Research Article

Molecular cloning and characterization of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (*hmgr*1) gene from rubber tree (*Hevea brasiliensis* Muell. Arg.): A key gene involved in isoprenoid biosynthesis

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ABSTRACT

Natural rubber (*cis*-1,4-polyisoprene) is a secondary metabolite produced in the laticiferous tissue of *Hevea* tree. Mevalonate synthesis, which is the first step in isoprenoid biosynthesis, is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (*hmgr*1). We have cloned and characterized a full-length cDNA as well as genomic DNA for *hmgr*1 gene from an elite Indian rubber clone (RRII 105). The nucleotide sequence of the genomic clone comprises 4 exons and 3 introns, giving a total length of 2440 bp. The sequences of 42 bp 5' UTR and 69 bp of the 3' UTR were also determined. The *hmgr*1 cDNA contained an open reading frame of 1838 bp coding for 575 amino acid protein with a theoretical pI value of 6.6 and the calculated protein M_W was 61.6 kDa. The deduced amino acid sequence showed high identity with other plant *hmgr*1 sequences. The amino acid sequence of the *Hevea hmgr*1 revealed several motifs which are highly conserved and common to the other plant species. These sequence conservations suggest a strong evolutionary pressure to maintain amino acid residues at specific positions, indicating that the conserved motifs might play important roles in the structural and/or catalytic properties of the enzyme. Southern blot analysis of genomic DNA from *Hevea* probed with a genomic fragment indicated that there were at least three isoforms of *hmgr* in *Hevea*. This result reveals that *hmgr*1 is one of the members of a small gene family. (Northern blot analysis showed that *hmgr*1 mRNA transcripts were noticed in all tissues - latex, leaf, immature leaf, and seedlings), however, the abundance of transcript level was higher in latex cells. As one step towards a better understanding of the role that this enzyme plays in coordinating isoprenoid biosynthesis in plants, *hmgr*1 cDNA was over expressed in transgenic *Arabidopsis* plants. Transgenic plants were morphologically distinguishable from control wild-type plants and an increased expression level of *hmgr*1 mRNA was detected. These data suggest that *hmgr*1 gene expression is playing an important role in isoprenoid biosynthesis. **[Physiol. Mol. Biol. Plants 2009; 15(2) : 133-143]** *E-mail : pvenkat67@yahoo.com* or *chalampv@yahoo.co.in*

Key words : *Hevea brasiliensis, hmgr*1, HMG-CoA reductase, *cloning, rubber biosynthesis gene*

Abbreviations : *HbHMGR – Hevea brasiliensis 3-hydroxy – 3-methylglutaryl-coenzyme A reductase, PCR – Polymerase chain reaction, RT-PCR – Reverse transcription polymerase chain reaction, RRII – Rubber Research Institute of India, RRIM – Rubber Research Institute of Malaysia*

INTRODUCTION

Natural rubber (cis-1,4-polyisoprene) is a mixture of high molecular weight polymers synthesized in at least 2000 plant species. However, the rubber tree (*Hevea brasiliensis*) has been the only economically viable source of natural rubber due to its good yield of rubber and the excellent physical properties of the rubber products (Asawatreratanakul *et al*., 2003). The mevalonate pathway, which starts with the synthesis of mevalonate by HMG-CoA reductase (HMGR), provides precursors for the diverse spectrum of isoprenoid compounds produced by a cell (Stermer *et al*., 1994). Hydroxymethylglutaryl-CoA reductase (HMGR) is a key regulatory enzyme, which provides the backbone for the synthesis of isoprenoids (Chappell *et al*., 1995). Also, HMGR has been shown to be involved in early steps of rubber biosynthesis (Hepper and Audley, 1969).

