Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Plant Science 171 (2006) 470–480
(4)
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis

P. Priya [1], P. Venkatachalam [1,*], A. Thulaseedharan

Biotechnology Division, Rubber Research Institute of India, Kottayam 686009, India

Received 24 February 2005; received in revised form 8 March 2006; accepted 12 May 2006
Available online 13 June 2006

Abstract

Hevea rubber tree (*Hevea brasiliensis*) is the only plant species being cultivated for commercial production of rubber in the world. In order to meet ever increasing rubber demand, it is a prerequisite to identify and characterize a key gene involved in rubber biosynthesis and over-expression of rubber biosynthesis gene will eventually lead to enhance the latex (rubber) production in transgenic *Hevea* plants. Rubber elongation factor (REF) is a major protein located on the surface of large rubber particles in latex and is involved which is involved in rubber biosynthesis in *H. brasiliensis*. We report here cloning and characterization of REF gene as well as its 5′ promoter region from *Hevea*. REF gene (1367 bp) has three exons interrupted by two introns and encoded a 138 amino acid peptide containing an open reading frame of 414 bp with a calculated M_W of 14,700 Da. Nucleotide sequence analysis showed that 1.3 kb genomic DNA showed 100% homology to REF cDNA from *Hevea*. Southern blot hybridization of genomic DNA with REF gene probe revealed that REF gene is encoded by a small gene family consisting of two members. RNA blot analysis indicated that REF transcript is highly expressed in high yielding clone than in low yielder. The cloned 5′ promoter region has a putative TATA element at −150 and CAAT box at −221 position. To identify the regulatory role of REF promoter, chimaeric fusion between REF promoter sequence and the β-glucuronidase (GUS) coding, *uidA* gene was constructed and used to transform tobacco and Arabidopsis. Expression of the *uidA* reporter gene was detected histochemically in the transformed tobacco plants where, GUS activity was detected in the leaf and petiole of transformed plants. The stable integration of REF:*uidA* fusion into the tobacco genome was further confirmed by PCR amplification and Southern blot analysis. A histochemical study of stable transformants demonstrated that the 5′ upstream region of REF can drive strong GUS gene expression specifically in the vascular tissues (xylem and phloem) of leaf, stem and midribs of transgenic Arabidopsis. GUS staining revealed that REF:GUS expression was also induced by wounding. The results suggested that the cloned REF promoter is capable of directing gene expression. Our ultimate goal is to produce transgenic *Hevea* plants with enhanced latex yield by over expression of REF protein.

Keywords: *Hevea brasiliensis* (Hb); Rubber elongation factor (REF) gene; Cloning; Promoter; Tobacco; Arabidopsis; Transformation

1. Introduction

Natural rubber (*cis*-1,4-polyisoprene) is an important industrial raw material. Primarily due to its molecular structure and high molecular weight, this rubber has high performance properties that cannot be easily mimicked by artificially produced polymers. Although rubber or *cis*-1,4-polyisoprene is formed in over 2000 species of plants distributed amongst 300 genera of seven families [1], today only one species of plant—*Hevea brasiliensis* Muell. Arg is used extensively for the commercial production of natural rubber (NR). One of the reasons for this is that a high yield of technologically acceptable rubber can be obtained over many years by simple excision of tree bark of mature rubber tree made at frequent intervals.

H. brasiliensis Muell. Arg belonging to the family Euphorbiaceae originated in South America primarily in the Amazon basin, but it is now mainly cultivated in South East Asia [1]. In *Hevea*, NR is obtained from the latex, which is produced and stored in highly specialized cells called laticifers or latex

* Corresponding author. Present Address: Centre for Plant Molecular Biology, Department of Horticulture, Purdue University, West Lafayette, IN 47907, USA. Tel.: +91 481 2353311; fax: +91 481 2353327.
E-mail addresses: chalampv@yahoo.co.in, pvenkat@purdue.edu (P. Venkatachalam).
[1] The first two authors contributed equally to the work.

0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2006.05.009

vessels located in the outer bark overlaying the cambium [2]. The articulated laticifers of *Hevea* form a tube like network through the plant. Latex is present in all parts of the plant, but the laticifers exploited commercially are those in the bark. When severed during tapping, the laticifers expel latex. The latex, which is the milky cytoplasm of the laticiferous cells, contains all the cell constituents plus rubber particles (RPs) and characteristic organelles (Lutoids and Frey Wyssling particles). It has been estimated that latex contains 30–50% (w/w) of *cis*-1,4-polyisoprene [3].

Laticifers have been shown to exhibit differential gene expression and it has been demonstrated that genes involved in rubber biosynthesis and genes induced by wounding and ethylene treatment are more highly expressed in laticifers [4,5]. It is now known that rubber biosynthesis takes place on the surface of rubber particles suspended in the latex. Among the proteins associated with the lipid membrane surrounding the RPs, the most abundant is a 14.6 kDa protein tightly bound to the large RPs termed by Dennis and Light as the rubber elongation factor [6]. The polymerization of rubber chain is considered to consist of three distinct processes: initiation, elongation and termination. The first biochemical step essential for rubber biosynthesis is the isomerization of the C5 isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP) by the enzyme IPP-isomerase. This is followed by prenyl transferase-catalysed synthesis of the C10 (geranyl pyrophosphate, GPP), C15 (farnesyl pyrophosphate, FPP) and C20 (geranyl geranyl pyrophosphate, GGPP) allylic pyrophosphates which are essential initiators of rubber formation by a series of additions of IPP (non-allylic pyrophosphate) in the *trans* configuration to DMAPP [7]. The elongation of rubber chain proceeds by sequential condensation of IPP, the building block of rubber to the initiating molecules. The presence of an abundant protein called the rubber elongation factor (REF), which is associated with the large RPs in the latex, is required for rubber molecule elongation. REF interacts with prenyl transferase to alter the stereochemistry of IPP addition from the normal *trans* addition to *cis* and overrides the normal termination after two *trans* additions to affect the formation of *cis*-polyisoprene [8].

In *Hevea*, identification of genes or proteins directly involved in rubber biosynthesis and the cloning of these are of critical importance to understand the molecular mechanism of rubber biosynthesis. This in turn will allow rubber production systems in potential domestic rubber producing—crops to be modified as needed to produce commercially viable yields of high quality natural rubber. Recently there has been growing interest in using plants for the production of biopharmaceutical proteins and peptides because they are easily transformed and provide a cheap source of protein. In this aspect the commercial rubber tree has great potential to produce foreign proteins in the latex, which can be easily purified from the serum phase of the latex. The availability of regulatory sequences or promoters to target expression to laticifers is an essential component for engineering traits in transgenic *Hevea* plants as the constitutive expression of foreign proteins is deleterious to the plants. One way to identify such promoters would be to study the upstream region of genes like REF, HMGCoA reductase etc. that are highly expressed in latex [9]. Besides promoters, other regulatory elements like introns and enhancers can also play important roles in gene expression. As a first step, potentially useful promoters need to be evaluated in view of their specificity and expression levels. The aim of the investigation reported here is cloning and characterization of REF gene from *H. brasiliensis* and the functional characterization of its promoter by expression in transgenic tobacco and Arabidopsis plants.

