MOLECULAR CLONING, CHARACTERIZATION AND EXPRESSION OF RUBBER ELONGATION FACTOR GENE FROM *HEVEA BRASILIENSIS* MUELL. ARG

THESIS

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DOCTOR OF PHILOSOPHY
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By

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DECLARATION

I hereby declare that this thesis entitled **"Molecular cloning, characterization and expression of rubber elongation factor gene from *Hevea brasilienis* Muell. Arg"** is an authentic record of the research work carried out by me under the supervision of Dr. A. Thulaseedharan at Rubber Research Institute of India, Kottayam. The work presented in this thesis has not been submitted earlier for any other degree or diploma at any university.

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular cloning, characterization and expression of rubber elongation factor gene from *Hevea brasiliensis* Muell. Arg"** is an authentic record of original research work carried out by Miss Priya P. at Rubber Research Institute of India, Kottayam-9 under my supervision for the award of the degree of **Doctor of Philosophy in Botany**, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been submitted for any other degree or diploma earlier.

31.3.05

(Dr. A. Thulaseedharan)

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Contents

ABBREVIATIONS

bp	:	Base pair(s)
BAP	:	6- benzylaminopurine
BSA	:	Bovine serum albumin
°C	:	Degree centigrade
CTAB	:	Hexadecyltrimethylammonium bromide
cDNA	:	Complementary DNA
Ci	:	Curie
DEPC	:	Diethyl pyrocarbonate
dNTPs	:	Deoxynucleotide triphosphates
DTT	:	Dithiothreitol
EST	:	Expressed sequence tags
EDTA	:	Ethylene diamine tetraacetic acid
FDP (FPP)	:	Farnesyl di (pyro) phosphate
g	:	Gram
GDP (GPP)	:	Geranyl di (pyro) phosphate
GGPP	:	Geranylgeranyl pyrophosphate
GST	:	Gutathione- S- transferase
Hb	:	*Hevea brasiliensis*
h	:	Hour(s)
HMGCoA	:	3-hyroxy-3-methylglutaryl Coenzyme A
IPP (IDP)	:	Isopentenyl pyro(di)phosphate
IPTG	:	Isopropylthio-β-D-galactoside
Kb	:	Kilobase
kDa	:	Kilodalton
LB broth	:	Luria-Bertani broth
Min	:	Minute(s)
ml	:	Millilitre
M	:	Mole
mM	:	Millimole
µl	:	Microlitre
µg	:	Microgram
µM	:	Micromole
MOPS	:	[N-Morpholino] propane sulphonic acid
mRNA	:	Messenger RNA
MS	:	Murashige and Skoog
mg/l	:	Milligram per litre
mg/ml	:	Milligram per millilitre
nt	:	Nucleotide(s)
NR	:	Natural Rubber
ng	:	Nanogram
NAA	:	Naphthalene acetic acid
NCBI	:	National Centre for Biotechnology Information

ORF	:	Open reading frame
OD	:	Optical density
PCR	:	Polymerase chain reaction
PVPP	:	Polyvinlypolypyrolidone
PEG	:	Polyethylene glycol
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PMSF	:	Phenyl methyl sulfonyl fluoride
REF	:	Rubber elongation factor
RT	:	Reverse transcriptase
RRII	:	Rubber Research Institute of India
RP	:	Rubber particle
SRPP	:	Small rubber particle protein
SDS	:	Sodium dodecyl sulphate
SSC	:	Sodium chloride-Sodium citrate buffer
TBE	:	Tris borate EDTA buffer
Tris	:	Tris(hydroxymethyl) aminoethane
TE	:	Tris: EDTA
U	:	Unit(s)
UTR	:	Untranslated region
v/v	:	Volume per Volume
w/v	:	Weight per Volume
X-gal	:	5- bromo-4- chloro-3-indolyl- β-D-galactoside
X-gluc	:	5- bromo-4- chloro-3-indolyl- β-D-glucuronide

CHAPTER – 1

GENERAL INTRODUCTION

1. 1. Natural Rubber-An Overview

Natural rubber (NR) present in numerous plant species commonly known as laticiferous plants has been an essential product for mankind since several centuries. Rubber is the most fascinating material, known to mankind both on account of its range of applications in everyday life including defence and civilian purposes. At the turn of the 19th century, a series of technological developments in rubber processing revolutionized the uses and applications of rubber, which vastly expanded the range of products made from it. Today, India is the fourth highest producer of Natural Rubber in the World having a total of 5.7 lakh ha under rubber cultivation (Rubber Statistical Bulletin, 2004) with a total annual production of 6.5 lakh tons. The rubber molecule consists of long chains of monomer, isoprene (C_5H_8). Despite the availability of petroleum-based synthetics, NR is highly valued because no synthetic substitute has comparable elasticity, resilience and resistance to high temperature (Davies, 1997).

1. 2. Para Rubber Tree - *Hevea brasiliensis*

Natural rubber (*cis*-1, 4-polyisoprene) is synthesized and accumulated in over 2000 species of plants, representing about 300 genera from approximately seven families (Backhaus, 1985). Rubber content may vary widely in different species, which limits their consideration as sources of NR (Raghavendra, 1991). Finally, a limited number of rubber- producing plants can be exploited and only a few species are now cultivated and have economic importance. For reasons of high yield and low impurities, *Hevea brasiliensis* (Willd. Ex A Juss) Mull. Arg. (Para rubber tree) now accounts for over 99% of NR produced and consumed. One of the main reasons for this is that a high yield of

technologically acceptable rubber can be obtained over many years by simple excision of the trunk bark of mature rubber trees made at frequent intervals.

Hevea brasiliensis Muell. Arg belongs to the family Euphorbiaceae and is a native of Amazon river basin of South America. It was introduced to tropical Asia in 1876 through Kew garden in the UK through the seeds brought from Brazil by Sir Henry Wickam (Dijkman, 1951). The tree is now grown in the tropical regions of Asia, Africa and America. However, the major share of total production comes from tropical Asia. The rubber tree is sturdy and tall. It is monoecious and it grows best at temperatures of 20-28°C with an annual rainfall of not less than 200 cm. Latex, the source of natural rubber is obtained from the bark of the rubber tree by tapping. It is a process of controlled wounding during which thin shavings of barks are removed. Rubber represents 30-50% by weight of the latex exuded by mature trees in regular tapping (Kekwick, 1989). Latex upon coagulation and further processing yields natural rubber.

1. 3. Latex and Laticifers

In *Hevea*, rubber is largely formed and stored in the bark, in rings of latex vessels or laticifers. Dickinson published the first electron micrographs of *H. brasiliensis* latex vessels and described their initiation, development, structure and contents (Andrews and Dickenson, 1961; Dickenson, 1965; 1969). The latex vessels are organized in concentric layers around the cambium and are connected by anastomoses (Dickenson, 1969), which allow withdrawal of latex at each tapping and can be exploited for several years. Observations with the electron microscope on laticifers in species from several plant families have shown that the latex is a highly specialized cytoplasm containing typical subcellular structures such as nuclei and mitochondria as well as polyisoprene or resin particles (Dickenson, 1963; 1965; 1969; Sarkany *et al.*, 1965; Sassen, 1965). In addition, *H. brasiliensis* latex contains two other sub cellular particles which are not found generally in plant cells, but which may be present in the laticifers of other plants. One of these organelles, the Lutoid particles (Homans and Van Gils, 1948; Ruinen, 1950) plays an important part in the stability and flow of latex. The second non-rubber component much less numerous than the lutoid is the Frey- Wyssling particle (Frey Wyssling, 1929). Fresh natural rubber latex derived from *H. brasiliensis* contains about 0.95% proteins of which 27.2% is in rubber fraction, 47.5% in the serum fraction and 25.3% in the bottom fraction (Tata, 1980).

Rubber particles are the more typical latex components and are the essential components in rubber biosynthesis *in vitro* (Lynen, 1969). Each rubber particle contains hundreds to thousands of

rubber molecules within its enclosing interphase. Rubber particles are globular particles in which the hydrophobic rubber polymers are surrounded by a monolayer membrane containing various kinds of lipids, proteins and other components needed for rubber biosynthesis (Hasma and Subramaniam, 1986; Hasma, 1991; 1992; Benedict *et al.*, 1990; Cornish and Backaus, 1990; Cornish, 2001; Siler *et al.*, 1997; Cornish *et al.*, 1999; Wood and Cornish, 2000). Extensive investigations have been carried out on the types of RPs obtained from centrifuged fresh natural rubber latex (Gomez and Moir, 1979) as well as on the molecular weight (MW) and molecular weight distribution (MWD) of the rubber molecules they contain (Chen, 1979; Subramaniam, 1980).

Many investigators have speculated on the role of rubber in plants. In the case of the laticiferous rubber producers, it has been suggested that the rubber may protect the plant against attack by animals. But the available evidence shows that rubber is not a reserve food and this conclusion is supported by the fact that enzymes catalysing the degradation of rubber have never been found in plants (Bonner and Galston, 1947). There is no evidence that polyisoprenes have any biological functions whatsoever and these polymers are best regarded as inert end products of metabolism.

1. 4. Rubber Biosynthesis

Natural rubber biosynthesis is a side - branch of the ubiquitous isoprenoid pathway (Chappel *et al.*, 1995). Isoprenoids represent a large family of natural compounds with a wide variety of structure and function found mainly in higher plants, but also in prokaryotes, fungi, lower plants and animals (Klenig, 1989). These isoprenoid compounds are produced via a biosynthetic pathway that leads from acetyl-CoA via mevalonate to a central intermediate isopentenyl pyrophosphate (IPP) (McGarvey and Croteau, 1995). IPP is the building block from which these compounds are derived. In *Hevea*, besides the synthesis of the usual array of isoprenoid compounds normally present in higher plants, rubber is also synthesized. In most eukaryotes, IPP for sterol biosynthesis is synthesized from three molecules of acetyl - CoA via the mevalonic acid pathway whereas the plastidic isoprenoids are synthesized via the alternate 1-deoxy xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway (Lichtenthaler, 1999; Lichtenthaler *et al.*, 1997a; 2000). This separate mevalonate- independent pathway was discovered in bacteria and plant (Lichtenthaler, 1997b; Rohmer *et al.*, 1993). Rubber biosynthesis takes place on the surface of rubber particles suspended in the latex of *H. brasiliensis* (Figure 1).

Rubber biosynthesis requires four distinct biochemical processes:

- The formation of isopentenyl diphosphate (IDP), the monomer which polymerises to produce *cis*- rubber;
- The initiation of rubber biosynthesis which refers to the production of dimethylallyl diphosphate (DMAPP) followed by the production of other initiator molecules such as geranyl diphosphate (GDP), farnesyl diphosphate (FDP), geranylgeranyl diphosphate (GGDP);
- The propagation of rubber biosynthesis, achieved by the successive addition of IDP to initiator molecules to produce *cis*- rubber;
- The termination of rubber biosynthesis, which refers to the release of rubber from the enzyme rubber transferase (Yosuf *et al.*, 2000).

IPP is formed by the condensation of three molecules of acetyl- CoA followed by reduction, phosphorylation and decarboxylation (Kush, 1994; Sterner *et al.*, 1994; Venkatachalam *et al.*, 1992). Acetyl-CoA has been shown to be the precursor of isoprenoids and *Hevea* rubber (Archer and Audley, 1967; Archer *et al.*, 1961; Weeks and Kekwick, 1965). In *Hevea*, this 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, catalysed by pyruvate dehydrogenase complex localised in the mitochondria and the transport of the acetyl-CoA via citrate to the cytoplasm. Many of the enzymes involved in the pathway have been identified in many rubber-producing plants including *Hevea*. Condensation of two molecules of acetyl-CoA results in the formation of acetoacetyl CoA (Lynen, 1969). Next stage is the production of 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) by means of a HMG-CoA synthase combining a molecule of acetoacetyl CoA and a molecule of acetyl-CoA. The reduction of HMG-CoA to mevalonate is catalyzed by HMG-CoA reductase (Wititsuwannakul *et al.*, 1986), a key regulatory enzyme of this pathway that has been extensively studied (Bach, 1986; Rodwell *et al.*, 2000). HMG-CoA reductase, a highly conserved enzyme occurring commonly in eukaryotes catalyses the rate limiting step of IPP biosynthesis in animals and possibly also in the cytosolid terpene biosynthesis in plants. 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) also reported its occurrence in the bottom fraction of centrifuged latex obtained from *Hevea* seedlings. Sipat (1982),

Figure 1. General pathway of rubber biosynthesis

Sucrose

Glycolytic pathyway

Pyruvate

Acetyl CoA

3-hydroxy-3-methylglutaryl CoA

Mevalonate (MVA)

MVA-PP

Dimethylallyl Pyrophosphate

Isopentenyl pyrophosphate

Geranyl Pyrophosphate (GPP)

Prenyl transferase (*trans*-)

IN CYTOSOL

Farnesyl Pyrophosphate (FPP)

ON RUBBER PARTICLES

Geranylgeranyl Pyrophosphate (GGPP)

Rubber particle membrane

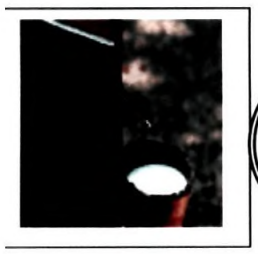
cis-Prenyl Transferase

Associated with

$OP_2O_6H_3$

REF

Give *cis*-form links



Primer in trans form

cis

RUBBER CHAIN
cis-POLY ISOPRENE

has studied the distribution of HMG-CoA reductase amongst the fractions obtained by centrifuging fresh latex. Wititsuwannakul *et al.* (1986) have described the preparation of HMG-CoA reductase from the bottom fraction of latex. The properties of HMG-CoA reductase like distribution of enzyme activity in the various fractions of latex, its cofactor requirements, thiol requirements, pH optima etc. were studied by Sipat (1985).

Mevalonate is then activated as phosphomevalonate by a cytosolic mevalonate kinase (Skilleter *et al.*, 1966). The next stage consists of fresh 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 DMAPP and then a series of prenyl transfers occurs. The enzyme isopentenyl diphosphate isomerase catalyses the interconversion of IPP to its highly electrophillic isomer, dimethylallyl diphosphate (DMAPP) (Oh *et al.*, 2000). These two isomers serve as substrates for the synthesis of isoprenoid compounds. In *Hevea* latex, IDP isomerase appears to be distributed between the serum and the surface of rubber particles (Barnard, 1965; Archer and Audley, 1987). It has also been found associated with the washed rubber particles (Lynen, 1969). Tangpakdee *et al.* (1997), reported the presence of IDP isomerase and prenyltransferase activity in the bottom and c-serum of *Hevea* latex. IDP isomerase activity has been demonstrated in the cytosol fraction of ground guayule stems and leaves (Madhavan and Benedict, 1984).

Sequential condensation of IPP with DMAPP results in the formation of a 10-carbon intermediate called geranyl diphosphate (GDP). GDP is then condensed with another IPP molecule yielding farnesyl diphosphate (FDP), a 15-carbon intermediate. Farnesyl diphosphate synthase mediates the production of FDP from DMAPP or GDP (Poulter and Rilling, 1981). FDP synthetase activity has been demonstrated in the serum from *Hevea* latex (Archer and Audley, 1987) and in the soluble fraction from ground guayule stems (Benedict, 1983) and leaves (Madhavan and Benedict, 1984). FDP synthase is a dimeric enzyme with a monomeric molecular mass of 38 kDa (Light and Dennis, 1989). Further condensation of GDP with initiator molecules result in the formation of a C_{20} molecule, the geranylgeranyl diphosphate (GGDP) using geranylgeranyl diphosphate synthase. 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. An outline of the isoprenoid biosynthesis pathway was given in

Figure 1. The successive head - to - tail condensation reactions of the five carbon intermediates catalysed by the enzyme (s), referred to as the rubber transferase (or polymerase) has been assumed to yield rubber. A protein called rubber elongation factor (REF), which is tightly bound to the rubber particles suspended in the latex is involved in the final rubber elongation step (Dennis and Light, 1989; Dennis *et al.*, 1989). REF plays an important role in rubber elongation by docking and positioning prenyltransferases on the rubber molecules. The isolation of the *H. brasiliensis* particle bound rubber transferase has not been achieved yet.

Mechanisms controlling molecular weight of rubber must exist because molecular weight of rubber in different rubber synthesizing plant species varies considerably (Archer and Audley, 1973). Many different plant species produce natural rubber, but only a few produce the high molecular weight required for high performance commercial products. The molecular weight distribution of *Hevea* rubber shows considerable variation from clone to clone (Subramaniam, 1980) and *in vitro*, the chain length of the product synthesised by a particular transferase can depend on the type of detergent (including phospholipid) present (Baba and Allen, 1980; Keenan and Allen, 1978), the structure of initiator molecule and even the Mg+ concentration (Fuji *et al.*, 1980). *In vitro* biochemical investigations carried out by Cornish (2001) have shown that a combination of factors must contribute to the molecular weight of rubber produced *in vivo*. Thus the molecular weight probably will be affected by endogenous substrate concentrations, the intrinsic properties of rubber transferases involved and by other as yet unknown endogenous factors.

1. 5. Significance of Molecular Interventions for *Hevea* Crop Improvement

Major improvements have been made over the recent years in the productivity of rubber from *Hevea* through hybridization and selection. However, subsequent yield gain achieved through hybridization during the recent breeding phases has been rather slow which has been attributed mainly to the narrow genetic diversity available to the breeders. The genetic base for the millions of rubber plantations in the east is very narrow, limited to a few seedlings originally collected from a miniscule of the genetic range in Brazil (Wycherly, 1968; Schultes, 1977). This lack of genetic diversity also leaves the crop highly susceptible to pathogenic attack and failure. Moreover, *H. brasiliensis* has strict climatic requirements, limiting its cultivation to specific tropical regions (Cornish, 2001). The perennial nature of the crop, seasonal nature of flowering, low fruit set, long breeding and selection cycle of about 30 years, the heterozygous nature of the species and lack of early prediction parameters are also serious constraints in *Hevea* breeding programmes. All this has necessitated the search for

various biotechnological as well as molecular biology techniques to enhance yield to the maximum level by the development of high yielding and disease tolerant *Hevea* cultivars. Molecular approaches hold great potential for plant breeding as it promises to expedite the time taken to develop crop varieties with desirable characteristics.

Efforts have been made worldwide to use molecular biology methods to transform crop plants by introduction of agronomically desirable traits. The development of novel biotechnological tools of direct gene transfer has added new dimension to plant improvement programmes. This new approach offers the potential to make relatively quick and specific changes in elite cultivars of *Hevea* without disrupting their otherwise desirable genetic constitution. The ability to obtain specific expression of foreign or native genes in *Hevea* opens up the possibility of improving this crop commercially by genetic manipulation. The potential limiting factor to rubber yield in *Hevea* is to be identified. This allows one to perceive some precise enzymes or proteins for which a modification of the expression of their corresponding gene may induce an increase in latex yield. Thus the prospect of developing transgenic rubber trees with enhanced latex yield through over expression of such enzymes or proteins is quite promising.

Recently, there has been a growing commercial interest in using plants as vehicles for synthesizing the enzymes used in the food industry, industrial oils, polymers and antibodies. Exciting possibilities have been predicted in using the laticiferous system of *H. brasiliensis* for producing novel plant products by introduction of specific genes into this system (Kush, 1994). The presence of all essential cytoplasmic components in latex as well as the ease with which the transformed latex can be extracted through tapping is considered as aspects of great economic advantage. A requisite for the production of transgenic plants is the availability of suitable promoters. At present, the CaMV 35S constitutive promoter is often used for transformation. As it may not be beneficial for *Hevea* plants to have foreign proteins expressed indiscriminately, it would be desirable to isolate promoters involved in latex vessel specific expression to direct the expression of foreign proteins in desired location within the plant without adversely affecting the whole plant (Arokiaraj, 2000).

Another attractive possibility will be to use transgenic technology to manipulate the rubber biosynthesis pathway, so as to bring about changes in the molecular properties of the natural rubber particle such as molecular weight and the associated protein content. Rubber molecules are produced and aggregated or packaged as rubber particles in laticifer cells of the rubber tree. Gene expression patterns in the latex are therefore of interest for the study of rubber biosynthesis. A detailed

investigation on rubber biosynthesis process at the molecular level is important as latex production involves complex mechanism. Even though a number of proteins involved in rubber biosynthesis have been characterized, the precise mechanism for the biosynthesis of rubber molecules has not yet been established. Among the rubber biosynthesis genes, REF is playing major and significant role in the rubber molecule formation.

1. 6. Objectives

In view of the above, the present work was carried out with the following objectives:

1) Molecular cloning and characterization of the genomic sequence coding for the rubber elongation factor protein from *Hevea brasiliensis*
2) Cloning and characterization of the cDNA encoding REF protein from *H. brasiliensis* and its expression in *E. coli* and in transgenic tobacco (*Nicotiana tabacum*)
3) Differential expression studies of rubber elongation factor gene in *H. brasiliensis*
4) Cloning and functional characterization of the promoter sequence of REF gene from *H. brasiliensis*.

CHAPTER – 2

MOLECULAR CLONING AND CHARACTERIZATION OF THE GENOMIC SEQUENCE CODING FOR THE RUBBER ELONGATION FACTOR PROTEIN FROM *HEVEA BRASILIENSIS*

2.1. INTRODUCTION

In *Hevea brasiliensis*, rubber biosynthesis takes place on the surface of rubber particles suspended in the latex. The surface of rubber particles contains the enzymes and other factors necessary for rubber biosynthesis (Cornish, *et al.*, 1998; Cornish *et al.*, 2000). Rubber constitutes about 90% of the dry weight or about 30% of the total weight of the latex exuded on tapping the anastomosing laticifers (Grilli *et al.*, 1980). Since latex is a specialized cytoplasmic fluid producing the highly valuable rubber molecules, much effort has been made to isolate and characterize the latex proteins involved in rubber biosynthesis. In order to identify the key enzymes involved in rubber biosynthesis, the proteins and genes associated with rubber particles have been investigated in many rubber- producing plants including *Hevea* (Dennis and Light, 1989; Light and Dennis, 1989; Attanyaka *et al.*, 1991; Goyvaerts *et al.*, 1991; Oh *et al.*, 1999; Cornish *et al.*, 1993) and guayule (Cornish and Backhaus, 1990; Benedict *et al.*, 1990; Madhavan and Benedict, 1984; Backhaus *et al.*, 1991; Backaus and Bess, 1986; Siler and Cornish, 1993; Cornish *et al.*, 1998). Rubber is synthesised by the sequential condensation of IDP to the initiating allylic diphosphates such as GDP, FDP and GGDP. Polymerisation of IPP by the prenyltransferase, assisted by REF, a protein bound to large rubber particle membrane, gives rise to long chains of *cis*- polyisoprene, with an average molecular weight ranging from 0.7 to 40 x 10^5 (Westall, 1968). The polyisoprene chains

are grouped together and form aggregates called the rubber particles, surrounded by a lipoprotein membrane.

Dennis and Light (1989) identified a low molecular weight protein bound to rubber particles of *H. brasiliensis* and named this protein as rubber elongation factor (REF). REF bound to rubber molecules can be purified from all other proteins in whole latex by treatment of rubber particles with low concentrations of detergent. REF has a molecular mass of 14,600 Da and is associated specifically with the rubber particles (Dennis *et al.*, 1989). It makes up between 10 - 60% of the total proteins in whole latex but is absent in C-serum. The amount of REF in whole latex is proportional to the rubber content (Dennis and Light, 1989). Quantitative analysis of REF and rubber in whole latex reveals a ratio of one molecule of REF to one molecule of *cis*-1,4-polyisoprene (Dennis and Light, 1989).

The primary structure of REF protein has been elucidated by Dennis and Light (1989). REF is present in stoichiometric amounts relative to the number of growing rubber molecules. Dennis *et al.* (1989) reported that presence of this protein on rubber particles is required for rubber chain elongation. REF interacts with *Hevea* prenyltransferase to alter the stereochemistry of IPP addition from the normal *trans* addition to *cis* and overrides the termination after two *trans* additions to affect the formation of *cis*- polyisoprene (Light *et al.*, 1989). Enzymatic, chemical or physical removal of REF from the rubber particles renders them incapable to accept further IPP additions during incubation with a prenyltrasferase (Dennis and Light, 1989).

Genomic sequences coding for proteins are characterized by the presence of introns interrupting the coding region that are removed from the nascent mRNA in a multistep process collectively called splicing. Including introns in the transcribed portion of a gene has been found to increase heterologous gene expression in both animal (Borenstein *et al.*, 1987) and plant systems (Callis *et al.*, 1987; Maas *et al.*, 1991; Mascarenhas *et al.*, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1989). While many homologous as well as heterologous cDNA sequences have been successfully expressed as transgenes in plants, there are some cases where the presence of introns has been shown to improve the level of transgene expression (Luehrsen *et al.*, 1994; Sinibaldi and Mettler, 1992). Koziel *et al.* (1996) have recommended the inclusion of some introns in a transgene as a means to optimize its expression in plants. It has also been proposed that inclusion of introns in the transgene construct may reduce the chances of co suppression and silencing (Baulcombe, 1996). Introns are not only important for normal gene expression, they also affect the pattern of gene

expression in plants. For example, when a 5' UTR intron was removed from a sucrose synthase (*sus4*) construct, its expression was highly reduced in potato tubers but much less in the roots (Fu *et al.*, 1995). It is further proposed that the regulatory effect of the intron is affected by the promoter and the 3' UTR elements. The stimulation of expression by introns is position dependent, although the magnitude of this effect depended on the coding sequence tested. Since it was demonstrated that the presence of introns could have a profound effect on the level of expression of genes in maize cells, introns are commonly used to increase expression of foreign genes in transgenic monocot plants.

In *H. brasiliensis*, HMG-CoA reductase, an important enzyme involved in isoprenoid pathway is encoded by a small gene family comprised of three members, *hmg 1, hmg 2* and *hmg 3*. Chye *et al.* (1991, 1992) reported the isolation and characterization of genomic sequences of the three HMG-CoA reductases from *H. brasiliensis*. Schaller *et al.* (1995) used a genomic fragment encoding one of the three 3-hydroxy-3-methylglutaryl coenzyme A reductases (HMGRs), *hmg*1 from *H. brasiliensis* to transform tobacco via *Agrobacterium* - mediated transformation to study the influence of the *hmg1* gene product on plant isoprenoid biosynthesis (Schaller *et al.*, 1995). So far there is hardly any report on the isolation and characterisation of the genomic sequence encoding REF from *H. brasiliensis*.

Despite the importance of rubber as an industrial raw material, the molecular mechanisms of rubber chain elongation and chain length determination in plants have not been studied in detail. In order to understand what determines the size of rubber in rubber producing plants that synthesize a defined molecular mass of rubber, it is of critical importance to investigate and characterise the genes involved in rubber biosynthesis in different rubber producing plant species.

The present work was carried out with the objective of isolation and characterization of the genomic sequence of REF from *H. brasiliensis*. The long-term goal is to use this REF genomic sequence to transform into *Hevea* as part of the effort to study the effect of intron sequence on its expression in transgenic *Hevea* plants. The present study contributes towards an understanding of the molecular mechanisms involved in regulating the gene expression of a key enzyme in rubber biosynthesis. The availability of the cloned gene from *Hevea* would be useful for future investigation of the regulation of rubber biosynthesis.

2.2. MATERIALS AND METHODS

2.2.1. Plant Material

Leaves for genomic DNA isolation was collected from *H. brasiliensis* clone RRII105 growing at RRII nursery. Young, expanded and light green healthy leaves were collected and used for DNA isolation.

2.2.2. PCR Amplification of REF Gene

2.2.2.1. Genomic DNA isolation

Genomic DNA was isolated from the leaf tissues of *H. brasiliensis* as described by Doyle and Doyle (1990) with modifications. The following steps were involved in genomic DNA isolation.

- The leaves were cleaned with sterile distilled water. One-gram leaf tissue was weighed into a pre-cooled mortar and frozen in liquid nitrogen. The tissue was pulverized to a fine powder using pestle.
- Ten ml CTAB extraction buffer and 1% PVPP (w/v), 1% (v/v) 2- mercaptoethanol were added to the ground tissues. Incubated in a water bath at 65° C for 30 min with occasional mixing.
- The homogenate was spun at 8000xg for 20 min at room temperature to recover the supernatant.
- The supernatant was extracted with equal volume of phenol: chloroform: Isoamyl alcohol (24: 24: 1), spun at room temperature (8000xg) for 20 min and collected the upper aqueous phase.
- The aqueous phase was treated with 5 µl of DNase free RNase A (Sigma, USA) and incubated at 37° C for 1 h in a water bath.
- This was followed by proteinase K (Sigma, USA) treatment at 50° C for 30 min in a water bath.
- Chloroform extraction was carried out and spun at 8000xg for 20 min at room temperature.
- The aqueous phase obtained after centrifugation was mixed with 0.6 volume of isopropyl alcohol for precipitation of DNA and incubated on ice for 20 min.
- Precipitated DNA was pelleted by 20 min centrifugation at 10000xg at 4 ° C.

- Finally DNA pellet was washed with 70% alcohol, dried and resuspended in TE buffer. The concentration of the isolated DNA was determined spectrophotometrically.
- The quality and concentration of the isolated DNA was also analyzed by agarose gel (0.8% w/v) electrophoresis.

CTAB Extraction buffer 2X	**RNAase A** - 10 mg/ ml stock
2% CTAB	**Proteinase K** - 20 mg/ml stock
1.4 M NaCl	**TE buffer** pH- 8.0
20 mM EDTA, (pH- 8.0)	10 mM Tris.Cl - pH- 8.0
0.1M Tris-Cl (pH- 8.0)	1 mM EDTA - pH- 8.0

2.2.2.2. Quantification of DNA

The isolated DNA was diluted with distilled water and the concentration was determined. For a cuvette with a 1- cm path length, the concentration of DNA was calculated by the following formula:

Total DNA (mg) = A_{260} x [0.040 mg/(1A_{260} x 1 ml)] x dilution factor x volume (ml).