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The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyses the synthesis of mevalonate from HMG-CoA. Mevalonate is converted to isopentenyl pyrophosphate (IPP), which acts as precursor to a wide range of isoprenoid compounds including natural rubber *via* the cytoplasmic mevalonate (MVA) pathway (Stermer *et al*., 1994; Newman and Chappell, 1999). It has been well documented that isoprenoids including natural rubber, sesquiterpenes, triterpenes, sterols and brassinosteroids are biosynthesized via the MVA pathway (Newman and Chappell, 1999), whereas gibberellins, abscisic acid, carotenoids, and chlorophyll side chains are biosynthesized via the MEP pathway (Lichtenthaler, 1999). Harker *et al*. (2003) showed that HMGR is a key enzyme controlling overall flux into the sterol biosynthesis pathway in seed tissue by over expression of *Hevea Hmgr*1 gene in transgenic tobacco. HMGR has been examined in a wide variety of plant species at the biochemical and molecular levels. Chye *et al*. (1992) reported that independent isoprenoid pathways do occur and the pathway for rubber biosynthesis in *Hevea* is distinct from the pathway(s) leading to the biosynthesis of other isoprenoid compounds in plants. Most recently, Sando *et al*. (2008) reported that biosynthesis of natural rubber takes place biochemically by the mevalonate (MVA) pathway in *Hevea*. Genes encoding HMGRs have been isolated and characterized from many plant species including *Hevea* (Chye *et al*., 1991, 1992), *Arabidopsis* (Enjuto *et al*., 1994, 1995), tomato (Park *et al*., 1992), potato (Korth *et al*., 1997) and mulberry (Jain *et al*., 2000). Unlike animals, which have single-copy *hmgr* genes (Gertler *et al*., 1988), plant *hmgr* usually occurs in small gene families and the number of genes encoding HMGR in plants vary depending on the species. Although the proteins they encode may share high sequence identity, the expression patterns of individual family members are generally distinct (Stermer *et al*., 1994). HMGR is encoded by at least two and/or even larger multigene families in plants. The presence of multiple genes is consistent with the hypothesis that different isoforms of HMGR are involved in separate subcellular pathways for isoprenoid biosynthesis.

In *Hevea*, different members of *hmgr* gene were cloned and characterized from RRIM 600 clone by Chye *et al*. (1991, 1992). It has been reported that *hmgr*1 is involved in rubber biosynthesis whereas *hmgr*3 in isoprenoid biosynthesis of a housekeeping nature. Northern blot results suggested that *hmgr*1 is more highly expressed in laticifers than in leaves, suggesting strongly that this member is specifically involved in rubber biosynthesis (Chye *et al*., 1992). Though, the *hmgr*1 gene has been cloned and characterized from RRIM 600 clone, it is not a popular clone cultivated in India. Also few nucleotide differences were noticed with *hmgr*1 genes between RRIM600 and RRII105 clones. The clone RRII105 is the most popular and high latex yielding Indian clone which is cultivated in the traditional rubber growing area across the country. Therefore we are interested in cloning and characterization of *Hevea hmgr*1 gene from RRII 105 because of its pivotal role in rubber biosynthesis. Ji *et al*. (1993) demonstrated that hmgr1 activity was positively correlated with rubber biosynthesis as well as latex yield. As in the case with most other crops, major improvements have been made over the last century in the productivity of rubber, as the yield of dry rubber per acre have been increased significantly by releasing elite clones through conventional breeding. However, it is quite possible that the rate of rubber biosynthesis within the tree becomes the limiting factor (Arokiaraj, 2000). At this point, latex yield could only be enhanced either by treatments or by over-expression of a key rubber biosynthesis gene in transgenic *Hevea* plants. It was reported that the constitutive level of HMGR1 enzyme may be a limiting factor in rubber biosynthesis. Based on this hypothesis, Arokiaraj *et al*. (1995) initiated genetic transformation experiment to over express *HMGR*1 in *Hevea* where, hmgr1 activity of transformed callus ranged from 70-410 % of the value of wild-type control and the activity of transformed embryos ranged from 250-300 %. However, they failed to produce transgenic plants. Our ultimate goal is to over express this gene under the control of a constitutive promoter for enhanced latex biosynthesis in transgenic *Hevea* clone RRII105. In the present report, we describe the isolation and characterization of an *hmgr*1 gene from genomic DNA as well as cDNA from RRII 105 clone by PCR amplification. We also demonstrated the expression pattern of *hmgr*1 gene in different tissues from *Hevea* plant by Northern blot analysis and over-expression of this gene under constitutive promoter in *Arabidopsis* by transgenic approach.