2. Materials and methods

2.1. Plant material

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 collected and stored in polybags before proceeding to the lab for DNA isolation. For RNA extraction, latex samples from mature rubber trees of *H. brasiliensis* clones; RRII 105 and KRS 25 were obtained from field.

2.2. Cloning and characterization of REF gene

Genomic DNA was isolated from the leaf tissues of *H. brasiliensis*, clone RRII 105 as described by Doyle and Doyle [10] with modifications. The collected leaves were frozen in liquid nitrogen and pulverized to fine powder using mortar and pestle. Extraction buffer (2× CTAB buffer—2% CTAB, 1.4 M NaCl, 20 mM EDTA, (pH 8.0), 0.1 M Tris–Cl (pH 8.0), 1% PVPP, 1% 2-mercaptoethanol) was added to the ground tissues and incubated in a water bath at 65 °C for 30 min with occasional mixing. The homogenate was then spun at 8000 × *g*, 20 min at room temperature. The supernatant was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) at room temperature (8000 × *g*). The upper aqueous phase collected after centrifugation was treated with RNase A (Sigma, Sigma St. Louis, MI, USA) and incubated at 37 °C for 1 h followed by proteinase K (Sigma, USA) treatment at 50 °C for 30 min in a water bath. This was followed by extraction with chloroform twice and the aqueous phase obtained after centrifugation was mixed with 0.6 volume of isopropyl alcohol for precipitation of DNA. Precipitated DNA was pelleted by 20 min centrifugation at 10,000 × *g* at 4 °C. Finally DNA pellet was washed with 70% alcohol, dried and resuspended in TE buffer. The quality and concentration of the isolated DNA was checked on 0.8% agarose gel. For PCR analysis, the isolated DNA was diluted appropriately with sterile distilled water.

PCR amplification of REF gene from *H. brasiliensis* was performed with gene specific primers designed based on sequences deposited earlier in GenBank database (X56535) and genomic DNA was used as template.

- Forward primer: 5′-CGA TTA TGG CT GAA GAC GAA GAC AAC C-3′
- Reverse primer: 5′-GGC CAA TAA TTC AAT TGG CCC TTT ATT C-3′

Amplification was carried out in a 20 μl reaction volume, which was composed of 10× buffer, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.5 U of Taq DNA polymerase, 25 ng template DNA and 250 nM of primer 1 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 amplification profile consisted of a first cycle at 94 °C for 4 min followed by 30 cycles at 94 °C for 1 min/55 °C, 1.30 min/72 °C, 2 min and a last cycle at 72 °C for 10 min.

The PCR amplified DNA fragment was purified and cloned into plasmid vector. Presence of the insert in the recombinant plasmid was further confirmed through insert release by restriction digestion with enzymes *Eco*RI and *Hin*dIII and by PCR analysis. The nucleotide sequence of the cloned DNA fragment was determined using the automated sequencing facility at Indian Institute of Science, Bangalore. The nucleotide 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 [11]. Sequence alignment and comparison was made using the ClustalW program (http://www.ebi.ac.uk/clustalw). Signal peptides and cleavage sites were predicted using pSORT and signal P programs.

2.3. DNA blot analysis

For Southern blot analysis, 10 μg of genomic DNA isolated from leaves of *H. brasiliensis* was digested with various restriction enzymes *Hin*dIII, *Eco*RI, *Eco*RV, and *Xba*I. The digested DNA was then separated on 1% agarose gel and then transferred to nylon membrane (Hybond N+, Amersham, UK). PCR amplified 1.3 kb REF gene fragment radiolabelled with α-P^{32} dCTP according to manufacturers instructions was used as probe for hybridization (Multiprime Labelling Kit, Amersham, UK). After hybridization, the DNA blot was washed twice in 2× SSC, 0.1% SDS for 5 min and 0.1× SSC and 0.1% SDS for 15 min at 65 °C. Subsequently the blot was exposed to X-ray film (Kodak X-Omat) with intensifying screens for signal detection at −80 °C.

2.4. RT-PCR analysis

RT-PCR was performed to obtain cDNA fragments and to identify the exact sequence of the putative cloned REF gene. Total RNA was extracted from latex as described [12]. About 2 μg of total RNA was reverse transcribed with AMV reverse transcriptase (Promega Inc., Madison, WI, USA) using oligo dT primer. The reverse transcription (RT) reaction was performed for 1 h at 42 °C. PCR was carried out with Taq DNA polymerase (Promega Inc., Madison, WI, USA) using 2 μl of RT reaction as template and with REF gene specific primers. PCR amplification was carried out in a 25 μl reaction volume which composed of 10× buffer, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTPs, 0.5 U Taq DNA polymerase and 250 nM of primers and the PCR cycles were similar as described previously. PCR products were analysed on 1.5% agarose gel and purified. Subsequently, the cDNA fragments were cloned into plasmid vector and sequenced.

2.5. RNA gel blot hybridization

Regularly tapped trees were selected in a plot of 18-year-old trees submitted to half spiral tapping every 2 days without stimulation. Total latex RNA was isolated from two different clones of *H. brasiliensis* (RRII 105 (high yielding) and KRS 25, (low yielding) according to the method of Venkatachalam et al. [12]. The isolated latex RNA was fractionated on a 1% (w/v) formaldehyde/MOPS agarose gel and transferred by capillary blotting onto a nylon membrane (Hybond N+, Amersham International, UK). Cloned REF genomic DNA labelled with α-P^{32} dCTP multiprime labelling system (Amersham Biosciences, UK) was used as probe for Northern hybridization. The blots were prehybridized for atleast 2 h in the hybridization buffer (6× SSC, 5× Denhardt's solution, 0.5% SDS) [13], and hybridization was carried out at 42 °C for 16 h. The blots were washed twice in 2× SSC and 0.5% (w/v) SDS for 10 min at 42 °C followed by 1× SSC and 0.1% (w/v) SDS for 10 min at 55 °C. The membrane was exposed to X-ray film with two intensifying screens for signal detection at −80 °C.

2.6. Cloning and characterization of REF gene promoter

The proximal promoter region of the REF gene was obtained by PCR amplification of the genomic DNA prepared from leaves of *H. brasiliensis* [10]. PCR was performed with oligonucleotide primers designed based on sequences deposited earlier in GenBank database (Accession nos. AY134670, AF380139). The following two primers were used:

- 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 25 ng of DNA as template, 2 μl of 10× buffer, 2 μl of $MgCl_2$ (1.5 mM) and 2 μl dNTPs (2.5 mM), 1 μl 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 for 1 min, 48 °C for 1 min, 72 °C for 1 min. After PCR, the amplified products were analysed on 1.5% agarose gel. 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 [11].

2.7. Construction of REF promoter:GUS fusion

The 750 bp REF promoter which was cloned in the plasmid vector was subcloned to the upstream of the coding region of the *uidA* gene in pGPTV binary vector between *Hin*dIII and *Sal*I sites to generate the REF promoter:GUS fusion construct designated pGPTVRP (Fig. 6). The REF promoter:GUS fusion was sequenced to verify the reading frame of the gene construct. The construct was then mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by freeze-thaw method [14] and was selected for resistance to kanamycin (50 mg/l).

