevea* clones with varied latex yield potential, both in normal tapped and ethylene stimulated trees. The expression of *HbSUT3*, a sucrose transporter was found to be significantly high in high yielding *Hevea* clones. *H. brasiliensis* exploits sucrose as the primary substrate for rubber biosynthesis. The gene expression of enzymes

like HMGR1, HMGS and MVD were also significantly higher in high yielding clones. Up regulation of these genes in the latex might result in an increased supply of total IPP. The down stream enzymes like FPPS, RuT and REF2 were also showed a higher gene expression in high yielding clones. The gene expression of other enzymes like HMGR2, HMGR3, GGPPS, REF2, REF3, RBSP and RBIP showed no significant difference between the high and low yielding clones. This study suggest that high yield of rubber tree is likely the result of a high IPP supply and more IPP distribution in the laticiferous tissues of *H. brasiliensis* for rubber biosynthesis.

The study on the gene expression in response to ethylene action suggest that the sucrose transporter gene, *HbSUT3* plays an important role in the sucrose loading of the laticifer tissue and finally leading to enhanced rubber yield in trees stimulated with ethylene compounds. Rubber productivity depends on sufficient regeneration of the expelled latex before the next harvesting, which requires an efficient sucrose supply. The gene expressions of *hmgr*1 and *MVD* were induced by ethylene treatment and this result suggests that both these enzymes are involved in enhanced IPP supply for rubber biosynthesis.

Identification of enzymes that catalyses the rate limiting steps in the isoprenoid pathway is an important task for developing markers related to yield potential in *Hevea*. Expression of genes involved in rubber biosynthesis were analysed in different *Hevea* clones with varied yield potential, both in unstimulated and stimulated trees. The expression of *HbSUT3*, a sucrose transporter was found to be significantly higher in high yielding compared to

low yielding *Hevea* clones. The gene expression of enzymes like HMGS, HMGR1, MVD, FPPS, RuT and REF2 was also significantly higher in high yielding clones than the low yielders. The expression of HbSUT3, HMGR1 and MVD was significantly higher in stimulated trees than unstimulated trees in different *Hevea* clones. The up regulation of these genes might result in an increased supply of total IPP. The present study suggests that high rubber yield is associated with higher rate of expression of these genes and is useful markers for determining yield potential in *Hevea.*

Chapter 4

HMG-CoA reductase activity in high and low yielding clones of *Hevea brasiliensis*

4.1. Introduction

The mevalonate pathway, which starts with the synthesis of mevalonate by HMG-CoA reductase (HMGR), provides precursors for a diverse spectrum of isoprenoid compounds. Isoprenoids are widespread in eukaryotes and they play key roles in plant growth and development, photosynthesis and resistance to pests. The isoprenoids are produced from acetyl-CoA *via* mevalonate to a central intermediate isopentenyl pyrophosphate (IPP) (McGarvey and Croteau, 1995). Evidence supporting this cytosolic pathway for rubber formation in *Hevea brasiliensis* was derived from incorporation of radiolabeled intermediates of the pathway such as mevalonate (Skilleter and Kekwick, 1971) and HMG-CoA (Hepper and Audley, 1969).

One among the major factors determining the yield of rubber in *Hevea brasiliensis* is the rate of synthesis of rubber in the isoprenoid pathway. Since HMG-CoA reductase (HMGR) is a rate limiting enzyme in many polyprenoid synthesis (Bach and Lichtenthaler, 1987; Goldstein and Brown, 1990), the activity of this enzyme may be an indicator of yield potential of *Hevea*. Wititsuwannakal and Sukonrat (1984) observed diurnal variations in the activity of the enzyme and a positive correlation with rubber content in the latex. Their results suggested correlation between the regulation of HMG-CoA reductase activity and rubber biosynthesis. Nair *et al.* (1990) studied the activity of the enzyme in the bark of different *Hevea* clones and observed significantly higher enzyme activity in high yielding clones compared to low yielding clones. Except these few reports, no

attempts have been made to correlate the activity of the enzyme to rubber yield in different *Hevea* clones. Also, no studies have been made on the activity of this enzyme upon ethylene treatment. In view of this, studies were conducted to quantify HMG-CoA reductase activity in different *Hevea* clones with varying yield potential and in ethylene stimulated trees. The role of C-serum (centrifuged latex serum) on the enzyme activity was also studied to detect possible presence of inhibitors and / or stimulators of the enzyme in the latex.

4.2. Materials and methods

4.2.1. Chemicals

DL-3-Hydroxy-3-methyl-(3-^{14}C) glutaryl CoA (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, U.S.A.

4.2.2. Plant material

The study was carried out in mature trees of two high yielding (RRII 105 and PB 217) and two low yielding (RRII 38 and Tjir 1) clones of *Hevea brasiliensis* grown at Rubber Research Institute of India, Kottayam. The trees were regularly tapped for harvesting latex in every three days using S/2, d3 6d/7 tapping system and the trees were in the 13th year of tapping.

4.2.3. Ethylene stimulation

Ethylene was administered by applying commercially available Ethephon (2-chloro ethyl phosphonic acid) as mentioned in 3.2.2.2. Three to six trees were used for the stimulation treatment in each clone.

4.2.4. Sample collection

Bark samples of two centimeter square size were collected from the tapping panel of the trees, brought to the laboratory in ice and stored at -80°C. The inner soft bark tissues from the bark samples were used for the assay of HMG-CoA reductase activity.

4.2.5. Latex yield

Total latex yield in each tree was recorded from the control and experimental groups as mentioned in 3.2.1.2.

4.2.6. HMGR enzyme extraction

The HMGR enzyme extraction was done as described by Witisuwannakul *et al.*, (1990). Two gram of soft bark tissue was homogenized in 5 ml solubilization buffer (0.1M K-phosphate buffer (pH 7.0), 10 mM DTT, 0.1M KCl, 5mM EDTA containing 1% Brij 35 (detergent) in prechilled motar and pestle using liquid nitrogen and the homogenate was collected in a centrifuge tube. PVP was added to the homogenate and kept for 30 min on ice followed by centrifugation at 12,000 rpm for 30 min. The supernatant containing the crude enzyme was used for the assay.

4.2.7. Latex collection and preparation of C-serum

The first 5 ml latex flowing immediately after tapping was discarded and the subsequent latex was collected in vials in an ice box. The latex samples were centifuged at 4^0C for 45 min. (59000 x g, Sorvall OTD 55B

Ultracentrifuge, T865 rotor). The top cream consisting of rubber particles were discarded and the middle aqueous layer (C-serum) was collected using a syringe.

4.2.8. Protein quantification

The soluble proteins in the enzyme extracts were quantified using Bradford method (Bradford, 1976). Serum sample (50 µl) was made up to 1 ml with 0.15 M NaCl, 3 ml of Bradford reagent was added to the above mixture and kept for color development. The absorbance of the reaction mixture was measured at 595 nm using a UV-visible spectrophotometer using BSA as the standard.

4.2.9. HMGR enzyme assay

HMGR activity was assayed according to Chappell *et al.* (1995). The bark extract containing 0.01 mg protein in 0.23 ml buffer (0.1M KCl, 10 mM DTT, 5 mM EDTA, 0.1M K-phosphate, pH 7.0) containing 2 mM NADPH and 20 µm (^{14}C) HMG-CoA (55 mCi/mmol), was incubated at 37°C for one hour. The assay was terminated by the addition of 10 µl each of 1mg ml^{-1} mevalonolactone and 6 N HCl and the mixture was incubated at 37 °C for one hour for the lactonization of the radiolabeled mevalonate. Assay controls included the omission of NADPH and the assays were performed in duplicate. For studying the effect of C-serum on HMGR activity, 10 µl of C-serum from respective *Hevea* clones was added to the incubation medium and performed the assay as per the above protocol.

4.2.10. Separation of (^{14}C) mevalonate and measurement of radioactivity

The presence of mevalonate in organic phase and HMG-CoA in aqueous phase was confirmed first by thin layer chromatography (TLC) using standards of mevalonolactone and HMG-CoA (Sigma). The TLC was carried out on silica gel 60 using solvents benzene and acetone (C_6H_6:Me_2CO) 1:1 (v/v) and iodine vapour for detection (Iijima *et al.*, 1973). The (^{14}C) mevalonolactone formed in the assay was separated from the (^{14}C) HMG-CoA by organic partitioning method of Feyereisen and Farnsworth (1987). 800 µl saturated K-phosphate (pH 6.0) and 2 ml ethyl acetate was added to the assayed samples. The samples were vortexed, briefly centrifuged and allowed to stand for 30 min for the separation. The upper organic phase (1 ml) and 5 ml of liquid scintillation cocktail (RPI, U.S.A) were used to determine the radioactivity using a liquid scintillation counter (LKB). HMGR activity was calculated as cpm mg^{-1} protein and the values were subjected to statistical analysis using ANOVA. The ratio with P-value ≤ 0.05 was adopted as significant.

4.3. Results

Four *Hevea* clones were selected, based on rubber yield (Fig. 4.1.), for studying the HMGR activity associated with varying yield potential. The thin layer chromatography confirmed mevalonolactone in upper organic phase and HMG-CoA in lower aqueous phase (Fig. 4.2.). The R_F values for HMG-CoA and mevalonolactone were 0.0 and 0.9 respectively.

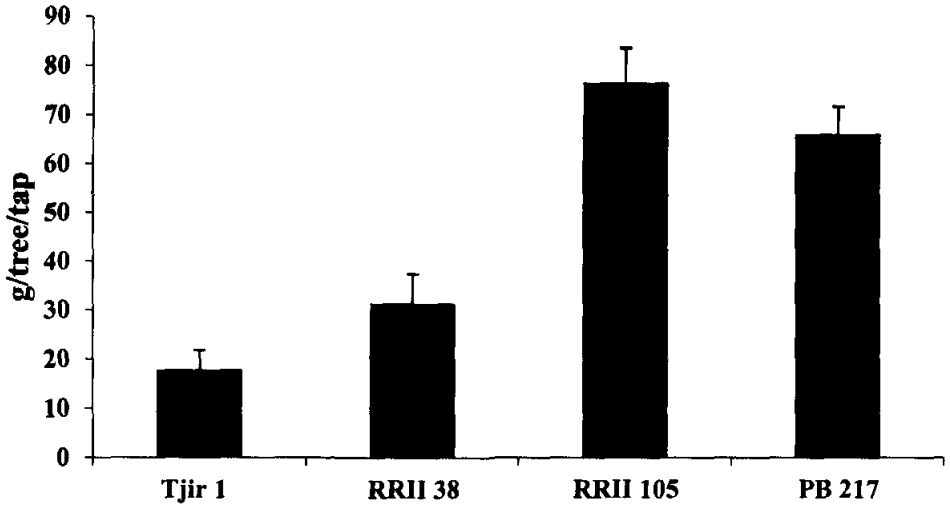


Fig. 4.1. Dry rubber yield (g/tree/tap) of *Hevea* clones.

The activity of HMGR in the bark extract of high and low yielding clones of *Hevea* is given in Table (4.1). The HMGR activity was found to be significantly higher in the bark extract of high yielding *Hevea* clones (RRII 105 and PB 217) than the low yielders (Tjir 1 and RRII 38) indicating that the enzyme activity is directly related to the yield potential of *Hevea* clones. When C-serum was added to the bark extract, HMGR activity increased significantly in all the clones (Table 4.1). The presence of an activator of HMGR enzyme present in the C-serum of latex may be stimulating the activity of the enzyme. Adding C-serum in the assay medium, HMGR activity was increased 55, 87,120 and 154 % in clones Tjir 1, RRII 38, RRII 105 and PB217 respectively. The HMGR activity showed positive correlation with rubber yield with an R value of 0.796 (Fig 4.3).

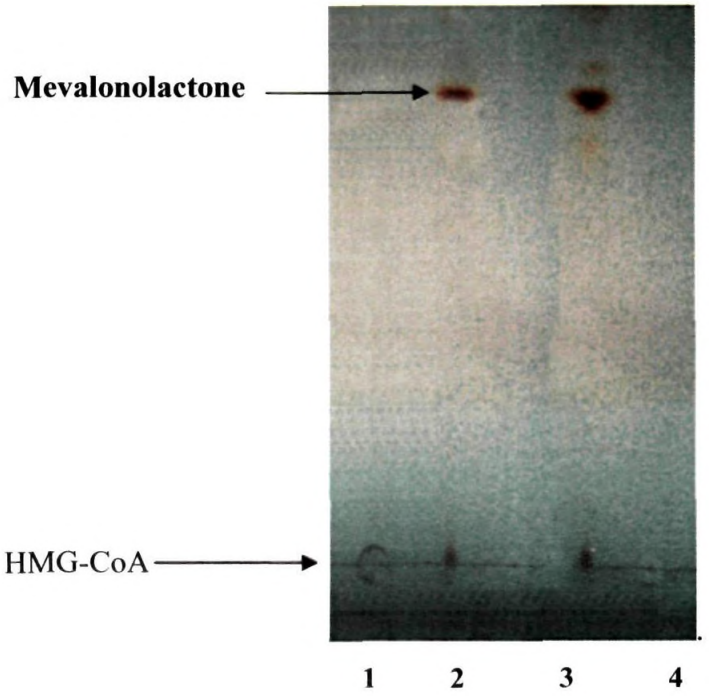


Fig. 4.2. Thin layer chromatography of standards (HMG CoA and Mevalonolactone), lower aqueous phase and upper organic phase

Table. 4.1. HMGR activity of different *Hevea* clones. (Radioactivity recovered as ^{14}C mevalonate)

Clones	HMGR activity (cpm mg^{-1} protein)		Increase in HMGR activity with addition of C-serum (%)
	Bark extract only	Bark extract + C-serum	
Tjir 1	1584	2466	55
RRII 38	1686	3156	87
RRII 105	1891	4154	120
PB 217	1916	4866	154
CD (P≤0.05)	112	416	

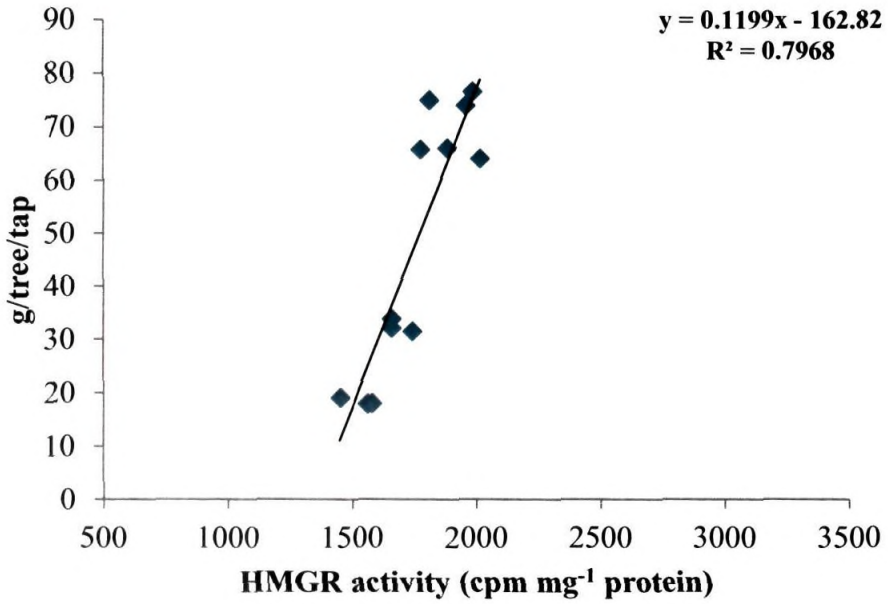


Fig. 4.3. Correlation of HMGR activity and dry rubber yield

Table. 4.2. Dry rubber yield of stimulated and control trees of different *Hevea* clones (g/tree/tap)

Clones	Dry rubber yield (g/tree/tap)	
	Control	Stimulated
Tjir 1	17	21.0
RRII 38	31	49.4
RRII 105	66	111.2
PB 217	76	147.8
CD(P≤ 0.05)	1.95	6.27

Table 4.3. Effect of stimulation on HMGR activity in different *Hevea* clones (Radioactivity recovered as ^{14}C mevalonate).

Clones	HMGR activity (cpm mg^{-1} protein)	
	Control	Stimulated
Tjir 1	1645	1721
RRII 38	1686	1779
RRII 105	1891	1985
PB 217	1969	2043
CD ($P \leq 0.05$) (Clone × Stimulation) 100.66		

The commercially available ethylene inducing compound, ethephon, was applied on both high and low yielding *Hevea* clones. This could enhance latex yield in all the clones (Table 4.2). No significant difference was observed on HMGR activity between the stimulated and unstimulated control trees belonging to high and low yielding *Hevea* clones (Table 4.3) and hence no change in HMGR activity noticed by ethephon stimulation.