MATERIALS AND METHODS

Plant material

Rubber clone RRII 105 (Rubber Research Institute of India, RRII) trees were grown in the experimental field and trees were tapped regularly (d/2 system). Leaves were collected and used for genomic DNA isolation. Laticifer RNA was extracted from the latex of field-tapped trees.

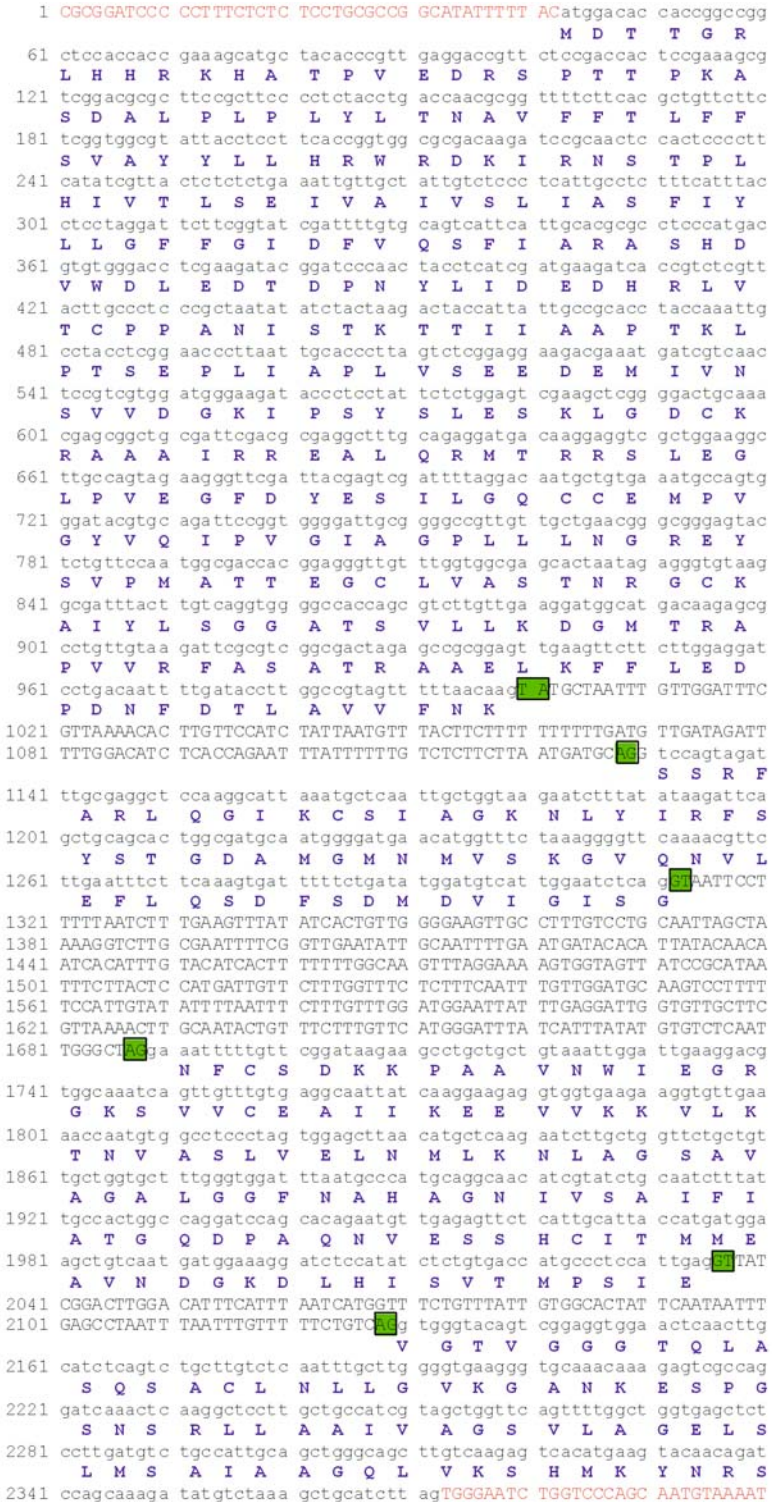

Fig. 1. Nucleotide and deduced amino acid sequence of 2.4 kb genomic DNA fragment encoding *HbHMGR*1. Upper case letters represent 5' and 3' UTR regions (marked in red colour) and introns (black colour). Lower case letters represent exons (blue colour) and splice donor and acceptor (highlighted). The translated amino acid sequence is shown in single letter code below the exon sequences. The termination codon is marked with an asterisk.