2.8. Plant transformation and molecular analysis of tobacco and Arabidopsis

Transformation of tobacco via *A. tumefaciens* was carried out according to Horsch et al. [15]. Leaf discs were prepared from *in vitro* grown tobacco plants and pre-cultured on MS medium containing NAA (0.5 mg/l) and BAP (2 mg/l) for 2 days. The explants were co-cultivated with *Agrobacterium* for 3 days, washed five times with sterile water and transferred onto MS medium [16] containing 50 mg/l kanamycin and 200 mg/l carbenicillin. Shoots regenerated from putatively transformed calli selected by 50 mg/l kanamycin were excised and transferred to fresh medium. The presence of the REF promoter:GUS fusion in the transgenic plants was confirmed by PCR using promoter specific primers as well as GUS primers with genomic DNA extracted from young leaves as template. To detect the integration of *uidA* gene in the PCR confirmed transgenic tobacco plants, Southern blot analysis was conducted with genomic DNA isolated from transgenic tobacco plants. The isolated genomic DNA (20 μg) was digested with *Hin*dIII alone and double digested with *Sac*I + *Sal*I and *Hin*dIII + *Sac*I enzymes and separated by agarose gel electrophoresis. Then the DNA was transferred onto nylon membrane and probed with α-P^{32} dCTP labelled *uidA* gene probe (internal fragment amplified by PCR). Hybridization and washing of blot was carried out as described previously.

In another experiment, the reporter gene construct was introduced into *A. tumefaciens* strain PGV 3101 and transformed bacteria were used to obtain transgenic Arabidopsis (ecotype: Columbia) plants by the floral dip procedure [17]. Transformed plants were selected on the basis of kanamycin resistance and PCR was carried out on genomic DNA from transgenic plants with specific primers to REF promoter and uidA gene. Three to five independent lines were further reproduced and homozygous plants were used to analyse GUS expression. Young leaves of Arabidopsis transgenic plants containing REF:GUS construct were wounded with a scalpel in water and subsequently transferred into GUS staining solution.

2.9. Histochemical analysis of GUS activity

Histochemical detection of β-glucuronidase (GUS) activity was visualized by staining using X-gluc (5-bromo-4-chloro-3-indoyl glucuronide). Transgenic tissues were placed in a solution of X-gluc (50 mM Sodium phosphate pH 7.0, 1 mM EDTA, 0.001% Triton-X-100, 10 mM 2-mercaptoethanol, 2 mM X-Gluc) and incubated at 37 °C overnight [18] along with negative control (non-transgenic control). After staining, chlorophyll was cleared from green plant tissues by immersing them in ethanol (70%) and the tissue was viewed under microscope.

3. Results and discussion

We cloned and characterized the REF gene from *H. brasiliensis* to understand the molecular mechanism and expression pattern in respect to rubber biosynthesis. Genomic DNA was used for PCR analysis and two amplicons of sizes 800 bp and 1.3 kb were obtained when 55 °C was used as the primer annealing temperature with 35 cycles. Further, when the number of cycles was reduced to 30, the 800 bp fragment was not amplified. No discrete bands were observed at 45 °C probably because of non-specific primer hybridization due to the high nucleotide homology between and within the REF gene. The present communication reports further characterization of the 1.3 kb amplicon. PCR amplified 1.3 kb DNA fragment was purified and cloned into pBS vector. The presence of insert in the recombinant plasmid was confirmed through insert (1.3 kb) release by restriction digestion with *Hin*dIII and *Eco*RI enzymes as well as by PCR amplification.

The nucleotide sequence of REF gene was elucidated and is presented in Fig. 1. Sequence analysis of the cloned 1.3 kb REF genomic DNA revealed that it comprised of 1367 bases. The complete sequence of REF cloned in this study is available from the DDBJ/GenBank/EMBL database under Accession no. AY299405. The alignment of the nucleotide sequence of the REF gene with cDNA of REF revealed that it consists of three exons, which perfectly matched the cDNA sequence, is separated by two introns of 298 and 455 bp in length. The 1.3 kb REF gene consists of a 414 bp coding region (ORF, stop codon not included) and a 193 bp 3′ untranslated region. Both the introns of the REF gene contain splice site consistent with the consensus sequence 5′ GT————————AG ′3 [19]. An open reading frame of 414 bases encodes a single polypeptide of 138 amino acids with a predicted molecular mass of 14.7 kDa. A computer analysis using the pSORT program (K. Nakai, Osaka University Japan) for protein localization sites suggested that REF is localized in cytoplasm. The deduced protein is acidic with an isoelectric point of 5.04 (pI 5.04). The REF genomic sequence showed 100% similarity with the REF cDNA sequence after deletion of intron sequences. The deduced amino acid sequence of *H. brasiliensis* REF shared a considerable degree of identity with other sequences from *Hevea* species, showing 51% identity with *H. brasiliensis* isoform of REF (AY430052), 51% with *H. brasiliensis* stress related REF like protein-1 (AY221988), and 42% with *H. brasiliensis* SRPP protein (AF051317). In addition, it showed 39% sequence identity to the *Hevea* REF like stress related protein-2 and 28% sequence identity to *Arabidopsis thaliana* REF related protein (NM130345) (Fig. 2).