The ratio between the readings at 260 nm and 280 nm (OD_{260}/ OD_{280}) was also determined.

2.2.2.3. Agarose gel electrophoresis

For a 100 ml gel of 0.8%, 0.8 g of agarose was weighed into a conical flask containing diluted running buffer (0.5 X TBE- pH- 8.0). The contents of the flask were mixed by swirling and boiled in a microwave oven until all the agarose was melted. The melted agarose was cooled to 50°C and ethidium bromide solution (1 mg/ml- stock) was added to give a final concentration of 5 µg / ml. The gel mixture was then poured into a clean gel casting tray and left to solidify. When the gel was solidified, the comb was removed carefully and placed the gel into the electrophoresis tank filled with running buffer (0.5 X TBE, pH- 8.0) just to cover the wells. DNA sample mixed with 5X gel loading buffer was loaded into the wells of the gel and electrophoresis was carried out. After electrophoresis, the gel was removed from the apparatus and the separated DNA was viewed under a UV transilluminator and photographed.

5X TBE Buffer, pH. 8.0	-	0.45 M Tris Borate 0.1 M EDTA
5X gel loading buffer	-	Bromophenol Blue - 0.25 %, Xylene Cyanol FF - 0.2 %, Glycerol - 30% in distilled water

2.2.2.4. PCR amplification

PCR amplification of REF gene from *H. brasiliensis* was performed with gene specific primers synthesised based on previously published cDNA of REF (Attanyaka *et al.*, 1991). The primers used for amplification were:

Forward primer, REF 1 -5' CGA TTA TGG CTG AAG ACG AAG ACA ACC - 3' Reverse primer, REF 2 -5'GGC CAA TAA TTC AAT TGG CCC TTT ATT C - 3'.

PCR cocktail was prepared in 20 µl reaction volume, which was composed of 10X buffer, 1.5 mM $MgCl_2$, 100 µM dNTPs, 0.5 U of Taq DNA polymerase, 10 ng template DNA and 250 nM of primer1 and primer 2. The reaction mixture was overlaid with mineral oil. The amplification was carried out in a thermal cycler (Perkin Elmer, USA). The PCR products were separated on 1.5% (w/v) agarose gel.

The PCR amplification conditions were:

Initial denaturation at 94° C for 4 min	- 1 Cycle
Denaturation at 94° C for 1min Annealing at 55°C for 1.30 min Extension at 72° C for 2 min	- 30 Cycles
Final extension at 72° C for 10 min	- 1 Cycle

2.2.2.5. Purification of PCR products from low melting temperature agarose gel

- The amplified products were separated on 1% (w/v) low melting temperature agarose gel.
- The slice of agarose containing the DNA band of interest was excised from the gel and transferred to a fresh sterile eppendorf tube.
- To the tube containing agarose gel slice, 5 vols of 20 mM Tris. Cl (pH-8.0) and 1 mM EDTA (pH- 8.0) was added and the tube was incubated at 65°C for 5 min to melt the agarose.
- Equal volume of Tris-buffered phenol was added to the melted agarose solution and mixed thoroughly. The contents of the tube were spun at room temperature.
- The aqueous phase was collected into a fresh sterile tube after centrifugation and re-extracted once with equal volume of chloroform.

- DNA was precipitated by the addition of 0.2 vol of 10 M ammonium acetate and 2 vol of ethanol at 4°C. After mixing thoroughly, the mixture was stored at –20°C.
- DNA was pelleted by centrifugation and washed the pellet with 70% (v/v) ethanol. Dried the DNA pellet under vacuum and resuspended in 20 µl sterile water.
- The concentration and purity of the DNA was checked by agarose gel electrophoresis.

2.2.3. Cloning of PCR Amplified REF Gene into Plasmid Vector

2.2.3.1. Digestion of plasmid DNA

The plasmid DNA, pBluescript (Stratagene, GmBH, Germany) was digested with *Eco*RV enzyme (Amersham, UK).

The following components were taken into an Eppendorf tube

Plasmid DNA in TE	- 10 µg
10X Restriction enzyme buffer	- 10 µl
Restriction enzyme, *Eco*RV	- 10 U
Water to make up	- 100µl

The components were mixed thoroughly and incubated at 37°C for 3 h.

2.2.3.2. Purification of restriction digests

After completion of digestion, the digested sample was extracted with equal volume of phenol and centrifuged at 8000xg for 15 min at room temperature. The upper aqueous phase was collected and extracted with equal volume of chloroform. DNA was precipitated by adding 2 volumes of ethanol to the aqueous phase and spun at 8000xg for 20 min at 4 °C. The DNA pellet was washed with 70% (v/v) ethanol. Then the DNA pellet was dissolved in sterile distilled water after drying. The concentration of DNA was then checked on 0.8% (w/v) agarose gel and was dephosphorylated.

2.2.3.3. Dephosphorylation of linearized vector DNA

Dephosphorylation of linearized vector DNA was carried out using shrimp alkaline phosphatase enzyme (SAP) (Amersham, UK). Reaction mixture was prepared in a sterile tube as follows:

Linearized vector DNA (100 ng/µl)	- 5 µl
10 X SAP buffer	- 1 µl
Shrimp alkaline phosphatase enzyme	- 1 U
Sterile water to	- 10 µl

The components were mixed and incubated at 37°C, for 30 min in a water bath.

At the end of the incubation period, the reaction was terminated by heating the reaction mixture at 75°C for 10 min in the presence of 5 mM EDTA (pH-8.0) and then purified by phenol: chloroform extraction followed by alcohol precipitation of DNA.

2.2.3.4. Ligation of REF gene into plasmid

Ligation mixture was prepared as follows:

Components	Reaction volume
10X ligase buffer	- 1 µl
pBS vector (50 ng)	- 1 µl
PCR product (50 ng)	- 1 µl
T_4 DNA ligase (Amersham)	- 1 µl
Water to a final volume of	- 10µl

The above said reaction components were taken in microfuge tube, mixed properly and the reaction mixture was incubated at 16°C overnight in a water bath.

2.2.3.5. Preparation of competent cells

Fresh competent bacterial cells were prepared with calcium chloride according to the modified method of Cohen *et al.* (1972). DH5α strain of *Escherichia coli* was used as the host. Using a sterile platinum wire loop, *E. coli* cells taken directly from a frozen stock was streaked onto the surface of LB agar plate incubated at 37°C overnight.

Luria Bertani Medium (LB)	
Bacto- tryptone	- 10 g/L
Yeast extract	- 5 g/L
NaCl	- 10 g/L
Bacto- agar	- 15 g/L
pH	- 7.0

Competent cells were prepared as follows:

- ➔ A single colony was aseptically transferred from the freshly grown plate into 10 ml of LB broth and incubated overnight at 37°C with agitation (200 rpm).
- ➔ Inoculated 500 µl of the overnight culture into 20 ml of fresh LB broth and incubated at 37°C with vigorous shaking until the OD_{600} reached 0.3 - 0.5 (about 2 - 2.5 hours).
- ➔ The cells were then transferred to sterile ice-cold polypropylene tubes and stored the tubes on ice for 10 min.
- ➔ The cells were harvested by centrifugation for 5 min at 2000xg at 4°C.

- ➔ After decanting the media completely, the pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ solution (freshly diluted from 1M $CaCl_2$ stock solution) and stored the cells on ice for 20 min.
- ➔ The cells were harvested by centrifuging at 2000xg for 10 min at 4°C and the $CaCl_2$ solution was decanted completely from the cell pellet.
- ➔ The pellet was again resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$.
- ➔ The cell suspension was dispensed (200 µl) into sterile eppendorf tubes and immediately used for transformation.

2.2.3.6. Transformation

- ➔ The competent cells were thawed and placed on ice immediately.
- ➔ The ligation mix (3 µl) was added to 200 µl of competent cells and mixed gently.
- ➔ Incubated the reaction mixture on ice for 15 - 20 minutes.
- ➔ The transformed cells were heat shocked at 42°C for 90 seconds and returned to ice for 1 minute.
- ➔ LB broth (800 µl) was added to the heat shocked cells and incubated with agitation at 37°C for one hour.
- ➔ Transformed cells were plated on the LB agar plates with ampicillin (50 µg/ml) and for blue - white screening of the transformants, IPTG (for the induction of the lac operon) and X- Gal were also added to the LB plate.
- ➔ Incubated at 37°C overnight for the appearance of colonies.

X-gal - 20 mg/ml (Stock solution), 35 µl/Plate IPTG - 200 mg/ml (Stock solution), 20 µl/Plate

2.2.3.7. Confirmation of REF gene cloning

2.2.3.7.1. *Plasmid preparation*

White colonies that developed on LB agar plate were selected for confirmation studies. Plasmid DNA was isolated from the recombinant white colonies by alkaline lysis method (Birnboim and Dolly, 1979).

- ❖ Individual bacterial colonies were picked using sterile toothpicks and inoculated into 3 ml LB medium containing ampicillin (50 µg/ml) in 15 ml tubes. Incubated at 37°C with vigorous agitation overnight.

- The cells were pelleted by centrifugation at 12,000xg for 5 min at 4°C and the medium was decanted off completely from the pellet.
- Resuspended the bacterial pellet in 200 µl of solution I by vigorous vortexing and the tubes were stored on ice.
- Solution II (300 µl) was added to the tubes and invert mixed five times rapidly and stored the tubes at room temperature.
- Solution III (300 µl) was then added to the tubes and the contents were vortexed gently to disperse the solution through the viscous bacterial lysate. The tubes were then returned to ice for 3-5 min.
- Centrifuged at 12000xg for 5 min at 4°C to collect the clear supernatant into a fresh tube.
- An equal volume of phenol: chloroform was added to the collected supernatant and centrifuged at 8000xg for 10 min at room temperature to collect the upper aqueous phase.
- Plasmid DNA was then precipitated by the addition of 2 vol of ethanol and spun at 8000xg for 10 min at room temperature.
- DNA pellet was washed with 70% alcohol (v/v), dried the pellet and dissolved in sterile distilled water.
- The isolated plasmid DNA was then checked on 0.8% (w/v) agarose gel. Super coiled, uncut pBS vector was also loaded into the gel as control to differentiate from the recombinant plasmid with cloned gene.

Solution I	Solution II	Solution III
50 mM glucose 25 mM Tris.Cl (pH- 8.0) 10 mM EDTA (pH-8.0) Autoclaved at 10 lb/sq. Stored at 4°C	0.2 NaOH (Freshly diluted from 10 N stock) 1% SDS	5 M potassium acetate - 60 ml Glacial acetic acid -11.5 ml Distilled water - 28.5 ml

2.2.3.7.2. *Restriction analysis of recombinant plasmid DNA*

Selected white colonies were grown in 3 ml cultures and the isolated plasmids were subjected to double digestion with *Eco*RI and *Hind*III to release the cloned insert DNA if present. Digestion was performed as described below.

Plasmid DNA	- 3 µg
*Eco*RI enzyme	- 3 U
*Hind*III enzyme	- 3 U
Restriction enzyme buffer (10X)	- 1 µl
Sterile distilled water upto	- 10µl

Plasmid DNA without insert linearized with *Eco*RI enzyme was used as a negative control. Digested DNA samples were separated on 0.8 % (w/v) agarose gel.

2.2.3.7.3. *Amplification of cloned gene by PCR*

The recombinant plasmids (with insert) isolated from white colonies were further screened by PCR. Plasmid DNA without insert was used as a negative control. The amplification was carried out using REF gene specific primers. The conditions for PCR amplification were similar as described above (section **2.2.2.4**). But the number of cycle was reduced to 25. After amplification, the PCR products were analyzed by 1.5 % (w/v) agarose gel electrophoresis.

2.2.4. Nucleotide Sequencing and Analysis

The plasmid DNA isolated through alkaline lysis procedure was purified by PEG precipitation for sequencing purpose. To 32 µl of plasmid DNA, 8 µl of 4 M NaCl was added followed by 40 µl of 13% PEG_{8000}. The sample was incubated for 20 min on ice for precipitation of plasmid DNA and pelleted the DNA by centrifugation at 10000xg for 20 min at 4°C after through mixing. Finally DNA pellet was rinsed with 70% (v/v) ethanol and the dried pellet was dissolved in 20 µl sterile distilled water.

The nucleotide sequence of the cloned DNA fragment was determined using the automated sequencing facility at Indian Institute of Science, Bangalore. The genomic DNA sequence of REF was edited to discard the vector sequences at either ends and compared with published sequences in the NCBI database using BLASTN programmes (Altschul *et al.*, 1990). Sequence alignment and comparison was made using the ClustalW program (Thompson *et al.*, 1994). Signal peptides and protein localization sites were predicted using signalP and pSORT programs.

2.2.5. Southern Blot Analysis

2.2.5.1. Restriction digestion of genomic DNA

For Southern blot analysis, 10 µg of genomic DNA isolated from leaves of *H. brasiliensis* (clone RRII 105) was digested with various restriction enzymes *Hind*III, *Eco*RI, *Eco*RV *and Xba*I

(Amersham, UK). The following components were taken into an eppendorf tube and incubated at 37°C for 4 h.

DNA in TE	- 10 µg
10X Restriction enzyme buffer	- 10 µl
Each restriction enzymes,	- 10 U
Water to make up to	- 100µl

2.2.5.2. Preparation of gel for blotting

Completely digested DNA samples were size fractionated on 1% (w/v) agarose gel. Gel casting and electrophoresis was described in previous sections (2.2.2.3). After electrophoresis, the gel was placed in a glass tray and washed with distilled water with gentle agitation. The gel was then subjected sequentially to depurination, denaturation and neutralization treatments. For depurination, gel was soaked for 10-15 min at room temperature in 0.25 M HCl. The gel was washed with distilled water and then soaked in denaturation solution (1.5 M NaCl / 0.5 M NaOH) for 45 min. The denatured gel was treated with neutralization solution (1.5 M NaCl / 0.5 M Tris Cl, pH- 7.0) for 45 min before blotting. Neutralized gel was washed with distilled water and then blotted.

Reagents	Composition
100x Denhardt's Solution	2% (w/v) BSA (Sigma Fraction V) 2% (w/v) Ficol (400,000 Mw) 2% (w/v) Polyvinylpyrrolidone (40,000 Mw)
20 X SSC	3 M NaCl 0.3 M Sodium citrate (pH- 7.0)
Pre-hybridization Solution	6X SSC 5X Denhardt's solution 0.5 % w/v SDS 100 µg/ ml salmon sperm DNA

2.2.5.3. Blot assembly

For assembling the blot, a glass tray was filled with transfer solution. A plastic platform was placed in the tray. A wick of whatman 3MM filter paper was kept on the platform, so that each end was dipped in the transfer solution. The gel was placed with the sample well face down on the wick. Nylon membrane (Hybond N+, Amersham, UK) was cut to the exact size of the gel and wetted with transfer solution (10X SSC). Nylon membrane was placed on top of the gel. Two pre-

wetted sheets of 3MM filter paper were placed on the top of the membrane. A pile of paper towels was kept on top of the filter paper. Weight was placed on the glass plate kept above the pile of papers. The blot assembly was left undisturbed overnight. Then the pile of paper towels and 3MM whatman filter papers were gently removed. The membrane was carefully lifted along with the gel. The position of wells was marked on the membrane using a soft lead pencil. The gel was gently peeled off and discarded. Membrane was then placed on a 3MM whatman filter paper and UV cross-linked. The nylon membrane was placed in a hybridization bottle and soaked in pre-hybridization solution. The membrane was incubated for 3 h at 65°C with gentle rotation.

2.2.5.4. Synthesis of labeled probe

PCR amplified 1.3 kb REF gene fragment radiolabeled with [α-^{32}P dCTP] (Megaprime DNA labeling systems, Amersham, UK) was used as probe for hybridization. The DNA to be labeled was diluted to a concentration of 10 ng/ µl in TE buffer.

The reaction mixture was prepared as follows :

Template DNA	: 25 ng
Primers	: 5 µl
Water to a volume of	: 50 µl

All the components were gently mixed and denatured by heating to 95- 100°C for 5 min in a boiling water bath. Then chilled on ice.

To this denatured mixture, the following components were added.

Unlabeled dNTPs (250µM)	: 4 µl each (dATP, dGTP and dTTP)
Reaction buffer (10X)	: 5 µl
[α-^{32}PCTP], 3000 Ci/m mol	: 5 µl
Klenow fragment	: 2.5 µl

Incubated the reaction mixture at 37°C for 10 min. After incubation, the reaction was stopped by the addition of 5 µl of 0.2 M EDTA. Before, hybridization, the labeled probe DNA was purified by spun column.

2.2.5.5. Purification of labeled DNA by spun column

- The bottom of a 1 ml disposable syringe was plugged with a small amount of sterile glass wool. The syringe was filled with sephadex G- 50 equilibrated in 1X TEN buffer (pH- 8.0)

1X TEN buffer- pH-8.0	10	mM	Tris.Cl	pH-8.0
	1	mM	EDTA	pH- 8.0
	100	mM	NaCl	

- Inserted the syringe into a 15 ml disposable plastic tube and centrifuged at 1600xg for 4 min at room temperature in a swinging bucket rotor.
- TEN buffer (0.1 ml) was applied to the column and centrifuged as before. Centrifugation was repeated twice as described above.
- Radiolabeled probe DNA to be purified was applied to the center of the column and placed it in a fresh disposable 15 ml tube containing a decapped microcentrifuge tube.
- Centrifuged and collected the effluent, which contained the DNA into the decapped microcentrifuge tube.
- The radioactivity was measured by a mini- monitor.

2.2.5.6. Hybridization and autoradiography

The purified probe was denatured by heating to 100 °C for 5 min, cooled and was added directly to the pre-hybridization solution and hybridization was performed at 65°C for 20 h under the same conditions as pre-hybridization. After hybridization, the filter was first washed in 2X SSC containing 0.1% SDS at 65°C for 15 min, twice at room temperature, followed by washes of 0.2X SSC or 0.2X SSC containing 0.1% SDS at 65°C for 15 min twice. After discarding the wash buffer, the filter was placed on a sheet of saran wrap and exposed to X-ray film (Kodak X-Omat) with intensifying screen to obtain autoradiographic image for about 16-24 h at –80 °C.

2.3. RESULTS

2.3.1. Genomic DNA Isolation and PCR Amplification of REF Gene

The genomic sequence coding for REF protein of *H. brasiliensis* was amplified by PCR using REF specific primers. Genomic DNA was isolated from the leaves of *H. brasiliensis*. The concentration of the isolated DNA was determined spectrophotometrically and 45 µg of DNA / g leaf tissue was obtained. The isolated genomic DNA was also analysed by agarose gel electrophoresis and it migrated through the gel as a single band (Figure 1). The genomic DNA was diluted and used for PCR amplification. PCR was performed with REF specific oligonucleotide primers synthesised based on previously published cDNA sequence of REF from *H. brasiliensis* (Attanyaka *et al.*, 1991).

Various PCR parameters like template DNA concentration, annealing temperature, concentration of $MgCl_2$, Taq DNA polymerase concentration and number of cycles were optimized for the specific amplification of REF gene. After PCR amplification, the PCR products were analyzed on 1.5% (w/v) agarose gel. Different annealing temperatures (45°C to 55°C) as well as $MgCl_2$ concentrations (1 mM to 4.5mM) were also tried. Raising the concentration of $MgCl_2$ from 1.5 mM to 4.5 mM in the reaction mixture significantly decreased the yield of PCR amplification and a concentration of 1.5 mM proved optimal. Enhancing the concentration of Taq DNA polymerase from 0.5 units to 2 units increased the number of bands and 0.5 units was found to be the optimum concentration for PCR amplification. Two amplicons approximately of 0.7 Kb and 1.3 Kb were visualized when 50°C and 55°C were tried for primer annealing in the PCR with 40 cycles (Figure 2 A). Further when the number of cycles was reduced to 30, in the PCR at 55°C, the 700 bp band disappeared and only the 1.3 Kb band was amplified as shown in Figure 2B. No discrete bands were observed at 45°C primer annealing temperature, probably because of nonspecific oligonucleotide primer hybridization due to high nucleotide homology between and within the REF gene. DNA concentrations of 10 ng to 25 ng were tried for PCR and 10 ng was found to be the optimum concentration for best PCR amplification. When 25 ng DNA was used for PCR, a streaking or smear like pattern was noticed along with PCR products. After standardization of PCR parameters, a single PCR product of 1.3 Kb size was obtained (Figure 2B). The amplified 1.3 Kb REF genomic fragment was gel purified and used for further studies.

2.3.2. Cloning of REF Gene

PCR amplified 1.3 Kb DNA fragment was cloned into *Eco*RV site of pBluescript vector. The vector (pBS) DNA was linearized with *Eco*RV enzyme and then purified by phenol: chloroform extraction. Digested vector DNA was subjected to dephosphorylation with SAP enzyme to avoid self-ligation of plasmid DNA during ligation. Purified PCR amplified REF gene fragment was ligated to linearized and dephosphorylated plasmid DNA and then transformed into competent DH5α cells. The transformed cells were plated on LB agar plates containing ampicillin. The plates also contained X- gal and IPTG for blue white screening through α complementation. About 150 white colonies or putative recombinants and a few blue colonies (non-recombinant) were developed on plates.

Plasmid DNA was isolated from the cultures of recombinant white colonies and analyzed by agarose gel electrophoresis. Undigested vector DNA without insert was loaded onto the gel as negative control. A number of plasmids from the selected white colonies showed the presence of

Figure 1

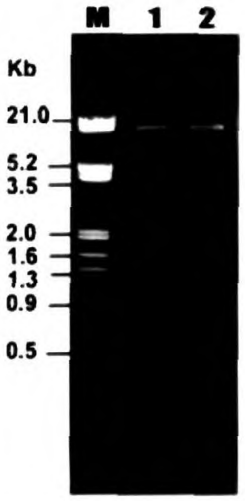


Figure 2A

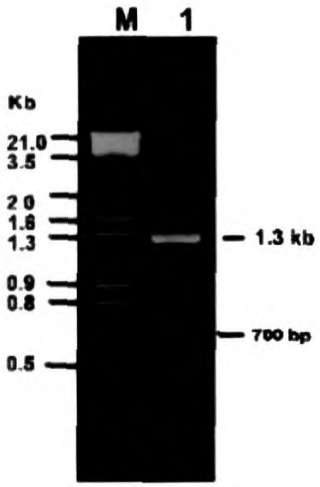


Figure 2B

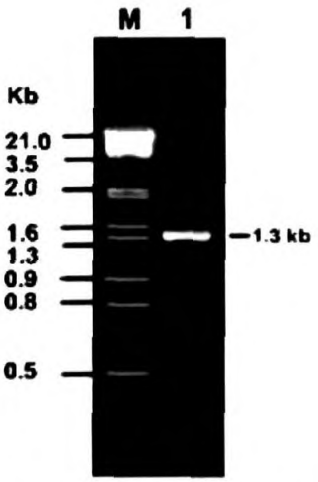


Figure 1. Genomic DNA isolated from the leaf tissues of *H. brasiliensis* . Lane M- DNA molecular weight markers, lane 1&2 - Genomic DNA isolated from leaf.

Figure 2A. PCR amplification of genomic sequence of REF from DNA of *H. brasiliensis* with REF specific primers. PCR products were analyzed by agarose gel electrophoresis. Lanes : M- molecular weight markers. Lane 1- PCR done at annealing temperature 50°C for 40 cycles.

Figure 2B - PCR amplification of genomic sequence of REF from DNA of *H. brasiliensis* with REF specific primers. PCR done at 55 °C and for 30 cycles. Lanes: M- molecular weight markers, Lane 1- PCR amplified 1..3 Kb REF gene fragment

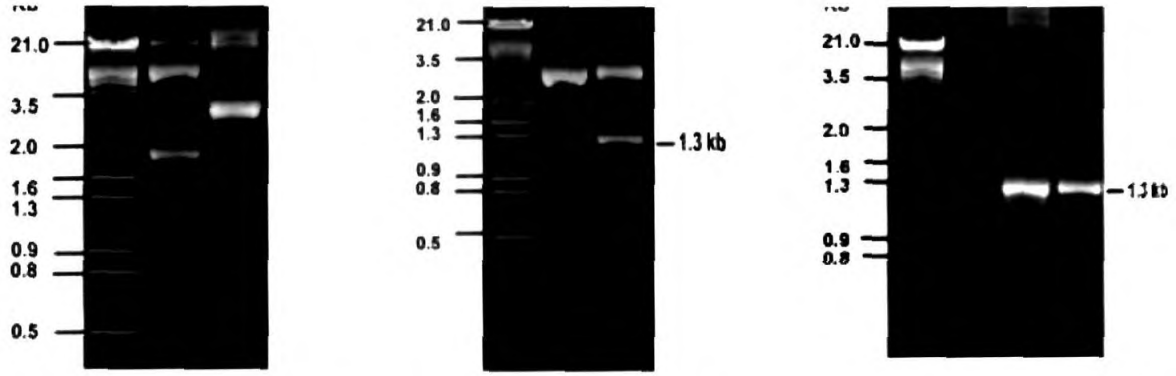


Figure 3. Cloning of REF gene into plasmid vector. Lanes: M- DNA molecular weight markers, 1- Supercoiled plasmid DNA without cloned insert DNA, 2- Plasmid DNA with cloned REF gene insert.

Figure 4. Restriction analysis of plasmid DNA. Lanes M- DNA molecular weight marker, 1- Plasmid DNA without any insert digested with *Eco*RI enzyme, 2- Plasmid DNA with cloned REF gene digested with *Eco* RI and *Hind* III enzymes to release the 1.3 Kb cloned REF gene insert.

Figure 5. PCR confirmation of cloning of REF gene into plasmid vector with REF specific primers to amplify the cloned REF gene. Lanes: M- DNA molecular weight marker, 1 - Plasmid DNA without any insert as template showing no amplification, 2 & 3- Plasmid DNA with cloned REF gene as template showing amplification of 1.3 Kb REF gene.

insert. Presence of insert in the vector resulted in an increase in molecular weight of the vector and they migrated slowly through the gel (Figure 3). In contrast, the control which was devoid of REF gene insert was unchanged in its molecular weight and migrated rapidly through the gel as shown in Figure 3 (Lane 2). The selected plasmids were subjected to further screening.

The selected plasmids which contained the cloned REF gene were further analysed by double digestion with *Hind*III and *Eco*RI enzymes to release the cloned 1.3 Kb REF gene fragment from the vector. Control vector without insert was digested with *Eco*RI enzyme to linearize it. The single and double digests were analyzed on 0.8% (w/v) agarose gel. Double digestion resulted in the cleavage of the vector on either side of the insert releasing the 1.3 Kb REF gene insert from the pBS vector harbouring it (Figure 4). This confirmed the success of cloning of REF gene.

Further confirmation of the presence of cloned REF gene was carried out by PCR (Figure 5). The recombinant plasmids were amplified with REF gene specific primers. Vector DNA without insert was included as negative control. The amplified products were electrophoresed on 1.5% (w/v) agarose gel. Results of electrophoresis showed that only recombinant plasmids with cloned REF gene insert amplified the 1.3 Kb REF gene fragment and no amplification was detected in the negative control (Figure 5). The cloned 1.3 Kb PCR amplified REF gene was then sequenced.

2.3.3. Sequence Characterisation of REF Gene

The nucleotide sequence of the cloned 1.3 Kb REF gene was elucidated. The nucleotide sequence and the deduced amino acid sequence were presented in Figure 6. The sequence length of REF gene was revealed to be 1367 bp with an ORF of 414 bp (without stop codon) encoding for a single polypeptide of 138 amino acid residues. The molecular mass of the predicted protein is 14,700 Da and the deduced protein is acidic with an isoelectric point of 5.04 (pI – 5.04). A computer analysis for protein localization sites suggested that REF is localized in the cytoplasm. The SignalP program predicted absence of a signal peptide. The nucleotide sequence of REF was aligned with previously published cDNA sequence of REF. Alignment showed that the open reading frame of 414 bp is interrupted by 2 introns. A perfect homology was noticed in the coding region between the cDNA and genomic sequence of REF. REF gene comprised of 2 introns and 3 exons. In the amplified REF gene, the introns of 298 bases and 455 bp long occur after nucleotide positions at 36-333 and 550-1004 respectively. The last or the second intron is the largest. All introns of REF gene contain splice sites consistent with the consensus sequence 5' GT——AG 3' (Hanley and Schuler, 1988). The three exons of sizes 30 bp, 216 bp and 171 bp are located at positions 6-35, 334-549 and 1005-1175

respectively. The first exon is 10 amino acids in length, the second code for 72 amino acids and is the largest. The third exon codes for 56 amino acids. The translation start site or ATG is located at 6 nt position. The sequence surrounding the ATG initiation codon (Lutcke *et al.*, 1987) partially fits with the plant consensus sequence. A translation termination codon (TGA) is identified at 1173 bp position. The coding region is flanked by 5 bp and 192 bp of untranslated sequences at the 5' and 3'region respectively. The sequence of REF gene cloned in this study is available from the DDBJ/GeneBank/ EMBL database under Accession no: AY299405 .

Comparison of the nucleotide sequence of REF and the predicted amino acid sequence against other known sequences in the GenBank, EMBL, Swiss Prot database were conducted using the appropriate BLAST programmes. These searches revealed that REF nucleotide sequence has some degree of homology to the cDNA sequence of *Hevea* REF- like stress related protein1, cDNA of isoform of REF from *H. brasiliensis* and SRPP gene sequences besides homology to REF cDNA. The deduced amino acid sequence of REF was aligned with other related sequences from database (Figure 7). The predicted amino acid sequence of REF shares some degree of homology with those of the isoform of REF from *H brasiliensis* (51% identity, Accession no. AY430052) and *Hevea* REF like stress related protein-1 (51% identity, Accession no. AY221988). REF protein also show similarity to *Hevea* small rubber particle protein (42% identity, Accession no. AF051317) and to *Hevea* REF like stress related protein-2 (39 % identity, Accession no. AY221989). Multiple sequence alignment of the deduced amino acid sequence of REF with other related sequences was shown in Figure 7.