4.4. Discussion

In *Hevea brasiliensis* the biosynthesis of rubber is being regulated by HMGR enzyme which is located in the bottom fraction of centrifuged latex (Lynen, 1969; Witisuwannakul, 1986). HMGR, provides backbone for isoprenoids by catalyzing mevalonate synthesis from HMG-CoA (Lynen, 1969).

The present study showed that HMGR activity was significantly high in high yielding *Hevea* clones than the low yielders. Earlier, Nair *et al.* (1990) observed similar results in low yielding and high yielding *Hevea* clones by taking the ratio of quantities of HMG-CoA to mevalonate as an index of the enzyme activity; a low ratio indicating high enzyme activity and *vice versa*. When C-serum was added to the reaction mixture, the enzyme activity increased significantly in all the clones. This suggests the involvement of some C-serum factors in the regulation of HMGR activity. Wititsuwannakul *et al.* (1990) reported a HMGR activator in the C-serum of latex similar to that of cadmodulin. The *Hevea* cadmodulin may indirectly activate HMGR *via*, regulation of enzymes involved in the phosphorylation and dephosphorylstion of HMGR, similar to those suggested for mammalian HMGR (Gibson *et al.*, 1987; Witisuwannakul *et al.*, 1990). Chappell *et al.* (1995) reported that constitutively expressed Hamster HMGR cDNA in tobacco plants (*Nicotiana tabacum* L.) resulted in 3-6 fold increase in total HMGR enzyme activity and an overall increase in the sterol accumulation.

The HMGR activity showed correlation with rate of sterol production in developing seeds of rape and tobacco (Harker *et al.*, 2003). HMGR activity and transcript levels were high in actively dividing *Arabidopsis*, tomato and tobacco culture cells (Hemmerlin *et al.*, 2003). Yang *et al.* (1991) reported that some solanaceous species challenged with pathogen or elicitor treatment induced HMGR activity and transcript levels that provided the accumulation of sesquiterpenoid phytoalexin. Thus the regulatory role of HMGR in mevalonate pathway seems to be important not only for normal

plant growth and development but also for the adaptation to the challenging environmental stress. In MVA pathway, plant HMGR is modulated by a variety of developmental and environmental signals such as phytohormones, calcium, cadmodulin, light, wounding, elicitor treatment and pathogen attack (Stermer *et al.*, 1994). The major changes in HMGR activity would be determined at the transcriptional level, whereas the post transcriptional control would allow a finer and faster adjustment (Chappell, 1995). Similarly, treatment with mevinolin (a competitive inhibitor of HMGR) can slow or inhibit plant growth and development (Gray, 1987; Narita and Gruissem, 1989). Witisuwannakul (1986) reported that the specific activity of HMGR was high in high yielding clones and the diurnal variations observed in the rubber content of the latex coincided with the variations in the HMGR activity.

Ethephon stimulation increased the latex volume in all the *Hevea* clones. In this study, HMGR activity was almost similar in all the stimulated and control trees irrespective of the clonal traits. Gene and protein expression of HMGR was inhibited by ethylene stimulation (Wang *et al.*, 2015). The *hmgr*1 gene, considered to be responsible for rubber biosynthesis, was induced by ethylene. But interestingly, ethylene could not influence the activity of HMGR (Witisuwannakul, 1986).

4.5. Summary and conclusions

The activity of HMG-CoA reductase studied in different *Hevea* clones with varying yield potential indicated that there was significant clonal

variation in the activity of HMG-CoA reductase in bark of high and low yielding clones. The HMGR activity was found to be significantly higher in high yielding *Hevea* clones (RRII 105 and PB217) and had significant positive correlation with rubber yield. Ethephon stimulation enhanced the total rubber yield in all the *Hevea* clones irrespective of their yield potential but the enzyme activity was not changed due to stimulation. A positive correlation obtained between the HMGR enzyme activity and dry rubber yield suggests the possibility of utilizing this enzyme for developing as a marker for determining yield potential in *Hevea.*

Chapter 5

Cloning and expression of *hmgr*1 gene from *Hevea brasiliensis*

5.1. Introduction

Biosynthesis of natural rubber takes place by mevalonate pathway in *Hevea* (Sando *et al.*, 2008a). Generation of acetyl-coenzyme A and its conversion to isopentenyl pyrophosphate (IPP) *via* mevalonic acid and polymerization of IPP to rubber are the major steps involved in the rubber biosynthetic process. The synthesis of mevalonate from HMG-CoA is one of the rate limiting steps in isoprenoid pathway catalysed by 3-hydroxy-3 methyl glutaryl coenzyme A reductase (HMGR). Wititsuwannakul (1986; 1990) reported a positive correlation between rubber biosynthesis and HMG-CoA reductase activity in *Hevea*.

A positive correlation between HMGR activity and rubber yield in *Hevea* clones already been shown in the previous chapter (chapter 4). Ji *et al.* (1993) demonstrated that HMGR activity was positively correlated with rubber biosynthesis as well as rubber yield. Different members of *hmgr* gene family were cloned and characterized from *Hevea* clone RRIM 600 (Chye *et al.*, 1991; 1992). They observed that *hmgr*1 was expressed more in laticifers than in leaves and was specifically involved in rubber biosynthesis. The increased production of rubber in modern *Hevea* clones may be due to the enhanced rubber biosynthetic ability and the mechanisms needs to be investigated.

The objective of the study was to clone *hmgr*1 from *H. brasiliensis* and *in vitro* expression of the HMGR protein for developing specific antibody which could be further utilized for screening the yield potential of

Hevea clones that are being developed through crop improvement programmes.

5.2. Materials and methods

5.2.1. Plant material

Latex samples were collected from the *Hevea* clone RRII 105. The trees were grown in the experimental field of Rubber Research Institute of India and they were harvested regularly under S/2 d2 tapping system.

5.2.2. mRNA isolation

Latex mRNA was isolated using magnetic beads as per manufacturer's instruction (Dyna Beads, Invitrogen, USA). Latex (5 ml) was mixed with 10 ml of 1x RNA extraction buffer and vortexed for few minutes to get homogenized thoroughly. The mixture was centrifuged at 12000 rpm for 30 minutes at room temperature and the supernatant was transferred to oakridge tube and centrifuged at 12000 rpm for 15 minutes. The clear supernatant was mixed with required quantity of magnetic beads saturated with the RNA extraction buffer. The mixture was kept at room temperature for 20-30 minutes to allow the annealing of polyA tail of mRNA with the oligo dT ends on the beads. After mixing, the tubes were kept in a magnetic separation stand for 15 minutes to get the beads attracted to the side wall of the tube. After the removal of buffer from the tubes, wash solution A (0.5 ml) was added to the beads followed by proper mixing. The wash solution was removed by magnetic separation. The washing with solution A was repeated twice followed by washing thrice with solution B. Finally the

mRNA was eluted with elution buffer twice by incubating at 95°C for 3 minutes. The eluted sample was mixed with 0.1 volume of sodium acetate and 2.5 volume of ethanol and kept at -80°C for 1 hour for precipitation. The mixture was then centrifuged at 12000 rpm for 15 minutes at 4°C. The pellet was washed with 85% ethanol and dried in a Centrivap concentrator. The mRNA was resuspended in nuclease free water and quantified using Nanodrop spectrophotometer.

5.2.3. cDNA synthesis

cDNA was synthesized from mRNA using Superscript™ III First strand synthesis kit of Invitrogen. 10 µl RNA mix was incubated at 65^{0}C for 5 min followed by incubation on ice for a minute and mixed with 10 µl of cDNA mix. After a brief spin, the mixture was incubated at 50^{0}C for 50 min and at 85^{0}C for 5 min. After a brief incubation in ice, 1 µl of RNase H was added to each tube and incubated for 20 min at 37^{0}C. The cDNA was quantified and stored at -20^{0}C.

5.2.4. PCR Amplification of *hmgr*1 gene

PCR amplification of *hmgr*1 gene from *H. brasiliensis* was performed with gene specific primers designed based on sequences deposited earlier in GenBank database (X54659; Chye *et al.*, 1991). The primers were designed and synthesized by Eurogentec, Belgium. The primers were flanked with restriction sites *Kpn*I in the forward primer and *Xho*I in the reverse primer.

Forward primer: 5'- CTC GAG ATG GAC ACC ACC GGC CGG C -3'

Reverse primer: 5'- GGT ACC ATT GCT GGG ACC AGA TTC CC -3'

The PCR amplification profile consisted of a first cycle at 94°C for 5 min followed by 35 cycles at 94°C for 30s; 69.2°C for 30 s; 72°C for 1 min and finally extension at 72°C for 10 min. The PCR cocktail was prepared in 20 µl reaction volume, which composed 2 µl of 10x buffer, 200 µM dNTP, 1.5 U of Taq DNA polymerase, 100 ng template DNA and 500 nM each of forward and reverse primers. The PCR amplification was carried out in a thermal cycler (Master gradient, Eppendorff, USA). The amplified cDNA fragments were separated by electrophoresis on 1% agarose gel and the amplified cDNA fragment (~ 1.8 kb fragment) was eluted using PCR clean – up kit (Sigma).

5.2.5. Cloning and DNA sequencing analysis

The PCR amplified *hmgr*1 fragments were cloned into pGEM-T Easy vector (Fig. 5.1). The ligated plasmids were transferred into *E. coli* cells (Gen Hunter) and plated onto LB agar plates containing 50 µg ml^{-1} ampicillin and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg ml^{-1} 5-bromo-4-indolyl-β-D-galacto-pyranoside (X-Gal).

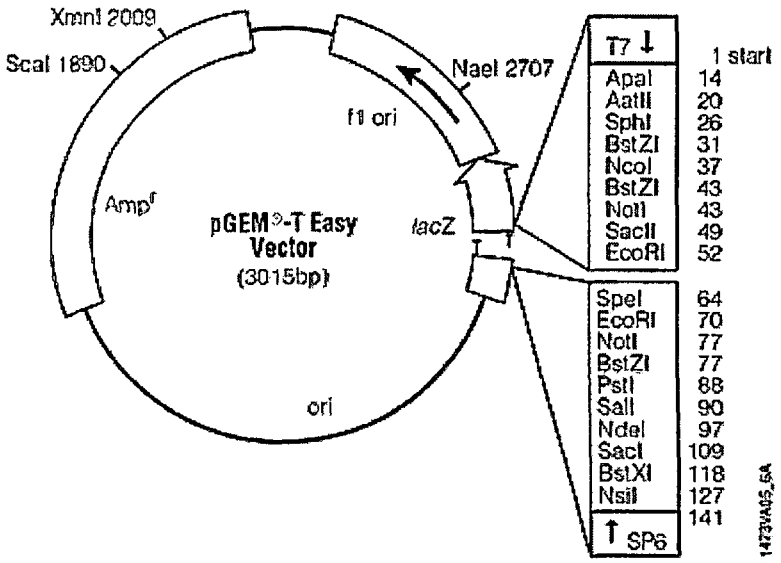


Fig. 5.1. Vector map of pGEM-T Easy vector

The presence of insert in the recombinant clone was confirmed by colony PCR as well as and further confirmed by restriction digestion analysis using *Kpn*I and *Xho*I. The nucleotide sequence of the cloned DNA fragment was determined by sequencing (Macrogen, Korea). The nucleotide sequence of *hmgr*1 was compared with the NCBI database using BLASTN programme.

5.2.6. Cloning in expression vector

The plasmid (pGEMT/*hmgr*1) was double digested using *Kpn*I and *Xho*I and purified the *hmgr*1 fragment. The expression vector (pRSET-A, Invitrogen) (Fig.5.2) was also double digested using *Kpn*I and *Xho*I and purified. The *hmgr*1 was ligated to pRSET-A using T4 DNA ligase and transformed in to *E.coli* cells (Gen Hunter). The transformed colonies were selected by colony PCR and restriction digestion.

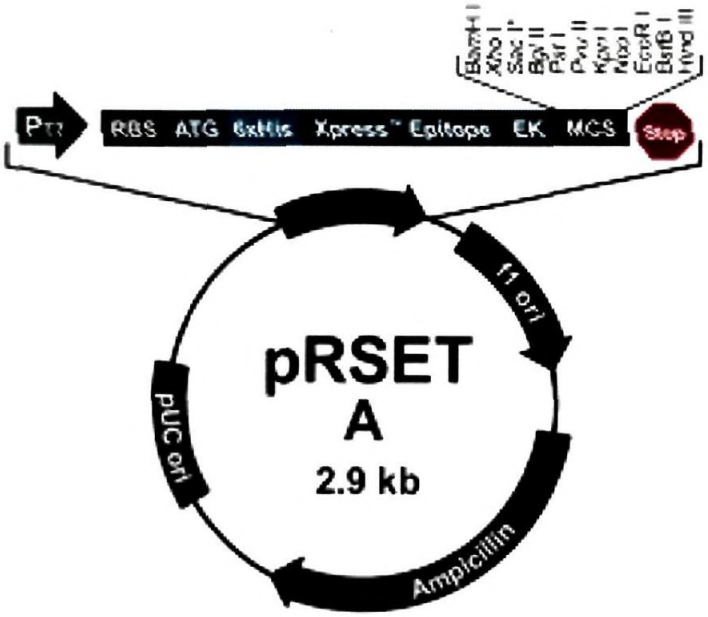


Fig.5.2. Vector map of pRSETA

The orientation of the insert was confirmed by sequencing (Macrogen, Korea). The sequencing results were analysed and the clone with *hmgr*1 in the right orientation was selected. Plasmid DNA isolated from these colonies was used to transform expression specific BL21 (DE3) pLysS cells and transformed colonies were selected by colony PCR.

5.2.7. Expression of protein

A single recombinant colony (pRSET-A/*hmgr*1 construct) in BL21 (DE3)pLysS was inoculated in 2 ml of SOB medium containing ampicillin (50 µg ml^{-1}) and chloramphenicol (35 µg ml^{-1}) and incubated at 37°C, overnight with shaking. The next day, inoculated 50 ml of SOB (with out antibiotic) with 1 ml of overnight culture (OD_{600} = 0.1). The culture was grown at 37°C with vigorous shaking to an OD_{600} = 0.4-0.6, and culture was

collected as zero hour samples. IPTG (1 mM) was added to the culture and samples were collected at different time intervals (0, 1.5 and 3 hours after induction). The samples were centrifuged and each pellet resuspended in 100 µl of 20 mM phosphate buffer at neutral pH. Proteins were collected from the cells by freeze-thaw method. The supernatant from the samples were mixed with four times the volume of acetone and incubated for 60 min at -20 °C. The samples were centrifuged at 15,000 rpm for 10 min. Resuspended the pellet in 0.5% SDS and the protein was quantified (Bradford, 1976).

5.2.8. SDS-PAGE analysis of proteins

The profiling of the expressed recombinant protein was carried out by SDS-PAGE according to the method of Laemmli (1970) using a 10% linear gel. Separation of 30 µg of protein samples were carried out in Bio Rad electrophoresis device with 10% polyacrylamide gel as resolving gel and 4% stacking gel. Electrophoresis was conducted at constant voltage (60 volts) for 30 min followed by voltage at 120 volts until the marker dye reached the bottom of the gel. The gel was stained with coomassie blue and documented.

5.3. Results

In this study, *hmgr*1 coding region was isolated and over expressed in an expression vector for protein production. For this purpose, mRNA was isolated from latex of clone RRII 105 (Fig. 5.3) and cDNA was synthesized.

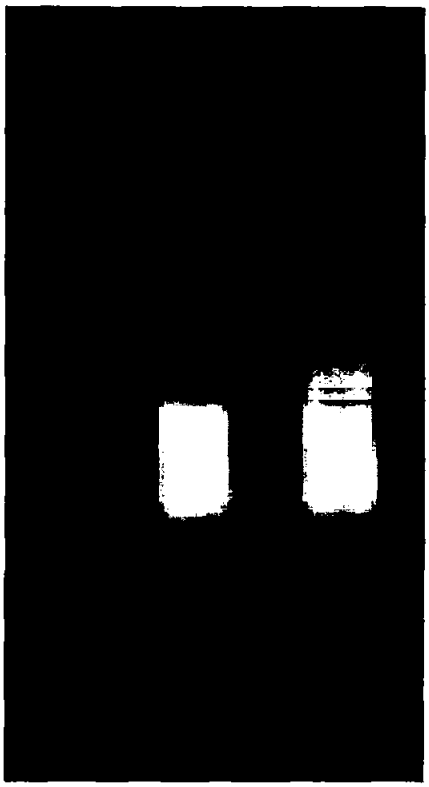


Fig. 5.3. mRNA isolated from latex of clone RRII 105 on 2% agarose

A PCR based approach was employed to isolate *hmgr*1 gene from *Hevea*. Primers were designed and synthesized based on the published sequences of *hmgr*1 from *Hevea* clone RRIM 600 by Chye *et al.* (1991). PCR amplicon with a size of 1.8 kb corresponding to the length of *hmgr*1 (Fig. 5.4) could be obtained which was later gel purified and cloned in to pGEM-T vector. The recombinant clones were subjected to colony PCR (Fig. 5.5) as well as restriction digestion (Fig. 5.6) and confirmed the presence of *hmgr*1 coding region.