Recently, Ko et al. [20] employed the EST and cDNA-AFLP analysis to study the genes expressed in latex of *Hevea* rubber tree. It is reported that among the transcripts expressed in latex, REF was the most abundant followed by small rubber particle protein (SRPP). In addition, denfense- or stress-related genes (REF isoform and stress related REF-like) were also expressed in latex. These proteins had certain homology with REF gene sequence but these proteins were involved in defense mechanism not in rubber biosynthesis. Among 2000 latex producing plant species, the REF gene has been reported from only *H. brasiliensis* that is why this species produces enormous amount of latex. However, in other rubber producing plants, the SRPP is involved in rubber biosynthesis where the latex volume is less [21]. REF, a 14 kDa protein tightly associated with large

```
5'-CGATTatggc tgaagacgaa gacaaccaac aaggg[redacted]ACG TGCTCTCTCA AAATTTATAT 60
       M  A   E  D  E   L  N  Q  Q   G
ATCTCAATCG CTCAATTTTC TTATTATATG GGTTTGAATA TATTATTTGA ACGGTTTCTA 120

GAGGTGTTTG GTTGCTTAGA AAAGTAATCA AAGACTCGTG CAGAAATTAC AATGGAAGAT 180

ATAAAAATCT ATGATGCTAT ACATGAGAAT AAAGACTTGT TTCATGCAGT TGTTCTTAGA 240

TGCAAACCAA GTTCCTCGTT ATCTTCTATT TTATGGGGTT GTTTTGTGAG ATAATTAAAT 300

ATTTGCTTAT TTTTTGAATA TCCAAATTAA C[redacted]cagggggg aggggttaaa atatttgggt 360
                                    Q  G  E   G  L  K   Y  L  G
tttgtgcaag acgcggcaac ttatgctgtg actaccttct caaacgtcta tctttttgcc 420
F  V  Q  D   A  A  T   Y  A  V   T  T  F  S   N  V  Y   L  F  A
aaagacaaat ctggtccact gcagcctggt gtcgatatca ttgagggtcc ggtgaagaac 480
K  D  K  S   G  P  L   Q  P  G   V  D  I  I   E  G  P   V  K  N
gtggctgtac ctctctataa taggttcagt tatattccca atggagctct caagtttgta 540
V  A  V  P   L  Y  N   R  F  S   Y  I  P  N   G  A  L   K  F  V
gacagcacg[redacted]GAGTCTTTT TCTTCTATGT TCAACTTTTG TGTTTAAATG TGAGGGGAGG 600
D  S  T
TTGAGATTCA AGGACCTAAG TGAGTAATTG ATTGGTTTCT GTTGTTACTA ATTCTGTGGG 660

TTGGAAAATA TTTGGGCTAG TTTGGATTTA AACAAAAATA GAAATAATTT TTTCAATACT 720

TAATAATGTT TTTCAAGCTA TGCTAATAAA GAAAAATGTT GCTTCATTTT CCCATTTGAG 780

TTTAGTTGCA ATTATACACC GATTTAAAGA GATTATGGAT ACTCCTAGTG CATTAAAAAA 840

TCAAATTCCA AACAATCTTA ATGTTGTCAT GATTAATATT ATACGGTTGA TGATAGGGGC 900

CTTAATAGCA AATTATGTGA TGATTTAATT AAACCTCTTG TCTTTCTTTG AATAAGCAGC 960

AGGGATGTTG AATGAAAATT GAAAGTTCTT TCACATGGTT GT[redacted]gttgtt gcatctgtca 1020
                                              V  V   A  S  V  T
ctattataga tcgctctctt cccccaattg tcaaggacgc atctatccaa gttgtttcag 1080
  I  I  D   R  S  L   P  P  I  V   K  D  A   S  I  Q   V  V  S  A
caattcgagc tgccccagaa gctgctcgtt ctctggcttc ttctttgcct gggcagacca 1140
  I  K  A   A  P  E   A  A  R  S   L  A  S   S  L  P   G  Q  T  K
agatacttgc taaggtgttt tatggagaga attgaGCCCC AATTTGCACC AATTGCTTCC 1200
  I  L  A   K  V  F   Y  G  E  N   *
AACTAAGCAA GTTAATGATA TGCTCAAGAA TATATATCTA TTGTGAGCTT TTTTTATGTT 1260

CTCATCCTGA GTGTTGAGAC TATGTTTTCG TTTGAATATT ACACTGTGTT TTATTATGTG 1320

TTTTGAATAT TCATAATGAG AATAAAGGGC CAATTGAATT ATTGGCC-3'                1367
```

Fig. 1. Nucleotide and deduced amino acid sequence of 1.3 kb genomic DNA fragment encoding HbREF. Upper case letters represent 5′and 3′ UTR regions were marked in green colour, introns in black colour. Lower case letters represent exons in blue colour and splice donor (GT) and accepter (AG) were highlighted. The translated amino acid sequence is shown in single letter code below the exon sequences. The termination codon is marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

rubber particles and SRPP encodes a 24 kDa protein which is tightly associated with small rubber particles in *Hevea*. Although REF and SRPP genes were highly expressed in latex tissue, the REF (14 kDa) is very crucial to polymerise cis-1,4-polyisoprene (rubber) in *Hevea*. Dennis and Light [6] proved that removal of REF protein was inhibited rubber biosynthesis *in vitro*. These results suggest that REF gene is playing a key role in rubber biosynthesis in *H. brasiliensis*, whereas the REF isoform and stress related REF genes are involved in defense mechanism within the laticiferous tissue. Even though REF gene sequence shared some homology with other genes, their nucleotide sequence size is quite different from REF gene (REF cDNA: 681 bp, REF isoform: 993 bp, stress related REF like protein: 763 bp and SRPP: 910 bp). In the present study, we designed two primers specific to REF cDNA and these primers should yield only 622 bp of cDNA fragment from *Hevea*. When we use these primers for RT-PCR analysis, we could amplify the 622 bp fragment as expected. The amplified REF cDNA was cloned and sequenced. The nucleotide sequence was similar to REF cDNA as well as the cloned genomic DNA fragment (after deleting the introns) [data not shown]. This result confirms that the cloned genomic DNA belongs to REF gene not REF isoform or stress related REF like protein.

To define the relative copy number of REF gene in *H. brasiliensis*, we performed Southern blot analysis using genomic DNA isolated from the leaf tissues digested with enzymes *Hin*dIII, *Eco*RV, *Eco*RI and *Xba*I using the REF gene probe. The *Hin*dIII, *Eco*RI and *Xba*I digested DNA revealed two hybridizing bands and when *Eco*RV was used, three bands were detected in the DNA (Fig. 3). Our results clearly indicate that the REF gene is present in more than one loci within *Hevea* genome. Sookmark et al. [22] concluded that there were atleast two REF loci within *Hevea* genome, one of which co-localized with SRPP locus in

```
SRPP          MAEE-----------------VEEE----------------------------RLKYLDF 15
AtREF         MAEDEIV--------------VEEEQSQPQEITPVPPSSSSSPSLVVEDDDEMKLKHLEF 46
HbREFIso      MAEGEEEVNIQEEANKGEENPQEEANIQEETNKGEENIQEEANIEEEANKEEESLKYLDF 60
RLP-1         MAEGEEEVNIQEEANKGEENPQEEANIREETNKGEANIQEEANIQEEANKEEESLKYLDF 60
HbREF         MAEDED-------------NQQGQG---------------------------EGLKYLGF 20
RLP-2         MAEGKEN-----------ENFQQEAN----------------------EQEEKLKYLEF 26
              ***                  :                              **:* *

SRPP          VRAAGVYAVDSFSTLYLYAKDISGPLKPGVDTIENVVKTVVTPVYY----IPLEAVKFVD 71
AtREF         IQVAAVYFAACFSTLYELAKDNAGPLKLGVENIEDCVRTVLAPLYEKFHDVPFKLLLFVD 106
HbREFIso      VQAATVYARASFSKLYLFAKDKSGPFKPGVNTVESRFKSVVRPVYNKFQPVPNKVLKFAD 120
RLP-1         VQAATLYARASFSKLYLFAKDKSGPFKPGVNTVESRFKNVVRPVYNKFQPVPNKVLKFAD 120
HbREF         VQDAATYAVTTFSNVYLFAKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGALKFVD 80
RLP-2         VQATTDNAVTALSNIYLYAKDNSGPLKPGVETIEGVAKTVVIPAS----KIPTEAIKFAD 82
              :: :       :*.:*  *** :**:: **: :*.  :.*  *       :*   : *.*

SRPP          KTVDVSVTSLDGVVPPVIKQVSAQTYSVAQDAPRIVLDVASSVFNTGVQEGAKALYANLE 131
AtREF         RKVDDVFFDVETYVPSLVKQASSQALTVATEVQR------TGVVDT-TKSIARSVRDKYE 159
HbREFIso      RRVDAYVTVLDRIVPPIVKRASIQAYS---VAPG---------------AALAVASYLP 161
RLP-1         RRVDAYVTVLDRIVPPIVKRASIQAYS---VAPG---------------AASAVASYLP 161
HbREF         STVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE---------------AARSLASSLP 124
RLP-2         RAVDASFTTLQNIVPSVLKQLPTQACD-----------------------TSVKESAE 117
                *   .  ::  :*.::*  . *.                          ::

SRPP          PKAEQYAVITWRALNKLPLVPQVANVVVPTAVYFSEKYNDVVRGTTEQGYRVSSYLPLLP 191
AtREF         PAAEYYAATLWRLLNQLPLFPEVAHLVIPTAFYWSEKYNDAVRYVGDRDYFGAEYLPMIP 219
HbREFIso      LHTKRLSKVLYGDG------------------------------------------- 175
RLP-1         LHTKRLSKVLYGDG------------------------------------------- 175
HbREF         GQTKILAKVFYGEN------------------------------------------- 138
RLP-2         ------------------------------------------------------------

SRPP          TEKITKVFGDEAS--- 204
AtREF         IEKISDILEQDQCRAD 235
HbREFIso      ---------------
RLP-1         ---------------
HbREF         ---------------
RLP-2         ---------------
```