2.3.4. DNA Blot Analysis

To determine the copy number of REF gene, Southern blot hybridization analysis was performed. Radiolabeled 1.3 Kb PCR amplified fragment of REF was used as hybridization probe. *H. brasiliensis* genomic DNA was isolated from leaf and digested with the restriction enzymes, *Hind*III, *Eco*RI, *Eco*RV and *Xba*I. Digested DNA was separated on 1% (w/v) agarose gel and transferred to nylon membrane. Specific hybridizing signals confirmed the identity of the REF gene with *H. brasiliensis* genome. The hybridization pattern showed 2-3 major hybridizing bands with digested DNA samples. Two bands each were obtained in the case of *Hind*III, *Eco*RI, *Xba*I digested DNA under high stringency conditions (Figure 8). Under the same conditions, in the case of *Eco*RV digested DNA, the REF gene probe hybridized to three bands as given in the Figure 8, Lane 3. The

Figure 6. Nucleotide and deduced amino acid sequence of 1.3 kb genomic DNA fragment encoding HbREF. 5'and 3' UTR regions were marked in green colour, Introns in black colour, Exons in blue colour and splice donor (GT) and splice accepter (AG) were highlighted. The translated amino acid sequence was shown in single letter code below the exon sequences. The termination codon was marked with an astrisk.

```
SRPP            MAEEVE-----------------------------------------EERLKYLDF 15
HbRLP2          MAEGKENENFQQEAN--------------------------------EQEEKLKYLEF 26
ISOREF          MAEGEEEVNIQEEANKGEENPQEEANIQEETNKGEENIQEEANIEEEANKEEESLKYLDF 60
HbRLP1          MAEGEEEVNIQEEANKGEENPQEEANIREETNKGEANIQEEANIQEEANKEEESLKYLDF 60
HbREF           MAEDED-------------NQQGQG---------------------------EGLKYLGF 20
                ***  :                                               * **** *
SRPP            VRAAGVYAVDSFSTLYLYAKDISGPLKPGVDTIENVVKTVVTPVY----YIPLEAVKFVD 71
HbRLP2          VQATTDNAVTALSNIYLYAKDNSGPLKPGVETIEGVAKTVVIPAS----KIPTEAIKFAD 82
ISOREF          VQAATVYARASFSKLYLFAKDKSGPFKPGVNTVESRFKSVVRPVYNKFQPVPNKVLKFAD 120
HbRLP1          VQAATLYARASFSKLYLFAKDKSGPFKPGVNTVESRFKNVVRPVYNKFQPVPNKVLKFAD 120
HbREF           VQDAATYAVTTFSNVYLFAKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGALKFVD 80
                *: :   *  ::*.:**:*** ***::***: :*.  *.*. *       :*  .:**.*
SRPP            KTVDVSVTSLDGVVPPVIKQVSAQTYSVAQDAPRIVLDVASSVFNTGVQEGAKALYANLE 131
HbRLP2          RAVDASFTTLQNIVPSVLKQLPTQAC------------------DTSVKESAE------- 117
ISOREF          RRVDAYVTVLDRIVPPIVKRASIQAYS---VAPGAALAVASY-LPLHTKRLSKVLYGDG- 175
HbRLP1          RRVDAYVTVLDRIVPPIVKRASIQAYS---VAPGAASAVASY-LPLHTKRLSKVLYGDG- 175
HbREF           STVVASVTIIDRSLPPIVKDASIQVVSAIRAAPEAARSLASS-LPGQTKILAKVFYGEN- 138
                  *  .* ::  :*.::*  . *.                    .:  ::
SRPP            PKAEQYAVITWRALNKLPLVPQVANVVVPTAVYFSEKYNDVVRGTTEQGYRVSSYLPLLP 191
HbRLP2          ------------------------------------------------------------
ISOREF          ------------------------------------------------------------
HbRLP1          ------------------------------------------------------------
HbREF           ------------------------------------------------------------

SRPP            TEKITKVFGDEAS 204
HbRLP2          -------------
ISOREF          -------------
HbRLP1          -------------
HbREF           -------------
```

Figure 7. Multiple alignment of predicted aminoacid sequence of HbREF gene with Hb SRPP (Accession no AF051317), Hb isoform of REF (Accession no. AY430052), Hb stress related REF like protein 1 (Accession no. AY221988), Hb stress related REF like protein 2 (AY221989)

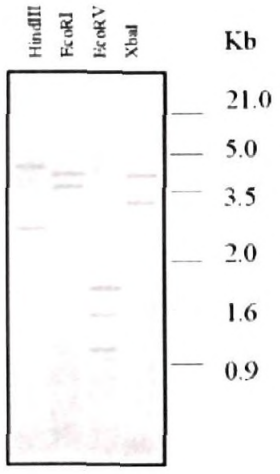


Figure 8. DNA gel blot hybridization of genomic DNA. Equal amounts of genomic DNA (10μg) digested with *Hind* III (lane 1), *Eco* RI (lane 2), *Eco* RV (lane 3) and *Xba* I (lane 4) were probed with radiolabeled REF gene. DNA molecular mass markers are indicated on the left.

results of DNA blot analysis supports the existence of at least two REF genes in the genome of *H. brasiliensis*.

2.4. DISCUSSION

2.4.1. PCR Amplification and Cloning of REF Gene

Rubber elongation factor is associated with the large rubber particles in the latex of *H. brasiliensis* in a ratio of one REF to one rubber molecule. This most abundant protein of the rubber particle plays a significant role in rubber polymerization (Dennis *et al.*, 1989). As a first approach to investigate the role of REF in rubber biosynthesis, the molecular characterization of the genomic sequences coding for REF protein of *H. brasiliensis* was undertaken. The cloning strategy for isolating REF gene from *H. brasiliensis* relied on PCR technique. Using PCR technique, the specific gene can be amplified more efficiently and less expensively. Genomic DNA isolated from leaves of *H. brasiliensis* was used as template for PCR amplification. REF gene specific primers were designed corresponding to the cDNA sequence of REF published in the database. The main disadvantage of the PCR is the sensitivity of DNA amplification to PCR conditions. The PCR parameters are very critical to amplify DNA fragments by PCR. Similar observations were also reported by Muraleedharan and Wakeland (1993) and Park and Kohel (1994). Therefore, various PCR parameters like DNA concentration, $MgCl_2$ concentration, Taq DNA polymerase concentration, annealing temperature and number of cycles were standardized for the specific amplification of REF gene. After extensive standardization of the optimum PCR conditions, a 1.3 Kb fragment of REF gene was amplified from the genomic DNA of *H. brasiliensis*. The amplified 1.3 Kb gene fragment was eluted from the gel and cloned into the pBluescript plasmid vector.

2.4.2. Sequence Characterization of REF Gene

The nucleotide sequence of REF gene was determined. The cloned REF gene was 1367 bp in length and an open reading frame of 414 bp encodes for a protein of 138 amino acids without any mismatches. The coding region of the cloned REF gene corresponds to the REF cDNA isolated previously (Attanyaka *et al.*, 1991 and Goyvaerts *et al.*, 1991). The deduced amino acid sequence of REF is identical to the primary protein sequence of REF determined by Dennis *et al.* (1989) and confirms that isolated REF gene does indeed encode REF protein. Alignment of cloned REF gene with the REF cDNA sequence reveals the presence of two introns in the coding sequence of REF gene. Introns can be located within the translated sequence or they may be present in the 5' and 3'

UTRs. The number of introns varies widely within different genes of the same species and within the same gene in different species (Minocha, 2000). Typically, the number of introns may be less than 10, however, some plant genes have been known to contain as many as 31 introns (Roesler *et al.*, 1994). The size of the introns is also quite variable, ranging from about 70 bases to as much as 7 Kb. A typical intron is 100-200 bases long (Filipowicz *et al.*, 1995, Simpson and Filipowicz, 1996). In the REF gene the two introns are 298 nt and 455 nt long. Most plant introns have conserved AG/GT sequences at the 5' and 3' splice sites: the /GT at the 5' end and the AG/ at the 3' end are conserved in nearly 100 % of all dicot introns, but the other bases are more variable (Filipowicz *et al.*, 1995). Both the introns in the REF gene contain AG/GT consensus at both splice sites (3' and 5'). While most dicot introns are highly AT- rich (60-70 % AT), monocot introns are less so (as low as 30-35% AT). The two REF introns contain 69% A+T, well within the range of compositions found in dicot introns.

There is no discernable signal peptide at the amino terminus in the deduced REF protein suggesting of its cytoplasmic localization in *Hevea*. Sequence analysis and comparison of the deduced amino acid sequence of REF with other sequences in the database reveals homology to other proteins reported from *H. brasiliensis*. The highest level of homology is with the isoform of REF (51% identity) and with the REF like stress related protein-1 from *H. brasiliensis* (51%). In addition, it also share significant homology to the small rubber particle protein (SRPP) from *H. brasiliensis* (42%) and 39% sequence identity to the *Hevea* REF like stress related protein-2.

2.4.3. Southern Blot Analysis

A DNA gel blot of *H. brasiliensis* genomic DNA digests was probed with a radiolabeled REF gene fragment. At high stringency conditions, the probe hybridized to 2-3 fragments. The results of DNA blot analysis indicate that REF is represented by a small gene family consisting of at least 2 members in the *Hevea* genome.

In *H. brasiliensis*, about a quarter of the total latex protein are in the rubber cream that comprises mainly the rubber particles. Also the amount of proteins associated with the rubber particles varies tremendously between species and the number of different proteins is remarkably different. The most complicated rubber particles currently known are those of *H. brasiliensis*, which produces high molecular weight rubber. These contain about 80 different proteins, but the rubber particles from other plant species contain far fewer proteins (Cornish, 2001). Among many latex associated proteins in *H. brasiliensis*, two main proteins associated with the rubber particles are REF (14.6 kDa) (Dennis and Light, 1989) with large rubber particles and SRPP (24 kDa) with small rubber particles. It has been

established that the SRPP has high amino acid sequence homology to the REF and plays a positive role in IDP incorporation into high molecular weight polymers (Oh *et al.*, 1999). Light and coworkers identified a soluble prenyltransferase from the latex of *H. brasiliensis* that possessed farnesyl pyrophosphate synthase (FPS) activity (Light *et al.*, 1989). They proposed that FPS underwent a stereochemical switch from a *trans*- to a *cis*- prenyltransferase converting it to rubber transferase, after complexing with rubber elongation factor located on the surface of rubber particles. They suggested that the FPS-REF complex was responsible for the *cis*-1,4 -polyprenol condensations observed in isolated rubber particles.

The molecular weight of the rubber biopolymer produced by different species varies widely, and most do not produce the high molecular weights required for commercial applications (Cornish *et al.*, 2000). Thus an important question in rubber biosynthesis is what controls the size or the molecular weight of rubber in plants. It has been suggested that high molecular weights were associated with smaller rubber particles (Yeang *et al.*, 1995). Singh *et al.* (2003) suggested that among the three rubber-producing plants, *Ficus carica, Ficus benghalensis* and *Hevea brasiliensis, Hevea brasiliensis* producing the high molecular weight rubber has the smallest rubber particles. However, more extensive analysis is required for a better understanding of the correlation between rubber particle size and the molecular weight in plants.

It is possible that rubber transferase itself or rubber transferase in complex with additional factors controls the molecular weight of rubber (Kim *et al.*, 2004). It is believed that the plants producing different sizes of rubber should contain specific factors that control the rubber biosynthesis and determine the molecular weight of the rubber. In this context, another interesting report is that guayule rubber particles do not possess REF or proteins that are immunologically similar to it (Pan *et al.*, 1996). This may one of the reasons that *Hevea*, which contains the abundant REF protein in the latex, produces high molecular weight superior quality rubber than the other rubber producing plants. Despite substantial amounts of the reports investigating the proteins associated with the rubber particles and involved in rubber biosynthesis, the report investigating the genes encoding rubber biosynthesis- related proteins is relatively scanty.

In this study, the cloning and characterization of the REF gene from *Hevea* was reported. To date, this is the first report on the isolation and characterization of genomic DNA of REF. The availability of cloned gene would be useful for future investigation on the regulation of rubber biosynthesis.

CHAPTER - 3

CLONING AND CHARACTERIZATION OF THE cDNA ENCODING REF PROTEIN FROM *HEVEA BRASILIENSIS* AND ITS EXPRESSION IN *E. COLI* AND IN TRANSGENIC TOBACCO (*NICOTIANA TABACUM*)

3.1. INTRODUCTION

Genetic transformation of crops is rapidly becoming the technique of choice for the production of new plant varieties. Plant genetic transformation has become an important tool for functional genomics and as an adjunct to conventional breeding programmes. The natural ability of the phytopathogenic *Agrobacterium* for gene transfer to plants has been exploited recently for the genetic engineering of several woody tree species, including rubber (Arokiaraj *et al.*, 2002). A successful molecular breeding approach through *Agrobacterium* - mediated genetic transformation would represent a significant step in overcoming the existing constraints in rubber improvement programmes. It is perceived to have several advantages over other forms of transformation including the ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies with lower cost.

It is feasible to express in plants, heterologous genes that encode high value products. Transgenic plants offer the potential to be one of the most economical systems for large- scale production of proteins for various uses. Examples abound for expression of foreign genes in transgenic plants (Benfey and Chua, 1989). Attempts were made to express useful proteins by transforming tobacco plants. There are a number of reports on the expression of foreign proteins in transgenic tobacco plants. For example, bovine

aprotinin, human erythropoietin and active human epidermal growth factor (hEGF) were expressed in tobacco (Zhong *et al.*, 1999; Matsumoto *et al.*, 1995; Higo *et al.*, 1993). Wirth *et al.* (2004) also reported the expression of human epidermal growth factor in tobacco plants by integrative and non-integrative systems. Similarly expression of the human milk protein in tobacco plant cell culture was also carried out (Girard *et al.*, 2004).

Transgenic tobacco and *Arabidopsis* have already been produced by the insertion of heterologous genes, which are known to be involved in isoprenoid rubber biosynthetic pathway such as HMG-CoA reductase, FDP synthase and REF. A constitutively expressing hamster HMG-CoA reductase gene was introduced into tobacco to obtain unregulated HMG-CoA reductase activity to evaluate the impact of the enzyme activity on the biosynthesis and accumulation of particular isoprenoids (Chappel *et al.*, 1995). A genomic fragment encoding one HMG-CoA reductase, the laticifer specific *hmg 1* from the *H. brasiliensis* was introduced into *Nicotiana* via *Agrobacterium* transformation to study the influence of the *hmg1* gene product on plant isoprenoid biosynthesis (Schaller *et al.*, 1995). Re *et al.* (1995) constructed a chimaeric HMG-CoA reductase gene consisting of the full- length *Arabidopsis* cDNA fused to the CaMV 35S promoter and introduced this construct into *Arabidopsis* by *Agrobacterium*- mediated transformation. Harker *et al.* (2003) reported generation of transgenic tobacco seeds substantially enhanced in phytosterol content by the expression of a modified form (N- terminal truncated) of one of the key sterol biosynthetic enzymes, HMG-CoA reductase from *H. brasiliensis*. Dadonnet *et al.* (1997) generated transgenic tobacco plants expressing the gene coding for FDP synthase of *Sacharomyces cerevisiae*. Attanayaka *et al.* (1998) reported the genetic transformation of tobacco with the gene coding for REF protein from *H. brasiliensis*.

Genetic transformation of plants or introduction of foreign genes to plants relies on the availability of cDNA, which is the source of coding region. Some of the cDNAs encoding the different rubber biosynthesis related enzymes were cloned and characterized from *Hevea* as well as from other rubber producing plants like guayule. In *H. brasiliensis*, HMG-CoA reductase is encoded by a small gene family comprised of 3 members, *hmg 1*, *hmg 2* and *hmg 3*. Chye *et al.* (1991 and 1992) isolated and characterised cDNA clones of HMG-CoA reductase from *H. brasiliensis*. Adiwilaga and

Kush (1996) isolated a full- length cDNA, which encode a 47 kDa protein, FDP synthase from *H. brasiliensis*. Oh *et al.* (1999) reported isolation and characterisation of cDNA clones that codes for a protein tightly bound on small rubber particles in the latex of *H. brasiliensis* known as small rubber particle protein (SRPP). The cDNA clones encoding isopentenyl pyrophosphate (IPP) isomerase was cloned and characterised from the latex of *H. brasiliensis* (Oh *et al.*, 2000). Nine unique cDNAs from a *H. brasiliensis* latex library encoding the rubber biosynthesis stimulator protein (RBSP) previously identified as the eukaryotic translation initiation factor 5A were isolated (Yusof *et al.*, 2000). A cDNA clone encoding HMG-CoA synthase was isolated from a cDNA library prepared from the C- serum of latex from *H. brasiliensis* using *Arabidopsis thaliana* HMG-CoA synthase cDNA as probe (Suwanmanee *et al.*, 2002). Takaya *et al.* (2003) cloned, over expressed and characterised the cDNA clone encoding GGPP synthase from cDNA libraries of leaf and latex of *H. brasiliensis*. A full- length cDNA from guayule encoding the protein homologous to the small rubber particle protein from *H. brasiliensis* has been isolated and characterised (Kim *et al.*, 2004). The deduced amino acid sequence is homologous to those of the SRPP and the REF. Asawatreratanakul *et al.* (2003) isolated the cDNA encoding the *cis*- prenyltransferase from *H. brasiliensis*.

REF is an enzyme involved in the final polymerization step of the rubber biosynthesis in *H. brasiliensis*. This most abundant rubber particle associated protein play a major functional role in rubber polymerization (Dennis and Light, 1989; Dennis *et al.*, 1989). The cDNA encoding the REF protein has also been cloned (Attanyaka *et al.*, 1991; Goyvaerts *et al.*, 1991). In this study the cDNA coding for the REF protein was cloned and characterised from *H. brasiliensis,* RRII 105 clone (a high yielding popular Indian clone). To express REF as a fusion protein in *E. coli* cells, the isolated cDNA was cloned into an expression vector. Using this cDNA, a chimaeric gene construct was synthesised in which the REF coding sequence was placed downstream of an active promoter in the pBIB binary vector. Stable transformation of *Nicotiana* was carried out with this gene construct with the aim of producing tobacco plants expressing the REF protein from *H. brasiliensis*. Since tobacco does not accumulate REF protein or rubber, expression of the introduced REF can be studied in transgenic tobacco plants. Attempts were also made to determine whether a *Hevea* REF gene which is involved in rubber

biosynthesis may be expressed in a plant unable to synthesize rubber (laticifers are absent in tobacco). Tobacco was utilized as a model system for the present work as it is easier to transform and quicker to regenerate than *Hevea* allowing to evaluate the functioning of the gene construct prepared in the lab. The ultimate aim is to produce genetically engineered *Hevea* plants through transgenic approach to enhance the latex yield in *Hevea* clones via over expression of the genes involved in rubber biosynthesis. A good strategy for increasing the activity of an enzyme is to express its structural gene under the control of the strong promoter in transgenic plants. Subsequently the genetic manipulation of *Hevea* clones with REF gene will be carried out to produce transgenic rubber plants over expressing it.

The isolation and characterisation of the cDNA encoding rubber elongation factor protein from *H. brasiliensis* and its cloning into an expression vector to over express in *E. coli* cells is reported in this study. The successful *Agrobacterium* – mediated stable transformation of tobacco using binary vector construct harbouring *H. brasiliensis* REF cDNA and screening of its expression was also attempted.

3.2. MATERIALS AND METHODS

3.2.1. Cloning of REF cDNA by RT-PCR

3.2.1.1. Plant material

For RNA isolation, mature trees of *H. brasiliensis* (clone, RRII 105) were tapped and latex was collected.

3.2.1.2. RNA extraction

Total RNA was isolated from the latex according to the method of Venkatachalam *et al.* (1999).

- Rubber trees were tapped and the fresh latex was collected into chilled containers. While collecting, the latex was continuously mixed with an equal volume of RNA extraction buffer and immediately frozen in liquid nitrogen.

RNA extraction buffer
0.2 M NaCl, 0.1M Tris HCl, pH- 7.0,
0.01M EDTA, 1.5% SDS,
0.021% PVPP and 2% β-mercaptoethanol

- After thawing, an equal volume of buffer saturated phenol was added to the collected latex and mixed thoroughly. The mixture was centrifuged at 8000xg for 30 min at room temperature to separate the phases.
- The upper aqueous phase collected after centrifugation was extracted with an equal volume of chloroform and spun at 8000xg for 20 min at room temperature to collect the upper phase.
- Total RNA was precipitated from the aqueous phase by the addition of 1/3 volume of 8M LiCl.
- The mixture was incubated at -20°C and the RNA was pelleted by centrifugation at 10000xg for 20 min at 4° C.
- RNA pellet was washed with 2 M LiCl and dissolved in DEPC treated water.
- Further purification of RNA was carried out by treating with 3 M Sodium acetate (pH - 5.2) and 2.5 volume of 100% ethanol.
- Finally RNA pellet was washed with 70% ethanol, dried and dissolved in sterile DEPC treated water and frozen immediately at –80° C.

3.2.1.3. Quantification of RNA

The isolated RNA was diluted with distilled water and the concentration was determined. For a cuvette with a 1- cm pathlength, the concentration of RNA was calculated by the following formula

Total RNA (mg) = A_{260} x [0.040 mg/(1A_{260} x 1 ml)] x dilution factor x volume (ml).

1 A_{260} O.D. Unit for RNA = 40 µg/ml

The ratio between the readings at 260 nm and 280 nm (OD_{260}/ OD_{280}) was also determined.

3.2.1.4. cDNA synthesis by RT-PCR

Total RNA isolated from latex was used as template for the reverse transcription reaction. RNA was heat denatured at 70°C for 5 min in a water bath. The first strand synthesis reaction was set up in a microfuge tube on ice. The reaction mixture was prepared as follows:

Components	Volume /reaction
Reaction buffer	4 µl
Sodium pyrophosphate solution	1 µl
RNase Inhibitor	1 µl
dNTP mix	2 µl
oligo dT primer	1 µl
Template RNA	3 µl
Reverse Transcriptase	1 µl
RNase free water	2 µl
Total volume	15µl
Incubated at 42°C for 60 min	

3.2.1.5. PCR amplification of REF cDNA

The first strand cDNA synthesis reaction mixture was used for the amplification of REF cDNA. PCR was performed with REF gene specific primers synthesized based on published cDNA sequences from database. The REF specific primers used were:

Forward primer, REF 1 -5'TCT TCG ATT ATG GCT GAA GAC GAA GAC- 3'
Reverse primer, REF 2 -5' TAT TGG CCA ATA ATT CAA TTG GCC CTT - 3'

PCR Cocktail	
Component	**Volume/ reaction**
Sterile water	34.5 µl
10X PCR buffer	5 µl
25 mM $MgCl_2$	3 µl
dNTPs (2 mM)	5 µl
Primer 1(250 nm)	1 µl
Primer 2 (250nm)	1 µl
Taq DNA polymerase	0.5 µl
Total volume	50 µl

Set up the following automated amplification cycles in the thermal cycler (Perkin Elmer).

1 Cycle	4 min	94°C
35 Cycles	1 min	94°C
	1 min	55°C
	2 min	72°C
1 Cycle	7 min	72°C

3.2.1.6. Cloning of REF cDNA

Purified PCR amplified REF cDNA fragment was cloned into *Sma*I site of pUC19 vector. The vector was digested with *Sma*I enzyme (blunt) and dephosphorylated with SAP (Amersham, UK) enzyme. Purified PCR product was ligated into the linearized and dephosphorylated vector and then transformed into competent DH5α *Escherichia coli* cells. Transformed colonies were selected by blue-white screening using X-gal and IPTG. Further screening of the transformants and confirmation of the presence of insert in the

vector was carried out by plasmid minipreparation, insert release by double digestion with *BamH*I and *EcoR*I enzymes and by PCR analysis. All the procedures involved in cloning of REF cDNA were performed as described in previous sections (Chapter 2, **section 2.2.3**).

3.2.1.7. Nucleotide sequencing

The sequence of the cloned REF cDNA was determined using the automated sequencing facility at Indian Institute of Science, Bangalore. Both strands of the inserts were sequenced using the M13 forward and reverse primers. The cDNA sequence of REF was edited to discard the vector sequences at either ends and compared with published sequences in the NCBI database using BLASTN programmes (Altschul *et al.*, 1990). The sequences were used to search the GenBank and the EMBL data library using the BLAST network service. Multiple sequence alignment of the deduced amino acid sequence of REF with other related sequences was performed with ClustalW (Thomson *et al.*, 1994). Signal peptides and protein localization sites were predicted using signalP and pSORT programs.

3.2.2. Heterologous Expression of Recombinant *H. Brasiliensis* REF Protein in *E. Coli*

3.2.2.1. Construction of expression vector

The coding region of the REF cDNA was cloned into the *Bam*HI and *Eco*RI sites of the expression vector pGEX-2T (Amersham Biosciences, UK). To facilitate directional cloning, *Bam*HI and *Eco*RI restriction enzyme recognition sites were introduced to the 5' and 3' end of the coding region of REF cDNA. REF cDNA was amplified by PCR and while designing primers to amplify REF gene, *Bam*HI site was introduced in frame of REF gene at 5' end of the forward primer and *Eco*RI site was made at 3' end of the reverse primer. The following two primers were used:

Forward primer- 5' CGC GGA TCC GCT GAA GAC GAA GAC AAC CAA 3'
Reverse primer- 5' CCG GAA TTC CCA TAA AAC ACC TTA GCA AGT A

The conditions for the reaction were the same as those described above for RT-PCR used for the amplification of REF cDNA (section, **3.2.1.5**). The resulting PCR product and the vector DNA was digested with *Bam*HI and *Eco*RI enzymes and the purified *Eco*RI-*Bam*HI restriction fragment of REF cDNA was ligated to the

corresponding sites of the digested expression vector pGEX-2T to generate a recombinant REF expression plasmid designated pGEX-REF. The recombinant plasmid was transformed into competent DH5α cells and the transformed cells were selected on LB agar ampicillin (100 μg /μl) plates according to standard protocols (Sambrook *et al.*, 1989). The presence of the cloned REF cDNA in the plasmid vector was confirmed through PCR amplification of REF cDNA from the plasmid isolated from the transformed colonies using REF cDNA specific primers. The resultant recombinant plasmid with cloned REF cDNA was sequenced with pGEX sequencing primers to verify the orientation of the reading frame.

3.2.2.2. Expression of REF protein in *E. coli*

→ The transformed *E.coli* cells harbouring the expression plasmid pGEX-REF were grown at 37°C with agitation (200 rpm) until the absorbance of the cultures at OD_{600} reached 0.4.

→ At this stage, IPTG (100 mM) was added to a final concentration of 0.3 mM to induce the expression of the recombinant REF protein.

→ The induced cells were further incubated for a period of 4 h at 30°C with vigorous agitation (250 rpm).

→ The bacterial cells were recovered by centrifugation at 10,000xg for 10 min at 4°C.

→ The cell pellet was resuspended in 50 mM Tris. HCl (pH- 7.5), 150 mM NaCl, 0.5 mM EDTA and 2 mM PMSF, the cells were disrupted by sonication (six cycles of 30 bursts) on ice to obtain whole cell fraction.

→ The homogenate was centrifuged at 10,000xg for 10 min and the supernatant was collected. The supernatant as well as the pellet fraction was analyzed by 12.5 % SDS-PAGE according to the method of Laemmli (1970).

→ Fractionated proteins were visualized by staining with coomassie Brilliant Blue R250. Crude extracts of uninduced bacterial cultures with empty plasmid were also examined by SDS-PAGE.

3.2.2.3. Purification of fusion protein

The expressed fusion protein was affinity purified using glutathione sepharose 4B resin (Microspin GST Purification Module, Amersham Biosciences) according to manufacturer's instructions. The purified recombinant fusion protein was analyzed by SDS - PAGE (12 %).

3.2.2.4. Immunoblot analysis of the recombinant REF protein

The purified fusion protein was separated on 12 % SDS-PAGE and transferred from the gel onto nitrocellulose membrane (Amersham, UK) by electro-transfer system (Biorad) at 40 V for 3 hr using transfer buffer composed of 25 mM Tris-Hcl (pH- 8.3), 192 mM glycine and 20% (v/v) methanol. The blot was incubated in blocking solution, 1 X TTBS (0.1% (v/v) Tween 20 in Tris buffered saline) for 2 h at 37°C and washed in 1X TTBS for 10 min. Polyclonal antibodies were raised in rabbit against REF protein isolated from the latex of *H. brasiliensis* (Bangalore Genei, Bangalore) and used as primary antibody. The blot was then incubated in primary antibody solution diluted to (1: 1000) times in blocking buffer for 1 h at 37 °C and washed in TTBS (thrice for a period of 15 min each at 37°C. Then the secondary antibody was diluted (anti- rabbit IgG- ALP conjugate, 1: 5000) in 1X TTBS and incubated for 1 h at room temperature with constant agitation. The membrane was removed and washed with 1X TTBS for 10 to 15 min. The presence of REF fusion protein/ antibody complex was detected using alkaline phosphatase system with NBT-BCIP as substrate. The immunoblot analysis of the fusion protein with anti-GST antibody was also performed using GST western blotting detection kit (Amersham pharmacia biotech) and the antibody binding was detected by an enhanced chemiluminiscence system (ECL, Amersham Biosciences) following manufacturer's instructions.