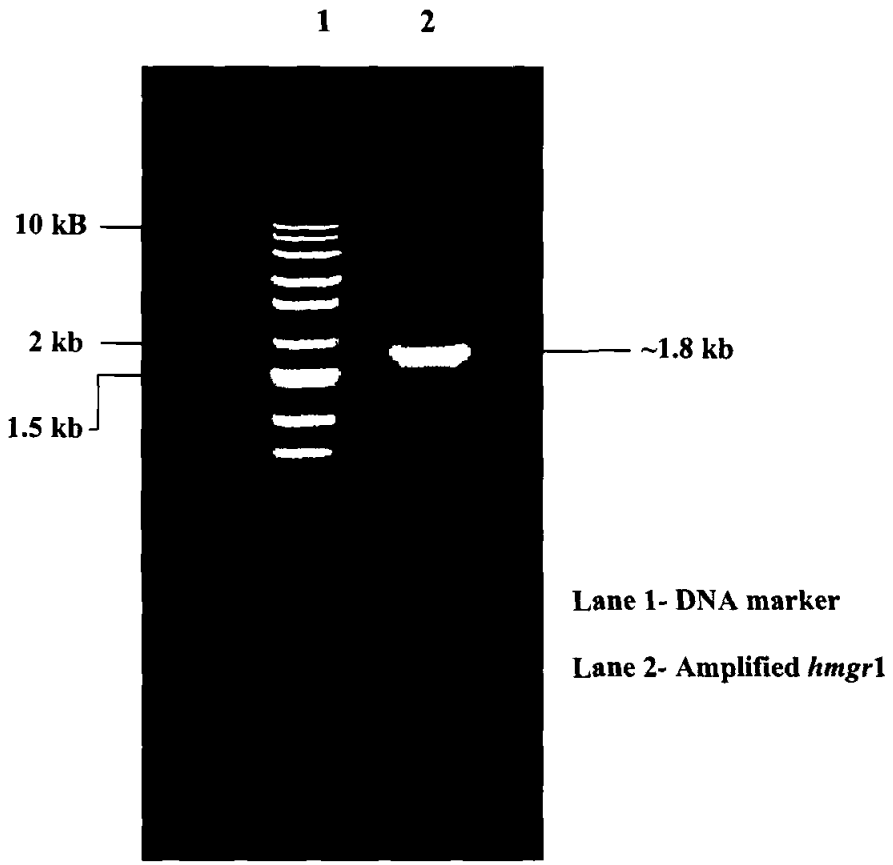


Fig. 5.4. Amplification of *hmgr1* gene from the latex of *H. brasiliensis*.

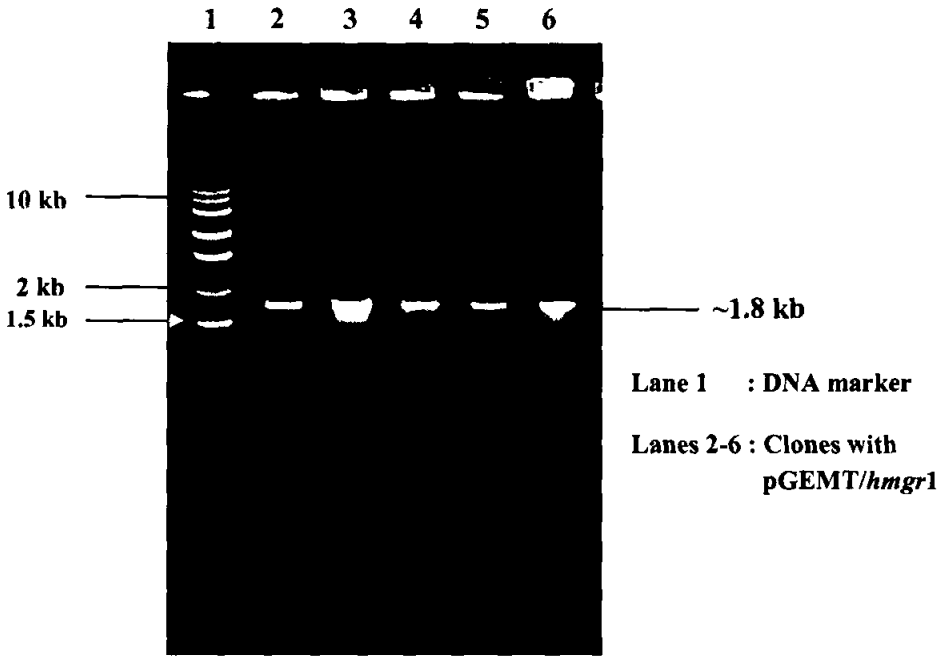


Fig. 5.5. Colony PCR of pGEMT/*hmgr*1

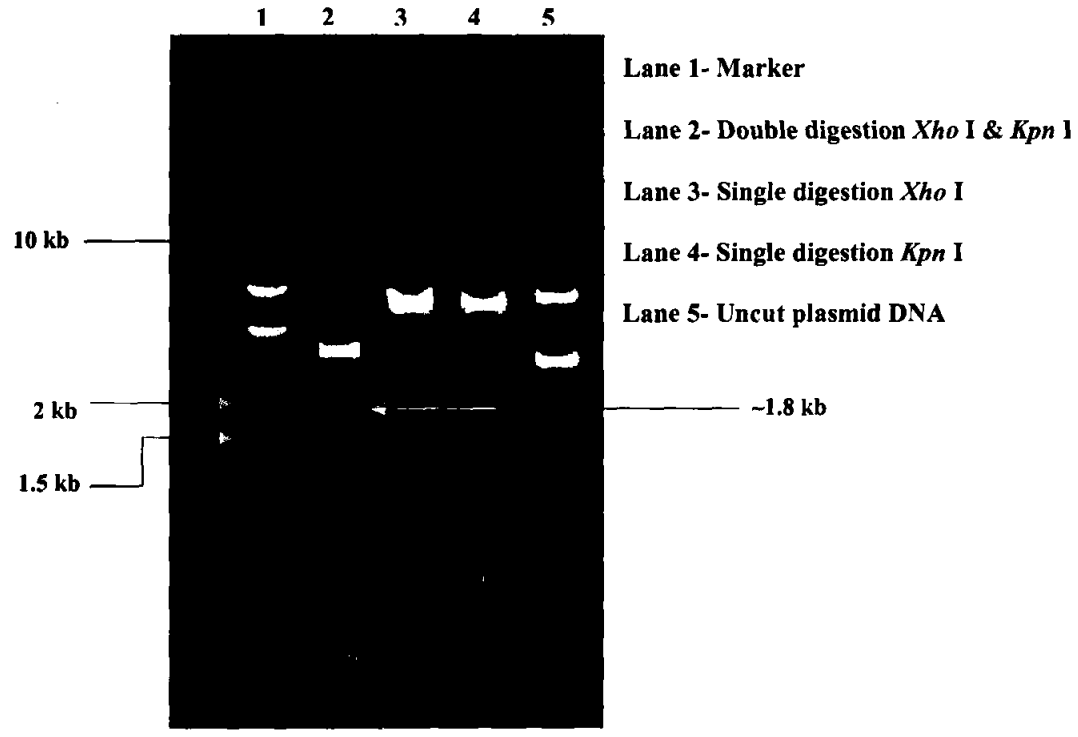


Fig. 5.6. Restriction digestion analysis of pGEMT/*hmgr1*

After confirmation, clones containing *hmgr*1 were selected and its nucleotide sequencing was carried out. The nucleotide sequence and the encoded amino acid sequence of *hmgr*1 cDNA are shown Figs. 5.7 and 5.8.

START

ATGGACACCACCGGCCGGCTCCACCACCGAAAGCATGCTACACCCGTTGAGGACCGTTCT
CCGACCACTCCGAAAGCGTCGGACGCGCTTCCGCTTCCCCTCTACCTGACCAACGCGGTTT
TCTTCACGCTGTTCTTCTCGGTGGCGTATTACCTCCTTCACCGGTGGCGCGACAAGATCCG
CAACTCCACTCCCCTTCATATCGTTACTCTCTCTGAAATTGTTGCTATTGTCTCCCTCATTG
CCTCTTTCATTTACCTCCTAGGATTCTTCGGTATCGATTTTGTGCAGTCATTCATTGCACGC
GCCTCCCATGACGTGTGGGACCTCGAAGATACGGATCCCAACTACCTCATCGATGAAGAT
CACCGTCTCGTTACTTGCCCTCCCGCTAATATATCTACTAAGACTACCATTATTGCCGCAC
CTACCAAATTGCCTACCTCGGAACCCTTAATTGCACCCTTAGTCTCGGAGGAAGACGAAA
TGATCGTCAACTCCGTCGTGGATGGGAAGATACCCTCCTATTCTCTGGAGTCGAAGCTCG
GGGACTGCAAACGAGCGGCTGCGATTCGACGCGAGGCTTTGCAGAGGATGACAAGGAGG
TCGCTGGAAGGCTTGCCAGTAGAAGGGTTCGATTACGAGTCGATTTTAGGACAATGCTGT
GAAATGCCAGTGGGATACGTGCAGATTCCGGTGGGGATTGCGGGGCCGTTGTTGCTGAAC
GGGCGGGAGTACTCTGTTCCAATGGCGACCACGGAGGGTTGTTTGGTGGCGAGCACTAAT
AGAGGGTGTAAGGCGATTTACTTGTCAGGTGGGGCCACCAGCGTCTTGTTGAAGGATGGC
ATGACAAGAGCGCCTGTTGTAAGATTCGCGTCGGCGACTAGAGCCGCGGAGTTGAAGTTC
TTCTTGGAGGATCCTGACAATTTTGATACCTTGGCCGTAGTTTTTAACAAGTCCAGTAGAT
TTGCGAGGCTCCAAGGCATTAAATGCTCAATTGCTGGTAAGAATCTTTATATAAGATTCA
GCTGCAGCACTGGCGATGCAATGGGGATGAACATGGTTTCTAAAGGGGTTCAAAACGTTC
TTGAATTTCTTCAAAGTGATTTTTCTGATATGGATGTCATTGGAATCTCAGGAAATTTTTGT
TCGGATAAGAAGCCTGCTGCTGTAAATTGGATTGAAGGACGTGGCAAATCAGTTGTTTGT
GAGGCAATTATCAAGGAAGAGGTGGTGAAGAAGGTGTTGAAAACCAATGTGGCCTCCCT
AGTGGAGCTTAACATGCTCAAGAATCTTGCTGGTTCTGCTGTTGCTGGTGCTTTGGGTGGA
TTTAATGCCCATGCAGGCAACATCGTATCTGCAATCTTTATTGCCACTGGCCAGGATCCAG
CACAGAATGTTGAGAGTTCTCATTGCATTCCATGATGGAAGCTGTCAATGATGGAAAGGA
TCTCCATATCTCTGTGACCATGCCCTCCATTGAGGTGGGTACAGTCGGAGGTGGAACTCA
ACTTGCATCTCAGTCTGCTTGTCTCAATTTGCTTGGGGTGAAGGGTGCAAACAAAGAGTC
GCCAGGATCAAACTCAAGGCTCCTTGCTGCCATCGTAGCTGGTTCAGTTTTGGCTGGTGAG
CTCTCCTTGATGTCTGCCATTGCAGCTGGGCAGCTTGTCAAGAGTCACATGAAGTACAAC
AGATCCAGCAAAGATATGTCTAAAGCTGCATCTTAG

STOP

Fig. 5.7. Nucleotide sequence of *hmgr*1cDNA from *H. brasiliensis*.

5'3' Frame

Met R G S H H H H H H G **Met** A S **Met** T G G Q Q **Met** G R D L Y D D D D K D R W

G S E L E **Met** D T T G R L H H R K H A T P V E D R S P T T P K A S D A L P L P L Y

L T N A V F F T L F F S V A Y Y L L H R W R D K I R N S T P L H I V T L S E I V A I

V S L I A S F I Y L L G F F G I D F V Q S F I A R A S H D V W D L E D T D P N Y L I

D E D H R L V T C P P A N I S T K T T I I A A P T K L P T S E P L I A P L V S E E D

E **Met** I V N S V V D G K I P S Y S L E S K L G D C K R A A A I R R E A L Q R **Met** T

R R S L E G L P V E G F D Y E S I L G Q C C E **Met** P V G Y V Q I P V G I A G P L L L

N G R E Y S V P **Met** A T T E G C L V A S T N R G C K A I Y L S G G A T S V L L K D

G **Met** T R A P V V R F A S A T R A A E L K F F L E D P D N F D T L A V V F N K S S

R F A R L Q G I K C S I A G K N L Y I R F S C S T G D A **Met** G **Met** N **Met** V S K G V

Q N V L E F L Q S D F S D **Met** D V I G I S G N F C S D K K P A A V N W I E G R G K

S V V C E A I I K E E V V K K V L K T N V A S L V E L N **Met** L K N L A G S A V A G

A L G G F N A H A G N I V S A I F I A T G Q D P A Q N V E S S H C I T **Met** **Met** E A

V N D G K D L H I S V T **Met** P S I E V G T V G G G T Q L A S Q S A C L N L L G V K

G A N K E S P G S N S R L L A A I V A G S V L A G E L S L **Met** S A I A A G Q L V K

S H **Met** K Y N R S S K D **Met** S K A A S **Stop**

Fig. 5.8. Aminoacid sequence of *hmgr*1 cDNAfrom *Hevea brasiliensis*

The clone that exhibited 100% homology to the previously submitted sequence in the GenBank database (Acc. No. X54659) was selected for further steps. The *hmgr*1 cDNA contained an open reading frame (ORF) of 1838 bp. The ORF of *hmgr*1 encoded 575 amino acids with a predicted molecular mass of 61.6 kDa and an isoelectric point of 6.6.

The selected clone of pGEM-T vector containing *hmgr*1 was double digested to release the *hmgr*1 insert and the gel purified insert DNA was cloned in to double digested pRSET-A expression vector at the *Xho*I and *Kpn*I restriction sites. The ligated products were transformed successfully into *E. coli* cells and the transformants were confirmed based on colony PCR (Fig. 5.9) and restriction digestion analysis (Fig. 5.10).