Fig. 2. Multiple alignment of predicted amino acid sequence of HbREF gene with Hb SRPP (Accession no. AF051317), *Arabidopsis thaliana* REF like 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).

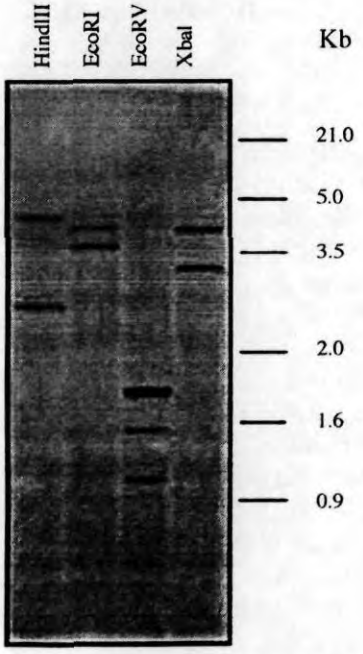

Fig. 3. DNA gel blot hybridisation of genomic DNA. Equal amounts of genomic DNA (10 μg) digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Eco*RV (lane 3) and *Xba*I (lane 4) were probed with radiolabelled REF gene. DNA molecular mass markers are indicated on the right.

genome maps. This may be one of the reasons that two strong bands and one lighter band were noticed in the Southern blot analysis, since REF is having some homology with SRPP gene.

In order to examine the level of REF gene expression in the latex of *H. brasiliensis*, RNA gel blot analysis was carried out against total RNA isolated from latex of two different clones of *H. brasiliensis* using REF gene probe. The expression pattern of REF gene was presented in Fig. 4. Northern analysis revealed that REF transcript was relatively more abundant in RRII 105 (a high yielding clone) than in KRS 25 (low yielder). The REF mRNA band was approximately 650 bp in size on blot. This indicates that the radiolabelled REF gene probe was hybridized with REF mRNA transcripts not with other genes. It is reported that the RNA content represents important latex characteristics related to clonal properties and significant correlation between mRNA level and latex yield was also established [23]. According to Dennis and Light [6], the amount of REF protein in whole latex is proportional to rubber content (yield). Further, they proved that rubber biosynthesis *in vitro* was inhibited by the addition of antibodies raised against REF protein. Based on these results we propose that REF may be one of the key factors responsible for yield determination in *Hevea*. Although these differences are likely to be linked with differences in gene expression, so far there is no report in this area.

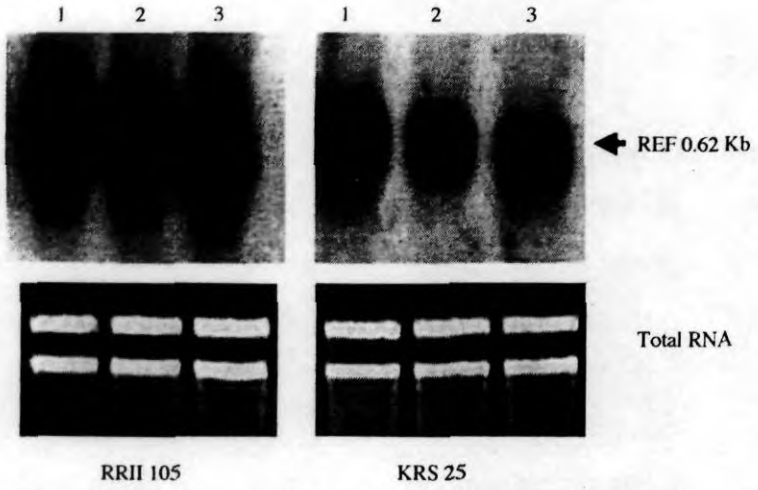

Fig. 4. Northern blot analysis of the REF gene. Total RNA from two clones (RRII 105 and KRS 25) of *Hevea brasiliensis* (15 μg/lane). Ethidium bromide stained rRNA as a control for the equal amount of RNA that was loaded. Lanes 1, 2, and 3: Three individual trees used for RNA isolation.

It has been suggested that REF plays a functional role in rubber polymerization [8,24]. Despite substantial number of reports investigating the proteins associated with the rubber particles and involved in rubber biosynthesis, the reports investigating the genes encoding rubber biosynthesis-related proteins is relatively scanty [25]. 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 [26]. REF is not reported from any other rubber producing plants other than *H. brasiliensis* and this may be the reason that *Hevea* produces high molecular weight superior quality rubber than the other rubber producing plants.

The upstream region of REF gene was amplified by PCR and two fragments of approximate sizes 750 and 500 bp were obtained. The 750 bp band was purified and cloned into plasmid vector. The cloned fragment was sequenced and identified as a fragment of REF gene from *H. brasiliensis* (Fig. 5). The nucleotide sequence of the 5′ upstream region of REF gene and the putative regulatory motifs are shown in Fig. 5. The amplified upstream region of REF was compared with sequences published in the database. The 378 bp sequence 5′ to the ATG translational start site was nearly identical to the other sequences with only five base differences. This may be due to clonal differences as the present sequence was amplified from RRII 105 (a popular Indian high latex yielding clone), whereas as other sequences reported in the database were from other clones. The consensus sequence of a putative TATA box (5′ TATAA 3′) was found at positions −150 to −155 upstream position of the translational initiation site (+1). A putative CAAT box motif was located at −221. 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. We searched the 5′ upstream region of REF gene for known motifs of other genes and found several potential regulatory elements using the PlantCARE program [27]. Within the 5′ upstream region of the *H. brasiliensis* REF gene, G-box motif was noticed and it comprises a family of *cis*-acting sequences that have been shown to be involved in the regulation of gene expression in response to a variety of factors in plants [28,29]. Additionally a W-box (TTGACT) like motif (AACTtA in antisense orientation in REF) [30], E-box with the consensus sequence of CANNTG known to be the recognition site of a class of transcription factors [31], a motif similar to ACCTTGCC [32] which is an elicitor—response related motif of potato *pin2* promoter (AgCTgGCC in REF), a sequence similar to ATTTCAAAA which is an ethylene response element (ATTTCcAtc in REF) [33], MYB recognition site, MYB1AT [34] and several ARE elements (GGTTTT) present in the upstream region of several anaerobically induced genes [35] were also identified in the REF promoter region. Another property of the REF gene promoter is the occurrence of several light responsive elements like GATA core elements [36], GT1 elements (GAAAT) [37] and Dofl protein binding motif (AAAAGG) [38] in the upstream region of REF. The nucleotide sequence of the REF promoter is available in GenBank database (Accession no. AY712939).