3.2.3. Genetic Transformation of Tobacco

3.2.3.1. Plant material

Cultures of *Nicotiana tabacum* maintained in the lab were used. Plants were grown in a culture room under a 16 h light/ 8 h dark photoperiod (maximum photon flux 30 μmol $m^{-2}s^{-1}$). All the *in vitro* plant materials were incubated at 25+2°C under a 16/8 h (light/dark) photoperiod.

3.2.3.2. Media composition

All the media used in the present experiment contained Murashige and Skoog's basal media (Murashige and Skoog, 1962) (Table 1), vitamins, sucrose 3% (w/v) and phytagel 0.2% (w/v) (Sigma) with different hormone and antibiotic concentrations. The pH of the medium was adjusted to 5.6 to 5.8 with NaOH and autoclaved for 15 min at 121°C. Various media used in the present experiment were given below.

MS – 1: Co-cultivation medium: 1mg/l BAP

MS –2: Regeneration and selection medium: 1mg/l BAP. Autoclaved to 121°C for 15 min, cooled to 50°C and added kanamycin (100 mg/l) and cephotaxime (400 mg/l) and poured into sterile petriplates and bottles

MS – 3: Rooting medium: 1mg/l BAP, 0.1 mg/ l NAA. Autoclaved to 121°C for 15 min, cooled to 50°C and added kanamycin (100 mg/l) and cefotaxime (400 mg/l) and poured into sterile bottles.

Agrobacterium culture medium (LB medium): 10 g /l bactotryptone, 5 g/l yeast extract, 5 g/l NaCl and autoclaved.

Table 1. Composition of MS medium

Nutrients	Concentrations	Nutrients	Concentrations
Macro	(mg/l)	**Vitamins**	
NH_4NO_3	1650	Myo-inositol	100
KNO_3	1900	Nicotinic acid	0.5
KH_2PO_4	170	Pyridoxine HCl	0.5
$MgSO_4.7H_2O$	370	Thiamine HCl	0.1
$CaCl_2.2H_2O$	440	Glycine	2.0
Micro		**Iron**	
KI	0.83	$FeSO_4.7H_2O$	27.85
H_3BO_3	6.20	$Na_2EDTA.2H_2O$	37.25
$MnSO_4.4\ H_2O$	22.30	Sucrose	20.0(g/L)
$ZnSO_4.7H_2O$	8.60	Phytagel	2.0 (g/l)
$Na_2MoO.\ 2H_2O$	0.25		
$CuSO_4.5H_2O$	0.025	pH 5.6-5.8	
$CoCl_2.6H_2O$	0.025		

3.2.3.3. Construction of binary vector

The full-length HbREF cDNA was previously isolated from *H. brasiliensis* and cloned into the pUC19 plasmid vector. This cDNA was cleaved from the plasmid and inserted into the binary vector pBIB between *Xba*I and *Kpn*I sites of the polylinker which

lie between super promoter and polyadenylation of nopaline synthase. The resulting plasmid was designated as pBIBREF. The resulting chimaeric gene includes the entire coding region of Hb REF cDNA as well as the 5' and 3' UTR. Translation initiation is directed by the ATG initiation codon used in the native *H. brasiliensis* REF cDNA. A schematic representation of the gene construct was given in Figure 13. The chimaeric plasmid was then transformed into *E. coli* strain DH5α by standard methods (Sambrook *et al.*, 1989). Transformed colonies were selected and the presence of the chimaeric plasmid was confirmed through PCR and insert release by restriction digestion of plasmid DNA isolated from the transformed colonies. The chimaeric plasmid was sequenced to verify the orientation of the reading frame. The chimaeric plasmid was then mobilized into the *Agrobacterium tumefaciens* strain, LBA 4404 by freeze thaw method (Holsters *et al.*, 1978).

3.2.3.4. Transformation of *Agrobacterium tumefaciens* by freeze- thaw method

- *Agrobacterium* was grown in 5 ml Luria broth supplemented with rifampicin (10 mg/l) at 28°C overnight in a rotary shaker.
- Two ml of this overnight culture was inoculated to 50 ml LB broth and incubated at 28°C overnight in rotary shaker. The cells were grown till OD_{600} reached 0.8 to 1.
- The cells were pelleted down by centrifugation at 2000xg for 5 min at 4°C.
- The bacterial cell pellet was suspended in 1 ml of 20 mM $CaCl_2$.
- The cell suspension (100 µl) was mixed with 0.1 µg of plasmid DNA and frozen in liquid nitrogen.
- Cells were thawed and then heat shocked at 37°C for 5 min.
- One ml LB medium was added to the heat shocked cells and incubated at 28°C for 2 days for the appearance of colonies

3.2.3.5. Bacterial culture

Agrobacterium strain LBA 4404 containing the binary plasmid pBIBREF was used as vector delivery system. The bacteria were cultured for 24 h at 28°C on solid Luria broth (Sambrook *et al.*, 1989) containing 100mg/ l kanamycin and 10 mg/ l rifampicin. A single colony from the LB agar plate was inoculated into liquid LB medium and cultured

overnight at 28°C in rotary incubator till OD $_{600}$ reached 1.3. This culture was used for transformation.

3.2.3.6. Transformation of tobacco

- Leaf discs were punched out from *in vitro* grown tobacco plants from 5-6 week old leaves with a 1 cm diameter cork borer.
- The leaf discs were pre-cultured on MS-1 medium in petridishes for 48 h. The petridishes were wrapped with parafilm and cultures were maintained at 25°C with 16 h photoperiod.
- After preculture, all the leaf discs were transferred to *Agrobacterium* culture and left immersed in the culture for 10 min.
- The inoculated leaf discs were recovered from the medium with forceps and excess liquid was eliminated from the leaf discs by blotting on sterile whatman filter paper.
- Dried leaf discs were placed on co-cultivation medium (MS-1) prepared in petridishes and stored for 2 days in the dark at 25°C.
- After co-cultivation, leaf discs were washed with sterile distilled water thrice to remove excess bacterial contamination.
- Wet leaf discs were blotted dry and transferred to regeneration and selection medium MS-2 containing kanamycin (100 mg/l) and cefotaxime (400 mg/l) as the selection agent.
- Petridishes were sealed with parafilm and transferred to 16 h day length at 25°C and continued incubation until shoots regenerate.

3.2.3.7. Recovery of transformed shoots

The developing shoots were removed and placed on the same regeneration and selection medium for further enlargement of shoots. The shoots were then transferred to root inducing medium, MS-3 for development of roots. Rooted transgenic plants were transferred to plastic cups containing soil and sand (1:1) and maintained in the green house.

3.2.3.8. Molecular analysis of transgenic tobacco plants

3.2.3.8.1. *Genomic PCR analysis of transformants*

Kanamycin resistant putative transgenic tobacco plants were screened for the presence of REF and *nptII* genes integrated in the tobacco genome by PCR using REF and *nptII* specific primers. Genomic DNA was isolated from the leaves of putative transgenic and nontransgenic tobacco plants by the method of Edward *et al.* (1991) and used as template for PCR amplification of transgenes. REF specific primers used were same as the one given above (**section 3.2.1.5**). The *nptII* specific primers used for PCR were:

Forward primer, *nptII*-1, 5'- GAG GCT ATT CGG CTA TGA CT- 3'
Reverse primer, *nptII*- 2, 5'- AAT CTC GTG ATG GCA GGT TG- 3'

The PCR mixture contained 0.5 U Taq DNA polymerase, 10X buffer (pH-9.0), 1.5 mM $MgCl_2$, 200 μm of dNTPs, 1μl of each primers and 10 ng of template DNA. Samples were covered by a drop of mineral oil to prevent evaporation. PCR was also performed with the plasmid DNA pBIBREF as positive control and DNA isolated from non transgenic plant served as negative control. PCR samples were subjected to an initial denaturation at 94°C for 4 min. This was followed by 40 cycles of denaturation, annealing and extension, which consisted of 1min at 94°C, 1 min at 55°C and 2 min at 72°C. Finally, an additional extension of 7 min at 72°C was performed in a Perkin Elmer Thermal Cycler. The amplified products were separated by 1.5% (w/v) agarose gel electrophoresis.

3.2.3.8.2. *Expression studies of REF gene in transgenic tobacco by RT-PCR analysis*

RT-PCR was carried out to study the expression of the transgene in various transgenic lines of tobacco. Total RNA was isolated from the leaves of transgenic plants and control plants by a one-step method using Trizol reagent (Invitrogen Life Technologies) according to manufacturer's instructions. RNA concentrations were determined by measuring the absorbance at 260 nm. The isolated total RNA was reverse transcribed using random primer. The resultant cDNA mixture was used as the template for the PCR amplification of REF and *nptII* cDNA using REF and *nptII* specific primers. Sequences of primers used for RT- PCR were given above in the genomic PCR section (**3.2.3.8.1**). The standard protocol used for RT- PCR and the amplification conditions were described in the previous section (**3.2.1.4**).

3.3. RESULTS

3.3.1. Cloning and Characterization of REF cDNA

3.3.1.1. Isolation and cloning of REF cDNA

In order to isolate and clone the cDNA encoding the REF protein from the latex of *H. brasiliensis*, an RT-PCR based approach was used. REF specific primers were designed based on the published cDNA sequence of REF (Attanyaka *et al.*, 1991). Total RNA was isolated from the latex of *H. brasiliensis* and it was quantified spectrophotometrically. The isolated RNA had an $OD_{260/280}$ of 2.0 and it was of good purity (Figure 1). The isolated latex RNA was used as template for the first strand cDNA synthesis. The single stranded cDNA template was subsequently used for the selective amplification of REF cDNA with REF specific primers. A cDNA product of approximately 600 bp was amplified by PCR when examined by agarose gel electrophoresis (Figure 2). The amplified REF cDNA was purified from the agarose gel.

The purified cDNA was cloned into the *Sma*I site of the pUC19 plasmid vector and transformed into competent DH5α cells. The transformed cells were plated on LB agar ampicillin plate containing X-gal and IPTG for blue-white screening of transformants. Plasmids isolated from the recombinant white colonies were checked on agarose gel using supercoiled pUC19 plasmid without insert as control. As shown in Figure 3, recombinant plasmids harbouring the cloned REF cDNA migrated slowly through the gel than the control plasmid without insert. For further confirmation, the recombinant plasmid was subjected to double digestion with restriction enzymes (*Bam*HI and *Eco*RI) to release the cloned REF cDNA from the plasmid. The digested plasmids were fractionated on agarose gel to visualize the insert. As shown in Figure 4, the recombinant plasmids with cloned REF cDNA released the insert on double digestion which was detected as a separate 600 bp band. The presence of REF cDNA insert in the selected plasmids was also confirmed by PCR. The plasmids isolated from white colonies were amplified by PCR with REF specific primers and pUC19 plasmid without insert was used as control. Only the recombinant plasmids with cloned REF cDNA amplified the 600 bp REF cDNA while no amplification was detected in control plasmid (Figure 5).

Figure 1

Figure 2

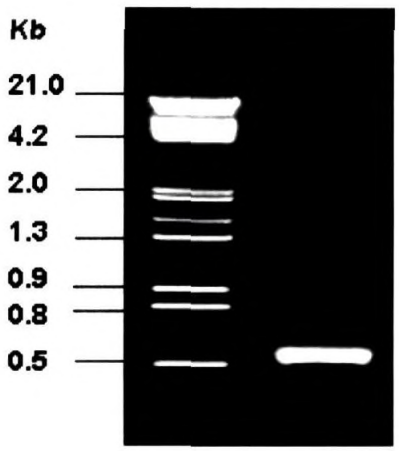


Figure 1. Total RNA isolated from the latex of *H. brasiliensis*.

Figure 2. Amplification of REF cDNA by PCR using REF specific primers. Total latex RNA was reverse transcribed with oligo- dT primers and REF cDNA was amplified by PCR. Lanes: M- DNA molecular weight markers, 1- REF cDNA of 0 .6 Kb.

Figure 3

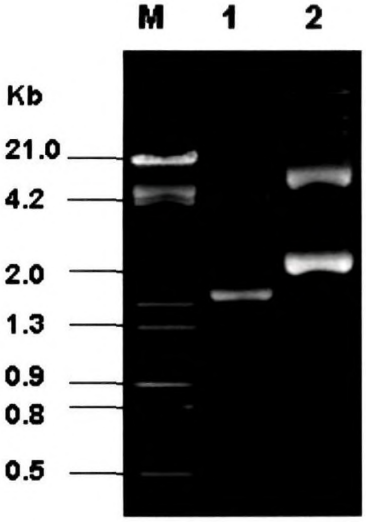


Figure 4

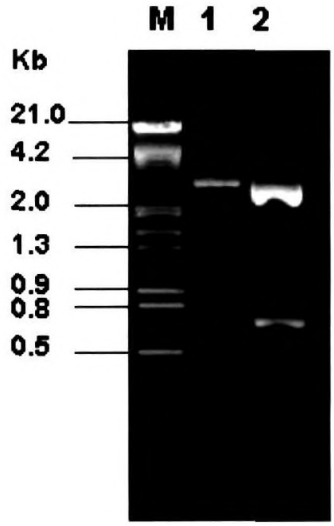


Figure 3. Cloning of REF cDNA into pUC19 plasmid vector. Lanes: M- DNA molecular weight markers, 1- Supercoiled pUC19 DNA without cloned REF cDNA insert, 2- pUC19 plasmid DNA with cloned REF cDNA insert.

Figure 4. Confirmation of cloning by restriction analysis of plasmid DNA. Digested products were analysed on 0.8% agarose gel. Lanes : M- DNA molecular weight marker, 1- Plasmid DNA without any insert digested with *Eco*RI enzyme, 2- Plasmid DNA with cloned REF cDNA digested with *Eco*RI and *Hind*III enzymes to release the 0.6 Kb cloned REF cDNA insert.

3.3.1.2. Sequence characterization of REF cDNA

The cloned REF cDNA was sequenced. The nucleotide sequence and the encoded amino acid sequence of REF cDNA were shown in Figure 6. Sequence analysis revealed that the amplified REF cDNA was 622 bp long. An open reading frame of 414 bp (except stop codon) encodes for a single polypeptide of 138 amino acids with a predicted molecular mass of 14,700 Da. The calculated pI- 5.04 of REF pointed to an acidic protein. The amplified REF cDNA contained a 5' UTR of 9 nt, a coding sequence of 414 nt and a 3' UTR of 196 nt. An ATG initiation codon is located at 10 bp in the amplified cDNA. The sequence surrounding the ATG of REF cDNA was examined and this region (GATTATGGC) matches the plant consensus sequence AACAATGGC in six out of nine positions (Lutcke *et al.*, 1987). A TGA translation termination codon is identified at 424 position. Two sequences homologous to a terminator transcription factor consensus (TGTGTTTT) are found between positions 556 and 563 and at positions 568/ 575 (TGTGTTTT) around 129 nucleotides downstream of the translational stop codon in the REF cDNA. Analysis of the deduced amino acid sequence with SignalP program (Neelsen *et al.*, 1997) predicted absence of a signal peptide and cytoplasmic localization of REF was predicted by pSORT. The REF cDNA isolated in this study was aligned with previously characterized cDNA of REF from database. Alignment revealed a perfect match between the various REF cDNA sequences. The amino acid sequence of REF as deduced from the cDNA sequence was identical to the primary protein sequence of REF determined by Dennis *et al.* (1989). In REF, the initiator methionine precedes the N-terminal alanine, which is ultimately modified. The sequence of REF cloned in this study was deposited in the DDBJ/GenBank/EMBL database (Accession no. AY120685).

The deduced amino acid sequence of REF was compared with sequences from the database. Considerable amino acid sequence similarity was observed between REF and some other sequences from *H. brasiliensis*. Other REF related sequences from *H. brasiliensis* deposited in the GenBank database include REF- like stress related protein1 (RLP1) (Acc.no AY221988), Stress- related REF like protein 2 (AY221989), isoform of HbREF (AY430052) and SRPP protein (AF51317). The predicted amino acid sequence of REF showed 51% identity to that of the isoform of REF (Accession No. AY430052) and to REF- like stress related protein-1 (51% identity, Accession No. AY221988). It also showed some degree of

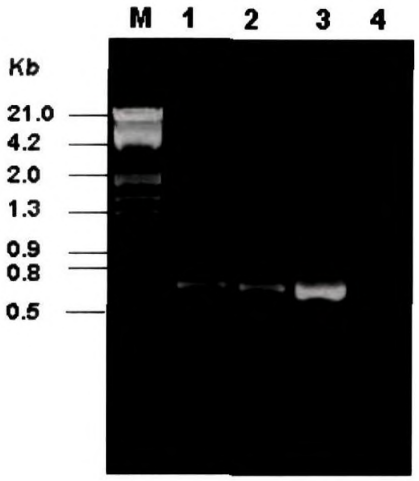


Figure 5. PCR confirmation of cloning of REF cDNA into pUC19 vector. PCR was conducted with REF gene specific primers to amplify the cloned REF cDNA with recombinant plasmids isolated from white bacterial colonies. Lanes : M- DNA molecular weight marker, 1-3 - Recombinant plasmid DNA with cloned REF cDNA as template showing amplification of REF cDNA, 4 - Plasmid DNA without insert as template showing no amplification.

```
  1 TCTTCGATTa tggctgaaga cgaagacaac caacaagggc agggggaggg gttaaaatat
              M   A  E  D   E  D  N   Q  Q  G  Q   G  E  G   L  K  Y
 61 ttgggttttg tgcaagacgc ggcaacttat gctgtgacta ccttctcaaa cgtctatctt
    L  G  F  V   Q  D  A   A  T  Y   A  V  T  T   F  S  N   V  Y  L
121 tttgccaaag acaaatctgg tccactgcag cctggtgtcg atatcattga gggtccggtg
    F  A  K  D   K  S  G   P  L  Q   P  G  V  D   I  I  E   G  P  V
181 aagaacgtgg ctgtacctct ctataatagg ttcagttata ttcccaatgg agctctcaag
    K  N  V  A   V  P  L   Y  N  R   F  S  Y  I   P  N  G   A  L  K
241 tttgtagaca gcacggttgt tgcatctgtc actattatag atcgctctct tcccccaatt
    F  V  D  S   T  V  V   A  S  V   T  I  I  D   R  S  L   P  P  I
301 gtcaaggacg catctatcca agttgtttca gcaattcgag ctgccccaga agctgctcgt
    V  K  D  A   S  I  Q   V  V  S   A  I  R  A   A  P  E   A  A  R
361 tctctggctt cttctttgcc tgggcagacc aagatacttg ctaaggtgtt ttatggagag
    S  L  A  S   S  L  P   G  Q  T   K  I  L  A   K  V  F   Y  G  E
421 aattgaGCCC CAATTTGCAC CAATTGCTTC CAACTAAGCA AGTTAATGAT ATGCTCAAGA
    N  *
481 ATATATATCT ATTGTGAGCT TTTTTTATGT TCTCATCCTG AGTGTTGAGA CTATGTTTTC

541 GTTTGAATAT TACACTGTGT TTTATTATGT GTTTTGAATA TTCATAATGA GAATAAAGGG

601 CCAATTGAAT TATTGGCCAA TA
```

Figure 6. Nucleotide and the encoded amino acid sequence of REF cDNA from *H. brasiliensis*. 5' and 3' UTR regions were indicted in blue colour. The termination codon was marked with an astrisk.

Figure 7. Multiple alignment of predicted aminoacid sequence of HbREF gene with Hb SRPP (Accession no AF051317), *Arabidopsis thaliana* REF protein (NM130345), Hb isoform of REF (Accession no. AY430052), Hb stress related REF like protein 1 (Accession no. AY221988), Hb stress related REF like protein 2 (AY221989).

similarity to SRPP (42% identity, Accession No. AF051317) and to *Hevea* REF like stress related protein-2 (39 % identity Accession no. AY221989). *H. brasiliensis* REF showed 28% sequence identity with *Arabidopsis thaliana* REF- related protein (NM130345). A comparison of the deduced amino acid sequence of REF with other related sequences was shown in Table 2. A multiple sequence alignment of the deduced amino acid sequence of REF with other related sequences was presented in the Figure 7.

Table 2. Comparison of deduced amino acid sequence of REF with other sequences from database

Plant Species	Gene	GenBank Acc No	Sequence identity %	References
H. brasiliensis	Isoform of REF	AY430052	51%	-
H.brasiliensis	REF like stress related Protein 1	AY221988	51%	Ko *et al.*, 2003
H. brasiliensis	SRPP Protein	AF051317	42%	Oh *et al.*, 1999
H. brasiliensis	REF like stress related Protein 2	AY221989	39%	Ko *et al.*, 2003
A. thaliana	*A. thaliana* REF like protein	NM130345	28%	-

3.3.2. Heterologous Expression of REF Fusion Protein in *E. Coli*

3.3.2.1. Expression and purification of REF fusion protein

To produce recombinant REF as a fusion protein in *E. coli* cells, an expression plasmid was constructed by cloning the REF cDNA into pGEX plasmid. This system permits the simple purification of foreign protein which is produced as a fusion protein of glutathione-S- transferase from crude bacterial lysates. The coding region of REF cDNA was amplified with PCR primers for the introduction of *Bam*HI and *Eco*RI restriction enzyme sites into their 5' and 3' ends (Figure 8). This facilitated its directional cloning into the corresponding sites of the expression plasmid to produce the clone, pGEX-REF. The amplified cDNA fragment was subjected to double digestion with *Bam*HI and *Eco*RI restriction enzymes and the digested cDNA fragment was ligated to the respective sites of the pGEX plasmid vector and this recombinant plasmid was designated as pGEX-REF (Figure 9). The recombinant plasmid was introduced into *E. coli* cells and the transformed colonies were selected on LB ampicillin agar plates. The presence of the recombinant plasmid in the bacterial colonies was confirmed by PCR amplification of the plasmids isolated from the recombinant colonies (Figure 10). The recombinant plasmid with cloned REF cDNA amplified the insert with gene specific primers, while no amplification was detected with empty plasmid pGEX (Figure 10). As the REF was to be

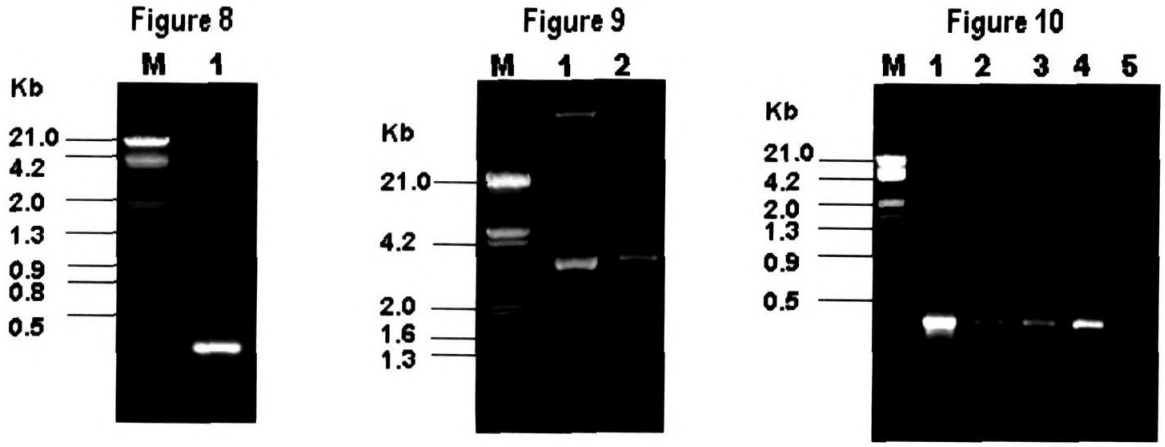


Figure 8. Amplification of REF cDNA by PCR with REF specific primers for cloning into pGEX. Lanes: M- DNA molecular weight markers, 1- REF cDNA.

Figure 9. Cloning of REF cDNA into pGEX expression vector. Lanes: M- DNA molecular weight markers, 1- Supercoiled pGEX plasmid DNA without REF cDNA insert, 2- Plasmid DNA with REF cDNA insert.

Figure 10. PCR confirmation of cloning of REF cDNA into pGEX vector with REF specific primers. Lanes: M- DNA molecular weight marker, 1 to 4 - Recombinant plasmid DNA with cloned REF cDNA as template showing amplification of REF cDNA, 5- pGEX plasmid DNA without any insert as template showing no amplification.

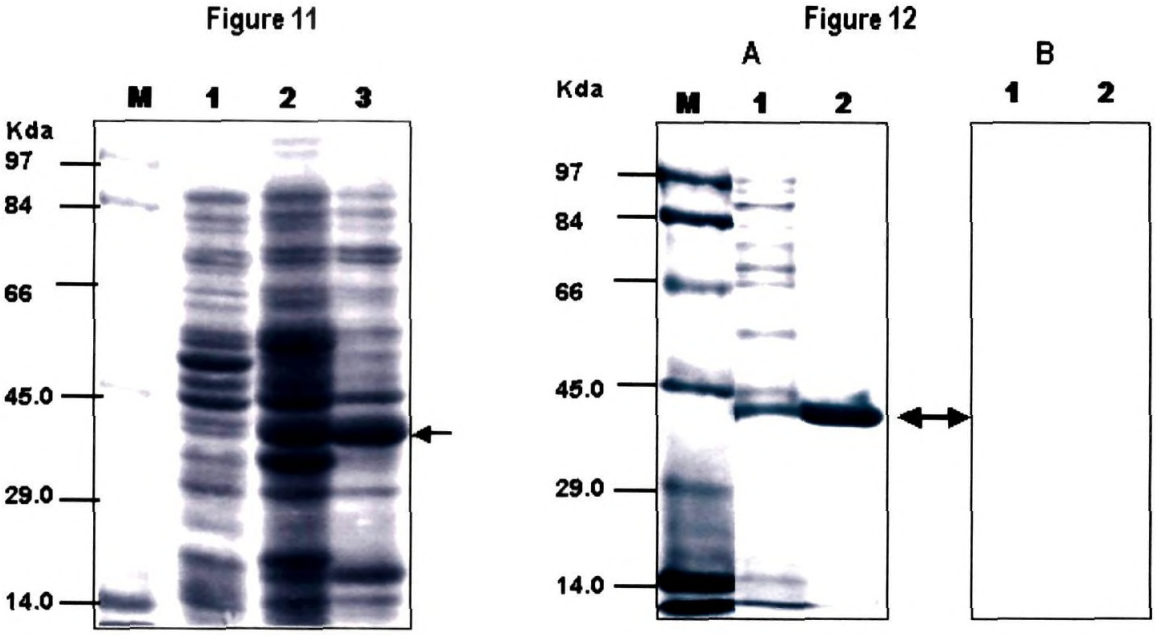


Figure 11. SDS/ PAGE coomassie- stained protein patterns. Lanes: M- Protein Marker; 1- Control bacterial cells with pGEX vector without IPTG induction. 2- Uninduced *E. coli* cells with pGEX-REF, 3-*E. coli* cells with pGEX-REF after IPTG induction showing REF fusion protein expression. Arrow indicates REF fusion protein.

Figure 12A. SDS/ PAGE analysis of proteins. Lanes: M- protein molecular weight markers, Lane 1- Induced bacterial cells with pGEX-REF expressing REF protein, 2- Purified REF recombinant protein.

Figure 12B. Immunoblot analysis of recombinant REF protein. Lanes: 1 with anti- GST antibodies, 2- with anti REF antibodies.

expressed from the coding region of cloned cDNA, the establishment of a correct reading frame is important before attempting expression of recombinant protein. For this, the bi-directional nucleotide sequencing of the REF cDNA insert in the expression plasmid was performed with primers that flank the multiple cloning sites of the vector. After sequence confirmation, the confirmed clone was used for further protein expression studies.

The *E. coli* cells harbouring the pGEX-REF were induced with IPTG for protein expression and the cells were sonicated. The soluble fraction of bacterial lysates from both uninduced and induced bacterial cells containing pGEX-REF were analysed on SDS-PAGE. The fractionated proteins were visualized by staining with coomassie brilliant blue R- 250. The REF fusion protein was detected in the protein fraction of the induced and uninduced bacterial cells with the recombinant expression plasmid pGEX-REF. But this protein was observed as a major band only in the protein fraction of induced bacterial cells. It was detected as a major protein with a molecular mass of approximately 40 kDa which was close to the expected size of fusion protein, GST- fusion (26 kDa) plus REF protein (14 kDa) (Figure 11). This protein band did not appear in the protein fraction of the bacteria containing the empty plasmid pGEX. The recombinant protein was also detected in the pellet fraction of bacterial lysates (Results not given). The crude bacterial lysate was subjected to GST affinity column for purification of the GST-tagged fusion protein. The GST- REF fusion protein was purified to homogeneity and it was detected as a band of 40 kDa on SDS-PAGE gel (Figure 12 A).

3.3.2.2. Immunoblot analysis of REF-GST fusion protein

Immunoblot analysis was carried out to check whether the antibody raised against native *H. brasiliensis* REF could recognize the recombinant REF protein. The purified recombinant REF protein separated on SDS-PAGE was transferred to PVDF membrane. The blot was reacted with the polyclonal antibodies raised against REF protein. The antibody recognized the recombinant REF protein showing that the expressed fusion protein was REF (Figure 12 B, Lane 2). Immunoblot analysis was also performed with anti-GST antibodies to recognize the REF-GST fusion protein. GST- antibodies hybridized to 40 kDa REF-GST fusion protein (Figure 12 B, Lane 1). The results of immunoblot analysis confirmed that the 40 kDa protein over expressed in the *E. coli* cells was indeed the GST- REF fusion protein and the isolated cDNA indeed encoded the REF protein of *H. brasiliensis*.