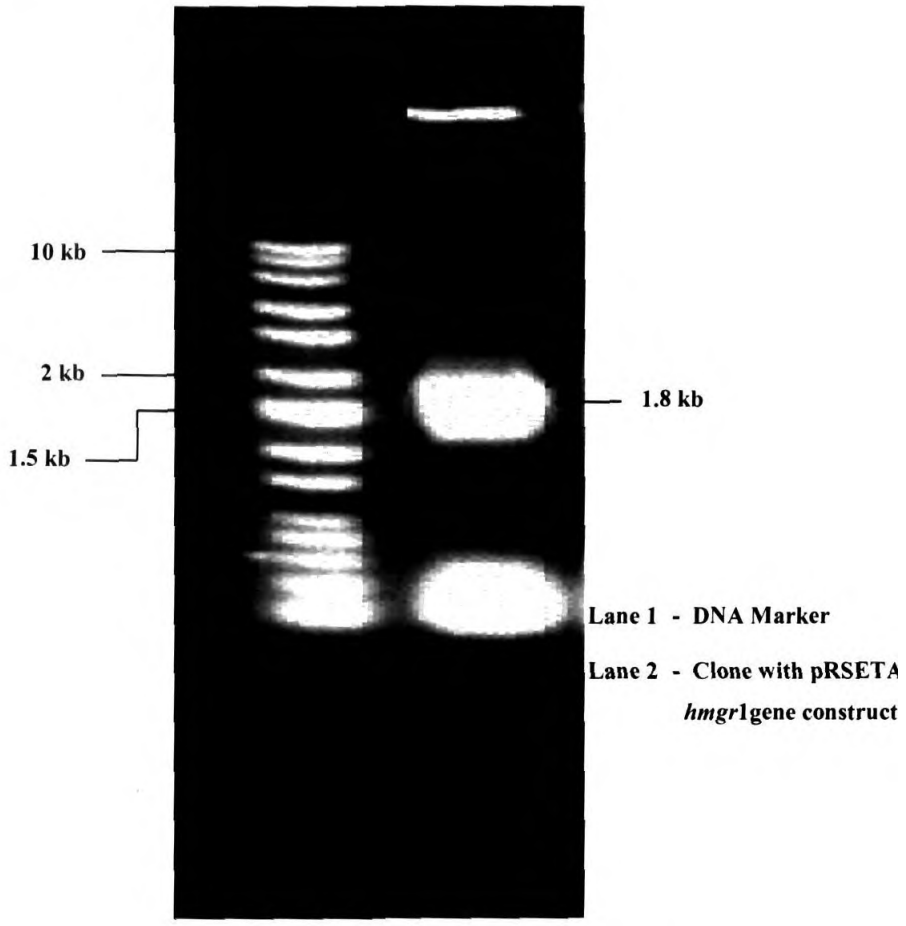


Fig. 5.9. Colony PCR of pRSET-A/*hmgr*1

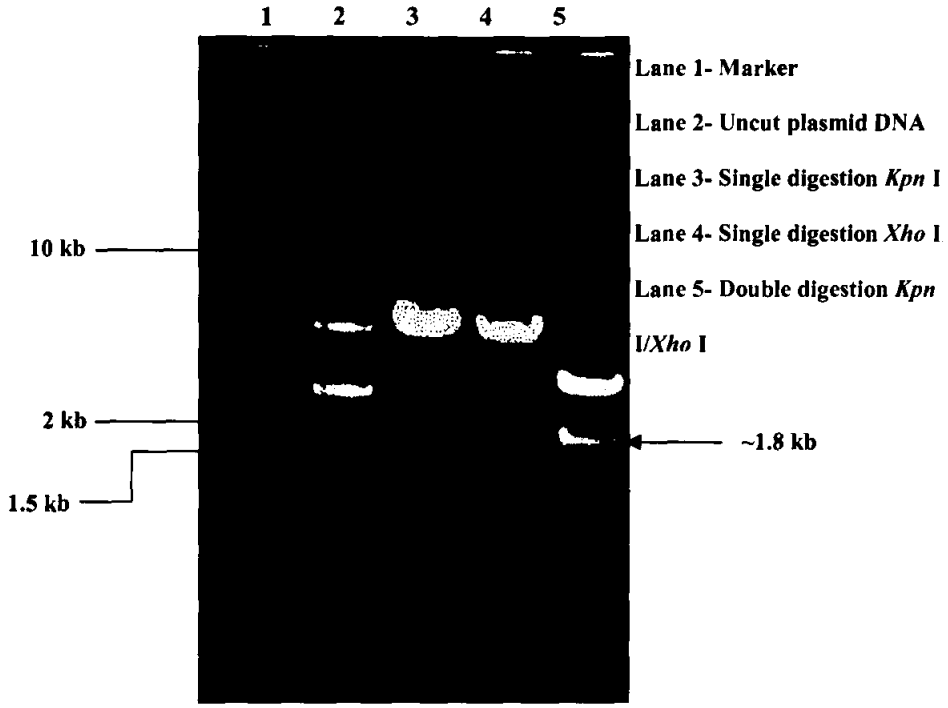


Fig. 5.10. Restriction digestion of pRSET-A/*hmgr*1

Orientation of the insert in the expression vector was also confirmed by verifying the sequence data (Fig. 5.11). The above results indicate the successful cloning of *hmgr*1 gene in pRSET-A expression vector. Protein expression in transformed cells when monitored by SDS-PAGE analysis indicated the presence of HMGR protein (Fig.5. 12). An increase in the intensity of protein band with a size of 64.6 kDa (including His Tag) corresponding to HMGR was obtained. The pRSET-A in BL21(DE3)pLysS was used as negative control. The maximum quantity of HMGR was observed in culture collected 3.5 hours after induction with IPTG.

TGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAA
TGGGTCGGGATCTGTACGACGATGACGATAAGGATCGATGGGGATCCGAGCTCGAGATG
GACACCACCGGCCGGCTCCACCACCGAAAGCATGCTACACCCGTTGAGGACCGTTCTCCG
ACCACTCCGAAAGCGTCGGACGCGCTTCCGCTTCCCCTCTACCTGACCAACGCGGTTTTCT
TCACGCTGTTCTTCTCGGTGGCGTATTACCTCCTTCACCGGTGGCGCGACAAGATCCGCAA
CTCCACTCCCCTTCATATCGTTACTCTCTCTGAAATTGTTGCTATTGTCTCCCTCATTGCCT
CTTTCATTTACCTCCTAGGATTCTTCGGTATCGATTTTGTGCAGTCATTCATTGCACGCGCC
TCCCATGACGTGTGGGACCTCGAAGATACGGATCCCAACTACCTCATCGATGAAGATCAC
CGTCTCGTTACTTGCCCTCCCGCTAATATATCTACTAAGACTACCATTATTGCCGCACCTA
CCAAATTGCCTACCTCGGAACCCTTAATTGCACCCTTAGTCTCGGAGGAAGACGAAATGA
TCGTCAACTCCGTCGTGGATGGGAAGATACCCTCCTATTCTCTGGAGTCGAAGCTCGGGG
ACTGCAAACGAGCGGCTGCGATTCGACGCGAGGCTTTGCAGAGGATGACAAGGAGGTCG
CTGGAAGGCTTGCCAGTAGAAGGGTTCGATTACGAGTCGATTTTAGGACAATGCTGTGAA
ATGCCAGTGGGATACGTGCAGATTCCGGTGGGGATTGCGGGGCCGTTGTTGCTGAACGGG
CGGGAGTACTCTGTTCCAATGGCGACCACGGAGGGTTGTTTGGTGGCGAGCACTAATAGA
GGGTGTAAGGCGATTTACTTGTCAGGTGGGGCCACCAGCGTCTTGTTGAAGGACGGCATG
ACAAGAGCGCCTGTTGTAAGATTCGCGTCGGCGACTAGAGCCGCGGAGTTGAAGTTCTTC
TTGGAGGATCCTGACAATTTTGATACCTTGGCCGTAGTTTTTAACAAGTCCAGTAGATTTG
CGAGGCTCCAAGGCATTAAATGCTCAATTGCTGGTAAGAATCTTTATATAAGATTCAGCT
GCAGCACTGGCGATGCAATGGGGATGAACATGGTCTCTAAAGGGGTTCAAAACGTTCTTG
AATTTCTTCAAAGTGATTTTTCTGATATGGATGTCATTGGAATCTCAGGAAATTTTTGTTC
GGATAAGAAGCCTGCTGCTGTAAATTGGATTGAAGGACGTGGCAAATCAGTTGTTTGTGA
GGCAATTATCAAGGAAGAGGTGGTGAAGAAGGTGTTGAAAACCAATGTGGCCTCCCTAG
TGGAGCTTAACATGCTCAAGAATCTTGCTGGTTCTGCTGTTGCTGGTGCTTTGGGTGGATT
TAATGCCCATGCAGGCAACATCGTATCTGCAATCTTTATTGCCACTGGCCAGGATCCAGC
ACAGAATGTTGAGAGTTCTCATTGCATTACCATGATGGAAGCTGTCAATGATGGAAAGGA
TCTCCATATCTCTGTGACCATGCCCTCCATTGAGGTGGGTACAGTCGGAGGTGGAACTCA
ACTTGCATCTCAGTCTGCTTGTCTCAATTTGCTTGGGGTGAAGGGTGCAAACAAAGAGTC
GCCAGGATCAAACTCAAGGCTCCTTGCTGCCATCGTAGCTGGTTCAGTTTTGGCTGGTGAG
CTCTCCTTGATGTCTGCCATTGCAGCTGGGCAGCTTGTCAAGAGTCACATGAAGTACAAC
AGATCCAGCAAAGATATGTCTAAAGCTGCATCTTAG

Fig. 5.11. Sequencing results of pRSETA/*hmgr*1 construct for the confirmation of orientation of insert. The start codon indicated in yellow color and termination codon in blue color.

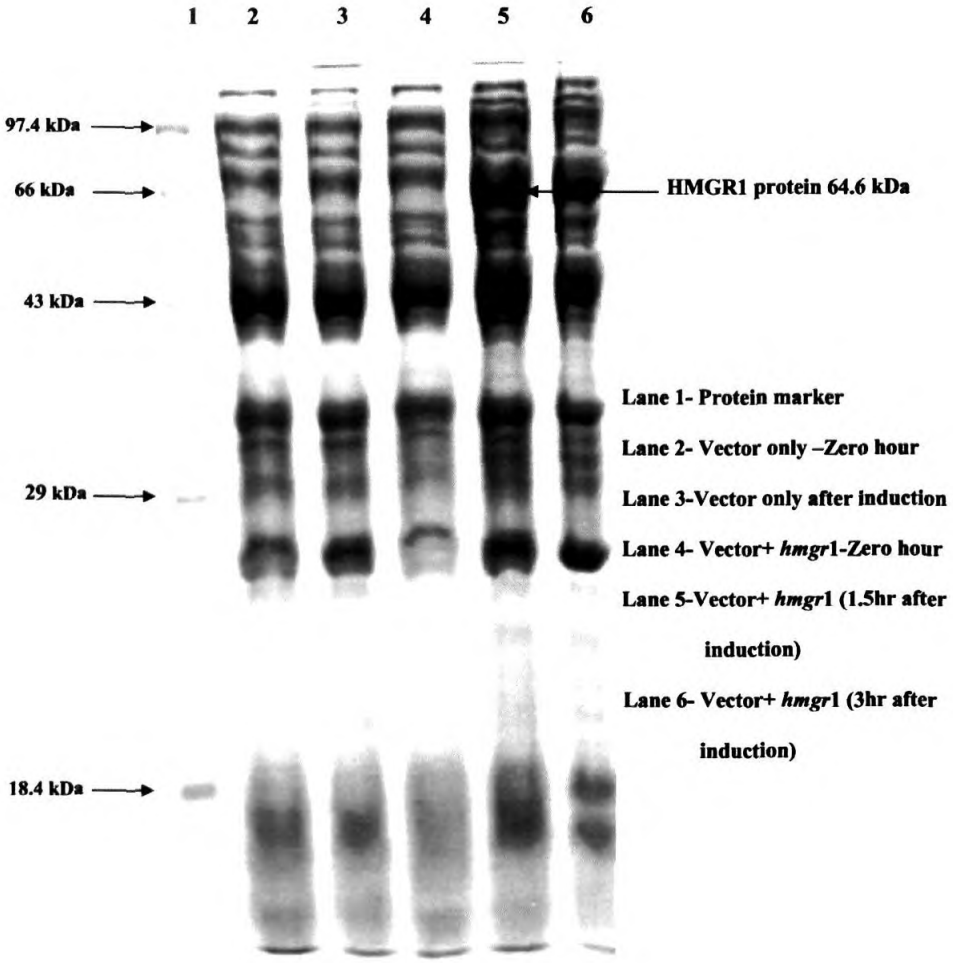


Fig. 5.12. SDS-PAGE analysis of proteins from transformed cells with pRSETA/*hmgr*1

5.4. Discussion

Biosynthesis of natural rubber takes place through mevalonate pathway in *Hevea*. The enzyme, HMG-CoA reductase, which catalyses the synthesis of mevalonate from HMG-CoA is a key regulatory enzyme in the pathway. HMG-CoA reductase regulates the carbon flux from primary to the secondary metabolism for biosynthesis of secondary plant metabolites. The regulation of HMGR enzyme leading to the increased production of isoprenoids has reported in a range of plant species (Schaller *et al.,* 1995; Chappell *et al.,* 1995; Ram *et al.,* 2010). Schalller *et al.* (1995) reported that the expression of *hmgr*1 mRNA was positively correlated with HMGR activity. Earlier studies showed a positive correlation between HMG-CoA reductase activity and rubber biosynthesis in *Hevea* (Nair *et al.,* 1990; Witisuwannakul *et al.,* 1990; Ji *et al.,* 1993)

In this study, in order to obtain the HMGR protein *invitro* and to further use this protein as a marker for yield potential in *Hevea*, cloning and expression of HMGR1 protein was carried out. For this purpose, mRNA was isolated from the latex of *Hevea* clone RRII 105. PCR amplification of coding region of *hmgr*1 was performed using *hmgr*1 specific primers and cloned the amplified product in to an expression vector. The protein expression when monitored by SDS-PAGE analysis indicated the presence of HMGR protein. The level of total phytosterol was increased about eight fold in leaves of tobacco plants by constitutive expression of either the *Hevea hmgr*1 gene (Schaller *et al.,* 1995) or the catalytic domain of hamster HMGR (Chappell *et al.,* 1995). The over

expression of the *Arabidopsis* HMGR isoforms in transgenic *Arabidopsis* plants also led to higher accumulation of leaf phytosterol (Manzano *et al.*, 2004; Leivar *et al.*, 2005). Disruption of *hmgr*1 and *hmgr*2 in *Arabidopsis* caused triterpenoid levels 65 and 25% repectivelely lower than that in wild type plants (Susuki *et al.*, 2004).

Tomato transformed with an *Arabidopsis hmgr*1 under the control of 35S constitutive promoter elevated phytosterol by up to 2.3 fold in ripe fruits of T_0 generation plants (Enfissi *et al.*, 2005). Schaller *et al.* (1995) reported that the transgenic tobacco lines expressing 35S promoter controlled *hmgr*1 from *Hevea brasiliensis,* increased total phytosterol in leaves up to six fold through production of phytosterol esters. Engineered tobacco seeds constitutivelely or seed specifically expressing an N-terminal truncated *Hevea brasiliensis* HMGR increased phytosterol by 2.4 or 3.2 fold respectivelely (Harker *et al.*, 2003). In *Taraxacum kok-saghyz,* a rubber producing plant, *Tkhmgr*1 is highly expressed in the root, the main tissue where rubber is synthesized and stored and a decline in gene expression of *Tkhmgr*1 in roots occurred when plants became mature (Ponicano and Chen, 2014). The expression of *hmgr*1 gene was correlated to the presence of more laticiferous cells in the corresponding tissue and the finding is in agreement with the earlier reports for HMGR in *Hevea* (Chye *et al.*, 1992; Suwanmanee *et al.*, 2002).

5.5. Summary and Conclusions

The cloning of *hmgr*1 gene in expression vector and in vitro synthesis of HMGR protein was successfully carried out. The protein would be useful for developing specific antibody and that could be further utilized for the quantification of HMG-CoA reductase in different clones of *Hevea* for developing this enzyme as a marker for yield potential in *Hevea.*

Chapter 6

General Conclusion

Natural rubber (NR) is an important industrial raw material with multifarious uses. NR consists of high molecular weight *cis*-polyisoprene, which is produced *via* the isoprenoid pathway in the laticiferous tissue of several plant species. *Hevea brasiliensis* is the only commercially cultivated species for harvesting NR. With the rapid development of automobile industry and world economy, NR production and productivity need to be increased to meet the rising market demand for this raw material. In recent decades great progress has been made in *Hevea* breeding programmes, which resulted in increased NR productivity from *Hevea brasiliensis* world over. In contrast to the progress made in rubber tree breeding through the classical approach, the molecular mechanisms underlying high yield traits in *Hevea* varieties are not well understood.

Even though rubber yield of a particular clone is attributed to many factors, the genes/ factors that are directly involved in contributing high rubber yield have not been studied in detail. Hence, the present work on biochemical and molecular studies on enzymes related to rubber biosynthesis in *Hevea brasiliensis* was conducted. In the present study, gene expression of some important enzymes involved in rubber biosynthesis was analysed in high and low yielding *Hevea* clones with and without stimulation. The activity of HMG-CoA reductase, a rate limiting enzyme of rubber biosynthesis pathway, was quantified in different *Hevea* clones with varying yield potential. The molecular cloning and expression analysis of *hmgr*1 from *Hevea* was also performed.

The expression analyses of genes showed that *HbSUT3*, *hmgs*, *hmgr*1, *MVD*, *FPPS*, *RuT* and *REF2* were significantly higher in high yielding *Hevea* clones than the low yielding clones. The magnitude of expression of genes corresponding to other enzymes and proteins like HMGR2, HMGR3, GGPPS, REF1, REF3, RBSP and RBIP showed no significant difference between the high and low yielding clones. The study suggest that high yield of rubber tree is likely the result of an increased supply and distribution of IPP for rubber biosynthesis. The study on the gene expression in response to ethylene stimulation suggests that the sucrose transporter, *HbSUT*3 plays an important role in sucrose loading of the laticifer tissue and thereby the rubber production in stimulated trees. The expression of *hmgr*1 and *MVD* was induced by ethylene treatment and this result suggests that both these enzymes are involved in enhanced IPP supply for rubber biosynthesis.

The enzyme assay of HMG-CoA reductase indicated that there was significant clonal variation in the activity in the bark tissue of different *Hevea* clones. The HMGR activity was found to be significantly higher in the bark extract of high yielding than the low yielding *Hevea* clones. HMGR activity showed an increase with the addition of latex C-serum to the bark extract indicating the presence of an activator of HMGR enzyme in the C-serum. HMG-CoA reductase activity showed a significant positive correlation with rubber yield. Stimulation with ethylene releasing compounds enhanced the total rubber yield in all the *Hevea* clones irrespective of the clonal trait, but the HMGR enzyme activity was not changed due to

stimulation. The results suggest that HMGR activity can be used as a marker for high yield in *H. brasiliensis*.