For functional analysis of the promoter region of the REF gene from *H. brasiliensis*, we generated transgenic tobacco plants containing chimaeric REF:*uidA* gene construct (pGPTVRP) in which a 750 bp fragment of the REF gene promoter region including the 5′ flanking region of REF gene, the 5′ untranslated leader sequence and the first few codons was fused in frame to the *uidA* coding sequence (GUS) (Fig. 6). Six independent kanamycin resistant transgenic tobacco lines were obtained and used for histological analysis of GUS activity. Histochemical analysis revealed that transgenic tobacco plants carrying the REF:*uidA* fusion construct exhibited GUS activity in the leaves and petioles (Fig. 7A and B) while GUS activity was absent in the leaves of non-transformed tobacco plants used as negative control (Fig. 7C). The stable integration of REF:*uidA* gene in transgenic tobacco was determined by molecular analysis in which specific primers for the *uidA* and REF promoter were used for genomic PCR analysis to amplify the DNA fragments of 650 bp of *uidA* gene and 750 bp of REF promoter fragment in transgenic tobacco plants and in the

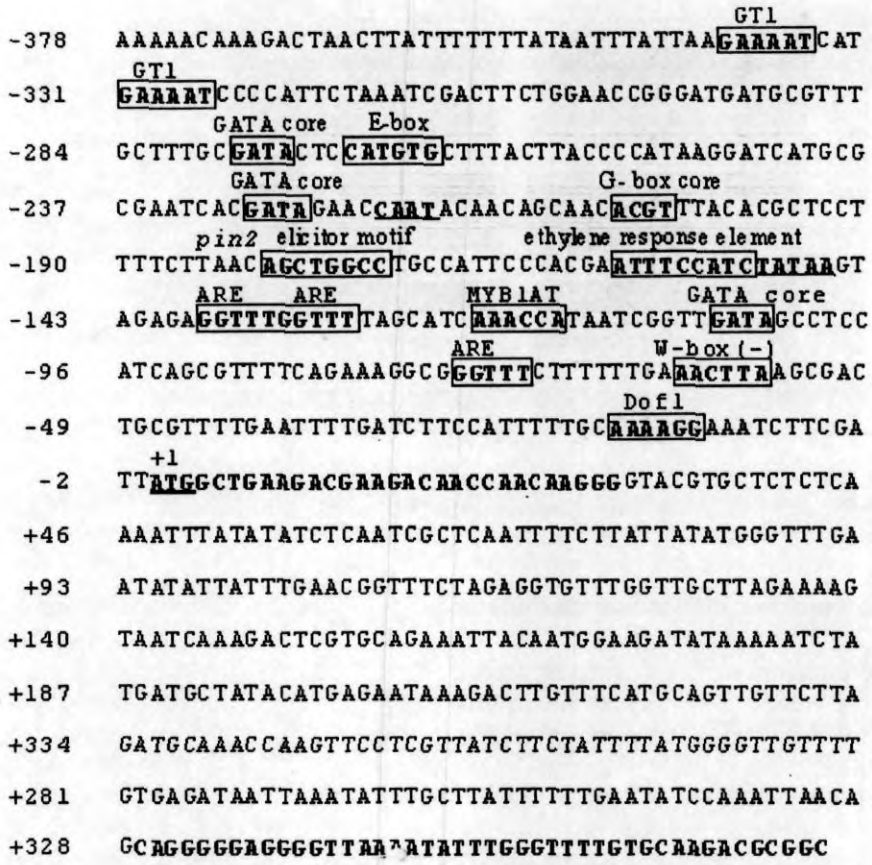

Fig. 5. The nucleotide sequence of the amplified 5′ upstream region of REF gene. The translational start site is labelled +1. The putative TATA and CAAT box and ATG codon are underlined. Putative regulatory motifs in the REF promoter region are boxed and labelled above the sequences. These motifs include GT 1 (GAAAAT), GATA core element, E-box, G-box/ABRE core (ACGT), *pin2* elicitor motif, ethylene responsive element (ERE), anaerobic responsive element (ARE), MYB1AT, Dof 1 protein biding site, W-box.

plasmid DNA (positive control), whereas corresponding bands were not detected in the non-transformed control (data not shown). Genetic transformation of tobacco plants was further confirmed by genomic Southern blot analysis (Fig. 8). Genomic DNA isolated from leaves of PCR screened transgenic tobacco plants was digested with *Hin*dIII alone and double digested with *Sac*I + *Sal*I and *Hin*dIII + *Sac*I enzymes. When probed with radiolabelled *uidA* gene fragment, gel blot of *Hin*dIII digested genomic DNA gave one hybridization band, 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 *Hin*dIII and *Sac*I

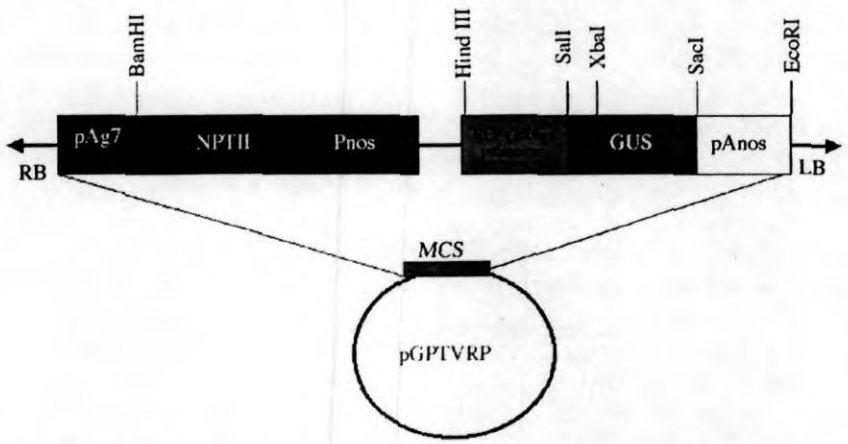

Fig. 6. Schematic representation of the binary vector pGPTVRP. Structure of chimeric construct of REF:GUS fusion used for tobacco transformation. The REF promoter sequence (750 bp) was fused to the GUS coding region between *Hin*dIII and *Sal*I sites which was followed by the polyadenylation signal from nopaline synthetase gene.