3.3.3. Genetic Transformation of Tobacco

3.3.3.1. Co-cultivation and development of transgenic plants

Nicotiana tabaccum leaf discs were transformed with *Agrobacterium* containing pBIBREF plasmid. The plasmid contained the *H. brasiliensis* REF gene under the control of a super promoter (Figure 13). It is assumed that the protein encoded by the *H. brasiliensis* REF transgene in tobacco is identical to the native *H. brasiliensis* REF protein. Tobacco leaf discs were co-cultivated with *Agrobacterium* harbouring the chimaeric plasmid for 2 days in the dark. After co-cultivation, the leaf discs were washed to remove *Agrobacterium* contamination and transferred to selection medium for regeneration of shoots. The number of leaf discs used for each transformation attempt ranged from 150 to 200. Kanamycin was used as the selection agent. Different concentrations of kanamycin ranging from 25 - 150 mg/l were applied to the medium to select the kanamycin resistant tobacco plants. A concentration of 100 mg/l kanamycin seemed to be optimal for the selection of putatively transformed shoots containing REF gene and using *nptII* as the marker gene. After 2 months, a number of transformed tobacco shoot lines were established. The transformed shoots were later transferred to root inducing medium for root development. The transgenic tobacco plants expressing *H. brasiliensis* REF gene showed no obvious morphological differences from the wild type tobacco plants (Figure 14).

3.3.3.2. Molecular confirmation of transgenic tobacco plants by PCR analysis

Preliminary screening of the kanamycin resistant putative transgenic tobacco plants transformed with the *H. brasiliensis* REF gene was conducted by genomic PCR. Four transgenic tobacco lines were selected for PCR analysis based on their rapid growth rate. These selected plants were analyzed for the presence of REF transgene in the genomic DNA using REF specific forward and reverse primers. Genomic DNA was extracted from the leaves of putative transgenic plants recovered from the kanamycin containing regeneration medium and used for PCR. DNA isolated from non-transgenic plant was used as negative control. Plasmid DNA, pBIBREF was used as a positive control. A PCR product of expected size, 600 bp REF gene fragment was amplified from the genomic DNA samples of all the four transgenic tobacco plant lines and in the positive control. This suggests that the *H. brasiliensis* REF gene was stably integrated

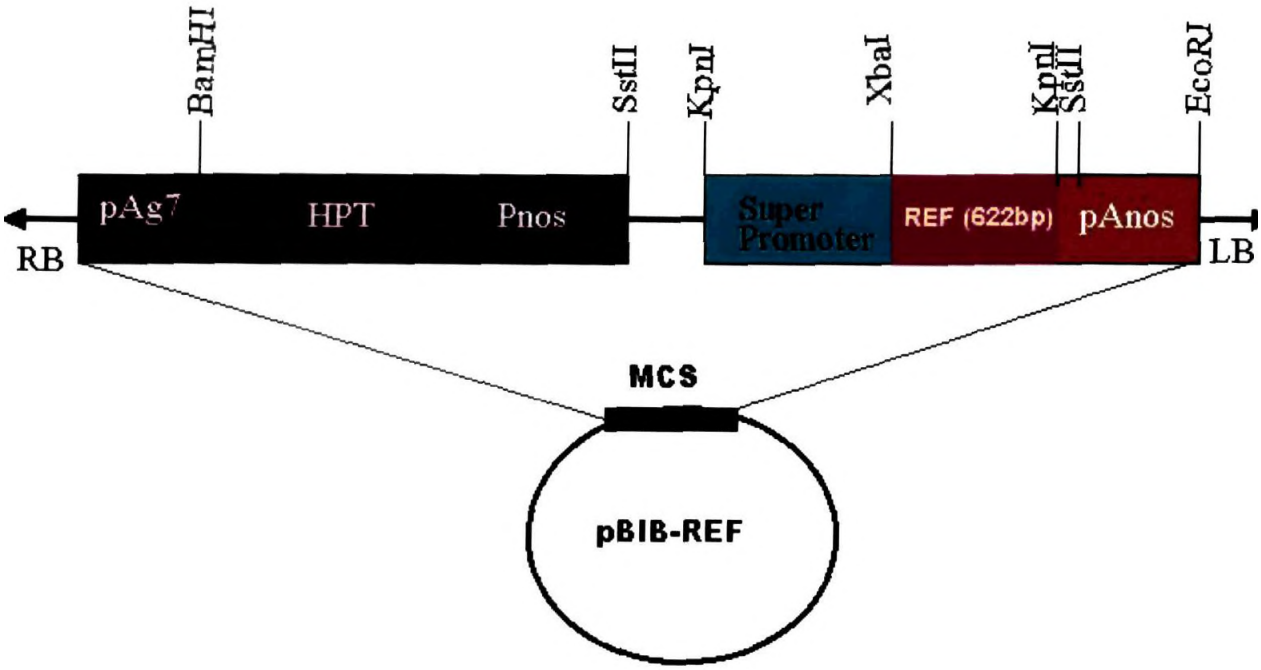


Figure 13. Schematic representation of the Hb REF chimaeric gene construct used in the transformation experiments. REF cDNA cloned between the *Kpn*I and *Xba*I sites of the binary vector pBIB.

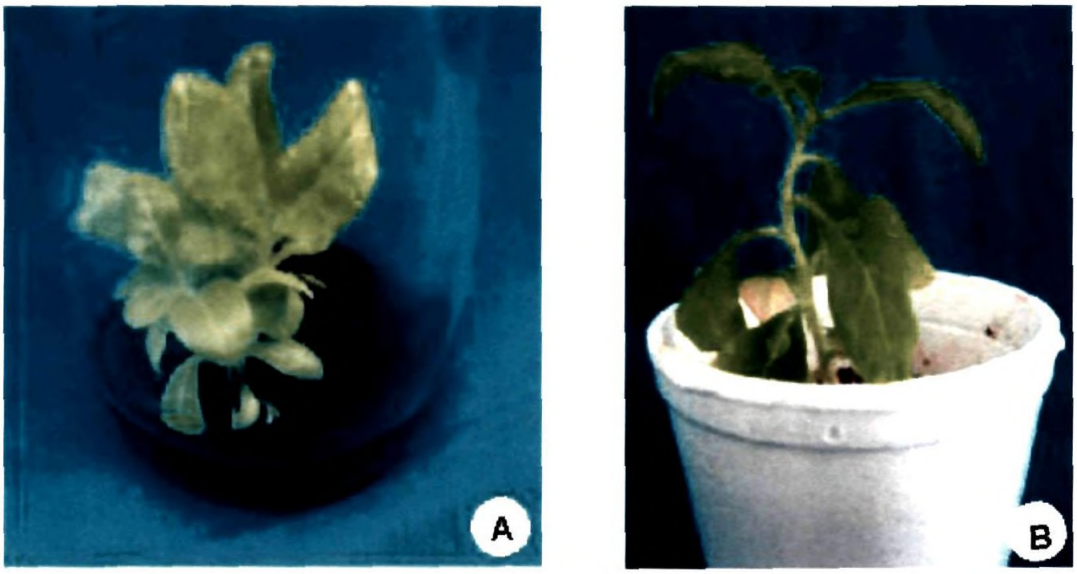


Figure- 14 A. Regenerated kanamycin- resistant transgenic tobacco plants harbouring pBIBREF gene construct.
Figure - 14 B. Rooted transgenic tobacco plants transferred to soil.

into the host genome of all four transgenic lines (Figure 15). As expected, the genomic DNA of negative control did not amplify the corresponding band. Non-specific amplification was not detected which indicates the high specificity of the primers and PCR conditions.

To further confirm the success of transformation and transgene integration, PCR was also conducted with primers specific to *nptII* gene. Genomic DNA of all the transgenic plants and the positive control DNA amplified a DNA fragment of 800 bp. But the corresponding DNA band was not detected in the nontransgenic negative control. The results of genomic PCR conducted with *nptII* primers were shown in Figure 16.

3.3.3.3. RT-PCR analysis to study the transcript level in transgenic plants

RT-PCR was conducted to study the REF transgene expression at the transcriptional level in the PCR confirmed transgenic plants. Total RNA was isolated from the leaves of PCR positive transgenic tobacco plants. The quality and concentration of the isolated RNA was determined spectrophotometrically. Total RNA was reverse transcribed and REF cDNA was amplified from the total cDNA with REF specific primers. RT-PCR was also conducted with RNA isolated from the leaves of non-transgenic plants as negative control. REF transcript of 600 bp was readily amplified from the total RNA of transgenic plants. As seen in Figure 17, a distinct band of 600 bp was clearly visible in the RT-PCR products from transgenic plants. But the intensity of the RT-PCR product varied in different transgenic tobacco lines. Among the four transgenic tobacco lines, which tested positive for REF gene integration, REF transcript was detected in all the four transgenic lines. No RT-PCR product was detected in the negative control.

In a further experiment, the *nptII* transcript was analysed in the various transgenic plants through RT-PCR performed with *nptII* specific primers. Results of RT-PCR revealed that all the four tobacco lines tested positive for the *nptII* mRNA (Figure 18). A specific cDNA product of 800 bp was detected in the transgenic lines. The *nptII* transcript was not observed in the RT- PCR conducted with RNA from non-transgenic plants.

Figure 15

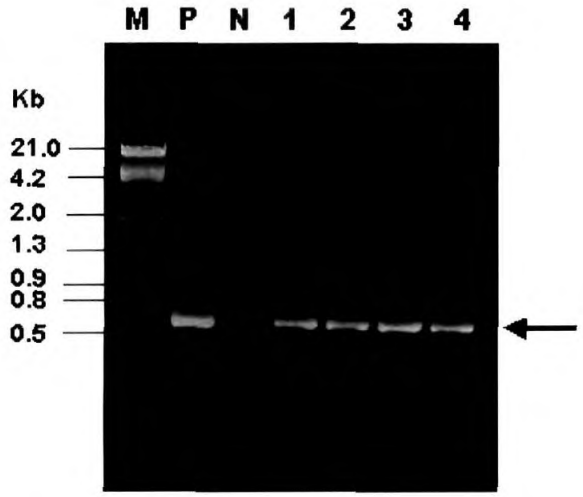
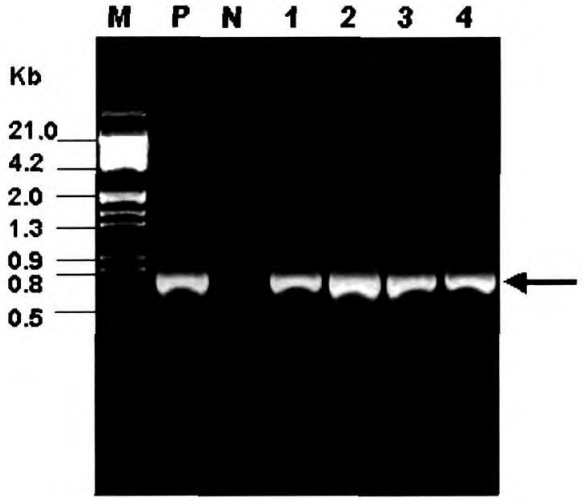


Figure 16



Molecular confirmation by genomic PCR screening of putative transgenic tobacco plants. Lanes: M - DNA molecular size marker, P- positive control, N- negative control, non-transgenic plants, 1-4 - Independent transgenic tobacco plants.

Figure 15. A 600 bp REF gene fragment was amplified from four representative transgenic tobacco lines (Arrow indicates the REF gene fragment).

Figure 16. A 800 bp *nptII* gene fragment was amplified from four representative transgenic tobacco lines (Arrow indicates *nptII* gene fragment).

Figure 17

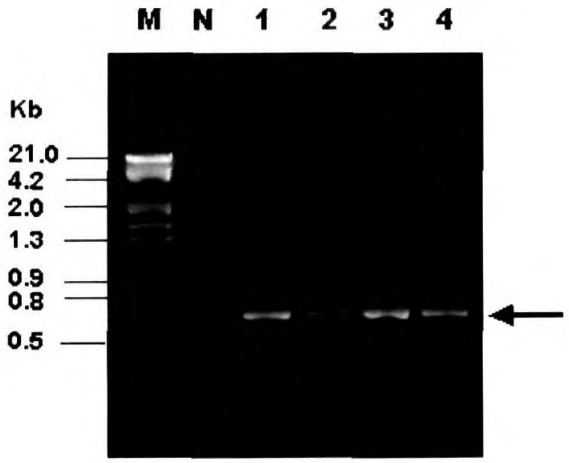
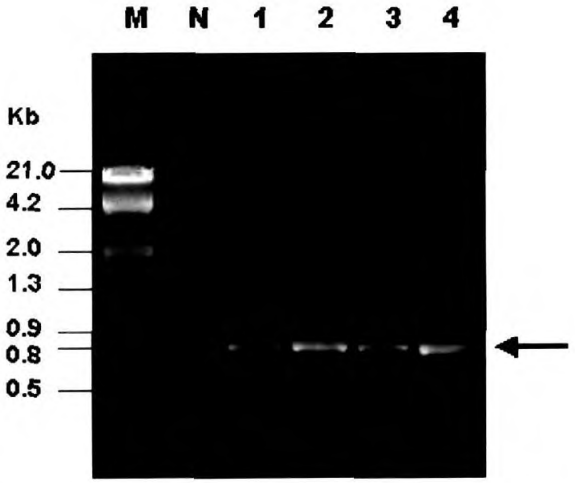


Figure 18



RT-PCR analysis of REF and *nptII* transcripts in transgenic tobacco plants. Total RNA from non-transgenic and transgenic tobacco plants were reverse- transcribed with oligo- dT and the cDNA product was amplified by PCR using gene specific primers. Lanes M- DNA molecular size markers, N- negative control (non transgenic tobacco plants), 1-4, Four independent transgenic tobacco plants.

Figure 17. Amplified REF cDNA of 600bp fragment.

Figure 18. Amplified *nptII* cDNA of 800 bp fragment.

3.4. DISCUSSION

3.4.1. Cloning and Sequence Characterisation of REF cDNA

The cDNA encoding the rubber elongation factor protein was cloned and characterized from *H. brasiliensis*. RT-PCR technique was used to clone the cDNA of REF. Total RNA isolated from the latex of *Hevea* was used as template for RT-PCR using oligonucleotide primers specific for REF. Gene specific primers were synthesised based on the cDNA sequences reported previously (Attanyaka *et al.*, 1991). A cDNA product of 622 bp was amplified from the RNA of latex. The amplified cDNA of REF was gel eluted and cloned into the plasmid vector. Nucleotide sequencing of the cloned cDNA of REF revealed that it is 622 bp in length. Comparison of the translated sequence with the published REF amino acid sequence confirmed that the cDNA of REF does indeed encode REF. The cloned REF cDNA encodes the entire coding region of mature REF. In the present study, REF cDNA was amplified from the *Hevea* clone RRII 105, which is a high yielding, and popular Indian clone. The cDNA of REF was compared with other previously characterised REF cDNAs (Attanyaka *et al.*, 1991; Goyvaerts *et al.*, 1991), which were isolated from other clones (RRIM 600). No difference was noticed between various cDNA sequences even though they were isolated from other clones.

Results of computer analysis using signalP program for prediction of signal peptide suggest the absence of a signal sequence in the deduced protein. Also, analysis of REF for protein localization sites using pSORT predicted cytoplasmic localization. These results are in agreement with previous report. According to Goyvaerts *et al.* (1991), REF is not made as a preprotein and no major glycosylation occurs *in vitro*. In the laticifers, translation of the REF mRNA probably occurs on free polysomes and a signal sequence was absent. In REF the post- translational modification is a two-step process: the removal of the N- terminal methionine and the acetylation of the newly exposed alanine. Goyvaerts *et al.* (1991), suggested a cytoplasmic assembly of the rubber particle whereby the starter molecules, short length polyisoprene pyrophosphates assemble with the requisite rubber particle surface components, lipids, phospholipids and proteins, mainly REF. Comparison of the deduced amino acid sequence of REF with other related sequence from database showed that REF protein has homology with protein sequences

from *H. brasiliensis*. REF has sequence identity of 51% with isoform of REF and REF like stress- related protein 1 from *H. brasiliensis (*Ko *et al.*, 2003*)*. With SRPP protein of *Hevea* the identity is 42% (Oh *et al.*, 1999). REF protein also shares some degree of similarity (39 % identity) with REF like stress related protein 2 from *Hevea* (Ko *et al.*, 2003). The predicted REF amino acid sequence is only distantly related to the *Arabidopsis thaliana* REF related protein (28% sequence identity).

3.4.2. Heterologous Expression of Recombinant REF Protein in *E. coli*

To further characterise REF cDNA, a GST-HbREF fusion gene was constructed to express REF protein in bacterial cells. The pGEX vector features a *tac* promoter for chemically inducible, high level expression and the *lac I9* gene allows the use of any *E. coli* host. For this, REF cDNA was cloned into pGEX expression plasmid and the gene construct was sequenced to verify the accuracy of the reading frame. Bacterial cells harbouring the GST-REF fusion were induced with IPTG to express the foreign protein as fusion protein. After extensive analysis of the induction conditions including incubation time, temperature and IPTG concentration, the GST-REF fusion protein could be expressed and purified. The bacterial cells expressed the fusion protein when induced with 0.3 mM IPTG at 30°C with vigorous agitation for 4 h. A reduction in the IPTG concentration as well as growth temperature enhanced the solubility of protein. Protein fraction of the soluble and pellet fraction of bacterial lysates were analysed by PAGE. A significant level of recombinant protein was found in the soluble part of the bacterial lysate, the protein was also detected in the pellet fraction. Under standard SDS/PAGE conditions, the GST- REF fusion protein appeared as a prominent band of approximately 40 kDa. The REF fusion protein was purified to homogeneity by using a GST-column. To test whether the antibody raised against the native REF protein from *H. brasiliensis* could recognize the recombinant REF protein, immunoblot analysis was carried out. The antisera of native REF protein hybridized to the recombinant REF protein. The REF recombinant protein also recognized the anti- GST antibody confirming the identity of the GST-fusion protein. These results thus confirm that the cloned cDNA codes for REF protein, which is identical to the native *H. brasiliensis* REF protein.

Obtaining sufficient amounts of purified REF from *Hevea* latex is difficult and time consuming; the recombinant REF presents an economic alternative to the

purification of REF from latex of *H. brasiliensis*. The heterologous expression system will facilitate further study of REF with regard to rubber biosynthesis by providing recombinant protein for *in vitro* rubber assay. The recombinant protein can also be utilized for the production of antibodies, which can be used as a latex diagnostic tool for early detection of latex yield in various clones.

3.4.3. Genetic Transformation of Tobacco

Transgenic tobacco plants expressing REF gene were generated via *Agrobacterium*-mediated genetic transformation. A binary vector was constructed such that the expression of the HbREF cDNA was driven by an active promoter and the resulting chimaeric gene construct was transformed into tobacco by *Agrobacterium* mediated transformation. The putative transgenic tobacco plants were selected by kanamycin. The resultant kanamycin resistant shoots were rooted on root inducing medium. Only those shoots that were transgenic rooted and in general the escapes did not root. According to Draper *et al.* (1988), roots are generally much more sensitive to antibiotics and thus the ability to root on selection medium containing high level of selection agent is strong indication of transformation.

Preliminary screening of the putative transgenic plants was conducted by genomic PCR. Initially genomic DNA from four representative putative transgenic tobacco lines was used in PCR reactions with primers specific to the coding region of REF gene from *H. brasiliensis*. All the four transgenic lines tested, amplified a specific DNA product of 600 bp. This indicated that the *H. brasiliensis* REF gene was successfully integrated into the genome of the tobacco plants screened. These plants were further subjected to PCR screening with *npt II* gene specific primers. In all the transgenic tobacco plants the *npt II* DNA fragment was amplified further confirming foreign gene integration into the host genome.

As a means of studying the expression of REF gene in lines of PCR positive transgenic tobacco plants, the PCR positive transformants were subjected to a secondary screening by RT-PCR using REF specific and *nptII* specific primers. Results of RT-PCR conducted with total RNA population from PCR positive transgenic tobacco plants clearly show accumulation of REF mRNA as well as *nptII* transcripts. But the intensity of the amplified RT- PCR product varies in different plants. Since the insertion of the

transferred DNA into the chromosomal DNA was random, different levels of the foreign gene expression in the independent transformants were observed. It is assumed that the protein encoded by the transgene was identical to the native REF protein. Attanayaka *et al.* (1998) also reported expression of *H. brasiliensis* REF gene in transgenic tobacco plants following genetic transformation of tobacco with REF gene from *H. brasiliensis*. Northern analysis of total RNA extracted from leaves of transgenic tobacco plants transformed with REF gene in a sense orientation revealed REF transcript, though at different levels of expression. But the transgenic tobacco plants transformed with REF gene in antisense orientation did not reveal any REF mRNA. Recently another protein from *H. brasiliensis*, the antifungal hevein protein was also successfully expressed in Indian mustard as a potential control against Alternaria blight (Kanrar *et al.*, 2002). A cDNA encoding hevein under the control of Ca MV 35S promoter was transferred into Indian mustard. Northern and western analyses proved successful expression of the transgene in the transgenic mustard plants.

A genetic transformation experiment reported by Chappel *et al.* (1995) provided evidence that introduction of a foreign gene encoding the mammalian HMG-CoA reductase into tobacco led to 3- to 5- fold increase in sterol content of the transgenic plants compared with untransformed plants. Previous studies employing constitutive expression of a full-length *H. brasiliensis* HMG-CoA reductase in tobacco reported enhanced levels of sterols in leaf tissues (Schaller *et al.*, 1995). Harker *et al.* (2003), reported the generation of transgenic tobacco seeds substantially enhanced in phytosterol content by expression of an N- terminal truncated *H. brasiliensis* HMG-CoA reductase. However, the constitutive expression of this gene resulted in only moderate increase in seed sterol levels in transgenic tobacco. Re *et al.* (1995) reported high levels of HMG-CoA reductase mRNA in transgenic *A. thaliana* following over expression of full- length *A. thaliana* HMG-CoA reductase, but only moderate increase in enzyme activity was detected.

Results of the present work showed that the heterologous REF protein expressed in *E. coli* was functional in terms of our immunoblot analysis. Genetic transformation experiments clearly demonstrate the utility of tobacco as a heterologous expression system to evaluate the functioning of gene constructs. Although, efficient plant

regeneration and genetic transformation systems are available for *Hevea* as described previously (Arokiaraj, 2000), it is more time consuming and labour intensive. Therefore, the tobacco transformation system was used for heterologous REF gene expression studies.

CHAPTER - 4

DIFFERENTIAL EXPRESSION STUDIES OF RUBBER ELONGATION FACTOR GENE IN *HEVEA BRASILIENSIS*

4.1. INTRODUCTION

In *H. brasiliensis*, latex is produced in specialized ducts called laticifers. A unique feature of the laticifers is the absence of cytoplasmic connections or plasmodesmata between these cells and their neighbouring cells (Hebant, 1981). Therefore, the exuded latex should represent only the cytoplasmic contents of the laticifers uncontaminated by those of other cells. Since, latex can be readily obtained in large quantities, it provides an opportunity to investigate the biochemical properties of a single, specialized cell type. Laticifers exhibit intense metabolic activity. Laticiferous cells actively translate the transcribed genes into proteins. The expression levels of various genes in laticifers of *Hevea brasiliensis* have been shown to be markedly different from those in leaves (Kush *et al.*, 1990). About 200 distinct polypeptides are present in latex (Posch *et al.*, 1997). Genes expressed in the latex of *Hevea* can be divided into three groups based on the proteins they encode: 1) defense related proteins such as hevein (Broekaert *et al.*, 1990), chitinase, HEVER etc. (Sivasubramaniam *et al.*, 1995), 2) rubber biosynthesis- related proteins such as REF (Goyvaerts *et al.*, 1991), HMG-CoA reductase (Chye *et al.*, 1992), HMG-CoA synthase (Suwanmanee *et al.*, 2002), FDP synthase etc. (Adiwilaga and Kush, 1996) and 3) latex allergens such as Hev b3 (Yeang *et al.*, 1993), Hev b4 (Akasawa *et al.*, 1996), Hev b5 (Slater *et al.*, 1996), Hev b7 (Sowka *et al.*, 1998) etc.

Tupy (1988) reported the presence of ribosomes and polysomes in laticifers. The expression pattern of various rubber biosynthesis as well as defense related genes in *H. brasiliensis* were studied by some of the earlier workers. Broekaert *et al.* (1990) studied

the accumulation of hevein mRNA in the laticifers of rubber tree by wounding as well as by the application of abscisic acid and ethylene. The expression of HMG-CoA reductase genes, *hmg1, hmg2 and hmg3* genes in different tissues of *H. brasiliensis* was studied by *in-situ* hybridization and by northern analysis (Chye *et al.*, 1991; 1992). Sivasubramaniam *et al.* (1995) characterised the expression of HEVER gene in the laticifers of *Hevea* tissues and also its inducible nature by stress treatment with salicyclic acid and ethephon. The mRNA expression pattern of the farnesyl diphosphate (FDP) synthase gene was determined by northern blot analysis and the tissue specific pattern of the encoded protein was studied using immunohistochemistry (Adiwilaga and Kush, 1996). Oh *et al.* (1999) used northern analysis to detect the accumulation of transcripts of small rubber particle protein (SRPP) gene in the tissues of *Hevea*. Expression of *cis*-prenyltransferase was examined in various tissues of *Hevea* by northern blot analysis (Asawatreratanakul *et al.*, 2003). Han *et al.* (2000) studied the genes expressed in the laticifers of *H. brasiliensis*. Oh *et al.* (2000) studied the effect of wounding on the transcript levels of IPP isomerase gene by northern blotting. The expression of *H. brasiliensis* HMG-CoA synthase gene in various tissues of *Hevea* seedlings and mature rubber tree was determined using gel blot analysis of total RNA isolated from various tissues (Suwanmanee *et al.*, 2002). Chow *et al.* (2003), characterised a family of elF-5A cDNAs from *H. brasiliensis* and studied the expression in tissues like latex and leaf using northern analysis. The expression levels of mRNAs of *Hevea* geranylgeranyl pyrophosphate synthase (GGPP) in various tissues like mature leaf, young leaf, latex and petiole was studied by RT-PCR (Takaya *et al.*, 2003). Ko *et al.*, (2003) established the transcriptome profile to gain insights into the molecular events occurring in the latex of *H. brasiliensis*.

Biosynthesis of natural rubber, like other secondary metabolites, is affected by various plant hormones. One of the extensively studied plant hormones in relation to rubber biosynthesis and latex production is ethylene, which is applied as ethephon. Ethephon treatment increases the production of latex between 1.5 - 2- fold (Pujade Renaud *et al.*, 1994). Even though the exact mechanism of action of ethylene on the rubber tree is poorly understood, it is presumed that ethylene affects the genes involved in rubber biosynthesis directly or indirectly by modifying general nitrogen metabolism in

the latex. So an attempt was made to study the effect of exogenous ethephon treatment on REF gene expression in the latex of stimulated trees.

The economic importance of natural rubber has prompted active investigations on the biochemical and cell biological aspects of rubber biosynthesis in *H. brasiliensis*. It is interesting to know about the regulation and expression of the genes involved in the biosynthesis of natural rubber. In the recent past, a number of investigations have been undertaken to study the differential expression of various rubber biosynthesis related genes in *H. brasiliensis*. However, to date there is no detailed report on REF expression profiles in *Hevea*. REF, being an abundant protein in the latex and since it also plays an important role in the final polymerization step of rubber biosynthesis, a detailed study of its expression pattern in *Hevea* by RNA blot analysis was undertaken. The objectives of the work are to analyse the REF transcript level by Northern blot analysis 1) in various tissues of rubber trees and seedlings 2) in tapped and untapped *Hevea* trees 3) in high yielding and low yielding clones of *H. brasiliensis* and 4) in response to exogenous ethylene treatment.

4.2. MATERIALS AND METHODS

4.2.1. Plant Material

Plant materials like bark, leaf and latex were collected from 18-year old trees of *Hevea brasiliensis* (RRII 105) and used for RNA isolation to study tissue specific expression of REF gene. Leaf, petiole, stem and root tissues were also collected from seedlings of *H. brasiliensis* for RNA isolation.

Regularly tapped trees were selected in a plot of 18-year-old trees subjected to half spiral tapping every 2 days without stimulation. Latex was collected from both the high yielding clones (RRII 105, PB 235 and PB 260) and low yielding clones (KRS 25, KRS 128, and KRS 163) and RNA was isolated for Northern analysis.

To study the effect of ethylene treatment on REF gene expression, mature rubber trees (RRII 105) were selected and ethephon (2- chloroethanephosphonic acid) treatment was performed on trees by spreading 2.5% (v/v) and 5% (v/v) ethephon in palm oil over the tapping cut after scraping. 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. Latex was collected from each tree at 0, 24, 48 and 72 h after ethephon application. The control as well as the stimulated trees was tapped at the same time to collect the latex for RNA isolation.

Latex was also obtained from untapped as well as regularly tapped trees (RRII 105) to study REF transcript accumulation.

4.2.2. RNA Extraction

Total RNA was extracted from various tissues of *H. brasiliensis* following the method of Venkatachalam *et al.* (1999). The collected tissue samples were cleaned with DEPC- treated water and frozen immediately in liquid nitrogen. The frozen tissues were finely powdered in a mortar and mixed with RNA extraction buffer and equal volume of buffer saturated phenol. The latex was mixed with extraction buffer and buffer saturated phenol. The details of RNA isolation were given in previous sections (Chapter 3, section 3.2.1.2). The concentration as well as the purity of the isolated RNA was determined by spectrophotometric method before RNA gel blotting. Equal amount of RNA was loaded into each wells.