The molecular cloning and *in-vitro* expression of *hmgr*1 from *Hevea* was also performed. The cloning of *hmgr*1 gene in expression vector and *in-vitro* synthesis of HMGR protein was successfully carried out. The protein would be useful for developing specific antibody and that could be further utilized for the quantification of HMGR as a marker for yield potential in *Hevea.*

References

Abraham, P.D., Blencowe, J.W., Chua, S.E., Gomez, J.B., Moir, G.F.J., Pakianathan, S.W., Sekhar, B.C., Southorn, W.A. and Wycherley, P.R. (1971). Novel stimulants and procedures in the exploitation of *Hevea*. II. Pilot trial using (2-chloroethyl)-phosphonic acid (ethephon) and acetylene with various tapping systems. *Journal of the Rubber Research Institute of Malaya,* **23**: 90-113.

Adiwilaga, K. and Kush, A. (1996). Cloning and characterization of cDNA encoding farnesyl diphosphate synthase from rubber tree (*Hevea brasiliensis*). *Plant Molecular Biology*, **30**: 935-946.

Alam, A., Britton, G., Powls, R. and Goad, J. (1991). Aspects related to 3-hydroxy-3-methylglutaryl-CoA synthesis in higher plants. *Biochemical Society Transactions,* **19:** 164S.

Alessandro, C.M., Luiz, E.M.O., Paulo, M. and Nelson, D.F. (2006). Anatomical characteristics and enzymes of the sucrose metabolism and their relationship with latex yield in the rubber tree (*Hevea brasiliensis* Muell. Arg.). *Brazilian Journal of Plant Physiology*, **18**: 263-268.

Alex, D., Bach, T.J. and Chye, M.L. (2000). Expression of *Brassica juncea* 3-hydroxy-3-methylglutaryl CoA synthase is developmentally regulated and stress-responsive. *Plant Journal*, **22**: 415-426.

Ament, K., Van Schie, C.C., Bouwmeester, H.J., Haring, M.A. and Schuurink, R.C. (2006). Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (E, E)-4, 8, 12-

trimethyltrideca- 1,3,7,11- tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways. *Planta*, **224**: 1197-1208.

Archer, B.L., Audley, B.G., Cockbain, E.G. and McSweeney, G.P. (1963). The biosynthesis of rubber. Incorporation of mevalonate and isopentenyl pyrophosphate into rubber by *Hevea brasiliensis*-latex fractions. *Biochemical Journal*, **89**: 565-574.

Archer, B.L. and Audley, B.G. (1967). Biosynthesis of rubber. In: *Advances in Enzymology and Related Areas of Molecular Biology*, Vol 29: (Ed. F.F. Nord), John Wiley & Sons, Inc., USA. pp. 221- 257.

Archer, B.L. and Cockbain, E.G. (1969). Rubber transferase from *Hevea brasiliensis* latex. *Methods in Enzymology*, **15**: 476-480.

Archer, B.L. and Audley, B.G. (1973). Rubber, Gutta-percha and Chicle. In: *Phytochemistry* 2, (Ed. L.P. Miller), Van Nostrand Reinhold Co., New York, pp. 310-343.

Archer, B.L. and Audley, B.G. (1987). New aspects of rubber biosynthesis. *Botanical Journal of the Linnean Society*, **94**: 181-196.

Arebalo, R.E. and Mitchell, E.D. (1984). Cellular distribution of 3-hydroxy-3-methylglutaryl coenzyme a reductase and mevalonate kinase in leaves of *Nepeta cataria*. *Phytochemistry*, **23**: 13-18.

Asawatreratanakul, K., Zhang, Y.W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayaporn, A. and Koyama, T. (2003).

Molecular cloning, expression and characterisation of cDNA encoding *cis*-prenyltransferases from *Hevea brasiliensis*. A key factor participating in natural rubber biosynthesis. *European Journal of Biochemistry*, **270**: 4671-4680.

Bach, T.J., Rogers, D.H. and Rudney, H. (1986). Detergent-solubilization, purification, and characterization of membrane-bound 3-hydroxy-3-methylglutaryl-coenzyme A reductase from radish seedlings. *European Journal of Biochemistry,* **154**: 103-111.

Bach, T.J. (1987). Synthesis and metabolism of mevalonic acid in plants. *Plant Physiology and Biochemistry*, **25**: 163-178.

Bach, T.J. and Lichtenthaler, H.K. (1987). Plant growth regulation by mevinolin and other sterol biosynthesis inhibitors. In: *Ecology and Metabolism of Plant Lipids.* (Eds. Fuller, G. and Nes W.D.), American Chemical Society Symposium Series, Vol 325 American Chemical Society, Washington, DC, pp 109-139

Bach, T.J. (1995). Some new aspects of isoprenoid biosynthesis in plants - A review. *Lipids* **30**: 191- 202.

Backhaus, R.A. (1985). Rubber formation in plants - a mini-review. *Israel Journal of Botany*, **34**: 283-293.

Balasubramaniam, S. Goldstein J.L. and Brown M.S. (1977). Regulation of cholesterol synthesis in rat adrenal gland through coordinate control of 3-hydroxy-3-methylgutaryl coenzyme A synthase and reductase

activities. *Proceedings of National Accademy of Sciences USA*, **74**: 1421-1425.

Bantignies, B., Liboz, T. and Ambid, C. (1996). Nucleotide sequence of a *Catharanthus roseus* geranylgeranyl pyrophosphate synthase gene. *Plant Physiology*, **110**: 336.

Baud, S., Wuillème, S., Lemoine, R., Kronenberger, J., Caboche, M., Lepiniec, L. and Rochat, C. (2005). The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in *Arabidopsis*. *Plant Journal*, **43**: 824-836.

Benedict, C.R. (1983). Biosynthesis of rubber. In: *Biosynthesis of Isoprenoid Compounds*; (Eds. Porter, J.W. and Spurgeon, S.L.) Wiley: New York, pp 355-369.

Benedict, C.R., Greer, P. and Foster, M.A. (2008). The physiology and biochemical responses of guayule to the low temperature of the Chihuahuan Desert in the biosynthesis of rubber. *Industrial Crops and Products*, **27**: 225-235.

Biardi, L. and Krisans, S.K. (1996). Compartmentalization of cholesterol biosynthesis: conversion of mevalonate to farnesyl diphosphate occurs in the peroxisomes. *Journal of Biological Chemistry*, **271**: 1784-1788.

Blackley, D.C. (1997). *Polymer Latices: Science and Technology*. Vol 2: Types of Lattices. Chapman and Hall. London, UK. 612 p.

Boatman, S.G. (1966). Preliminary physiological studies on the promotion of latex flow by plant growth regulators. *Rubber Research Institute of Malaya*, **19**(5): 243-258.

Bradford, M.M. (1976). A dye binding assay for protein. *Analytical Biochemistry,* **72**: 248-254.

Braun, D.M. and Slewinski, T.L. (2009). Genetic control of carbon partitioning in grasses: roles of sucrose transporters and tie-dyed loci in phloem loading. *Plant Physiology,* **149**: 71-81.

Broekaert, W.F., Lee, H.I., Kush, A., Chua, N.H. and Raikhel, N.V. (1990). Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree. *Proceedings of the National Academy of Sciences USA*, **87**: 7633-7637.

Brooker, J.D. and Russell, D.W. (1975). Subcellular localization of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Pisum sativum* seedlings. *Archives of Biochemistry and Biophysics,* **167**: 730-737.

Brooker, J.D. and Russell, D.W. (1979). Regulation of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase from pea seedlings: rapid posttranslational phytochrome-mediated decrease in activity and *in vivo* regulation by isoprenoid products. *Archives of Biochemistry and Biophysics,* **198**: 323-334.

Brzozowska-Hanower, J., Hanower, P. and Lioret, C. (1978). Étude du mècanisme de la coagulation du latex d'Hevea brasiliensis (Kunth)

Müll. Arg. II. systémes enzymatiques impliqués dans le processus. *Physiologie Végetale,* 16: 231-254. [in French]

Buttery, B.R. and Boatman, S.G. (1967). Effects of tapping, wounding and growth regulators on turgor pressure in *Hevea brasiliensis* Muell. Arg. *Journal of Experimental Botany,* **18**(57): 644-659.

Caelles, C., Ferrer, A., Balcells, L., Hegardt, F.G. and Boronat, A. (1989). Isolation and structural characterization of a cDNA-encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Molecular Biology*, **13**: 627-638.

Castillon, J. and Cornish, K. (1999). Regulation of initiation and polymer molecular weight of cis- 1,4-polyisoprene synthesized in vitro by particles isolated from *Parthenium argentatum* (Gray). *Phytochemistry*, **51**: 43-51.

Cataldo, F. (2000). Guayule rubber: a new possible world scenario for the production of natural rubber. *Progress in Rubber, Plastics, Recycling Technology*, **16**: 31-59.

Chamot, D and Kuhlemeier, C. (1992). Differential expression of genes encoding the hypusine-containing translation initiation factor, eIF-5A, in tobacco. *Nucleic Acids Research*, **20:** 665-669.

Chang, S., Puryear, J. and Carney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, **11**: 113-116.

Chao, J., Chen, Y., Wu, S. and Tian, W.M. (2015). Comparitive transcriptome analysis of latex from rubber tree clone CATAS8-79 and PR107 reveals new cues for the regulation of latex regeneration and duration of latex flow. *BMC Plant Biology*, **15**: 104. DOI: 10.1186/s12870-015-0488-3.

Chappell, J. (1995). Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* **46**: 521-547.

Chappell, J., Wolf, F., Proulx, J., Cuellar, R. and Saunders, C. (1995). Is the reaction catalysed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate limiting step for isoprenoid biosynthesis in plants? *Plant Physiology*, **109**: 1337-1343.

Chesterton, C.J. and Kekwick, R.G.O. (1968). Formation of 3-isopentenyl pyrophosphate by *Hevea brasiliensis*. *Biochemical Journal*, **98**: 26.

Chincinska, I.A., Liesche, J., Krügel, U., Michalska, J., Geigenberger, P., Grimm, B. and Kühn, C. (2008). Sucrose transporter StSUT4 from potato affects flowering, tuberization, and shade avoidance response. *Plant Physiology,* **146**: 515-528.

Chiou, T.J. and Bush, D.R. (1998). Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Sciences, USA*, **95**: 4784-4788.

Chong, F.C. (1981). The role of carbohydrate in the exploitation on latex flow of *Hevea*. *Journal of Rubber Research Institute of Malaysia*, **29**(2): 125-126.

Chow, K.S., Mohd.Lim, S. and Han, K.H. (2003) Characterisation of a Family of eIF-5A cDNAs from *Hevea brasiliensis*. *Journal of Rubber Research*, **6**(2): 107-120.

Chow, K.S., Yusof, F., Lim, S.M. and Abdullah, L. (2006). An assessment of stimulation of *Hevea brasiliensis* rubber biosynthesis by eIF-5A protein-enriched bacterial lysates. *Journal of Rubber Research*, **9(4)**: 251-259.

Chow, K.S., Wan, K.L., Mat Isa, M.N., Bahari, A., Tan, S.H., Harikrishna, K. and Yeang, H.Y. (2007). Insights into rubber biosynthesis from transcriptome analysis of *Hevea brasiliensis* latex. *Journal of Experimental Botany*, **58**: 2429-2440.

Chow, K.S., Mat-Isa, M.N., Bahari, A., Ghazali, A.K., Alias, H., Zainuddin, Z.M., Hoh, C.C. and Wan, K.L. (2012). Metabolic routes affecting rubber biosynthesis in *Hevea brasiliensis* latex. *Journal of Experimental Botany*, **63**: 1863-1871.

Chu, F., Guillot, J. and Guyot, A. (1998). Rheology of concentrated multi-sized poly (St/BA/MMA) latexes. *Colloid and Polymer Science*, **276**: 305-312.

Chye, M.L., Kush, A., Tan, C.T. and Chua, N.H. (1991). Characterization of cDNA and genomic clones encoding 3-hydroxy-3-methylglutaryl CoA reductase from *Hevea brasiliensis*. *Plant Molecular Biology,* **16**: 567-577.

Chye, M.L., Tan, C.T. and Chua, N.H. (1992). Three genes encode 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *Hevea brasiliensis*: hmg1 and hmg3 are differentially expressed. Plant *Molecular Biology*, **19**: 473-484.

Clément-Demange, A., Priyadarshan, P.M., Tran, T.T.H. and Venkatachalam, P. (2007). *Hevea* rubber breeding and genetics. In: *Plant Breeding Reviews*, (Ed. J. Janick), John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 177-283.

Clinkenbeard, K.D., Reed, W.D., Mooney, R.D. and Lane D. (1975a). Intracellular localization of the 3- hydroxy-3-methylglutaryl coenzyme A cycle enzymes in liver. Separate cytoplasmic and mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A generating systems for cholesterogenesis and ketogenesis. *Journal of Biological Chemistry,* **250**: 3108-3116.

Clinkenbeard, K.D., Sugiyama, T., Reed, W.D. and Lane, D. (1975b). Cytoplasmic 3-hydroxy-3- methylglutaryl coenzyme A synthase from liver. Purification, properties and role in cholesterol synthesis. *Journal of Biological Chemistry,* **250**: 3124-3135.

Closa, M., Vranova, Eva., Bortolotti, C., Bigler, L., Arro, M., Ferrer, A. and Gruissem, W. (2010). The *Arabidopsis thaliana* FPP synthase isozymes have overlapping and specific functions in isoprenoid biosynthesis, and complete loss of FPP synthase activity causes early developmental arrest. *The Plant Journal*, **63**: 512-525.

Cook, A.S. and Sekhar, B.C. (1953). Fractions from *Hevea brasiliensis* latex centrifugation at 59,000g. *Journal of Rubber Research Institute of Malaya*, **14**: 163.

Cordier, H., Karst, F. and Berges, T. (1999). Heterologous expression in *Saccharomyces cerevisiae* of an *Arabidopsis thaliana* cDNA encoding mevalonate diphosphate decarboxylase. *Plant Molecular Biology*, **39**: 953-967.

Cornish, K. and Backhaus, R.A. (1990). Rubber transferase activity in rubber particles of guayule, *Phytochemistry,* **29:** 3809-3813.

Cornish, K. (1993). The separate roles of plant *cis* and *trans* prenyl transferases in *cis*-1,4-polyisoprene biosynthesis. *European Journal of Biochemistry*, **218**: 267-271.

Cornish, K., Siler, D.J., Grosjean, O.K. and Godman, N. (1993). Fundamental similarities in rubber particle architecture and function in three evolutionarily divergent plant species. *Journal of Natural Rubber Research*, **8**: 275-285.

Cornish, K. and Bartlett, D.L. (1997). Stabilisation of particle integrity and particle-bound *cis*-prenyl transferase activity in stored, purified rubber particles. *Phytochemical Analysis*, **8:** 130-134.

Cornish, K. (2001). Similarities and differences in rubber biochemistry among plant species. *Phytochemistry*, **57**: 1123-1134.

Cornish, K. and Brichta, J.L. (2002). Some rheological properties of latex from *Parthenium argentatum* Gray compared with latex from *Hevea brasiliensis* and *Ficus elastica*. *Journal of Polymers and the Environment*, **10**: 13-18.

Coupé, M. and Chrestin, H. (1989). Physico-chemical and biochemical mechanisms of hormonal (ethylene) stimulation. In: *Physiology of Rubber Tree Latex.* (Eds. d'Auzac J., Jacob JL, Chrestin H). Boca Raton, FL: CRC Press; pp. 295-319.

Cunillera, N., Boronat, A. and Ferrer, A. (1997). The *Arabidopsis thaliana* FPS1 gene generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate isoform. *Journal of Biological Chemistry*, **272**: 15381-15388.

Cunillera, N., Boronat, A. and Ferrer, A. (2000). Spatial and temporal patterns of GUS expression directed by 5'regions of the *Arabidopsis thaliana* farnesyl diphosphate synthase genes FPS1 and FPS2. *Plant Molecular Biology*, **44**: 747-758.

Cunningham, F.X. and Gantt, E. (2007). A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. *Photosynthesis Research*, **92**: 245-259.