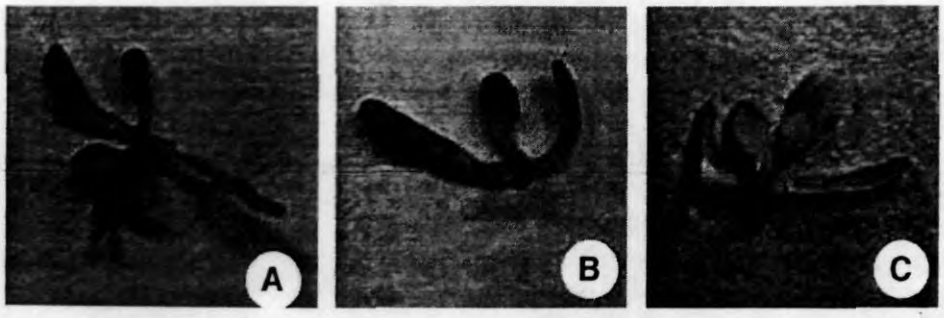

Fig. 7. Histochemical analysis of GUS activity in transgenic tobacco plants harbouring GUS:REF gene construct. (A and B) GUS activity is visible as blue colouration on leaves of transgenic tobacco plants. (C) Non-transgenic tobacco plants as negative control without any GUS staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

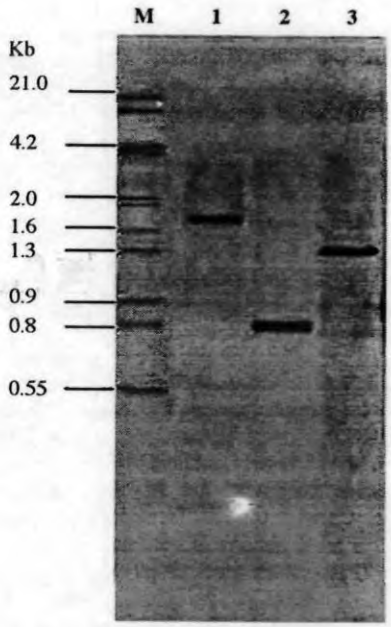

Fig. 8. Southern blot analysis of genomic DNA isolated from transgenic tobacco plants and hybridized to *uidA* specific probe. Lanes (M) DNA marker; DNA digested with (1) *Hin*dIII enzyme, (2) *Sal*I + *Sac*I and (3) *Hin*dIII + *Sac*I.

digest, the probe hybridized to a fragment approximately of 1.4 kb as expected. Since in the gene construct, the *Hin*dIII and *Sal*I restriction recognition sites delineate REF promoter and *uidA* gene, this 1.4 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.

To examine the tissue specific expression patterns of REF promoter in further detail, we produced transgenic Arabidopsis plants using the REF promoter:GUS plasmid. A detailed examination of GUS activity in leaf, stems and midribs by histochemical staining demonstrated that REF:GUS expression was largely restricted to the vascular system in the stem and midribs. By treatment with X-Gluc solution, vascular tissues (xylem and phloem) from all over the plant formed strong indigo blue products. In general, REF gene is highly expressed in laticiferous tissue which is modified phloem tissue in *Hevea*. Therefore, the present result clearly indicates that the tissue specific expression (phloem) of REF:GUS fusion reflects, REF promoter activity and that the expression of the fusion gene was specific to vascular tissues. Wounding is one of the stresses that produce signals which propagate from injured into adjacent

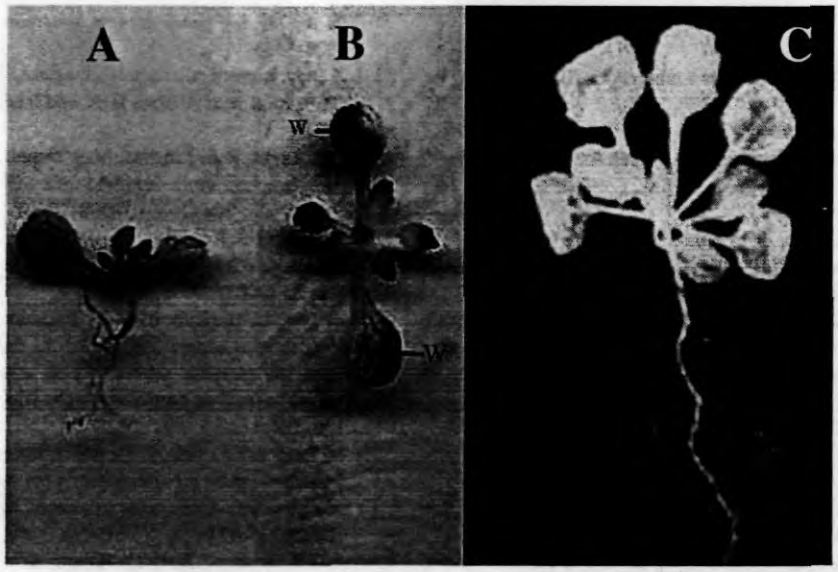

Fig. 9. Histochemical detection of the REF:GUS promoter activity in transgenic Arabidopsis plants. (A) Strong GUS staining in vascular tissues of leaf, stem and midribs; (B) GUS staining in wounded leaves (W-wounded sites) and (C) no GUS activity detected in non-transgenic plant.

non-injured tissues and the de novo synthesis of specific wound-induced proteins, which include some enzymes involved in defense mechanisms [39]. To investigate wound stress induced regulation, the plants were subjected to wound stress and GUS analysis was performed. As shown in Fig. 9, GUS rapidly accumulated at the wounding sites of wounded leaf. In contrast, mesophyl and epidermal cells adjacent to the wounded region were poorly stained by GUS expression. These results suggest that the REF gene from *Hevea* can be regulated by wounding stress at the transcriptional level, which involved the interaction of nuclear transcription factors with specific *cis*-elements. It was concluded that the expression of the REF:GUS fusion was not only tissue specific regulated but also triggered by environmental cues such as wounding.

In conclusion, we cloned and characterized the REF gene and its promoter region from *H. brasiliensis*. To date, this is the first report on the isolation and characterization of REF gene from genomic DNA. We are currently investigating the possibility of improving rubber productivity (latex yield) through transgenic approaches introducing REF gene into *Hevea* to over express REF protein. We also demonstrated that the cloned *H. brasiliensis* REF promoter is regulating foreign gene expression in transgenic tobacco and Arabidopsis. The results presented here are promising, but it would be interesting to elucidate the potential tissue specificity of this promoter in *Hevea*. This will provide the basis for targeting foreign protein production to the laticiferous system of *H. brasiliensis* for easy harvesting and manipulation.

Acknowledgements

The senior author (PP) is grateful to acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi for the award of Senior Research Fellowship. We wish to thank Prof. Dr. Michel Delseny for his valuable suggestions which enabled us to successfully complete this work.