Genomic DNA isolation and PCR amplification of *hmgr*1 gene fragment

Genomic DNA was isolated from the leaf tissues of *H. brasiliensis,* clone RRII 105 as described by Venkatachalam *et al*. (2002). PCR amplification of *hmgr*1 gene from *H. brasiliensis* was performed with gene specific primers designed based on sequences deposited earlier in GenBank database (X54657; Chye *et al*., 1991) (Forward primer : 5'-CGCGGATCCCCCTTTCTCTCTCCT GCGCCGGCA-3'; Reverse primer : 5'-AAATGTGGCGGA GATTGTTTGAATTCCGG-3') and genomic DNA was used as template. Amplification was carried out in a 20 µl reaction volume, which was composed of 10x 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 each of forward primer and reverse primer. 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.

Total RNA isolation and *hmgr*1 cDNA synthesis by RT-PCR

Total laticifer RNA was isolated as described previously by Venkatachalam *et al*. (1999), treated with DNaseI and subsequently reverse transcription of total RNA was carried out. The 50 µl RT reaction contained 5 µg total RNA, 50 pmol oligo dT primer, 50 mM dNTPs mix, 10 mM DTT, 1 µl RNase inhibitor and 1 µl (40 U) Superscript II reverse transcriptase (Invitrogen, CA) in 10x buffer supplied by the manufacturer. The RNA and primers were preheated to 70 ºC for 10 min and snap-cooled in ice before adding the remaining components, the RT reactions (1 h, 42 ºC), were terminated by heating at 70 ºC for 15 min. The cDNA (2 µl) was then used for PCR amplification in a solution with 2.5 µl 10x buffer, 2.5 µl dNTPs (2.5 mM), 2.5 µl $MgCl_2$ (1.5 mM), 0.5µM of gene specific primers as described above and 1U Taq DNA polymerse (Promega). RT-PCR amplification was carried out for 4 min at 94 ºC, followed by 30 cycles of 94 ºC for 30s, 55 ºC for 1 min, and 72 ºC for 2 min. The final extension was performed at 72 ºC for 7 min. cDNA samples were separated by electrophoresis on 1.5% (w/v) agarose gel and the images of the ethidium bromide-stained bands were obtained with Kodak Imaging system.

Cloning and DNA sequencing analysis

The PCR amplified *hmgr*1 fragments were purified and cloned into pGEM-T plasmid vector. The ligated plasmids were then transferred into *E. coli* cells (DH5α) and plated onto LB agar plates containing 50 µg/ml ampicilin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 µg/ml 5-bromo-4-indolyl-β-d-galacto-pyranoside (X-Gal) for blue-white screening. Presence of the insert in the recombinant plasmid was further confirmed through insert release by restriction digestion with enzymes *Not I* and by PCR analysis. The nucleotide sequence of the cloned DNA fragment was determined using the automated sequencing facility (Indian Institute of Science, Bangalore). The nucleotide sequence of *hmgr*1 was edited to discard the vector contamination sequences at either ends and compared with published sequences in the NCBI database using BLASTN programme (Altschul *et al*., 1990). Sequence alignment and comparison was made using the ClustalW (Thomson *et al*., 1994).

Southern blot analysis

For southern blot analysis, high molecular weight genomic DNA (15 µg) isolated from leaves of *H. brasiliensis* and was digested with various restriction enzymes *Hind*III, *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 1.8 kb *hmgr*1 gene fragment radiolabelled with ^{32}PdCTP according to manufacturers instructions was used as probe for hybridization (Ambion, US). After hybridization, the DNA blot was washed twice in 2x SSC, 0.1 % SDS for 5 min and 0.1x SSC and 0.1 % SDS for 15 min at 65°C. Subsequently the blot was air dried and exposed to X-ray film (Kodak X-Omat) with intensifying screens for signal detection.