Czuppon, A.B., Chen, Z., Rennert, S., Engelke, T. and Meyer, H.E. (1993). The rubber elongation factor of rubber trees (*Hevea brasiliensis*) is the major allergen in latex. *Journal of Allergy and Clinical Immunology,* **92**: 690-697.

d'Auzac, J., Jacob J.L. and Chrestin, H. (1989). Physiology of the latex flow. In: *Physiology of Rubber Tree Latex.* (Eds. d'Auzac, Jacob J.L and Chrestin, H.). CRC Press Inc. Boca Raton, pp. 219-285.

d'Auzac, J., Prevot, J.C. and Jacob, J.L. (1995). What's new about lutoids? A vacuolar system model from *Hevea* latex. *Plant Physiology and Biochemistry*, **33**: 765-777.

d'Auzac, J., Jacob, J.L., Prévôt, J.C., Clément, A. and Gallois, R. (1997). The regulation of *cis*-polyisoprene production (natural rubber) from *Hevea brasiliensis*, *In Recent research developments in plant physiology.* (Ed. Pandalai S.G.).Trivandrum, India: Rhaoesearch Singpost; pp 273-332.

da Costa, B.M.T., Keasling, J.D. and Cornish, K., (2005). Regulation of rubber biosynthetic rate and molecular weight in *Hevea brasiliensis* by metal cofactor. *Biomacromolecules,* **6**: 279-289.

da Costa, B.M.T., Keasling, J.D., McMahan, C.M. and Cornish, K. (2006). Magnesium ion regulation of *in vitro* rubber biosynthesis by *Parthenium argentatum* Gray. *Photochemistry,* **67**: 1621-1628.

Daraselia, N.D., Tarchevskaya, S. and Narita, J.O. (1996). The promoter for tomato 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene 2 has unusual regulatory elements that direct high-level expression. *Plant Physiology*, **112**: 727-733.

de Faÿ, E., Moraes, L.A.C. and Moraes, V.H.F. (2010). Cyanogenesis and the onset of tapping panel dryness in rubber tree. *Pesq uisa Agropecuaria Brasileira*, **45**: 1372-1380.

Decourteix, M., Alves, G., Brunel, N., Améglio, T., Guillio, A., Lemoine, R., Pétel, G. and Sakr, S. (2006). JrSUT1, a putative xylem sucrose transporter, could mediate sucrose influx into xylem parenchyma cells and be up-regulated by freeze-thaw cycles over the autumn-winter period in walnut tree (*Juglans regia* L.). *Plant, Cell and Environment*, **29**: 36-47.

Decourteix, M., Alves, G. and Bonhomme, M. (2008). Sucrose (JrSUT1) and hexose (JrHT1 and JrHT2) transporters in walnut xylem parenchyma cells: their potential role in early events of growth resumption. *Tree Physiology*, **28**: 214-224.

Delabarre, M.A. and Serrier, J.B. (2000). *The Tropical Agriculturalist.* Macmillan Education Ltd., London. 168 p.

Denbow, C.J. (1997). Membrane domain of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase: targeting, topology and function. *PhD thesis in Plant Physiology*, Blacksburg, Virginia.

Dennis, M.S. and Light, D.R. (1989). Rubber elongation factor from *Hevea brasiliensis*. Identification, characterization and role in rubber biosynthesis, *Journal of Biological Chemistry*, **264**: 18608- 18617.

Dian, K., Sangare, A. and Diopoh, J.K. (1995) Evidence for specific variation of protein pattern during tapping panel dryness condition development in *Hevea brasiliensis*. *Plant Science*, **105**: 207-216.

Dusotoit-Coucaud, A., Brunel, N., Kongsawadworakul, P., Viboonjun, U., Lacointe, A., Julien, J.L., Chrestin, H. and Sakr, S. (2009). Sucrose importation into laticifers of *Hevea brasiliensis,* in relation to ethylene stimulation of latex production. *Annals of Botany*, **104**: 635-647.

Dusotoit-coucaud, A., Kongsawadworakul, P., Maurousset, L., Viboonjun, U., Brunel, N., Pujade-renaud, V., Chrestin, H. and Sakr, S. (2010). Ethylene stimulation of latex yield depends on the expression of a sucrose transporter (HbSUT1B) in rubber tree (*Hevea brasiliensis*). *Tree Physiology*, **30**: 1586-1598.

Enfissi, E.M.A., Fraser, P.D., Lois, L-M., Boronat, A., Schuch, W. and Bramley, P.M. (2005). Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the

production of health-promoting isoprenoids in tomato. *Plant Biotechnology Journal,* **3**: 17-27.

Enjuto, M., Balcells, L., Campos, N., Caelles, C., Arró, M. and Boronat, A. (1994). *Arabidopsis thaliana* contains two differentially expressed 3-hydroxy-3-methylglutaryl-CoA reductase genes, which encode microsomal forms of the enzyme. *Proceedings of National Academy of Sciences of USA*, **91**: 927-931.

Enjuto, M., Lumbreras, V., Marin, C. and Boronat, A. (1995). Expression of the *Arabidopsis* HMG2 gene, encoding 3-hydroxy- 3-methylglutaryl coenzyme A reductase, is restricted to meristematic and floral tissues. *Plant Cell*, **7**: 517-527.

Feng, S.P., Li, W.G., Huang, H.S., Wang, J.Y. and Wu, Y.T. (2008). Development, characterization and cross-species/genera transferability of EST-SSR markers for rubber tree (*Hevea brasiliensis*). *Molecular Breeding*, **23**: 85-97.

Feyereisen, R. and Farnsworth, D.E. (1987). Characterization and regulation of HMG-CoA reductase during a cycle of juvenile hormone synthesis. *Molecular and Cellular Endocrinology,* **53**: 227-238.

Gertler, F.B., Chiu, C.Y., Richter-Mann, L., and Chin, D.J. (1988). Developmental and metabolic regulation of the *Drosophila melanogaster* 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Molecular Cell Biology*, **8**: 2713-2721.

Gibson, D.M. and Parker, R.A. (1987). Hydroxymethylglutaryl-coenzyme A reductase. In: *The enzymes*. (Eds. Boyer P.D. and Kerbs, E.G.). Academic Press, New York, pp. 179-215.

Gidrol, X., Chrestin, H., Mounoury, G. and D'Auzac, J. (1988). Early activation by ethylene of the tonoplast H-pumping ATPase in the latex from *Hevea brasiliensis*. *Plant Physiology*, **86**: 899-903.

Gidrol, X., Chrestin, H., Tan, H.L. and Kush, A. (1994). Hevein, a lectin like protein from *Hevea brasiliensis* (rubber tree) is involved in the coagulation of latex. *Journal of Biological Chemistry*, **25**: 9278-9283.

Gillaspy, G., Ben-David, H. and Gruissem, W. (1993). Fruits: a developmental perspective. *Plant Cell,* **5**: 1439-1451.

Goldstein, J.L and Brown, M.S. (1990). Regulation of the mevalonate pathway. *Nature,* **343**: 425-430.

Gomez, J.B. (1982). Anatomy of *Hevea* and its influence on latex production. *Malaysian Rubber Research and Development Board Publication no.* 15, 54.

Gomez, J.B. and Moir, G.F.J. (1979). The ultracytology of latex vessels in *Hevea brasiliensis*. Malaysian Rubber Research and Development Board, Kuala Lumpur, Malaysia. Monograph, No.4.

Goyvaerts, E.M. Dennis, M., Light, D. and Chua, N.H. (1991). Cloning and sequencing of the cDNA encoding the rubber elongation factor of *Hevea brasiliensis*. *Plant Physiology*, **97**: 317-321.

Gray, J.C. (1987). Control of isoprenoid biosynthesis in higher plants. *Advances in Botanical Research*, **14**: 25-91.

Greenwood, R., Luckham, P.F. and Gregory, T. (1995). The effect of particle size on the layer thickness of a stabilizing polymer adsorbed onto two different classes of polymer latex, as determined from rheological experiments. *Colloid Surface*, **98**: 117-125.

Guo, D., Li, H.L. and Peng, S.Q. (2015). Structure conservation and differential expression of farnesyl diphosphate synthase genes in Euphorbiaceous plants. *International Journal of Molecular Sciences*, **16(9)**: 22402-22414.

Han, K.H., Shin, D.H., Yang, J., Kim, I.J. and Oh, S.K. (2000). Genes expressed in the latex of *Hevea brasiliensis*. *Tree Physiology*, **20**: 503-510.

Hao, B.Z. and Wu, J.L. (2000). Laticifer differentiation in *Hevea brasiliensis*: infection by exogenous jasmonic acid and linolenic acid. *Annals of Botany*, **85**: 37-43.

Harker, M., Holmberg, N., Clayton, J.C., Gibbard, C.L., Wallace, A.D., Rawlins, S., Hellyer, S.A., Lanot, A. and Safford, R. (2003). Enhancement of seed phytosterol levels by expression of an N-terminal truncated *Hevea brasiliensis* (rubber tree) 3-hydroxy-3-methylglutaryl-CoA reductase. *Plant Biotechnology Journal*, **2**: 113-121.

Hayes, M.A., Davies, C. and Dry. I.B. (2007). Isolation, functional characterization, and expression analysis of grapevine (*Vitis vinifera* L.) hexose transporters: differential roles in sink and source tissues. *Journal of Experimental Botany*, **58**: 1985-1997.

Hebant, C. (1981). Ontoge'nie des lacticife'rs du systeme Primaire *d'Hevea brasiliensis*:une etudeultrastructurale et cytochimique. *Canadian Journal of Botany*, **59**: 974-985.

Hefner, J., Ketchum, R.E. and Croteau, R. (1998). Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyl transferase in cells induced for taxol production. *Archives of Biochemistry and Biophysics*, **360**: 62-74.

Hemmerlin, A., Hoeffler, J.F., Meyer, O., Tritsch, D., Kagan, I.A., Grosdemange-Billiard, C., Rohmer, M. and Bach, T.J. (2003). Cross talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. *Journal of Biological Chemistry*, **278**: 26666-26676.

Hepper, C.M. and Audley, B.G. (1969). The biosynthesis of rubber from beta-hydroxy-beta-methyl gluaryl coenzyme A in *Hevea brasiliensis* latex. *Biochemical Journal*, **114**: 379-386.

Ho, C.Y. and Paardekooper, E.G. (1965). Application of stimulants to the virgin bark in clone trials. First Bulletin. *Rubber Research Institute of Malaya*, No. **80**: 150

Hopkins, M.T., Lampi, Y., Wang, T.W., Liu, Z. and Thompson, J.E. (2008). Eukaryotic translation initiation factor 5A is involved in pathogen-induced cell death and development of disease symptoms in *Arabidopsis*. *Plant Physiology*, **148**: 479-489.

Hugueney, P., Bouvier, F., Badillo, A., Quennemet, J., d'Harlingue, A. and Camara, B. (1996). Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathway in pepper fruits. *Plant Physiology*, **111**: 619-626.

Iijima, S. and Muruyama, M. (1973). Lipids, **15**: 265.

Jacob, J.L. (1970). Particularité de la glycolyse et de sa régulation au sein du latex d'*Hevea brasiliensis*. *Physiologie Vegetale*, **8**: 395-411.

Jacob, J.L., Prévôt, J.C., Roussel, D., Lacrotte, R., Serres, E., d'Auzac, J., Eschbach. J.M. and Omont, H. (1989). Yield-limiting factors, latex physiological parameters, latex diagnosis and clonal typology, In: *Physiology of Rubber Tree Latex.* (Eds. J. d'Auzac, J.L. Jacob and H. Chrestin). CRC Press Inc. Boca Raton, FL, USA. pp. 345-382.

Jacob, J.L. and Prevot, J.C. (1992). Metabolism of the laticiferous system and its biochemical regulation. In: *Natural Rubber: Biology, Cultivation and Technology* (Eds. M.R. Sethuraj and N.M. Mathew), Elsevier, Amsterdam pp. 116-136.

Ji, W., Benedict, C.R. and Foster, A. (1993). Seasonal variations in rubber biosynthesis, 3-hydroxy-3-methylglutarylcoenzyme A reductase and

rubber transferase activities in *Parthenium argentatum* in the Chihuahuan desert. *Plant Physiology,* **103**: 535-542.

Kang, H., Kim, Y.S. and Chung, G.C. (2000). Characterization of natural rubber biosynthesis in *Ficus benghalensis*. *Plant Physiology and Biochemistry,* **38**: 979-987.

Kasahara, H., Hanada, A., Kuzuyama, T., Takagi, M., Kamiya, Y. and Yamaguchi, S. (2002). Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. *Journal of Biological Chemistry*, **277**: 45188-45194.

Kishimoto, S. and Ohmiya, A. (2006). Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium*). *Physiologia Plantarum*, **128**: 436-447.

Kloer, D.P., Welsch, R., Beyer, P. and Schulz, G.E. (2006). Structure and reaction geometry of geranylgeranyl diphosphate synthase from *Sinapis alba*. *Biochemistry*, **45**: 15197-15204.

Ko, J.H., Chow, K.S. and Han, K.H. (2003). Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree). *Plant Molecular Biology,* **53**: 479-492.

Kondo, K. and Oba, K. (1986). Purification and characterization of 3-hydroxy-3-methylglutaryl CoA reductase from potato tubers. *Journal of Biochemistry*, **100**: 967-974.

Kongsawadworakul, P. and Chrestin, H. (2003). Laser diffraction: A new tool for identification and studies of physiological effectors involved in aggregation/coagulation of the rubber particles from *Hevea* latex. *Plant Cell Physiology*, **44**: 707-717.

Korth, K.L., Stermer, B.A., Bhattacharyya, M.K. and Dixon, R.A. (1997). HMG-CoA reductase gene families that differentially accumulate transcripts in potato tubers are developmentally expressed in floral tissues. *Plant Molecular Biology*, **33**: 545-551.

Koyama, T. (2003). Molecular cloning, expression and characterization of cDNA encoding *cis*-prenyl transferases from *Hevea brasiliensis*. A key factor participating in natural rubber biosynthesis. *European Journal of Biochemistry*, **270**: 4671-4680.

Krishnakumar, R., Cornish. K. and Jacob. J. (2001). Rubber biosynthesis in tapping panel dryness affected *Hevea* trees. *Journal of Rubber Research*, 4(2): 131-139.

Kush, A. (1994). Isoprenoid biosynthesis: the *Hevea* factory. *Plant Physiology and Biochemistry*, **32**: 761-767.

Kush, A., Goyvaerts, E., Chye, M.L. and Chua, N.H. (1990). Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *Proceedings of the National Academy of Sciences of USA*, **87**: 1787-1790.

Laemmli, U.K. (1970). Clevage of structural proteins during the assembly of the head of bacteiophage T4. *Nature,* **227**:680-685.

Laskaris, G., VanDer heijden, R. and Verpoorte, R. (2000). Purification and partial characterization of geranylgeranyl diphosphate synthase, from *Taxus baccata* cell cultures: An enzyme that regulates taxane biosynthesis. *Plant Science*, **153**: 97-105.

Le, G.V., Gay, C., Xiong, T.C., Souza, L.M., Rodier-Goud, M. and Seguin M. (2011). Development and characterization of 296 new polymorphic microsatellite markers for rubber tree (*Hevea brasiliensis*). *Plant Breeding*. **130**: 294-296.

Leivar, P., Gonzalez, V.M., Castel, S., Trelease, R.N., Lopez-lglesias, C., Arro, M., Boronat, A., Campos, N., Ferrer, A. and Fernandez-Busquets, X. (2005). Subcellular localization of *Arabidopsis* 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Plant Physiology*, **137**: 57-69.

Lemoine, R., Burkle, L., Barker, L., Sakr, S., Kuhn, C., Regnacq, M., Gaillard, C., Delrot, S. and Frommer, W.B. (1999). Identification of a pollen-specific sucrose transporter-like protein NtSUT3 from tobacco. *FEBS Letters*, **454**: 325-330.

Lespinasse, D., Rodier-Goud, M., Grivet, L., Leconte, A., Legnate, H. and Seguin, M. (2000). A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellite, and isozyme markers. *Theoretical and Applied Genetics*, **100**: 127-138.

Li, D., Deng, Z., Chen, C., Xia, Z., Wu, M., He, P. and Chen, S. (2010). Identification and characterization of genes associated with tapping panel dryness from *Hevea brasiliensis* latex using suppression subtractive hybridization. *BMC Plant Biology*, **10**: 140.

Li, D., Deng, Z., Qin, B., Liu, X. and Men, Z. (2012). De novo assembly and characterization of bark transcriptome using Illumina sequencing and development of EST-SSR markers in rubber tree (*Hevea brasiliensis* Muell. Arg.). *BMC Genomics*, **13**: 192.

Li, D., Hao, L., Liu, H., Zhao, M., Deng, Z., Li, Y., Zeng, R. and Tian, W. (2015). Next-generation sequencing, assembly, and comparative analyses of the latex transcriptomes from two elite *Hevea brasiliensis* varieties. *Tree Genetics and Genomes*, **11**: 1-9.

Lichtenthaler, H.K., Schwender, J., Disch, A. and Rohmer, M. (1997a). Biosynthesis of isoprenoids in higher plant chloroplasts proceeds *via* a mevalonate-independent pathway. *FEBS Letters*, **400**: 271-274.

Lichtenthaler, H.K., Rohmer, M. and Schwender, J. (1997b). Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiologia Plantarum*, **101**: 643-652.