References

[1] R.A. Backaus, Rubber formation in plants: a mini review, Israel J. Bot. 34 (1985) 283–293.

[2] P.B. Dickenson, The ultra structure of the vessel of *Hevea brasiliensis*, in: Proceedings of the National Rubber Product Research Association Jubilee Conference, Cambridge, MacLaren and Sons Ltd., London, 1965, pp. 52–66.

[3] R.G.O. Kekwick, The formation of isoprenoids in *Hevea* latex, in: J.D. Auzac, J.L. Jacob, H. Chrestin (Eds.), Physiology of Rubber Tree Latex, CRC Press, Boca Raton, 1989, pp. 145–164.

[4] A. Kush, E. Goyvaerts, M.L. Chye, N.-H. Chua, Laticifer specific gene expression in *Hevea brasiliensis* (rubber tree), Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 1787–1790.

[5] K.-H. Han, D.H. Shin, J. Yang, I.J. Kim, S.K. Oh, K.-S. Chow, Genes expressed in the latex of *Hevea brasiliensis*, Tree Physiol. 20 (2000) 503–510.

[6] M.S. Dennis, D.R. Light, Rubber elongation factor from *Hevea brasiliensis*: identification, characterization and role in rubber biosynthesis, J. Biol. Chem. 264 (1989) 18608–18617.

[7] K. Cornish, Z. Pan, R.A. Backaus, Engineering new domestic sources of natural rubber, in: J. Janick, J.E. Simon (Eds.), New Crops, Wiley, New York, 1993, pp. 192–196.

[8] M.S. Dennis, W.J. Henzel, J. Bell, W. Kohr, D.R. Light, Amino acid sequence of rubber elongation factor protein associated with rubber particles in *Hevea* latex, J. Biol. Chem. 264 (1989) 18618–18626.

[9] P. Arokiaraj, Genetic transformation of *Hevea brasiliensis* (rubber tree) and its applications towards crop improvement and production of recombinant protein of commercial value, in: S.M. Jain, S.C. Minocha (Eds.), Molecular Biology of Woody Plants, Kluwer Academic Publishers, The Netherlands, 2000, pp. 305–325.

[10] J.J. Doyle, J.L. Doyle, Isolation of DNA from small amounts of plant tissues, Focus 12 (1990) 13–15.

[11] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.

[12] P. Venkatachalam, I. Thanseem, A. Thulaseedharan, A rapid and efficient method for isolation of RNA from bark tissues of *Hevea brasiliensis*, Curr. Sci. 77 (1999) 635–637.

[13] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 1989.

[14] M. Holsters, A. de Walaele, E. Depicker, M. Messens, J. Van Montagu, Schell transformation of *Agrobacterium tumefaciens*, Mol. Gen. Genet. 163 (1978) 181–187.

[15] R.B. Horsch, J.E. Fry, N.L. Hoffman, D. Eichholtz, S.G. Rogers, R.T. Fraley, A simple and general method for transferring genes into plants, Science 227 (1985) 1229–1231.

[16] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.

[17] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.

[18] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.

[19] B.A. Hanley, M.A. Schuler, Plant intron sequences: evidence for distinct groups of introns, Nucl. Acids Res. 16 (1988) 7159–7174.

[20] J.H. Ko, K.S. Chow, K.H. Han, Transcriptome analysis reveals novel features of the molecular events occurring in the laticifiers of *Hevea brasiliensis* (Para rubber tree), Plant Mol. Biol. 53 (2003) 479–492.

[21] H. Kang, M.Y. Kang, K.H. Han, Identification of natural rubber and characterization of rubber biosynthetic activity in Fig tree, Plant Physiol. 123 (2000) 1133–1142.

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

[23] J. Tupy, Ribosomal and polyadenylated RNA content of rubber tree latex, association with sucrose level and latex pH, Plant Sci. 55 (1988) 137–144.

[24] D.R. Light, R.A. Lazarus, M.S. Dennis, Rubber elongation by farnesyl pyrophosphate synthases involves a switch in enzyme stereospecificity, J. Biol. Chem. 264 (1989) 18598–18607.

[25] J.J. Kim, S.B. Ryu, Y.S. Kwak, H. Kang, A novel cDNA from *Parthenium argentatum* Gray enhances the rubber biosynthetic activity *in vitro*, J. Exp. Bot. 55 (2003) 277–385.

[26] Z. Pan, L. Herickhoff, R.A. Backaus, Cloning, characterization and heterologous expression of cDNAs for farnesyl diphosphate synthase from the guayule rubber plants reveals that this prenyltransferase occurs in rubber particles, Arch. Biochem. Biophys. 332 (1) (1996) 196–204.

[27] M. Lescot, Y. Dehais, B. De Moor, P. Rouze, S. Rombauts, PlantCARE: a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences, Nucl. Acids Res. Database Issue 30 (2002) 325–327.

[28] R.G.K. Donald, A.R. Cashmore, Mutation of either G-box or I-box sequences profoundly affects expression from the *Arabidopsis rbcS-1A* promoter, EMBO J. 9 (1990) 1717–1726.

[29] U. Schindler, A.E. Menkens, H. Beckmann, J.R. Ecker, A.R. Cashmore, Heterodimerization between light-regulated and ubiquitously expressed *Arabidopsis* GBF bZIP proteins, EMBO J. 11 (1992) 1261–1273.
[30] T. Eulgem, P.J. Rushton, S. Robatzek, I.E. Somssich, The WRKY superfamily of plant transcription factors, Trends Plant Sci. 5 (2000) 199–206.
[31] C.O. Pabo, Transcription factors: structural families and principles of DNA recognition, Annu. Rev. Biochem. 61 (1992) 1053–1095.
[32] C.J. Palm, M.A. Costa, G. An, C.A. Ryan, Wound—inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 603–607.
[33] J. Deikman, Molecular mechanisms of ethylene regulation of gene transcription, Physiol. Plant. 100 (1997) 561–566.
[34] H. Abe, T. Urao, T. Ito, M. Seiki, K. Shinoaki, K. Yamaguchi-Shinozaki, Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abcisic acid signaling, Plant Cell 15 (2003) 63–78.
[35] P. Sarni-Manchado, C. Verries, C. Tesniere, Molecular characterization and structural analysis of one alcohol dehydrogenase gene (GV *Adh*1) expressed during ripening of grapevine (*Vitis vinifera* L.), Plant Sci. 125 (1997) 177–187.
[36] D. Gidoni, P. Brosio, D. Bond-Nutter, J. Bedrook, P. Dunsmuir, Novel *cis*-acting elements in petunia cab gene promoters, Mol. Gen. Genet. 215 (1989) 1989337–1989344.
[37] P. Villain, R. Mache, D.Y. Zhou, The mechanism of GT element—mediated cell type-specific transcriptional control, J. Biol. Chem. 271 (1996) 32593–32598.
[38] S. Yanagisawa, Dofl and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize, Plant J. 21 (2000) 281–288.
[39] M.E. Saltveit, Wound induced changes in phenolic metabolism and tissue browning are altered by heat shock, Postharvest Biol. Technol. 21 (2000) 61–69.