4.2.3. RNA Gel Blot Analysis

4.2.3.1. Electrophoresis of RNA

Reagents	Composition
MOPS buffer 20X	0.4 M MOPS 0.1 M sodium acetate 20 mM EDTA
Gel loading buffer	0.1% (v/v) Xylene cyanol 0.1% (w/v) Bromophenol blue 1X MOPS 50% (v/v) glycerol
SSPE 20X	3.6 M sodium chloride 200 mM sodium phosphate 20 m M EDTA pH- 6.8
Denhardt's 100X	2% BSA (w/v), fraction V 2% (w/v) Ficoll 2%(w/v) Polyvinylpyrrolidone
Prehybridization solution	50% (v/v) Formamide 6X SSPE 5X denhardt's 0.5% (w/v) SDS

- For a 1% (w/v) gel (200 ml), 2 g of agarose was weighed into a 500 ml conical flask containing 156 ml of DEPC- treated water and boiled till the agarose was completely melted.
- The agarose solution was cooled to 70°C and 10 ml of 20 X MOPS buffer and 34 ml formaldehyde (37 %) (v/v) was added to it.
- After mixing, the solution was poured into a gel casting tray and allowed to solidify at room temperature.
- The isolated RNA samples were prepared by mixing the following components in a 1.5 ml microfuge tube.

MOPS buffer 20 X	-	1 µl
Formamide	-	10 µl
Formaldehyde (37%)	-	3.5 µl
Gel loading buffer	-	2 µl
RNA	-	5 µg

- Heat denatured RNA at 60°C for 5 min in a water bath.
- The denatured RNA samples were spun briefly and then loaded carefully into the solidified gel.
- Electrophoresis was carried out for 5-7 h at 60 V in 1X MOPS running buffer.

4.2.4. REF cDNA Synthesis by RT- PCR

Total RNA isolated from latex was used as template for the reverse transcription reaction to amplify REF cDNA, which was used for, radiolabeled probe preparation. The first strand cDNA synthesis reaction was performed in 20 µl volume using oligo dT primer in presence of Mu-MLV reverse transcriptase at 42° C for 60 min. After the reverse transcription step, the first strand cDNA served as template in the PCR amplification of REF cDNA using gene specific primers (section, **3.2.1.5**). PCR amplification was performed with 2 µl of the first strand cDNA mixture and 2 unit of Taq DNA polymerase along with other PCR reagents in a reaction volume of 50 µl. The PCR amplification profile consisted of a first cycle at 94° C for 4 min followed by 30 cycles at 94° C for 1min / 55°C, 1.30 min / 72° C – 2 min and a last cycle at 72° C for 7 min. The reaction mixture was overlaid with mineral oil. The amplification was carried out in a thermal cycler (Perkin Elmer, USA). The amplified cDNA was checked on 1.5% (w/v)

agarose gel and the cDNA was purified from low melting point agarose gel (section **2.2.2.4**).

4.2.5. REF Probe Synthesis

The purified cDNA was radiolabeled with α^{32}P- dCTP and then used as probe in the subsequent hydridization step. The detailed procedure of probe labeling and purification of labeled probe was given in previous sections (Chapter 2, **section 2.2.5.4**).

4.2.6. Washing and Hybridization

- After electrophoresis, the gel was rinsed several times in DEPC-treated water to remove formaldehyde.
- The fractionated RNA was transferred overnight to a nylon membrane (pre-wet in 1X MOPS buffer) with a 10X SSPE stock solution by capillary blotting according to standard protocols (Sambrook *et al.*, 1989).
- The blot was rinsed in 4X SSPE and then UV crosslinked.
- Prehybridization of the blot was carried out at 42°C in prehybridization buffer for 3-4 h with gentle rotation.
- Radiolabeled REF cDNA probe was added to fresh prehybridization buffer and hybridization was performed overnight at 42°C.
- After hybridization, the blot was rinsed twice with 2X SSC for 15 min at 60°C followed by washes with 0.2 X SSC containing 0.1% (w/v) SDS for 30 min at room temperature twice.
- The washed blot was covered with saran wrap and exposed to X- ray film with intensifying screens at –70°C for signal detection.

4.3. RESULTS

4.3.1. Differential Expression of REF Gene in Various Tissues of *Hevea*

The level of REF mRNA transcript in different tissues of *H. brasiliensis* was studied by RNA gel blot analysis. Total RNA was isolated from different tissues like latex, bark and leaf of mature rubber trees. The concentration of the isolated RNA was determined and the value of $OD_{260/280}$ was 2.0 indicating that the isolated RNA was pure. Equal amount of total RNA isolated from various tissues were loaded onto a 1% (w/v) denaturing agarose gel and fractionated electrophoretically (Figure 1). The expression

pattern of REF was analyzed by RNA blot analysis using radiolabeled REF cDNA as probe. Results demonstrated that REF transcript was relatively abundant in latex followed by bark of mature rubber trees and least abundant in leaf (Figure 1). REF accumulation was about 10- 20 fold higher in latex compared with leaf. The expression level of REF in *Hevea* seedling tissues, viz., leaf, petiole, stem and root was also analysed. Total RNA extracted from different seedling tissues was separated on gel and then blotted onto a nylon membrane. The blot was hybridized with radiolabeled REF cDNA as probe. RNA blot results indicated that REF gene expression was tissue specific in seedlings also. As shown in Figure 1, the transcript level was found to be predominant in stem tissues and petiole whereas it was barely detectable in leaf and root tissues of *Hevea* seedlings.

4.3.2. Effect of Tapping on REF Gene Expression

The accumulation of REF transcripts in the latex of regularly tapped as well as untapped rubber trees was also examined. Total RNA extracted from the latex of regularly tapped and untapped rubber trees were subjected to RNA blot analysis (Figure 1). Radiolabeled REF cDNA was used as probe. The level of REF gene expression in tapped trees was found to be high compared with untapped rubber trees (Figure 1). Thus regular tapping seems to have an effect on REF gene expression.

4.3.3. REF Gene Expression in High Yielding and Low Yielding Clones

REF gene expression pattern was also compared in different clones of *H. brasiliensis*. Six different clones of *Hevea* were selected for this study (RRII 105, PB 235, PB 260, KRS 25, KRS 128, KRS 163). These clones are belonging to 2 groups, high yielding (RRII 105, PB 235, PB 260) and low yielding (KRS 25, KRS 128, KRS 163). RNA blot analysis with latex RNA isolated from different clones revealed significant difference in the REF transcript level. The REF mRNA level was predominantly higher in high yielding clones than in low yielding clones (Figure 2 and 3). Among the high yielding clones examined, REF mRNA accumulation was most abundant in RRII 105, a high yielding clone and a slightly lower level of REF transcript was noticed in the other two clones, PB 235 and PB 260 (Figure 2). Significantly low level of REF expression was detected in the low yielding clones, KRS 25, KRS 128 and KRS 163 (Figure 3) compared with high yielding clones. Among the low yielding clones, REF transcript level was higher in the clone KRS 25 followed by the other clones, KRS 128 and KRS 163.

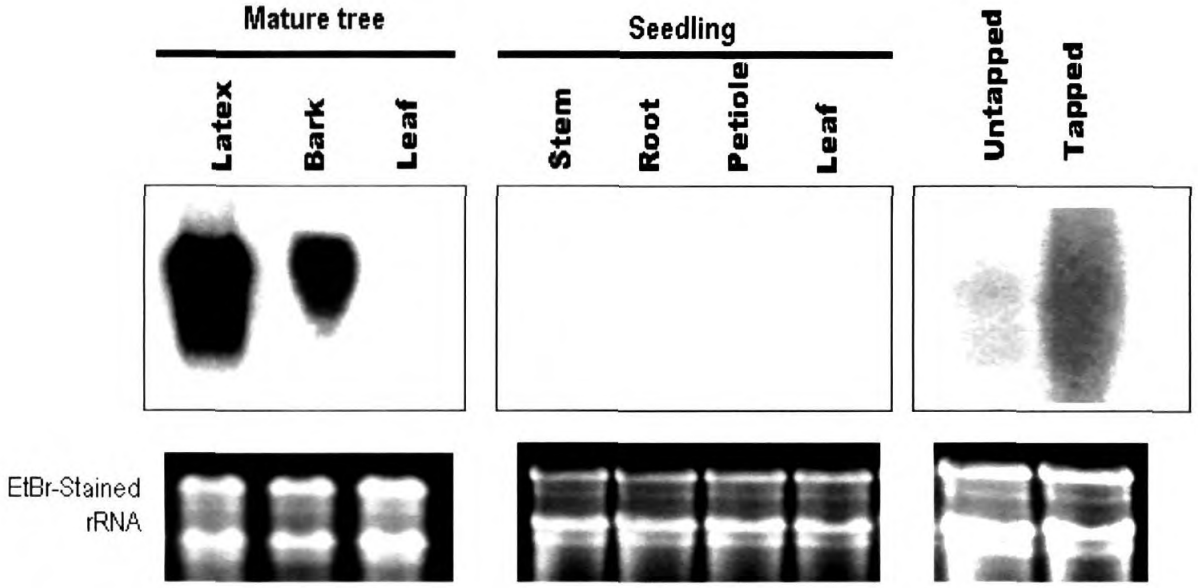


Figure 1. Differential expression pattern of REF gene in various tissues as well as latex collected from untapped and tapped trees of *H. brasiliensis*. Northern blots were hybridized with REF cDNA probe. Each lane contained 15 μg of total RNA isolated from different tissues. Equal amount of RNA loaded in each lane was confirmed by EtBr staining.

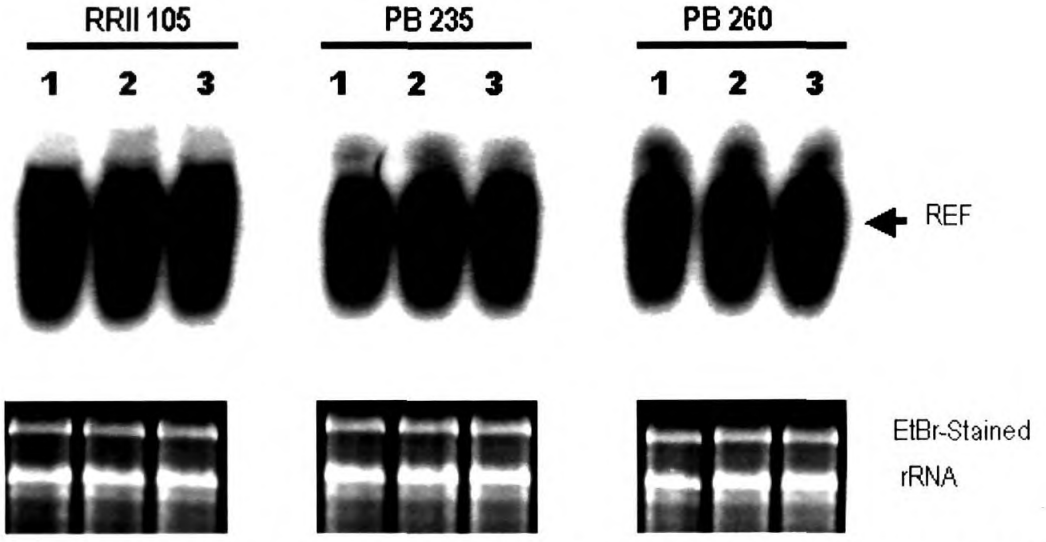


Figure 2. Northern blot analysis carried out with RNA extracted from latex of three high yielding clones. Total RNAs (15 μg) were transferred to a nylon membrane and hybridized with REF cDNA probe. The 28 S and 18 S rRNA bands in EtBr stained gel is shown as loading control. Lanes 1 – 3, RNA samples from three individual trees.

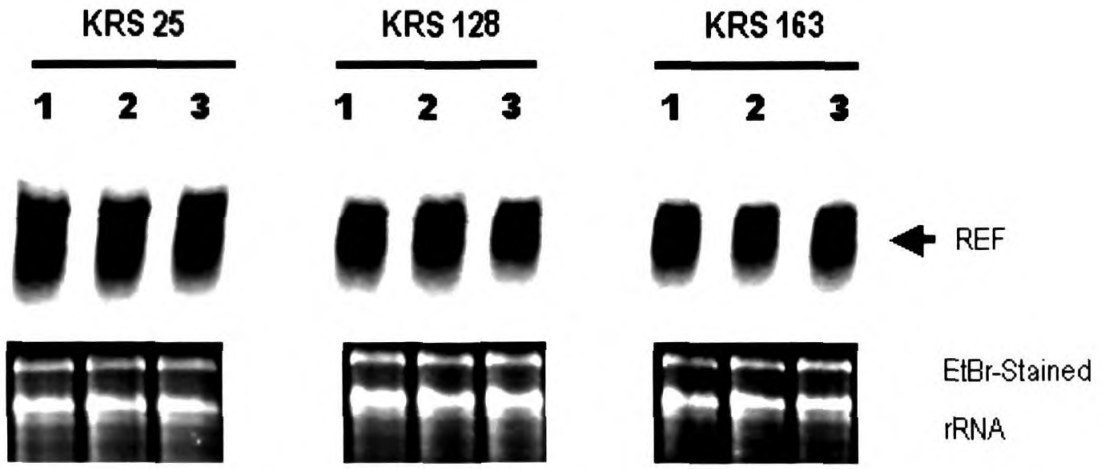


Figure 3. Northern blot analysis carried out with RNA extracted from latex of three low yielding clones. Total RNAs (15 μg) were transferred to a nylon membrane and hybridized with REF cDNA probe. The 28 S and 18 S rRNA bands in EtBr stained gel is shown as loading control. Lanes 1 – 3, RNA samples from three individual trees.

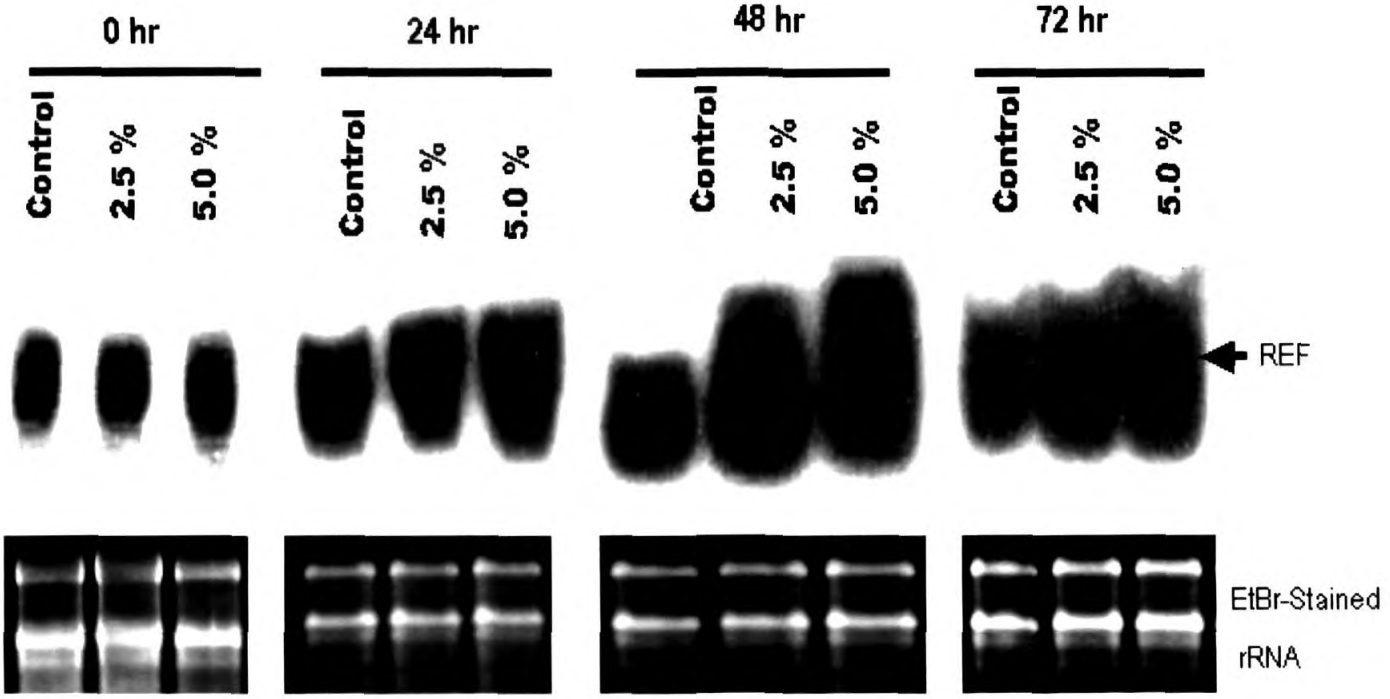


Figure 4. Accumulation of REF transcripts upon ethephon treatment (2.5% and 5.0%). Total RNA was prepared from latex of control as well as ethephon treated trees at 0, 24, 48 and 72 h after stimulation. Total RNAs (15 μg) were separated on 1% gel and hybridized with REF cDNA probe. The 28 S and 18 S rRNA bands in EtBr stained gel is shown as loading control.

About 10- 20 fold rise in mRNA could be noticed in the high yielding clones. In all the three low yielding clones, REF expression was much low.

4.3.4. Effect of Exogenous Ethephon Application on REF Transcript Level

RNA blot analysis was performed to study the effect of exogenous ethylene application on REF transcript level in the latex of ethephon-stimulated rubber trees. Rubber trees were treated with two different concentrations of ethephon (2.5 % and 5 % (v/v)), an ethylene-releasing agent. Total RNA was isolated from the extruded latex collected at 0, 24, 48 and 72 h after stimulation from ethephon treated trees as well as control trees. RNA blot analysis was carried out with REF cDNA as probe. Northern blot results demonstrated a clear accumulation of the REF transcripts in the latex of ethephon treated trees at all concentrations. A noticeable increase was detected in the latex of stimulated trees at 24 and 48 h of treatment compared with the control trees (Figure 4). The stimulatory effect was maximal at 48 h of treatment and a 10- 15 fold increase in mRNA level could be detected at 48 h compared with 0 h treatment. In control trees also, the expression of REF increased with tapping (0, 24, 48, 72h). At 72 h stimulation, at both concentrations of ethephon, no significant rise in the REF expression level was noticed in the treated trees compared with 48 h treatment. Also the trees stimulated with 5% (v/v) ethephon exhibited a higher level of REF transcripts than the trees treated with 2.5% ethephon. The stimulatory effect was specific for ethephon, since the basal level of REF transcripts remained low in control trees subjected to bark scraping and palm oil treatment alone (Figure 4). In control trees also, REF mRNA levels increased dramatically with the number of tapping.

4.4. DISCUSSION

The aim of the present work was to study the expression pattern of REF gene, a rubber biosynthesis- related gene in *H. brasiliensis*. Analysis of expression of genes in a given tissue can provide information about the biochemical and molecular events occurring in the tissue at the time of investigation. Here we employed the technique of RNA blot analysis to study the pattern of REF gene expression in various tissues and also in the latex of different clones of *H. brasiliensis*. The effect of exogenous ethylene application on REF gene expression in the latex of *H. brasiliensis* was also investigated.

4.4.1. Differential Expression of REF Gene in Various Tissues of *Hevea*

Analysis of RNA samples from different tissues of *Hevea* showed a higher mRNA level in latex and bark of mature rubber trees than in leaves. This suggests that the expression of REF gene is higher in laticifer cells than in leaves. This finding is similar to the report that the transcripts of REF and SRPP are highly accumulated in the latex of *H. brasiliensis* but not in the leaves (Ko *et al.*, 2003). Chye *et al.* (1992) reported that HMG-CoA reductase a key rubber biosynthesis related gene, *hmg1*, *hmg2* and *hmg 3* are also differentially expressed in *H. brasiliensis*. The *hmg1* which predominantly expresses in laticifers and hardly in leaves (Chye *et al.*, 1992) is involved in rubber biosynthesis. Recently, a *Hevea* gene encoding *cis*- prenyltransferase, involved in rubber biosynthesis is identified and is shown to be highly expressed in the latex (Asawatreratakul *et al.*, 2003). Northern and RT-PCR analysis of the *cis*-prenyltransferase genes in various *Hevea* tissues showed predominant expression of the genes in the latex (Asawatreratanakul *et al.*, 2003). Further, Suwanmanee *et al.* (2002), reported a higher mRNA expression of HMG-CoA synthase gene in latex and petiole of mature rubber trees than in leaves. In mature trees also there is higher mRNA level of HMG-CoA synthase in latex and petiole than in leaves. This is in contrast to the expression of IPP isomerase, another gene involved in isoprenoid biosynthesis, which was not detected as an abundant transcript in the latex of *H. brasiliensis*. (Oh *et al.*, 2000). Similarly the mRNA expression of the GGPP synthase, another isoprenoid biosynthesis gene was found to be higher in flower and leaf than in petiole and latex of mature rubber trees, where a large quantity of natural rubber is produced (Takaya *et al.*, 2003).

Among the tissues studied from *Hevea* seedlings, stem and petiole tissues exhibited a higher level of REF expression than in other tissues like root and leaf where the expression level was barely detectable. These results are in agreement with a recent study, which reported that HMG-CoA synthase gene is highly expressed in the stem, and petiole of *Hevea* seedlings than in leaves (Suwanmanee *et al.*, 2002). The higher level of REF expression in stem and petiole tissue may be due to the presence of more number of laticifers in petiole and stem than in leaves and roots of seedlings.

In *Hevea brasiliensis*, since rubber biosynthesis takes place on the surface of rubber particles suspended in latex, the genes uniquely or preferentially expressed in the latex might be important for rubber biosynthesis. Earlier, Kush *et al.* (1990) demonstrated that laticifer RNA is 20- 100 fold enriched in transcripts encoding enzymes involved in rubber biosynthesis. Han *et al.* (2000), generated 245 expressed sequence tags (ESTs) to study gene expression profile in the latex of *H. brasiliensis*. Rubber biosynthesis genes like REF and SRPP comprised 25% of the ESTs from the regular latex library. Transcriptome analysis of laticifers (Ko *et al.*, 2003) revealed that transcripts from two most abundant genes REF and SRPP comprised 29% of the transcriptome. Thus the specific and abundant expression of REF in latex strongly suggests a major role of REF in rubber biosynthesis.

4.4.2. Effect of Tapping on REF Gene Expression

Results of RNA blot analysis conducted with RNA isolated from the latex of tapped and untapped trees reveals a clear accumulation of REF transcripts in the latex of regularly tapped trees than in untapped trees. The over expression of REF in tapped trees could be ascribed to the effect of metabolic activation associated with latex regeneration within the laticifers due to regular tapping. Adiwilaga and Kush (1996), also reported a similar increase in the expression of FDP synthase gene in the latex of regularly tapped trees in comparison with untapped trees. An interesting feature of latex production in *H. brasiliensis* is the increase in metabolism in laticifers in response to tapping or wounding. The *in situ* latex regeneration mechanism allows reconstitution of the exported latex before the next tapping. Hence, tapping acts as a trigger for metabolic switch to turn on. In the studies on the expression pattern of REF in response to tapping, it is evident that tapping has a direct effect on the level of expression of REF mRNA. Moreover, tapping which is a process of controlled wounding will turn on a number of genes resulting in the accumulation of their gene products. This accounts for the abundance of REF mRNA in tapped trees than in the untapped trees.

4.4.3. REF Gene Expression in High Yielding and Low Yielding *Hevea* Clones

A significant variation in REF transcript accumulation could be detected in 6 different clones of *H. brasiliensis* used in the present study. REF gene expression was found to be relatively higher in high yielding clones than in the low yielding clones.

Previously Tupy *et al.* (1969) reported that total RNA and rRNA/ tRNA ratio are higher in high yielding trees than in low yielders (Tupy, 1969). Significant clonal variability in rRNA and poly A RNA content in latex and its relation to variability in latex sucrose has been suggested (Tupy, 1988). Tupy (1988) also reported that the contents of rRNA and polyA RNA represent important latex characteristic related to clonal properties and to physiological state of latex vessels (Tupy, 1988). According to Dennis and Light (1989), the amount of REF in whole latex is proportional to the rubber content. REF represents between 10-60% of the total protein in whole latex (Dennis and Light, 1989). In high yielding clones, rubber content as well as the rate of rubber biosynthesis will be more relative to low yielding clones. REF being a rubber biosynthesis related gene and since it is closely associated with the rubber particles, its level in the latex is proportional to the rubber content in various clones. This accounts for the increased accumulation of REF gene in the high yielding clones of *H. brasiliensis* where the rubber content as well as the yield is high. This is only a preliminary study and more investigations in this direction are needed.

4.4.4. Effect of Exogenous Ethephon Stimulation on REF Gene Expression

The effect of ethephon application on REF gene expression in the latex of stimulated trees was studied. The results indicate that exogenous application of ethylene lead to increased steady state levels of REF mRNA in the latex from the stimulated trees. It is not known at present whether the accumulation of REF transcript is confined to the laticifers of stimulated trees or whether other tissues are also involved as well. The expression of another gene HMG-CoA reductase which is a key enzyme in rubber biosynthesis in *Hevea* is also induced by ethylene treatment (Chye *et al.*, 1992). Furthermore, the level of transcripts of several genes like chitinase, hevein, glutamine synthetase are also increased by ethylene treatment (Brockaert *et al.*, 1990; Pujade-Renaud, *et al.*, 1994). The transcript of MnSOD could be induced 3-5 fold in response to sucrose, ethephon and MS salts (Miao and Gaynor, 1993). These results are in contrast to the expression of other rubber biosynthesis genes like FDP synthase (Adiwilaga and Kush, 1996), small rubber particle protein (SRPP) (Oh *et al.*, 1999) and IPP isomerase (Oh *et al.*, 2000), which are not influenced by ethephon treatment. This is particularly interesting in rubber tree as ethephon is routinely applied to the bark of rubber tree to

increase latex yield. Ethephon treatment increases latex flow presumably by preventing or delaying the rupture of lutoid membranes and thus preventing premature coagulation of latex. Ethylene generated by ethephon (2-chloroethanephosphonic acid) stimulates latex production by increasing the duration of latex flow after tapping and by activating the metabolism involved in latex regeneration (Coupe and Chrestin, 1989). *In situ* latex regeneration mechanism allows reconstitution of exuded latex before the next tapping. The mechanism of ethylene action in increasing latex production has not been completely elucidated yet. Physiological and biochemical studies show that in general, ethylene act on membrane permeability leading to prolonged latex flow as well as an effect on general regenerative metabolism, acceleration of glycolysis (Tupy, 1973), increasing the adenylate pool, polysome and rRNA content (Amalou *et al.*, 1992). Pujade- Renaud *et al.* (1994) reported that ethephon treatment increases the production of latex between 1.5- 2 fold. Thus it is likely that the increase in the expression of REF results in an increase in rubber synthesis, although the effect of ethephon treatment on rubber trees appeared to be mainly an increase in latex volume.

CHAPTER - 5

CLONING AND FUNCTIONAL CHARACTERIZATION OF THE PROMOTER REGION OF REF GENE FROM *HEVEA BRASILIENSIS*

5.1. INTRODUCTION

The promoters are sequences that are present upstream to the transcriptional start site of genes (Datla *et al.*, 1997). The promoters of a gene contain the information required to direct when, where and to what extent this gene will be expressed. A large majority of the plant nuclear gene promoters contain a highly conserved TATA box in their promoters (Joshi, 1987). The availability of regulatory sequences or promoters to target expression to appropriate cells, tissues or developmental stages is an essential component for engineering traits in transgenic plants. The availability of promoters played a critical role in the development of gene transfer vector systems and the production of transgenic plants. In transgenic plants, promoters are used for the expression of marker genes and the specific gene introduced. These developments were instrumental in providing the tools for studying plant gene expression and regulation in general and for analyzing plant promoters in depth. *Agrobacterium tumefaciens* mediated gene transfer to plants followed by *in planta* activation of a number of genes associated with the transferred DNA (T-DNA) provided an early insight into plant gene structure and regulation (Ream and Gordon, 1982). Promoters of octopine synthase, mannopine synthase and nopaline synthase genes of *Agrobacterium tumefaciens* were studied and were used in early transformation experiments (An *et al.*, 1990; Velten *et al.*, 1984). Guilley *et al.* (1982) identified the promoter element directing the synthesis of 35S RNA in Cauliflower mosaic virus. Odell *et al.* (1990) identified the DNA sequences required for the activity of the CaMV 35S promoter and it was found that CaMV 35S promoter is expressed constitutively in most dicots (Rogers *et al.*, 1987).

More recently promoters from the rice *actin1* gene were isolated by McElroy *et al.* (1990). Christensen *et al.* (1992) isolated the maize *ubiquitin* gene and used for monocot transformation (Christensen and Quail, 1996). Norris *et al.*, 1993, isolated a strong constitutive dicot promoter from *A. thaliana* ubiquitin gene. Several cell and tissue specific promoters were also isolated and characterised. The use of fusion between promoters and reporter genes has allowed a detailed monitoring of the activity of the numerous plant promoters. Selectable and reporter genes have also been a critical component for advancing plant promoter research (Schrott, 1995). The genes specifying β-glucuronidase (*gus*) and luciferase (*luc*) have been widely used in promoter studies (Jefferson *et al.*, 1987; Ow *et al.*, 1986). More recently, the gene- encoding green fluorescent protein (*gfp*) is getting more attention as a nondestructive method for studying gene activity (Chiu *et al.*, 1996).

The fruit specificity of apple polygalacturonase and ACC oxidase promoter was investigated in transgenic tomato plants by promoter- GUS fusion (Atkinson *et al.*, 1998). The dihydroflavanol reductase *(dfr)* gene was fused to the *uidA* gene and endogenous *dfr* expression was studied in transformed grape fruits (Gollop *et al.*, 2002). Moon *et al.* (2004) reported isolation of peach ACC oxidase gene and identified the regulatory elements in the peach ACC oxidase gene by generating transgenic tomato plants with ACC oxidase promoter- GUS fusions.