Lichtenthaler, H.K. (1999). The l-deoxy-d-xylulose-5- phosphate pathway of isoprenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **50**: 7-65.

Licy, J., Saraswathyamma, C.K., Premakumari, D., Meenakumari, T., Meenattoor, J.R. and Nazeer, M.A. (2003). Genetic parameters and hetrosis in rubber (*Hevea brasiliensis* Muell. Arg), V. Hybrid vigor for yield and yield components among the RRII 400 series clones in small scale cultivation. *Indian Journal of Natural Rubber Research*, **16**(1&2): 75-80.

Light, D.R. and Dennis, M.S. (1989). Purification of a prenyl transferase that elongates *cis*-polyisoprene rubber from the latex of *Hevea brasiliensis*, *Journal of Biological Chemistry*, **264**: 18589-18597.

Liu, J-P., Xia, Z-Q., Tian, X-Y. and Li, Y-J. (2015). Transcriptome sequencing and analysis of rubber tree (*Hevea brasiliensis* Muell.) to discover putative genes associated with tapping panel dryness (TPD). *Journal of BMC Genomics*, **16**: 398.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, **25**(4): 402-408.

Low, F.C. (1978). Distribution and concentration of major soluble carbohydrates in latex. The effect of ethephon stimulation and the possible role of these carbohydrates in latex flow. *Journal of Rubber Research Institute of Malaysia*, **26**: 21-32.

Luo, M.W., Deng, L.H., Yi, X.P., Zeng, H.C. and Xiao, S.H. (2009). Cloning and sequence analysis of a novel *cis*-prenyltransferases gene

from *Hevea brasiliensis*. *Journal of Tropical and Subtropical Botany*, **17**: 223-228.

Lynen, F., Agranoff, B.W., Eggerer, H., Henning, U. and Moslein, E.M. (1959). Dimethylallyl-pyrophosphate and geranyl-pyrophosphate, biologische Vorstufen des squalens. Zur biosynthese for terpene VI, *Angewandte Chemie*, **71**: 657-663.

Lynen, F. and Henning, U. (1960). Biological path to natural rubber. *Angewandte Chemie International*, 72.

Lynen, F. (1967). Biosynthetic pathways from acetate to natural products. *Pure and Applied Chemistry,* **14**:137-168.

Lynen, F. (1969). Biochemical Problems of rubber synthesis. *Journal of Rubber Research Institute of Malaya*, **21**: 389-406.

Madhavan, S. and Benedict, C.R. (1984). Isopentenyl pyrophosphate *cis*-1,4-polyisoprenyl transferase from guayule (*Parthenium argentatum* Gray). *Plant Physiology*, **75**: 908-913.

Madhavan, S., Greenblatt, G.A., Foster, M.A. and Benedict, C.R. (1989). Stimulation of isopentenyl pyrophosphate incorporation into polyisoprene in extracts from guayule plants *(Parthenium argentatum* Gray) by low temperature and 2-(3,4-dichloro-phenoxy) triethylamine, *Plant Physiology*, **89**: 506- 511.

Mantello, C.C., Suzuki, F.I., Souza, L.M., Goncalves, P.S. and Souza, A.P. (2012). Microsatellite marker development for the rubber tree (*Hevea*

brasiliensis): characterization and cross-amplification in wild *Hevea* species. *BMC Research Notes*, **5**: 329.

Manzano, D., Fernandez-Busquets, X., Schaller, H., Gonzalez, V., Boronat, A., Arro, M. and Ferrer, A. (2004). The metabolic imbalance underlying lesion formation in *Arabidopsis thaliana* over expressing farnesyl diphosphate synthase (isoform 1S) leads to oxidative stress and is triggered by the developmental decline of endogenous HMGR activity. *Planta,* **219**: 982-992.

Mau, C.J.D., Garneau, S., Scholte, A.A., Van Fleet, J.E., Vederas, J.C. and Cornish, K. (2003). Protein farnesyltransferase inhibitors interfere with farnesyl diphosphate binding by rubber transferase. *European Journal of Biochemistry*, **270**: 3939-3945.

McGarvey, D.J. and Croteau, R. (1995). Terpenoid metabolism. *The Plant Cell*, **7**: 1015-1026.

Mcmullen, A.I. and Mcsweeney, G.P. (1966). The biosynthesis of rubber. Incorporation of isopentenyl pyrophosphate into purified rubber particles by a soluble latex serum enzyme. *Biochemical Journal,* **101**: 42-47.

Miao, Z. and Gaynor, J.J. (1993). Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (*Hevea brasiliensis*). *Plant Molecular Biology*, **23**: 267-277.

Milford, G.F.J., Paardekooper, E.C. and Ho, C.Y. (1969). Latex vessel plugging, its importance to yield and clonal behavior. *Journal of Rubber Research Institute of Malaya,* **21**: 274-282.

Mooibroek, H. and Cornish, K. (2000). Alternative sources of natural rubber. *Applied Microbiology and Biotechnology,* **53**: 355-365.

Mydin, K.K. (2014). Genetic improvement of *Hevea brasiliensis*: Sixty years of breeding efforts in India. *Rubber Science*, **27**(2): 153-181.

Nagata, N., Suzuki, M., Yoshida, S. and Muranaka, T. (2002). Mevalonic acid partially restores chloroplast and etioplast development in *Arabidopsis* lacking the non-mevalonate pathway. *Planta,* **216**: 345-350.

Nagegowda, D.A., Bach, T.J. and Chye, M.L. (2004). *Brassica juncea* 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 1: expression and characterization of recombinant wild-type and mutant enzymes. *Biochemical Journal*, **383:** 517-527.

Nagegowda, D.A., Ramalingam, S., Hemmerlin, A., Bach, T.J. and Chye, M.L. (2005). *Brassica juncea* HMG-CoA synthase: localization of mRNA and protein. *Planta*, **221**: 844-856.

Nair, N.U., Thomas, M., Sreelatha, S., Simon, S.P., Vijayakumar, K.R. and George, P.J. (1990). Clonal variations in the activity of 3-hydroxy-3-methyl glutaryl-CoA reductase in bark of *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, **3**(1): 40-42.

Narita, J.O. and Gruissem, W. (1989). Tomato hydroxy-methylglutaryl-CoA reductase is required early in fruit development but not during ripening. *Plant Cell*, **1**: 181-190.

Newman, J.D. and Chappell, J. (1999). Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway. *Critical Reviews in Biochemistry and Molecular biology*, **34**: 95-106.

Nuntanuwat, W. (2006). Relationship between the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR-1) and rubber content in *Hevea brasiliensis* (Willd. ex A. Juss. Muell. Arg.) RRIM 600, BPM 24 and PB 235. An abstract presented to the Thaskin University.

Ogura, K., Shinka, T. and Seto, S. (1972). The purification and properties of geranylgeranyl pyrophosphate synthetase from pumpkin fruit. *Journal of Biochemistry*, **72**: 1101-1108.

Ogura, K., Koyama, T. and Sagami, H. (1997). Polyprenyl diphosphate synthases. *Subcellular Biochemistry,* vol. **28**, *Cholesterols: its Functions and Metabolism in Biology and Medicine.* (Ed. Bittman R.), Plenum Press New York. pp. 57-87.

Oh, S.K., Kang, H., Shin, D.H., Yang, J. and Chow, K.S. (1999). Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*. *Journal of Biological Chemistry*, **274**: 17132-17138.

Oh, S.K., Kang, H., Shin, D.H., Yang, J. and Han, K.H. (2000a). Molecular cloning and characterization of a functional cDNA clone encoding isopentenyl diphosphate isomerase from *Hevea brasiliensis. Journal of Plant Physiology*, **157**: 549-557.

Oh, S.K., Han, K.H., Ryu, S.B. and Kang, H. (2000b). Molecular cloning, expression, and functional analysis of a *cis*-prenyltransferase from *Arabidopsis thaliana*: implication in rubber biosynthesis. *Journal of Biological Chemistry,* **275**: 18482-18488.

Ogura, K. and Koyama, T. (1998). Enzymatic aspects of isoprenoid chain elongation. *Chemical Reviews,* **98:** 1263-1276.

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M. and Kamiya, Y. (2000). Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in *Arabidopsis*. *Plant Physiology*, **122**: 1045-1056.

Paardekooper, E.G. and Samosorn, S. (1969). Clonal Variation in latex flow pattern. *Journal of Rubber Research Institute of Malaya*, **21**(3): 264.

Pan, Z., Herickhoff, L. and Backhaus, R.A. (1996). Cloning, characterization, and heterologous expression of cDNAs for farnesyl diphosphate synthase from the guayule rubber plant reveals that this prenyltransferase occurs in rubber particles. *Archives of Biochemistry and Biophysics*, **332**: 196-204.

Pang, Y., Shen, G-A., Bergès, T., Cardier, H., Wu, W., Sun, X. and Tang, K. (2006). Molecular cloning, characterization and heterologous expression in *Saccharomyces cerevisiae* of a mevalonate diphosphate decarboxylase cDNA from Ginkgo biloba. *Physiologia Plantarum,* **127**: 19-27.

Polhamus, L.G. (1962). *Rubber- Botany, Production, and Utilization.* Leonard Hill Limited, London. pp-179-180.

Ponciano, G. and Chen, G.Q. (2014). Identification of a highly expressed 3-hydroxy-3-methylglutaryl-CoA reductase gene in the root tissue of *Taraxacum kok-saghyz. American Journal of Plant Sciences,* **5**: 3603-3608.

Posch, A., Chen, Z., Wheeler, C., Dunn, M.J., Raulf-Heimsoth, M. and Baur, X. (1997). Characterization and identification of latex allergens by two-dimensional electrophoresis and protein micro sequencing. *Journal of Allergy and Clinical Immunology,* **99**: 385-395.

Poulter, C.D. and Rilling, H.C. (1981). Prenyltransferase and isomerase. In: *The Biosynthesis of Isoprenoid Compounds.* (Eds. Porter J.W. and Spurgeon S.L.). Vol.1, John Wiley and Sons, U.S.A. pp. 163-224.

Priya, P., Venkatachalam, P. and Thulaseedharan, A. (2006). Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis. *Plant Science*, **171**(4): 470-480.

Priya, P., Venkatachalam, P. and Thulaseedharan, A. (2007). Differential expression pattern of rubber elongation factor (REF) mRNA transcripts from high and low yielding clones of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Plant Cell Reports*, **26**: 1833-1838.

Pujade-Renaud, V., Clement, A., Perrot Rechenmann, C., Prévôt, J.C., Chrestin, H., Jacob, J.L. and Guern, J. (1994). Ethylene-induced increase in glutamine synthetase activity and mRNA levels in *Hevea brasiliensis* latex cells. *Plant Physiology*, **105**: 127-132.

Pujade-Renaud, V., Perrot-Rechenmann, C., Chrestin, H., Lacrotte, R. and Guern, J. (1997). Characterization of a full-length cDNA clone encoding glutamine synthetase from rubber tree latex. *Plant Physiology and Biochemistry*, **35**: 85-93.

Rahman, A.Y., Usharraj, A.O., Misra, B.B., Thottathil, G.P., Jayasekaran, K., Feng, Y., Hou, S., Ong, S.Y., Ng, F.L., Lee, L.S., Tan, H.S., Sakaff, M.K., The, B.S., Khoo, B.F., Badai, S.S., Aziz, N.A., Yuryev, A., Knudsen, B., Dionne-Laporte, A., Mchunu, N.P., Yu, Q., Langston, B.J., Freitas, T.A., Young, A.G., Chen, R., Wang, L., Najimudin, N., Saito, J.A., and Alam. M. (2013). Draft genome sequence of the rubber tree *Hevea brasiliensis. BMC Genomics,* **14**: 75.

Ram, M., Khan, M.A., Jha, P., Khan, S., Kiran, U. and Ahmad, M.M. (2010). HMGCoA reductase limits artemisinin biosynthesis and accumulation in *Artemisia* annual plants. *Acta Physiologiae Plantarum*, **32**: 859-866.

Rodwell, V.W., Beach, M.J., Bischoff, K.M, Bochar, D.A, Darnay, B.G., Friesen, J.A., Gill, J.F., Hedl, M,, Jordan-Starck, T., Kennelly, P.J., Kim, D.Y. and Wang, Y. (2000). 3-Hydroxy- 3-methylglutaryl-CoA reductase. *Methods in Enzymology,* **324**: 259-280.

Rogers, D.H., Panini, S.R. and Rudney, H. (1983). Properties of HMG-CoA reductase and its mechanism of action. In: *3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase* (Ed. J.R. Sabine) CRC Press, Inc., Boca Raton, FL pp.57-75

Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S. and Sahm, H. (1996). Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *Journal of the American Chemical Society*, **188**: 2564-2566.

Rosche, E., Blackmore, D., Tegeder, M., Richardson, T., Schroeder, H., Higgins, T.J.V., Frommer, W.B., Offler, C.E. and Patrick, J.W. (2002). Seed-specific over expression of a potato sucrose transporter increases sucrose uptake and growth rates of developing pea cotyledons. *Plant Journal*, **30**: 165-175.

Ruderman, S., Kongsawadworakul, P., Viboonjun, U., Mongkolporn, O. and Chrestin, H. (2012). Mitochondrial/cytosolic Acetyl CoA and rubber biosynthesis genes expression in *Hevea brasiliensis* latex and rubber yield. *Kasetsart Journal (Natural Science)*, **46**: 346-362.

Saha, T. and Priyadarshan, P.M. (2012). Genomics of *Hevea* rubber. In: *Genomics of Tree Crops.* (Eds. Schnell R.J and Priyadarshan P.M). Springer New York, Ny, USA, pp. 261-298.

Sakr, S., Noubahni, M., Bourbouloux, A., Riesmeier, J., Frommer, W., Sauer, N. and Delrot, S. (1997). Cutting, ageing and expression of plant membrane transporters. *Biochimica Biophysica Acta,* **1330**: 207-216.

Sando. T., Takaoka, C., Mukai, Y., Yamashita, A., Hattori, M., Ogasawara, N., Fukusaki, E. and Kobayashi, A. (2008a). Cloning and characterization of mevalonate pathway genes in a natural rubber producing plant, *Hevea brasiliensis. Bioscience, Biotechnology and Biochemistry*, **72**: 2049-2060.

Sando, T., Takeno, S., Watanabe, N., Okumoto, H., Kuzuyama, T., Yamashita, A., Hattori, M., Ogasawara, N., Fukusaki, E. and Kobayashi, A. (2008b). Cloning and characterization of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway genes of a natural-rubber producing plant *Hevea brasiliensis. Bioscience, Biotechnology and Biochemistry*, **72**: 2903-2917.

Sando, T., Hayashi, T., Takeda, T., Akiyama, Y., Nakazawa, Y., Fukusaki, E. and Kobayashi, A. (2009). Histochemical study of detailed laticifer structure and rubber biosynthesis-related protein localization in *Hevea brasiliensis* using spectral confocal laser scanning microscopy. *Planta*, **230**: 215-225.

Sanmiya, K., Ueno, O., Matsuoka, M. and Yamamoto, N. (1999). Localization of farnesyl diphosphate synthase in chloroplasts. *Plant Cell Physiology*, **40**: 348-354.

Sauer, N. (2007). Molecular physiology of higher plant sucrose transporters. *FEBS Letters,* **581**: 2309-2317.

Schaller, H., Grausem, B., Benveniste, P., Chye, M.L., Tan, C.T., Song, Y.H. and Chua, N.H. (1995). Expression of the *Hevea brasiliensis* Mull. Arg. 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiology,* **109**: 761-770.

Schultes, R.E. (1990). *A Brief Taxonomic View of the Genus Hevea.* Malaysian Rubber Research and Development Board, Kuala Lumpur, Malaysia. Monograph,14, ISSN 0127-7782.

Scott, D.J., da Costa, B.M.T., Espy, S.C., Keasling, J.D. and Cornish, K. (2003). Activation and inhibition of rubber transferases by metal cofactors and pyrophosphate substrates. *Phytochemistry*, **64**: 123-134.

Sekhar, A.C. (1989). *Rubber Wood Production and Utilisation.* RRII, Kottayam. 224p.

Sipat, A. (1985). 3-Hydroxy-3-methylglutaryl-CoA reductase in the latex of *Hevea brasiliensis. Methods in Enzymology*, **110**: 40-51.

Sirinupong, N., Suwanmanee, P., Doolittle, R.F. and Suvachitanont, W. (2005). Molecular cloning of a new cDNA and expression of 3-

hydroxy-3-methylglutaryl-CoA synthase gene from *Hevea brasiliensis*. *Planta,* **221**(4): 502-12.

Sivasubramaniam, S., Vanniasingham, V.M., Tan, C.T. and Chua, N.H. (1995). Characterization of HEVER, a novel stress induced gene from *Hevea brasiliensis*. *Plant Molecular Biology,* **29**: 173-178.