Genetic engineering opens a new route to increase the content of secondary metabolites in the producing plant species or even to produce the desired compounds in a heterologous easily cultivable host plant. The production of therapeutic or diagnostic proteins in a host plant is popularly known as molecular farming. Plants have considerable potential for the production of biopharmaceutical proteins and peptides because they are easily transformed and provide a cheap source of protein (Giddings *et al.*, 2000). The expression of recombinant proteins used as pharmaceuticals, industrial enzymes or fine chemicals in plants is now routine for many species and offers an exciting challenge. Plants offer various advantages for the production of recombinant proteins over conventional production systems such as bacterial or mammalian cell culture. This aspect becomes increasingly important in case where large amounts of protein need to be produced. Numerous heterologous proteins have been expressed in different plant organs and plant cell compartments (Fiedler and Conrad, 1995). However, the high costs of protein extraction and purification from biochemically complex plant tissues is an important

factor for large-scale protein production in plants. To overcome this problem, one such crop that could be usefully exploited is *Hevea brasiliensis*, which is an important industrial tree crop that provides large amounts of latex. *Hevea* can potentially be engineered to produce foreign proteins in the specialized laticifers. The proteins can then be easily purified from the serum phase of the latex, which is harvested by a simple tapping procedure. Hence transgenic rubber tree becomes a living factory for the production of the said protein where the recombinant protein can be extracted continuously and non-destructively by tapping the rubber tree. In transgenic *Hevea*, clonal copies can be vegetatively propagated from high expressing transformants. Thus, from a commercial standpoint transgenic rubber trees have a huge potential as a production system for heterologous proteins in bulk quantities. To explore the potential to produce commercially interesting proteins in *Hevea*, an attempt has already been made to produce transgenic rubber plants expressing human serum albumin (HSA) in the serum fraction of rubber latex using a binary vector containing HSA cDNA with its putative leader sequence in *Agrobacterium tumefaciens* (Arokiaraj *et al.*, 2002).

At present, the CaMV 35S constitutive promoter is often used to confer constitutive and high level expression of specific gene in transgenic plants. It may not be beneficial for *Hevea* plants to have foreign proteins or native homologous proteins expressed constitutively, as it could be deleterious (Arokiaraj, 2000). The choice of promoters can influence the expression level and targeted expression in localized areas of *Hevea*. Therefore, it would be desirable to isolate promoters involved in latex vessel specific expression. If a laticifer specific element could be identified within the upstream region of these genes, then latex vessel specific expression vectors can be designed. Tandem repeats of a putative latex enhancer element may give strong latex vessel specific expression compared to the native promoter. One way to identify such promoters would be to study the upstream regions of genes like, HMG-CoA reductase (Chye *et al.*, 1992), REF etc. which are highly expressed in the latex. Even though a number of genes involved in the isoprenoid rubber biosynthetic pathway have been isolated and their mode of expression have been characterised, the molecular structure and function of their promoter sequences are largely unknown and in fact such important information is only available for a few genes. Pujade- Renaud *et al.* (2000) isolated the 5' upstream region of hevein genes from rubber tree and the promoter-GUS fusion was introduced into rice by *Agrobacterium* mediated genetic transformation.

The objectives of the present work include, isolation and cloning of the 5' flanking region of the REF gene encoding the rubber elongation factor protein from *Hevea brasiliensis*. Chimaeric gene fusion of 5' flanking region of REF gene with the coding region of β-glucuronidase was prepared and the resultant gene construct was delivered into tobacco by *Agrobacterium* - mediated genetic transformation. The expression of *uidA* gene in different transgenic lines of tobacco was also analysed by RT-PCR.

5.2. MATERIALS AND METHODS

5.2.1. Plant Materials

Leaf material for genomic DNA isolation was collected from *H. brasiliensis* clone RRII 105 growing at RRII nursery. Young, expanded and light green healthy leaves were used for DNA isolation.

5.2.2. PCR Amplification of Promoter Region of REF Gene

The proximal promoter region of the REF gene was obtained by PCR amplification of the genomic DNA prepared from leaves of *H. brasiliensis* (Doyle and Doyle,1990). PCR was performed with oligonucleotide primers designed based on sequences from GenBank database (Accession no. AY134670, AF380139). The primers used for the amplification of promoter were:

Forward primer - 5'CCC AAG CTT GAA AAA CAA AGA CTA 3' Reverse primer - 5'ACG CGG TCG ACC CAG CAT AAG TTG C 3'

Reaction was carried out in a volume of 20 µl containing 15 ng of DNA as template, 2 µl of 10 X buffer, 2 µl of $MgCl_2$ (1.5 mM) and 2 µl dNTPs (2.5 mM), 1µl primers (10 µM) and 0.5 U Taq DNA polymerase. PCR was performed according to the following parameters: initial denaturation at 94°C for 4 min and 30 cycles of 94°C for1min, 52°C for 1 min, 72°C for 2 min followed by a last cycle of extension at 72°C for 7 min. After PCR, the amplified products were analysed on 1.5 % (w/v) agarose gel.

5.2.3. Cloning and Sequencing of REF Gene Promoter

Purified PCR product was cloned into plasmid vector. Cloned PCR fragment was sequenced and compared with published sequences in the NCBI database using BLASTN

program (Altschul *et al.*, 1990). Sequencing of the promoter sequence was carried out at Indian Institute of Science, Bangalore. The putative regulatory elements were identified by PLACE (Higo *et al.*, 1999) and PlantCare (Lescott *et al.*, 2002) programmes.

5.2.4. Construction of REF Promoter/ GUS Fusion

The 750 bp REF promoter which was cloned into the plasmid vector was subcloned to the upstream of the coding region of the *uidA* gene in pGPTV binary vector between *Hind* III and *Sal*I sites to generate the REF promoter:GUS fusion construct designated pGPTVRP. The REF promoter: GUS fusion was sequenced to verify the reading frame of the gene construct. A schematic representation of the gene construct used for genetic transformation experiments was shown in Figure 4. The construct was then transformed into *Agrobacterium tumefaciens* strain LBA 4404 by freeze-thaw method (Holsters *et al.*, 1978) and was selected for resistance to kanamycin (50 mg/l).

5.2.5. Genetic Transformation of Tobacco

5.2.5.1. Media composition

All the media used in the present experiment contained Murashige and Skoog's basal media (Murashige and Skoog, 1962), vitamins, sucrose 3% (w/v) and phytagel 0.2% (w/v) (Sigma) with different hormone and antibiotic concentrations. The pH of the medium was adjusted to 5.6 to 5.8 with NaOH and autoclaved for 15 min at 121°C.

5.2.5.2. Plant transformation

- Transformation of tobacco via *Agrobacterium tumefaciens* was carried out according to Horsch *et al.* (1985).
- Leaf discs were prepared from *in vitro* grown tobacco plants and pre-cultured on MS medium containing BAP (1mg/ l) for 2 days.
- The explants were co-cultivated with *Agrobacterium* for 3 days, washed 5 times with sterile water and transferred onto MS medium (Murashige and Skoog, 1962) containing 100 mg/l kanamycin and 200 mg/l carbenicillin.
- Regenerated shoots, selected by 100 mg/l kanamycin were excised and transferred to fresh medium. The shoots were transferred to root inducing medium to develop roots and rooted plantlets were finally planted in soil and sand (1:1) mixture in plastic cups.

5.2.5.3. Histochemical analysis of GUS Activity

Histochemical GUS activity was detected by staining with X- gluc (5-bromo-4-chloro-3-indoyl β-D-glucuronide). Transgenic tissues were placed in a solution of X-gluc and incubated at 37°C overnight (Jefferson *et al.*, 1987). The stained tissues were cleared of chlorophyll by washing with 70% alcohol (v/v) and then viewed under microscope and photographed.

X- gluc Solution	
50 mM	Sodium phosphate pH-7.0
1 mM	EDTA
0.001%	Triton-X-100
10 mM	2- mercaptoethaanol
2 mM	X-gluc

5.2.5.4. PCR screening of regenerated transgenic tobacco plants

The presence of the REF promoter- GUS fusion in the kanamycin resistant putative transgenic tobacco plants was confirmed by PCR. Genomic DNA was isolated from young leaves of tobacco plants (Dellaporta *et al.*, 1983) and used as template for PCR. PCR was performed with promoter specific and *uidA* gene specific primers. The promoter specific primers used for PCR was given above **(5.2.2).** The forward and reverse *uidA* specific primers used for PCR were:

uidA primers	
Forward primer	5'- TAG AGA TAA CCT TCA CCC GG- 3'
Reverse primer	5'- CGC GAA AAC TGT GGA ATT GA-3'

The conditions for PCR as well as the reaction components were given in the previous sections **(5.2.2)**. Only those plants with expected PCR products were used for further studies.

5.2.5.5. Southern blot analysis

Southern blot analysis was conducted with genomic DNA isolated from transgenic tobacco plants (Dellaporta *et al.*, 1983). The isolated genomic DNA (20 µg) was digested with *Hind*III alone and double digested with *Sac*I + *Sal*I and *Hind*III + *Sac*I enzymes. The digested DNA was then fractionated by agarose gel electrophoresis. Then the DNA was transferred onto nylon membrane and probed with α-P^{32}dCTP labeled *uidA*

gene probe (internal fragment amplified by PCR). Synthesis of probe, hybridization and washing of blot was carried out as described previously (sections, **2.2.5.4** and **2.2.5.5.**).

5.2.5.6. Expression studies by RT-PCR

The expression of GUS gene in PCR confirmed transgenic tobacco plants was further analyzed by RT-PCR. Total RNA was isolated from the leaves of transgenic tobacco plants by Trizol method according to manufacturer's instructions (Invitrogen Life Technologies). Total RNA was used for cDNA synthesis using oligo-dT primers and the cDNA mixture was then amplified by PCR using GUS specific primers as explained in the PCR section (5.2.5.3). The PCR parameters were the same as used for genomic PCR, but the number of cycles was increased to 45. The amplified products were fractionated on agarose gel.

5.3. RESULTS

5.3.1. PCR Amplification of Promoter Region of REF Gene

The upstream region of REF gene of *H. brasiliensis* was amplified by PCR. Specific oligonucleotide primers were synthesised based on sequences from database. Genomic DNA isolated from leaves was used as template for PCR. PCR at 55°C annealing temperature, performed for 30 cycles resulted in the amplification of two fragments of approximate sizes 750 and 500 bp from the genomic DNA of *H. brasiliensis*. The result of PCR amplification of REF promoter was given in Figure 1. The upper 750 bp band was purified from low melting point agarose gel and cloned into plasmid vector for further study.

5.3.2. Sequence Analysis of the Promoter Region of REF Gene

The cloned 5' upstream region of REF gene was sequenced and identified as a fragment of REF gene from *H. brasiliensis*. The nucleotide sequence of the 5' upstream region of REF was presented in Figure 2. The sequence of the REF gene promoter was compared with other promoter sequences of REF published in database. The promoter region of REF was nearly identical to the other REF promoter sequences deposited in the database with only five nucleotide differences. A comparison of the nucleotide sequence of 5' upstream region of REF gene with an earlier reported sequence was presented in Figure 3. The sequence 5' to the ATG translational start site showed five base

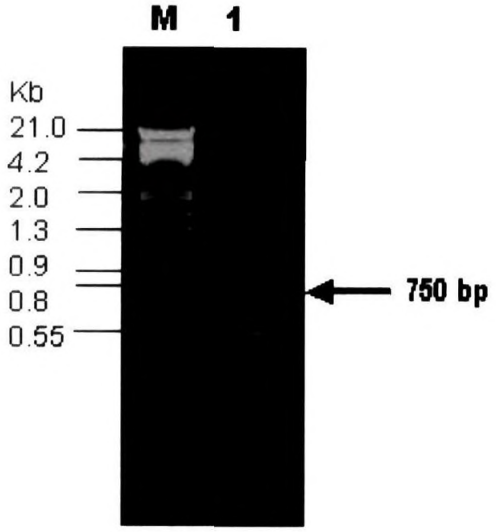


Figure 1. PCR amplification of 5' upstream region of REF gene of *H. brasiliensis*. Lanes: M - DNA molecular size marker, 1- Amplified REF promoter fragments

Figure 2. The nucleotide sequence of the amplified 5' upstream region of REF gene. The translational start site is labeled +1. The putative TATA and CAAT box and ATG codon are underlined. Putative regulatory motifs in the REF promoter region are boxed and labeled above the sequences.

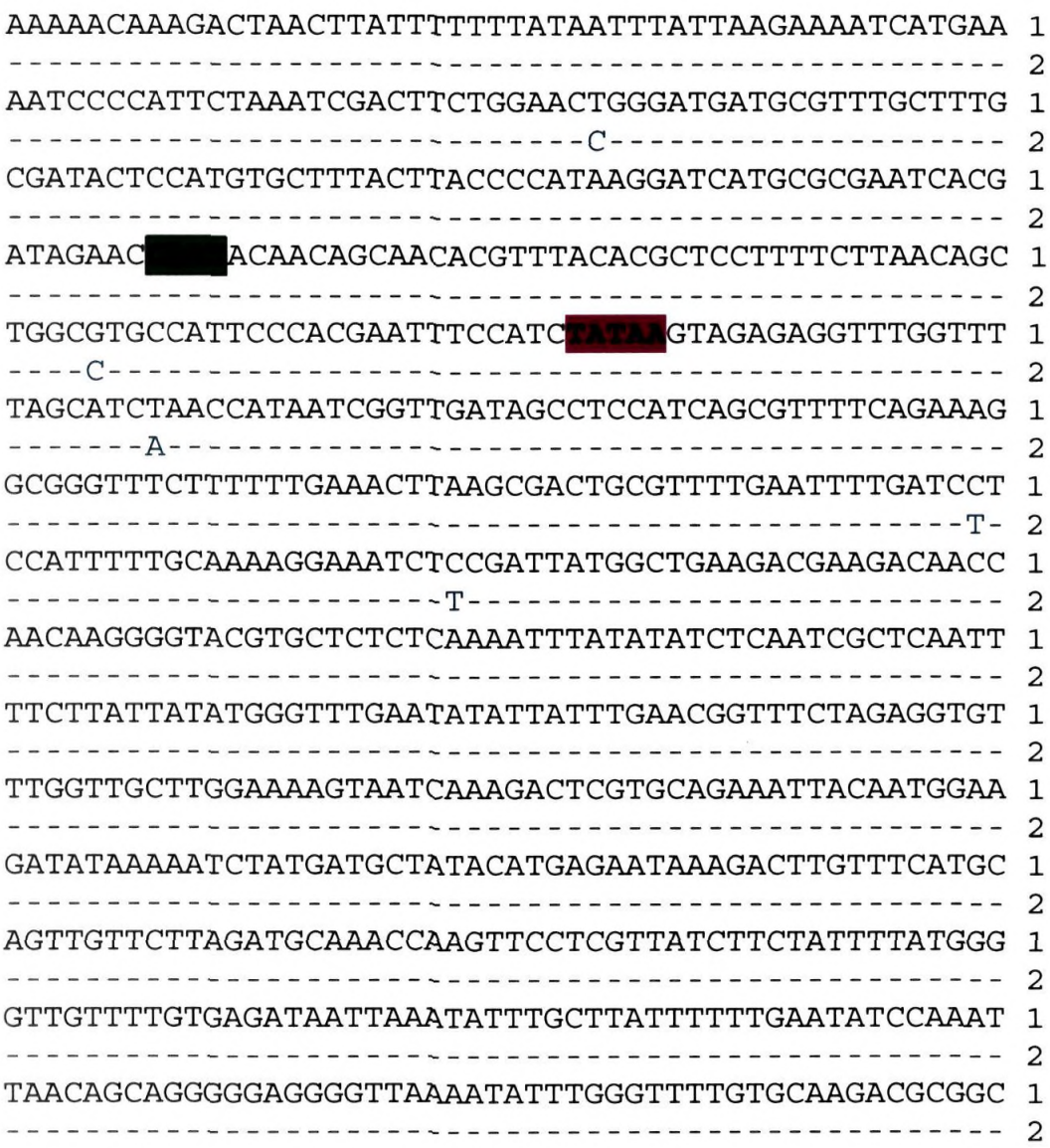


Figure 3. Comparison of nucleotide sequence of REF promoter: 1-REF promoter cloned in the present study and 2-Promoter sequence in GenBank (Accession No. AF380139). The places of identity were denoted by dash and differences were marked blue colour.

differences. These variations may be due to clonal differences as the present sequence was amplified from RRII 105 (a popular Indian clone) whereas the other sequences reported in the GenBank database were isolated from other clones. The nucleotide sequence of the proximal promoter region is available in database under Accession No. AY712939. Sequencing showed that the amplified upstream region of HbREF gene was 750 bp and the region upstream to the ATG codon was 378 bp in length. A putative TATA box was found between positions -150 and -146 with reference to the translational initiation site (ATG, +1). The TATA box sequence (CTATAA) closely corresponded to the plant consensus sequence (C/G) TATA (T/A) (A1-3)(C/T)A (Zhu *et al.*, 1995). A putative CAAT box motif was located at –221/ -218 position. Thus the proximal region of REF contains the elements shown to be necessary for an accurate initiation of basal transcription in promoters from other plants.

5.3.3. Putative Regulatory Elements in the REF Promoter

The 5' flanking region of REF was analyzed for known motifs of other genes and found several potential regulatory elements shown in Figure 2. Additional motifs were identified using the PlantCARE (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999). Motifs that control responses to JA, ABA, wounding stress and light were found in the REF promoter. A hexamer sequence CACGTT, closely resembling the consensus recognition sequence called G- box core element (CACGTG) was observed in the promoter between positions -207 and -202. This element was also similar to a conserved methyl jasmonic acid (MeJA) responsive domain CACGTG of soybean *VspB* gene (Williams *et al.*, 1992, Mason *et al.*, 1993). Another ACGT core element that is conserved in G- boxes, ABREs (Abscisic acid responsive elements) and E- boxes was also present in antisense orientation (TGCA) at -21/-18 position of this promoter. Sequences similar to ethylene response elements (ATTTCAAA) were also detected in REF promoter at -159 and -151 positions and adjacent to TATA element. REF promoter also had sequence resembling to *pin 2* elicitor motif (ACCTTGCC) found in the wound induced potato *pin2* promoter in six out of eight position between -181/ -174 region. A W- box (TTGACT) like element involved in response to plant defence signalling was identified at -61/-56 position in antisense orientation (AACTtA) and a cytokinin responsive ARRI binding element with a consensus GATCTT was also found in the

promoter at -34 and -29. Three GCC core elements (one in antisense orientation) involved in jasmonic acid (JA)- responsiveness were found at -102/-100, -172/-170 and -299/-297 positions. In the REF promoter sequence, three AREs (anaerobic responsive elements), the core consensus sequence present in the 5' upstream region of anaerobically induced genes were identified. These elements were located at -75/-71, -133/- 129 and -138/-134 positions down stream of the TATAA element and two of the ARE elements are closely located in this promoter (-133/- 129 and –138/-134).

Motifs similar to the Myb recognition site MYB 1AT having consensus sequence WAACA was identified at -121/-116 and at -136 / -131 (antisense orientation). A sequence element similar to the motifs of CANNTG also called E- box, which is involved in spatial and temporal specific expression was found in positions -270/-265 upstream of the TATAA box. This site (CATGTG) was also identified as MYC recognition site found in the promoters of dehydration- responsive gene and in many other genes in *Arabidopsis*. A MYB core motif (CNGTTR) was also located at -111/-106 positions in the REF promoter. The AAAAGG sequence located in the promoter between -18 and -13 position was identified as a Dof 1 protein-binding site. Two GATA core elements were also found at -229/-226 and at -277/-274 positions. GATA core were reported to be involved in light responsiveness. Two GT1 consensus motifs (GAAAAT) involved in light responsive expression were present at -340/-335 and -331/-326 regions. Binding consensus sequence of *A. thaliana* transcription factor RAVI, CAACA motif was located at -216/-212 position. Interestingly, the sequence GTTTTG (at -46 positions) conforms to the RTTTTR (R-A/G) consensus sequence, the binding site of a soybean enhancer for the regulatory nuclear protein, soybean embryo factor, SEF3 (Allen *et al.*, 1989). In addition, a pyramidine box like sequence (CCTTTT, at -193/-188 position) and a pollen specific element (AGAAA, at -84/-80 positions), which was characteristic of tomato gene were also detected in the REF promoter. A list of potential regulatory motifs or regions was shown in Table-1.

Table 1- Known regulatory motifs shared by REF gene promoter and other promoters

Regulatory motif	Consensus	Sequence in REF promoter	Position in REF	Function
G- box/ABRE/ E-box core	ACGT	ACGT TGCA (-)	-21/-18	Light, ABA & Stress responsive
MeJA responsive Element/G box	CACGTG	CACGTt	-207/-202	MeJA responsive
GCC core	GCC	GCC CGG (-)	-102/-100,-172/-170 -299/-297 (-)	MeJA responsive
Ethylene motif	ATTTCAAAA	ATTTCcAtc	-159/–151	Ethylene responsive
W- box	TTGACT	AACTtA (-)	-61/-56	Plant defence signalling
Pin2 elicitor motif	ACCTTGCC	AgCTgGCC	-181/-174	Plant defence signalling
ARRI binding Element	GATCTT	GATCTT	-34/-29	Cytokinin responsive element
ARE element	GGTTT	GGTTT	-75/-71 -133/-129 -138/-134	Anaerobically regulated expression
MYB1AT	WAACCA W-A/T	AAACCA TTTGGT(-)	-121/-116 -136/-131	Dehydration stress
MYB Core	CNGTTR	CGGTTG	-111/-106	Dehydration stress
MYCATERD 1	CATGTG	CATGTG	-270/-265	Dehydration stress
E- box	CANNTG N=A/T/G/C	CATGTG	-270/-265	Tissue specific and developmental expression
Dof1-protein binding site	AAAAGG	AAAAGG	-18/-13	Tissue specific and Light regulated Gene expression
GATA core/ I- box core	GATA	GATA	-229/-226, -277/-274	Light regulated Gene expression
GT1 consensus	GRWAAW R=A/G,W=A/T	GAAAAT GAAAAT	-340/-335 -331/-326	Light regulated gene expression
RAVI-A	CAACA	CAACA	-216/-212	Expression in leaves and roots
Pyramidine box	CCTTTT	CCTTTT	-193/-188	Sugar repression
SEF 4 motif	RTTTTR R-A/G	GTTTTG	-46/-41	Seed specific
Pollen specific Element	AGAAA	AGAAA	-84/-80	Pollen specific

Mismatches to the consensus sequence are shown in lower case letter

5.3.4. Construction of REF Promoter/ GUS Fusion

The 5' upstream region of REF, which was cloned into the PCR vector, was subcloned into the binary vector pGPTV between *Hind*III and *Sal*I sites in the multiple cloning site. The resultant chimaeric plasmid *REF:GUS* construct (pGPTVRP) contained a 750 bp fragment of the REF gene promoter region including the 5' flanking region of REF gene and the first few codons fused in frame to the *uidA* coding sequence (GUS). A schematic representation of the gene construct was shown in Figure 4. The chimaeric plasmid was introduced into *Agrobacterium tumefaciencs* strain- LBA4404 by freeze thaw method (Holsters *et al.*, 1978) and then used for genetic transformation of tobacco.

5.3.5. Transformation of Tobacco

To evaluate the functioning of the isolated promoter element of REF gene, a transgenic experiment using tobacco plants was conducted. Transgenic tobacco plants containing a chimaeric *REF:GUS* gene construct in which a *GUS* reporter gene was placed under the control of the 750 bp upstream region of REF gene from *H. brasiliensis* were generated. This construct was introduced into tobacco by *Agrobacterium tumefaciens*- mediated transformation of leaf discs. Leaf discs were co-cultivated with *Agrobacterium* for 2 days. The leaf discs were washed to remove contamination and placed on selection medium containing kanamycin as the selection agent. Shoots regenerated from leaf discs were subcultured and the regenerated shoots were transferred to root inducing medium. Six independent kanamycin resistant transgenic tobacco lines were obtained (Figures 5 A and B). The rooted transgenic tobacco plants were transferred to soil and sand mixture (1:1) in plastic cups (Figure 5C).

5.3.5.1. Histochemical GUS staining

A few representative plants from transgenic lines were analyzed for the presence of GUS activity by histochemical coloration of vegetative tissues with X- gluc along with non-transgenic tobacco plants as negative control. The transformed tobacco plants showed positive GUS activity. In transgenic tobacco plants GUS activity was detected on the leaves and petioles (Figures 6A & B) and no GUS activity was detected in non-transgenic plants used as negative control (Figure 6C). Thus the results presented in this study suggests that the promoter fragment of REF analyzed here is functional as

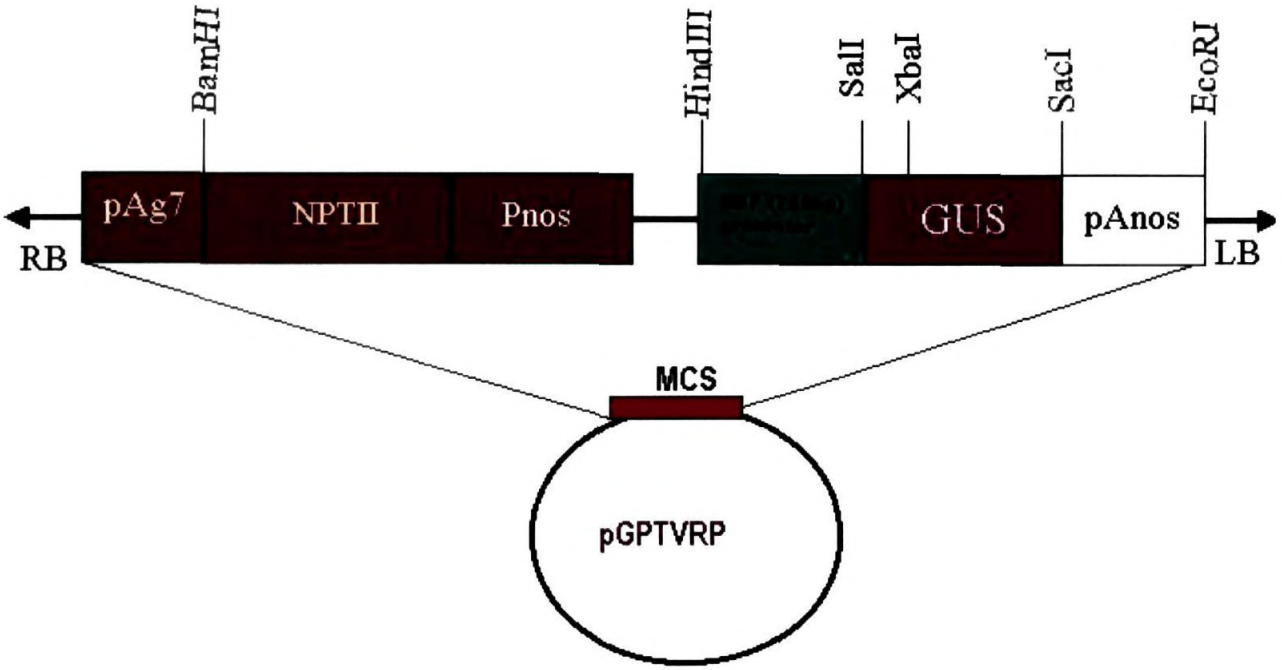


Figure 4. Schematic representation of the chimaeric construct of REF:GUS fusion used for tobacco transformation. The REF promoter sequence (750 bp) was fused to the GUS coding region in the vector pGPTV between *Hind* III and *Sal*I site.

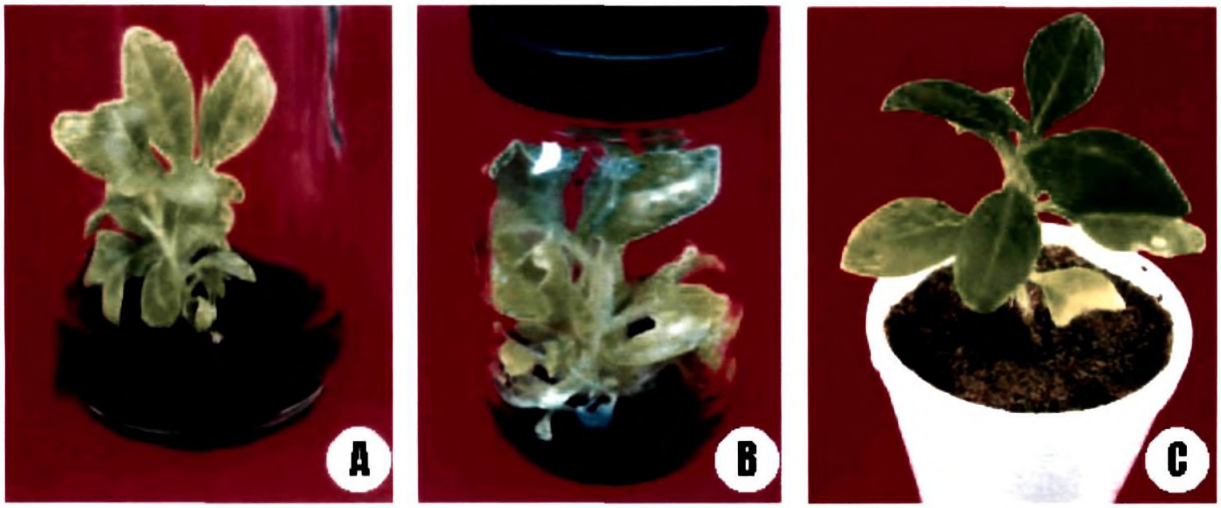


Figure 5. A - Regenerated kanamycin resistant transgenic tobacco plants harbouring REF:GUS gene construct. B- Rooted transgenic tobacco plants. C- Rooted trangenic tobacco plants established in soil.