Skilleter, D.N, Williamson, I.P. and Kekwick, R.G.O. (1966). Phosphomevalonate kinase from Hevea brasiliensis. Biochemical Journal, **98**: 279.

Skilleter, D.N. and Kekwick, R.G.O. (1971). The enzymes forming isopentenyl pyrophosphate from 5-phosphomevalonate (mevalonate-5-phosphate) in the latex of *Hevea brasiliensis*. *Biochemical Journal,* **124**: 407-415.

Sookmark, U., Pujade-Renaud, V., Chrestin, H., Lacote, R., Naiyanetr, C., Seguin, M., Romruensukharom, P. and Narangajavana, J. (2002). Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome. *Plant Cell Physiology*, **43**: 1323-1333.

Souza, L.M., Gazaffi, R., Mantello, C.C., Silva, C.C. and Garcia, D. (2013). QTL mapping of growth-related traits in a full-sib family of rubber tree (*Hevea brasiliensis*) evaluated in a sub-tropical climate. *Plos One*, **8**: e61238.

Spurgeon, S.L. and Porter, J. (1981). *Biosynthesis of Isoprenoid Compounds.* Vol. 1. John Wiley and Sons, New York. pp. 1-46.

Stermer, B.A., Bianchini, G.M. and Korth, K.L. (1994). Regulation of HMG-CoA reductase activity in plants. *Journal of Lipid Research*, **35**: 1133-1140.

Subramaniam, A. (1980). Molecular weight and molecular weight distribution of NR. In: *Technology Bulletin No.4,* Rubber Research Institute of Malaysia, pp. 1-25.

Sutherlin, A., Hedl, M., Sanchez-Neri, B., Burgner, J.W., Stauffacher, C.V. and Rodwell, V.W. (2002). Enterococcus faecalis 3-hydroxy-3-methylglutaryl coenzyme A synthase, an enzyme of isopentenyl diphosphate biosynthesis. *Journal of Bacteriology*, **184**: 4065-4070.

Suvachittanont, W. and Wititsuwannakul, R. (1995). 3-Hydroxy- 3-methylglutaryl coenzyme A synthase in *Hevea brasiliensis. Phytochemistry,* **40**: 757-761.

Suwanmanee, P., Suvachittanontand, W. and Fincher, G.B. (2002). Molecular cloning and sequencing of a cDNA encoding 3-hydroxy-3-methylglutaryl coenzyme A synthase from *Hevea brasiliensis* (HBK) Mull Arg. *Science Asia*, **28**: 29-36.

Suwanmanee, P., Sirinupong, N. and Suvachittanont, W. (2004). Regulation of the expression of 3-hydroxy-3-methylglutaryl-CoA synthase gene in *Hevea brasiliensis* (B.H.K.) Muell. Arg. *Plant Science*, **166**: 531-537.

Suwanmanee, P., Sirinupong, N. and Suvachittanont, W. (2013). Regulation of 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase and rubber biosynthesis of *Hevea brasiliensis* Mull. Arg. In: *Isoprenoid Synthesis in Plants and Microorganisms* (Eds. Bach, T.J. and Rohmer, M.). Springer, New York, pp-315-327.

Suzuki, M., Kamide, Y., Nagata, N., Seki, H., Ohyama, K., Kato, H., Masuda, K., Sato, S., Kato, T. and Tabata, S. (2004). Loss of function of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMG1) in *Arabidopsis* leads to dwarfing, early senescence and male sterility, and reduced sterol levels. *Plant Journal*, **37**: 750-761.

Takaya, A., Zhang, Y.W., Asawatreratanakul, K., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S. and Koyama, T. (2003). Cloning, expression and characterization of a functional cDNA clone encoding geranylgeranyl diphosphate synthase of *Hevea brasiliensis*. *Biochimica et Biophysica. Acta,* **1625**(2): 214-220.

Tanaka, Y., Aik-Hwee, E., Ohya, N., Nishiyama, N., Tangpakdee, J., Kawahara, S. and Wititsuwannakul, R. (1996). Initiation of rubber biosynthesis in *Hevea brasiliensis*: characterization of initiating species by structural analysis. *Phytochemistry*, **41**: 1501-1505.

Tang, C., Huang, D., Yang, J., Liu, S., Sakr, S., Li, H., Zhou, Y. and Qin, Y. (2010). The sucrose transporter HbSUT3 plays an active role in sucrose loading to laticifer and rubber productivity in exploited trees of

Hevea brasiliensis (para rubber tree). *Plant, Cell and Environment,* **33**: 1708-1720.

Tang, C., Xiao, X., Li, H., Fan, Y., Fan, Y., Yang, J., Qi, J. and Li, H. (2013). Comparative analysis of latex transcriptome reveals putative molecular mechanisms underlying super productivity of *Hevea brasiliensis*. *Plos One*, **8**(9): e75307.

Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Wititsuwannakul, R., Wititsuwannakul, D. and Asawatreratanakul, K. (1997). Isopentenyl diphosphate isomerase and prenyl transferase activities in bottom fraction and C-serum from *Hevea* latex. *Phytochemistry,* **45**: 261-267.

Tholl, D., Kish, C.M., Orlova, I., Sherman, D., Gershenzon, J., Pichersky, E. and Dudareva, N. (2004). Formation of monoterpenes in *Antirrhinum majus* and *Clarkia breweri* flowers involves heterodimeric geranyl diphosphate synthases. *Plant Cell*, **16**: 977-992.

Tholl, D. and Lee, S. (2011). Terpene specialized metabolism in *Arabidopsis thaliana. The Arabidopsis Book*, 9: e0143.

Thomas, M., Nair, D.B., Gopalakrishnan, J., Sreelatha,S. and Jacob, J. (1999). Ethephon application increases the rubber yield from *Hevea brasiliensis* by altering the water relations of the latex. *Journal of Plant Biology*, 26(**3**): 1-8.

Towaranonte, B., Chuenwarin, P., Kongsawadworakul, P., Viboonjun, U., Narangajavana, J. and Chrestin, H. (2010). Identification and

characterization of candidate genes related to latex yield in the laticifers of rubber tree (*Hevea brasiliensis*). *Thai Journal of Botany*, **2**: 199-206.

Triwitayakorn, K., Chatkulkawin, P., Kanjanawattanawong, S., Sraphet, S. and Yoocha, T. (2011). Transcriptome sequencing of *Hevea brasiliensis* for development of microsatellite markers and construction of a genetic linkage map. *DNA Research*, **18**: 471-482.

Tungngoen, K., Kongsawadworakul, P., Viboonjun, U., Katsuhara, M., Brunel, Sakr, S., Narangajavana, J. and Chrestin, H. (2009). Involvement of HbPIP2;1 and HbTIP1;1 aquaporins in ethylene stimulation of latex yield through regulation of water exchanges between inner liber and latex cells in *Hevea brasiliensis*. *Plant Physiology*, **151**: 843-856.

Tupy, J. (1973). The activity of the latex invertase and latex production of *Hevea brasiliensis*. *Physiologie Vegetale*, **11**: 633-641.

Tupy, J. (1988a). Ribosomal and polyadenylated RNA content of rubber tree latex, association with sucrose level and latex pH. *Plant Science*, **55**: 137-144.

Tupy, J. (1988b). The activity of latex invertase and latex production of *Hevea brasiliensis*. *Comptes rendus de I' Academie des Sciences*, **268:** 3046-3049.

Tupy, J. (1989). Sucrose supply and utilization for latex production. In: *Physiology of Rubber Tree Latex* (Eds. Jd'Auzac, J.L. Jacob and H. Chrestin), Boca Raton, FL: CRC Press pp: 179-199.

van Beilen, J.B. and Poirier, Y. (2007). Guayule and Russian dandelion as alternative sources of natural rubber. *Critical Reviews in Biotechnology*, **27**: 217-231.

Van Gils, G.E. (1951). Studies on the latex viscosity. I: influence of the dry rubber content. *Archives in Rubber Cultivation.* **28**: 61-66.

Venkatachalam, P., Geetha, N., Sangeetha, P. and Thulaseedharan, A. (2013). Natural rubber producing plants: An overview. *African Journal of Biotechnology*, **12**: 1297-1310.

Venkatachalam, P., Priya, P., Gireesh, T., Saraswathyamma, C.K and Thulaseedharan, A. (2006). Molecular cloning and sequencing of a polymorphic band from rubber tree (*Hevea brasiliensis* (Muell.). *Current Science,* **90**: 1510-1515.

Venkatachalam, P., Priya, P., Jayashree, R., Rekha, K. and Thulaseedharan, A. (2009a). Molecular cloning and characterization of a 3-hydroxy 3-methyl-glutaryl coenzyme A reductase 1 (*hmgr*1) gene from rubber tree (*Hevea brasiliensis* Muell. Arg.): A key enzyme involved in isoprenoid biosynthesis. *Physiology and Molecular Biology of Plants*, **15**: 133-143.

Venkatachalam, P., Thulaseedharan, A. and Raghothama, K. (2009b). Molecular identification and characterization of a gene associated with the onset of tapping panel dryness (TPD) syndrome in rubber tree (*Hevea brasiliensis* Muell.) by mRNA differential display. *Molecular Biotechnology,* **41**(1): 42-52.

Wang, T.W., Lu, L., Wang, D. and Thompson, J.E. (2001). Isolation and characterisation of senescence-induced cDNAs encoding deoxyhypusine synthase and eukaryotic translation initiation factor 5A from tomato. *Journal of Biological Chemistry*, **276**: 17541-17549.

Wang, T.W., Lu, L., Zhang, C.G., Taylor, C. and Thompson, J.E. (2003). Pleiotropic effects of suppressing deoxyhypusine synthase expression in *Arabidopsis thaliana*. *Plant Molecular Biology*, **52:** 1223-1235.

Wang, T.W., Zhang, C.G., Wu, W., Nowack, L.M., Madey, E. and Thompson, J.E. (2005). Antisense suppression of deoxyhypusine synthase in tomato delays fruit softening and alters growth and development. *Plant Physiology*, **138**: 1372-1382.

Wang, X., Wang, D., Sun, Y., Yang, Q., Wang, L., Meng, X., Huang, Q., Jin, X. and Tong, Z. (2015). Comprehensive proteomics analysis of laticifer latex reveals new insights in ethylene stimulation of natural rubber production. *Scientific Reports*, **5**: 13778.

Wegener, A., Gimbel, W., Werner, T., Hani, J., Ernst, D. and Sandermann, H. (1997). Molecular cloning of ozone-inducible protein from *Pinus sylvestris* L. with high sequence similarity to vertebrate 3-hydroxy-3-methylglutaryl-CoA- synthase. *Biochimica Biophysica Acta,* **1350**: 247- 252.

Whitworth, J.W. and Whitehead, E.E. (1991). Guayule natural rubber: a technical publication with emphasis on recent findings. Guayule

Administrative Management Committee and USDA Cooperative State Research Service.

Williamson, I. and Kekwick, R.G.O. (1965). The formation of 5-phosphomevalonate by mevalonate kinase in *Hevea brasiliensis*. *Biochemical Journal*, **96**: 862-871.

Wititsuwannakul, R. and Sukonrat, W. (1984). Diurnal variation of 3-hydroxy 3-methylglutaryl CoA reductase activity in the latex of *Hevea brasiliensis* and its relation to the rubber content. *Compte-Render da Colloque Exploitation Physiologie at Ameloiration de I' Hevea* Montpeller, France pp115-120.

Wititsuwannakul, R. (1986). Diurnal variation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in latex of Hevea brasiliensis and its relation to rubber content. *Cellular Molecular Life Sciences,* **42**: 44-45.

Wititsuwannkul, R., Wititsuwannkul, D. and Suwanmanee, P. (1990). 3-Hydroxy 3-methyl glutaryl coenzyme A reductase from the latex of *Hevea brasiliensis*. *Phytochemistry*, **29**: 1401-1403.

Wititsuwannakul, R., Pasitkul, P., Kanokwiroon, K. and Wititsuwannakul, D. (2008). A role for a *Hevea* latex lectin-like protein in mediating rubber particle aggregation and latex coagulation. *Phytochemistry,* **69**(2): 339-347.

Wong, R.J., McCormack, D.K. and Russell, D.W. (1982). Plastid 3-hydroxy-3-methylglutaryl coenzyme A reductase has distinctive kinetic and regulatory features: properties of the enzyme and positive phytochrome control of activity in pea seedlings. *Archives of Biochemistry and Biophysics*, **216**: 631-638.

Wycherley, P.R. (1992). The Genus *Hevea*: Botanical Aspects. In: *Natural Rubber: Biology, Cultivation and Technology*, (Eds. M.R. Sethuraj and N.M. Mathew), Elsevier, Amsterdam, pp 50-66.

Yang, Z., Park, H., Lacy, G.H. and Cramer, C.L. (1991). Differential activation of potato 3-hydroxy-3-methyl glutarylcoenzymeA reductase genes by wounding and pathogen challenge. *Plant Cell*, **3**: 397-405.

Yeang, H.Y., Cheong, K.F., Sunderasan, E., Hamzah, S. and Chew, N.P. (1996). The 14.6 kd rubber elongation factor (Hev b1) and 24 kd (Hev b3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy. *Journal of Allergy and Clinical Immunology*, **98**: 628-639.

Yusof, F. (1996). Factors affecting the formation of rubber from isopentenyl pyrophosphate in Hevea brasiliensis latex. *Ph. D. Thesis,* University of Hertfordshire, UK.

Yusof, F., Ward, M. A. and Walker, J.M. (1998). Purification and characterization of an inhibitor of rubber biosynthesis from C-serum of *Hevea brasiliensis* latex. *Journal of Rubber Research*, **1**: 95-110.

Yusof, Y., Chow, K.S., Ward, M.A. and Walker, J.M. (2000). A stimulator protein of rubber biosynthesis from *Hevea brasiliensis* latex. *Journal of Rubber Research*, **3**: 232-249.

Zhou, Y., Chan, K., Wang, T.L., Hedley, C.L., Offler, C.E. and Patrick. J.W. (2009). Intracellular sucrose communicates metabolic demand to sucrose transporters in developing pea cotyledons. *Journal of Experimental Botany*, **60**: 71-85.

Zhu, J. and Zhang, Z. (2009). Ethylene stimulation of latex production in *Hevea brasiliensis*. *Plant Signaling and Behavior*, **4**(11): 1072-1074.

Appendices

1. Media

1.1. Luria- Bertani (LB) Agar

Tryptone - 10g

Yeast Extract - 5g

NaCl - 10g

Agar - 15g

Mix the solution until dissolved with distilled water.

pH was adjusted to 7.0

Make up to 1 litre with distilled water.

Sterilize in the autoclave at 121^0C for 15 minutes.

1.2. SOB

Tryptone - 20g

Yeast Extract - 5g

NaCl - 0.5g

KCl - 186.0 mg

Mix the solution until dissolved.

pH was adjusted to 7.0

Make up to 1 litre with distilled water.

Sterilize in the autoclave at 121^0C for 15 minutes.

Once autoclaved, add 10 ml of sterile 1 M Mg^{2+}

(e.g. 10 ml of sterile 1M $MgCl_2$ or sterile 1M $MgSO_4$).

2. Buffer Solutions and Reagents

2.1. Phosphate Buffer (0.1M) pH-7

Stock Solutions:

A. 0.2 M solutions of $Na_2HPO_47H_2O$ = 53.6 litre water

B. 0.2 M solutions of $NaH_2PO_42H_2O$ = 27.8 litre water

Mix 61ml of solutions A and 39ml of solution B then dilute it with water to 200ml.

Store at room temperature.

2.2. Tris-HCl Buffer pH - 6.8

Tris Base - 60.6 g

dH_2O to 1L

pH was adjusted to 6.8

2.3. Tris-HCl Buffer pH - 8.8

Tris Base - 60.6 g

dH_2O to 1L

pH was adjusted to 8.8

2.4. Acrylamide stock solution

Acrylamide 30% - 30 g

Bisacrylamide 0.8% - 0.8 g

dH_2O to 100ml

2.5. Tris-Glycine running buffer (10x)

Tris Base - 30.3 g

Glycine - 144.1 g

dH_2O to 1L

pH ≈ 8.3

2.6. SOC Gel Loading dye (6x)

Bromophenol blue - 25mg

Xylene cyanol FF - 25mg

Sucrose - 4g

dH_2O to 10ml

List of Publications

- Krishnakumar, R., Ambily, P.K. and Jacob, J. (2009). Effect of stimulation in the stress responses in *Hevea brasiliensis*. *Journal of plantation Crops*, **37**(3): 217-220.
- Ambily, P.K., Thomas, M., Krishnakumar, R., Sathik, M.B.M. and Annamalainathan, K. (2014). Cloning and expression of *hmgr*1 gene from *Hevea brasiliensis*. *Journal of Plantation Crops,* **42**(3): 336-340.