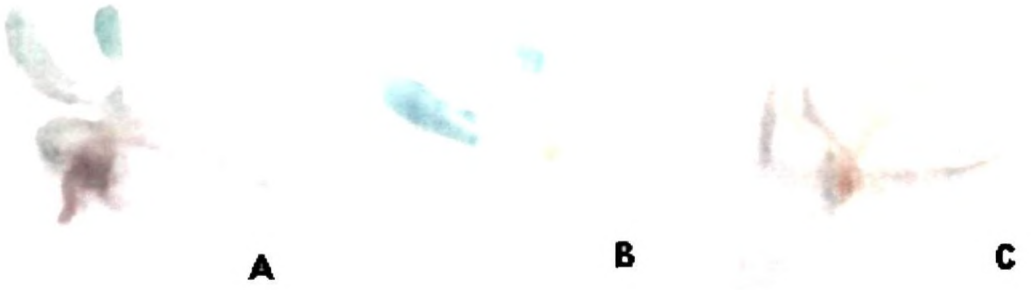


Figure 6. Histochemical analysis of GUS activity in transgenic tobacco plants harbouring GUS: REF gene construct. A & B- GUS activity is visible as blue coloration on leaves of transgenic tobacco plants. C- Non transgenic tobacco plants as negative control (no blue colour).

Figure 7

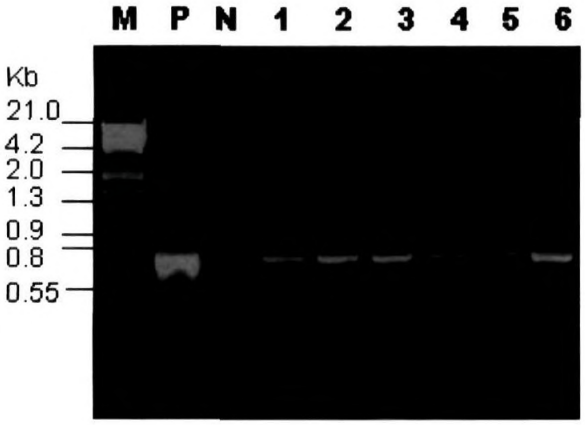
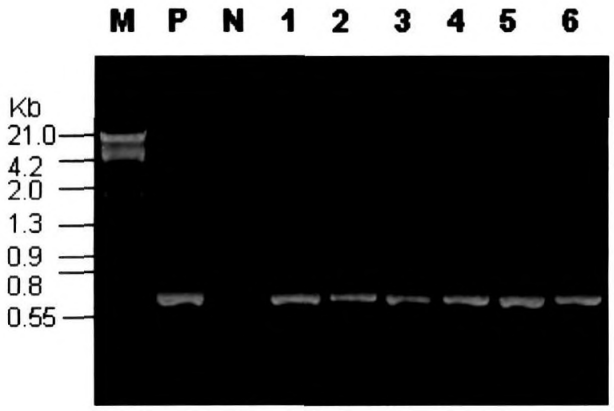


Figure 8



Molecular confirmation of putative transgenic tobacco plants, harbouring pGPTV-REF gene construct by PCR analysis. Lanes: M - DNA molecular size marker, P- Positive control, N- Negative control (non-transgenic plants), 1-6 - Independent transgenic tobacco plants.

Figure 7. A 750 bp REF promoter fragment was amplified from six representative transgenic tobacco lines with REF promoter specific primers.

Figure 8. A 650 bp *gus* gene fragment was amplified from six representative transgenic tobacco lines with *gus* gene specific primers.

determined in terms of their ability to direct expression of the GUS gene in transgenic tobacco plants.

5.3.5.2. Molecular confirmation of transformants by PCR analysis

The kanamycin resistant putative transgenic tobacco plants were initially screened by genomic PCR. Primers for the *uidA* and REF promoter were used to amplify the DNA fragments of 650 bp and 750 bp respectively. PCR was conducted with genomic DNA isolated from transgenic tobacco plants and from non-transgenic plants as negative control. The plasmid DNA pGPTVRP was used as positive control. Results of PCR showed that only the DNA from all transformed tobacco plants and the positive control amplified the 750 bp REF promoter fragment (Figure 7). No amplification was obtained in the untransformed negative control DNA. A 650 bp *uidA* gene was amplified in all the six transgenic lines of tobacco as well as in positive control when PCR was conducted with *uidA* specific primers (Figure 8). But the corresponding DNA product was not detected in the untransformed negative control. Thus the results of genomic PCR clearly revealed the integration of transgene in the genome of transgenic tobacco plants.

5.3.5.3. Genomic Southern blot analysis

Genetic transformation of tobacco plants and the integration of transgene in the host genome were further confirmed by genomic Southern blot analysis (Figure 9). Genomic DNA isolated from leaves of PCR screened transgenic tobacco plants was digested with *Hind*III alone and double digested with *Sac*I + *Sal*I and *Hind*III + *Sac*I enzymes. When probed with radiolabeled *uidA* gene fragment, gel blot of *Hind*III digested genomic DNA gave two hybridizing bands, confirming the presence of reporter gene in the host tobacco genome. In each case, the double digest produced internal fragment of predicted size that hybridized to the *uidA* probe. In *Sal*I + *Sac*I digests, the *uidA* probe hybridized to a fragment of approximately 0.8 Kb size whereas in the case of *Hind*III and *Sac*I digest, the probe hybridized to a fragment approximately of 1.6 Kb as expected. Since in the gene construct, the *Hind*III and *Sal*I recognition sites delineate REF promoter and *uidA* gene, this 1.6 Kb single band hybridization pattern suggested that during the process of *Agrobacterium*-mediated T- DNA transfer and host chromosome integration, no genetic rearrangement had occurred within the promoter: reporter gene.

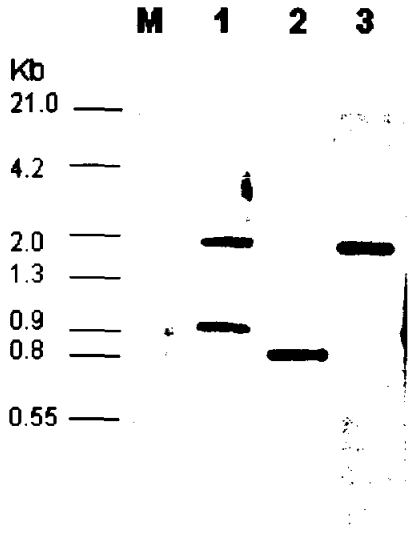


Figure 9. Southern blot analysis of genomic DNA isolated from transgenic tobacco plants and hybridized to *uidA* specific probe. Lanes: M- DNA marker; 1- DNA digested with *Hind*III, 2- DNA double digested with *Sal* I and *Sac* I and 3- DNA digested with *Sac* I and *Hind*III.

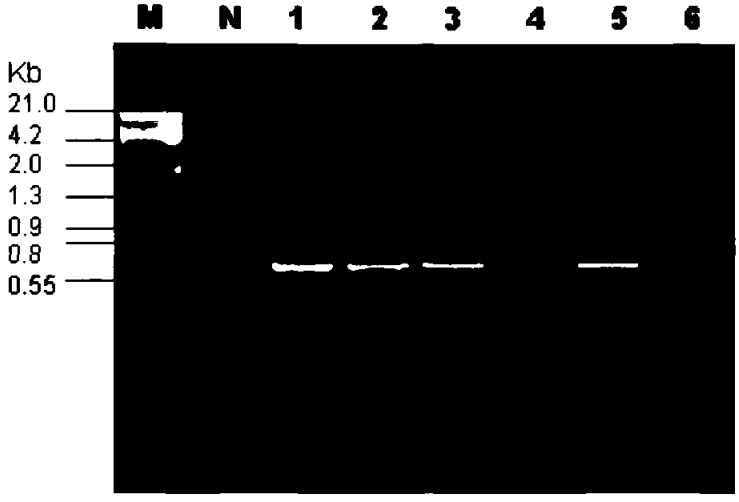


Figure 10. RT-PCR analysis of *gus* transcripts in transgenic tobacco plants. Total RNA from non-transgenic and transgenic tobacco plants were reverse- transcribed with oligo- dT and the cDNA product was amplified by PCR with *gus* gene specific primers. A 650 bp *gus* cDNA was amplified from six representative transgenic tobacco lines. Lanes: M- DNA molecular size markers, N- negative control (non transgenic tobacco plants), 1-6 -Independent transgenic tobacco plants.

5.3.5.4. Expression analysis of GUS gene by RT-PCR

RT-PCR technique was used to investigate the GUS reporter gene transcript levels in the RNA population isolated from leaf tissues of transgenic tobacco plants. Total RNA isolated from the leaves of transgenic tobacco and non-transgenic plants was reverse transcribed. The resultant cDNA was amplified with GUS specific primers. As seen in Figure 10, a specific cDNA product of 650 bp at the expected position for GUS was clearly visible in RT- PCR products from transgenic tobacco plants. But the same fragment was not observed in the untransformed negative control. These results show that the GUS transcript was accumulating in the transgenic tobacco plants, which were initially screened by genomic PCR.

5.4. DISCUSSION

5.4.1. Amplification and Cloning of the 5' Upstream Region of REF Gene

The 5' upstream element of REF gene was cloned and characterised from *H. brasiliensis*. PCR conducted with genomic DNA from *H. brasiliensis* could amplify a 750 bp fragment. This amplicon was cloned into a plasmid vector and then sequenced. Sequencing of the cloned promoter fragment showed that the amplified fragment is indeed a part of the REF gene from *H. brasiliensis*. The cloned REF promoter region was nearly identical to the other published REF promoter regions with only 5 nucleotide mismatches. The sequence length of the REF promoter is 750 bp.

5.4.2. Sequence Characterization of REF Promoter Fragment

The *H. brasiliensis* REF 5' flanking region was analyzed to identify promoter elements for which there is evidence of a functional role in plants. A putative TATAA box element at -150 and a CAAT motif is identified at -221 position of the REF promoter. From the comparison of the promoter region of the plant genes so far isolated, several consensus motifs emerge corresponding to putative *cis* elements. Analysis of the 5' region reveals a number of features, which include G- box motifs, E- box, W- box etc. G- box elements are involved in the regulation of a large number of plant genes. G- box element was first identified as a highly conserved protein-binding site upstream of many genes (Giuliano *et al.*, 1988). The original report defined a 12- bp sequence GACACGTGGC, necessary for efficient binding of the G- box factor (GBF) *in vitro*. The core hexanucleotide CACGTG become identified as G- box element. The G- box motif is required for expression of

differentially regulated genes such as light regulated (Donald and Cashmore, 1990; Schindler *et al.*, 1992), abscisic acid- induced (Mundy *et al.*, 1990), methyl jasmonic acid (MeJA) responsive (Williams *et al.*, 1992, Mason *et al.*, 1993) and stress- induced gene (Ferl and Laughner, 1989; Ferl and Nick, 1987). The core of the G-box, ACGT motif was shown to be the DNA binding site of the transcription factors belonging to the bZIP (basic domain/leucine Zipper) family (Foster *et al.*, 1994). Most functionally defined abscisic acid responsive elements, ABREs also contain the core sequence ACGT (Shinozaki and Yamaguchi, 2000; Straub *et al.*, 1994). DNA motifs with G- box cores (ACGT) mediate both ABA and JA responsiveness (Gultinan *et al.*, 1990; Kim *et al.*, 1992).

Motifs similar to the ethylene response elements found in fruit ripening and glutathione- S- transferase gene (ATTTCAAA), potato *pin2* elicitor response motif and one W- box like element are also detected in the upstream region of REF gene. The potato *pin2* promoter has a 10- bp sequence that binds a wound induced-protein and an elicitor- response related motif (Deikman, 1997), which is similar to the L- box motifs found in elicitor- and light- regulated genes (Palm *et al.*, 1990). W- box *cis* elements bind WRKY transcription factors. It is involved in response to plant defense signaling (Eulgem *et al.*, 2000; Yu *et al.*, 1993). A recent paper describing expression profiling of cytokinin action in *A. thaliana* identified a 5'- GATCTT-3' consensus sequence prevalent in cytokinin up-regulated gene promoters (Rashotte *et al.*, 2003). Several ARE elements are present in REF promoter. These ARE elements are present in the upstream region of several anaerobically induced genes. This GT- rich region motif is necessary and sufficient for anaerobically regulated gene expression (Sarni- Manchado *et al.*, 1997).

Presence of MYB IAT site, plant MYB binding site and MYCA TERD 1 sites in the REF promoter is another characteristic feature. MYB IAT is found in the promoters of the dehydration - responsive gene rd 22 and in many *A. thaliana* genes (Abe *et al.*, 2003), while MYC recognition sequences are necessary for expression of *erd 1* gene (early responsive to dehydration) in *A. thaliana* (Simpson *et al.*, 2003). The sequence (CNGTTR) or MYB core is the binding site for MYB proteins isolated from *A. thaliana* and is involved in regulation of genes that are responsive to water stress in *Arabidopsis* (Solano *et al.*, 1995). Myb gene family represents one of the largest regulatory factor families in plants and one of the important functions of Myb factors is to control development and determination of cell fate and identity (Stracke *et al.*, 2001). REF gene promoter is also characterised by the presence

of E- box. The E- box with the consensus sequence of CANNTG is known to be the recognition site of a class of transcription factors (basic region/ helix loop helix proteins) and can form homo and heterodimers to exert regulatory functions (Pabo, 1992). E- boxes have been shown to be involved in both tissue specific and developmental control of β-phaseolin promoter (Burrow *et al.*, 1992). It is a variation of the G- box motif (CCACGTG) (Li and Capetanaki, 1994).

Another property of the REF gene promoter is the occurrence of several light responsive elements like three GATA core elements and three GT1 elements. GATA box motif was identified in the promoter of petunia chlorophyll a/b binding protein and is required for high level, light regulated and tissue specific expression of gene (Gidoni *et al.*, 1989). GATA core elements also form part of I- box elements. The occurrence of I- box sequences in gene promoters has been closely correlated with light regulated gene expression (Giuliano *et al.*, 1988). GT1 is a consensus-binding site in many light -regulated genes of oat, pea, rice, spinach etc. GT1 element can stabilize the TF IIA-TBP-DNA (TATA box) complex. The activation mechanism of GT1 may be achieved through direct interaction between TF IIA and GT1 (Villain *et al.*, 1996). Presence of Dof 1 protein binding site required for the binding of Dof proteins is another characteristic of REF promoter. Dof 1 proteins (DNA one finger) are DNA binding proteins with presumably only one zinc finger and are unique to plants (Yanagisawa and Schimidt, 1999). They bind to AAAAGG core element motif (Yanagisawa and Sheen, 1998). Yanagisawa (2000) proposed that Dof protein can function as transcriptional activators or repressors of tissue specific and light regulated gene expression in plants.

In the upstream region of REF gene, motifs like CAACA (RAVI-A) and SEF4 binding site as well as pyramidine box and a pollen specific element (AGAAA) are identified. *A. thaliana* transcription factor, RAVI- A binds specifically to DNA with bipartite sequence motif CAACA (Kagaya *et al.*, 1999). Pyramidine box in rice α- amylase gene is partially involved in sugar repression (Mena *et al.*, 2002). It was also reported in barley α-amylase gene, which is induced in the aleurone layers in response to gibberellic acid and a protein called BPBF binds specifically to this site (Morita *et al.*, 1998). SEF 4 (soybean embryo factor 4) binding site is a consensus sequence found in 5' upstream region of soybean β-conglycinin gene and it binds with SEF4 (Allen *et al.*, 1989). Bate *et al.* (1998) reported that one of the two co-dependent regulatory elements responsible for pollen specific

activation of tomato lat 52 gene is AGAAA. Plants and animals respond to a wide range of internal and external stimuli by modulating transcription of diverse genes. Many plant DNA binding proteins can be grouped into distinct classes based on their conserved DNA binding domains (Paz-Ares *et al.*, 1987). Each member of these families interacts with closely related DNA sequence motifs that are often found in multiple gene promoters controlled by different regulatory signal. It has been proposed that the combination of different *cis* elements and *trans* acting factors may produce the diversity and specificity required for the regulation of gene expression (Menkens *et al.*, 1995; Martin and Paz- Ares, 1997).

5.4.3. Functional Characterization of REF Promoter in Transgenic Tobacco

A preliminary functional analysis of the REF 5' upstream region was performed in transgenic tobacco plants. Transgenic tobacco plants containing a translational fusion of a 750 bp 5' flanking region of REF gene with coding sequence of the GUS reporter gene were generated. Kanamycin resistant transformed lines were selected and the plants from the transformed lines were analyzed for GUS activity by histochemical coloration of vegetative tissues. The transgenic tobacco lines were stained blue by X- gluc, while blue coloration was not detected in the non-transgenic plants, which served as negative control. GUS activity was detected on the leaves and petioles of transgenic plants. The intensity of GUS expression varied among the different transgenic lines of tobacco. Transgenic tobacco lines were also analysed for the presence of REF promoter as well as GUS by PCR. DNA obtained from six independent kanamycin resistant transgenic tobacco lines revealed the specific predicted amplification products of 750 bp and 650 bp with REF promoter specific and GUS gene specific primers respectively. Positive control plasmid also amplified the corresponding bands. No amplification product was detected in DNA from untransformed plant when subjected to PCR amplification with either of the two primers. Genomic Southern blot analysis performed with the *uidA* gene as probe further confirmed the genetic transformation of tobacco and the integration of transgene in the host genome.

We used RT-PCR technique to study the expression of *uid*A transcript in various transgenic tobacco plants tested positive for the presence of GUS and promoter fragment by genomic PCR. RNA isolated from the leaves of transgenic tobacco plants were used as template for RT-PCR reaction. The resultant cDNA was amplified by PCR using *uidA* specific primers to amplify the specific cDNA of GUS. All transgenic tobacco plants amplified the GUS cDNA product, though the intensity of the cDNA product was very faint

in some transgenic plants. It also varied between various transgenic lines. These results show successful transcription of GUS gene. According to Finnegan and McElroy (1994), the levels of transgene expression are generally unpredictable and vary among independent transformants.

Our results of histochemical GUS analysis and RT-PCR analysis indicate that the Hb-REF 5' upstream region contains a functional promoter capable of driving foreign gene expression in transgenic tobacco plants. The promoter was directing GUS expression in leaf tissue of transgenic tobacco, although the level of expression was generally low. Recently Pujade- Renaud *et al.* (2000) isolated the 5' upstream region of hevein genes from rubber tree. The promoter of hevein was fused to the GUS reporter gene and introduced into rice by *Agrobacterium* mediated transformation. It appeared to be functional both in leaves, roots and anthers of rice and was activated in response to mechanical wounding. Furthermore the promoter fused to GUS reporter gene was found to be functional when introduced into rubber tree callus and embryoids. The 5' flanking regions of a potato *hmg1* (HMG-CoA reductase), a key enzyme involved in isoprenoid biosynthesis genomic sequence was used to drive the expression of β-glucuronidase gene (GUS) in transgenic tobacco and potato plants (Bhattacharya *et al.*, 1995). Strong activity of the GUS enzyme was observed in mature pollen grains. Jain *et al.* (2000) reported that the mulberry hmg1 promoter is differentially expressed and regulated by dark and abscisic acid in transgenic tobacco.

Promoter sequences that confer tissue specificity, temporal or developmental specificity, inducibility and alternative levels of activity are needed to regulate transgenic expression effectively. Efforts to genetically engineer rubber will benefit from availability of tissue specific promoters that control the expression of a transgene in a tissue. The well-established tobacco system was used for the analysis of the REF promoter from *H. brasiliensis*. This has successfully been used previously to analyse heterologous promoter elements. By using an alternate system such as tobacco, the promoter sequence of REF was able to drive GUS gene expression. The results presented here clearly demonstrate that tobacco is a useful model system for characterizing heterologous promoter elements. Further studies with REF gene promoter are required to understand the transcriptional control of REF gene expression in transgenic *Hevea*. This study represents a report on the functional characterization of promoter sequence of REF gene from rubber tree.

SUMMARY AND CONCLUSION

The genomic sequence coding for REF protein was amplified from the genomic DNA of *H. brasiliensis* using REF gene specific primers. The amplified 1.3 Kb REF gene fragment was cloned into plasmid vector and sequenced. The length of the amplified gene was 1367 bp with an ORF of 414 bp. Two introns were identified in the coding region of REF gene. The two introns are 298 bp and 455 bp long and the three exons are 30 bp, 216 bp and 171 bp in length respectively. The deduced protein is acidic (pI- 5.04) without a signal peptide. The coding region was similar to the cDNA of REF. Southern blot analysis performed with *Hevea* genomic DNA suggests that REF is encoded by a small gene family consisting of two members in *Hevea*. To date, this is the first report on the isolation and characterisation of the genomic sequence of REF protein from *H. brasiliensis*.

A cDNA encoding REF protein was amplified by RT-PCR from the latex of *H. brasiliensis* clone RRII 105 with gene specific primers. The amplified cDNA was cloned into plasmid vector and sequence characterised. The amplified REF cDNA was 622 bp in length with an ORF of 414 bp. The coding region of REF cDNA encodes for a protein of 138 amino acids with a predicted molecular mass of 14.7 kDa. The deduced protein was acidic with a pI of 5.04. The protein is localized in the cytoplasm and is without a signal peptide. Comparison of the REF cDNA sequence isolated in the present study revealed no differences with previously characterised REF cDNA sequences from *Hevea*. The deduced protein sequence of REF showed 51% sequence identity to *H. brasiliensis* isoform of REF, 51% to REF like stress- related protein 1 from *H. brasiliensis*, 42% to SRPP protein from *H. brasiliensis*, 39% identity to *Hevea* REF like stress related protein-2 and 28% to *A. thaliana* REF related protein.

The coding region of REF cDNA was cloned into an expression vector pGEX and transformed into *E. coli* cells to produce recombinant REF protein. Sequencing of the

recombinant vector with cloned REF cDNA ensured correct orientation of the reading frame. Conditions were optimized for the expression of the recombinant REF protein in soluble form. On induction with 0.3 mM of IPTG, the REF protein was expressed as a fusion protein of expected size (40 kDa) with GST N-terminal affinity tag. The GST-REF recombinant protein was purified to homogeneity using GST- affinity column. The identity of the recombinant protein was confirmed by immunoblot analysis with anti-GST antibodies.

The REF cDNA was cloned into the pBIB binary vector. Stable transformation of *Nicotiana tabacum* was carried out with this gene construct via *Agrobacterium*-mediated method to generate transgenic tobacco plants expressing REF protein of *H. brasiliensis*. Putative transgenic tobacco plants were selected using kanamycin as the selection agent. To confirm the transgene integration, the putative transgenic tobacco plants were initially screened by PCR using REF and *nptII* gene specific primers. PCR analysis revealed that a 600 bp REF as well as 800 bp *nptII* gene fragments were amplified from the genomic DNA of transgenic tobacco plants while non-transgenic tobacco plants (negative control) did not amplify the corresponding bands. The level of expression of REF and *nptII* genes in the transgenic tobacco plants was further studied by RT-PCR where both the 600 bp REF cDNA and 800 bp *nptII* cDNA fragments were detected and no amplification was obtained from non-transgenic control plants.

The expression pattern of REF gene in *H. brasilensis* was studied by RNA- blot analysis using radiolabeled REF cDNA as probe. Results of RNA blot revealed differential expression of REF gene in various tissues of mature rubber trees. Latex and bark showed a predominant expression of REF in comparison with leaf tissues. In *Hevea* seedlings, REF transcripts were accumulated more in the petiole and bark than in root and leaf. It is evident that tapping had an effect on the level of REF mRNA accumulation in *Hevea*. The REF transcripts accumulated more in regularly tapped trees than in untapped trees. RNA blot analysis performed in three different high yielding and low yielding clones suggests that there is significant clonal variation in REF transcript accumulation. In high yielding clones, REF gene expression was high in comparison with the low yielding clones. The effect of exogenous ethephon stimulation on REF transcript accumulation was also studied. Results of northern analysis revealed a detectable increase

in REF gene expression in the latex of ethephon stimulated trees than in the control unstimulated trees at all concentrations tried. Maximum stimulatory effect was noticed at 48 h of treatment. Also, the trees stimulated with 5% ethephon exhibited a higher expression level than in 2.5% treated trees.

The promoter region of REF gene was amplified by PCR with specific primers and a 750 bp fragment was obtained from the genomic DNA of *Hevea*. The amplified REF promoter region was cloned and sequence characterized. A putative TATA element at –150 and a CAAT box motif at –221 position were identified in the REF promoter. The 5' flanking region was analysed for the presence of different regulatory motifs identified in other plants. Several regulatory motifs like G- box core elements, GATA core, W- box, E- box, Dof 1 protein binding site, Ethylene response element, *pin 2* elicitor motif, GT 1 element and several Myb binding sites were identified in the REF promoter region. A preliminary functional analysis of the REF promoter was performed in transgenic tobacco plants containing a translational fusion of the 750 bp 5' upstream region with the coding region of *uidA* gene in the binary vector, pGPTV. Kanamycin resistant putative transgenic tobacco plants were generated and the plants were analysed for the presence of GUS activity by histochemical coloration of vegetative tissues with X- gluc. Transgenic tobacco plants showed positive GUS activity in the leaf and petiole, while no GUS activity was detected in the non-transgenic tobacco plants. Further screening of transgenic tobacco plants was carried out by PCR with GUS specific and promoter specific primers. Only the transgenic tobacco plants yielded the 750 bp REF promoter and 650 bp *uidA* gene fragments. Results of Southern blot analysis of the genomic DNA isolated from transgenic tobacco with *uidA* gene probe further confirmed stable integration of transgene in the tobacco genome. The presence of *uidA* transcripts in the PCR positive transgenic tobacco plants was further confirmed by RT-PCR using *uidA* gene specific primers. The transgenic tobacco plants amplified the *uidA* cDNA of 650 bp and no specific cDNA product was detected in nontransgenic control plant.

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Synopsis

Thesis Title: Molecular cloning, characterization and expression of rubber elongation factor gene from *Hevea brasiliensis*

Rubber elongation factor (REF) is an enzyme involved in the final polymerization step of rubber biosynthesis in *Hevea brasiliensis*. This most abundant protein in the latex is associated with the rubber particle membrane. The genomic sequence coding for REF protein was amplified by PCR from the genomic DNA of *Hevea* (Clone RRII 105) using REF specific primers. The 1.3 Kb REF gene fragment was cloned into plasmid vector and sequenced. The amplified gene was 1367 bp with an ORF of 414 bp interrupted by two introns of 298 bp and 455 bp. Southern blot analysis performed with *Hevea* genomic DNA suggests the presence of two copies of REF gene in *Hevea*. To date this is the first report on the isolation and characterization of the genomic sequence of REF from *Hevea brasiliensis*.

A cDNA encoding REF protein was amplified by RT-PCR from the latex RNA of *H. brasiliensis* clone RRII 105 with gene specific primers. Sequencing showed that the amplified REF cDNA was 622 bp with an ORF of 414 bp, which encodes a protein of 138 amino acids with a predicted molecular mass of 14.7 kDa. The coding region of REF cDNA was cloned into an expression vector pGEX and transformed into *E.coli* cells to produce recombinant REF protein. On induction with IPTG, the REF was expressed as a fusion protein of expected size (40 kDa) with GST– affinity tag. The GST-REF recombinant protein was purified using GST – affinity column and the identity of the recombinant protein was confirmed by immunoblot analysis with anti-GST antibodies and antibodies raised against native REF protein purified from *Hevea* latex.

The REF cDNA was cloned into pBIB binary vector and *Agrobacterium* mediated transformation of *Nicotiana tabacum* was carried out to generate transgenic tobacco plants expressing REF protein of *Hevea*. The kanamycin resistant putative transgenic tobacco plants were selected and screened by PCR using REF and *npt II* gene specific primers. Only the transgenic tobacco plants amplified the REF and *npt II* gene fragments while nontransgenic tobacco plants did not amplify the corresponding fragments. The expression of REF and *npt II* gene in the transgenic tobacco plants were further detected by RT-PCR. Both the 600 bp REF cDNA and 800bp *nptII* cDNA were detected only in transgenic tobacco plants.

The expression of REF gene in *H. brasiliensis* was studied by RNA – blot analysis using radiolabeled REF cDNA as probe. Results revealed differential expression of REF gene in different tissues of mature rubber trees and in *Hevea* seedlings. Latex and bark of rubber trees showed a predominant expression of REF in comparison with leaf tissues. In seedlings, REF transcripts accumulated more in the petiole and bark than in root and leaf. Also, the transcripts accumulated more in regularly tapped trees than in untapped trees, which revealed that tapping, had an effect on the level of REF transcripts. A significant clonal variation in REF gene expression was noticed by RNA blot. The REF gene expression was high in high yielding clones than in low yielding clones. Results of northern blot analysis revealed a detectable increase in REF gene expression in the latex of ethephon stimulated trees than in the control trees at all concentrations tried.

The promoter region of REF gene was amplified by PCR with specific primers and a 378 bp fragment upstream to the ATG size was obtained from the genomic DNA of *Hevea*. The amplified promoter fragment was cloned and sequenced. A putative TATA element at –150 and a CAAT box motif at –221 positions were identified in the REF gene promoter. The REF promoter fragment was fused upstream to the coding region of *uidA* gene in the binary vector pGPTV and used to transform tobacco via. *Agrobacterium* mediated method for a preliminary functional analysis. Kanamycin resistant transgenic tobacco plants were generated and the plants were screened for the presence of GUS activity by the histochemical coloration of vegetative tissues with x-gluc. Transgenic tobacco plants showed positive GUS activity in the leaf and petiole, while no activity was detected in the nontransgenic tobacco plants. Further screening of the transgenic plants was carried by PCR with GUS and promoter specific primers. Only the transgenic tobacco plants amplified the 750 bp REF promoter and 650 bp *uid A* gene fragments Results of southern blot analysis of the genomic DNA isolated from the transgenic tobacco plants with *uid A* gene probe further confirmed the stable integration of transgene in the tobacco genome. The presence of *uid A* transcripts in the PCR positive transgenic tobacco plants was further confirmed by RT-PCR using *uid A* specific primers. Only the transgenic tobacco plants amplified the *uidA* cDNA of 650 bp and no specific cDNA product was detected in nontransgenic control plant.