Analysis of *hmgr*1 gene expression

Regularly tapped trees were selected in a plot of 18-year-old trees submitted to half spiral tapping every 2 days without stimulation. Total RNA was isolated from latex, mature leaves, young leaves and seedlings of clone RRII 105 according to the method of Venkatachalam *et al*. (1999). The isolated latex RNA (10 µg) was denatured at 65 °C in the presence of 2.2 M formaldehyde and then separated on a 1 % (w/v) formaldehyde / MOPS agarose gel. The fractionated RNA was transferred onto a nylon membrane (Hybond N+ Amersham international, UK). Cloned *hmgr*1 cDNA labelled with a-P^{32}dCTP multiprime labeling system (Amersham Biosciences, UK) was used as probe for Northern hybridization. The blots were prehybridized for at least 2 h in the hybridization

Fig. 2. Multiple alignment of predicated amino acid sequence of *HbHMGR*1 gene with *Hevea* (AF429388, X54657), *Euphorbia* (EF062569), Litchi (DQ536143). A conserved region is boxed. Black stars denote essential residues of catalytic activity. (*) indicates a match across all sequences. (: and .) indicates a conservative and semi-conservative substitutes. (?) indicates the potential tyrosine phosphorylation of *HMGR*1 and (?) indicates the conserved Ser residue thought to be phosphorylated. The alignment was performed with the CLUSTAL W programme.

buffer (6 X SSPE, 5 X Denhardt's solution, 0.5 % SDS) and hybridization was carried out at 42 ºC for 1 h. The blots were washed twice in 2 X SSC and 0.5 % (w/v) SDS at 42 ºC followed by 1 X SSC and 0.1 % (w/v) SDS at 55 ºC. The membrane was exposed to X-ray film with 2 intensifying screens for signal detection.

Construction of plasmid pBIBHMGR1 and generation of *Arabidopsis* transgenic plants

The full-length *hmgr*1 cDNA isolated from *Hevea brasiliensis* (clone RRII105) was subcloned from pGEM-T to the *BamH*I and *EcoR*I sites of the binary plant transformation vector pBIB, placing the *hmgr*1 cDNA between the super promoter and nos terminator elements. Translational initiation is directed by the AUG initiation codon used in the native *hmgr*1 mRNA. The resulting plasmid was designated as pBIBHMGR1 and the details of the gene fusion are shown in Figure 7.

Arabidopsis thaliana plants (ecotype Columbia) were transformed using the in *planta* vacuum infiltration floral dip method (Clough and Bent 1998) using *Agrobacterium tumefacines*, strain pGV1301, harboring pBIBHMGR1 plasmid. Seeds from infiltered plants were surface-sterilized, sown in Petri dishes containing solid (0.8 % w/v agar) germination medium (GM:Murashige and Skoog medium supplemented with 10 g/L sucrose, 25 µg/ml Hygromycin) and incubated at 25 ± 2 °C for 10-12 days under 16/8 h light/dark illumination regime. Hygromycin-resistant seedlings (T_1) were transplanted into soil and grown to maturity under glass-house conditions. Leaf samples from wild and transgenic lines were collected and used for total RNA preparation as well as Northern blot analysis.

RESULTS

Isolation and cloning of *hmgr*1 gene

A PCR based approach was employed to isolate *hmgr*1 gene from *Hevea* tree. Primers were designed and synthesized based on the published sequences of *hmgr*1 from RRIM 600 clone (Malaysian clone) by Chye *et al*. (1991, 1992). Initially, total genomic DNA from leaf tissue was used as template for PCR amplification of *hmgr*1 gene where a 2.4 kb fragment was isolated. Total RNA from latex cells was reverse transcribed and used for PCR amplification of *hmgr*1 cDNA. Following the PCR amplification, a single 1.8 kb fragment corresponding to the predicted length for *hmgr*1 cDNA was detected. The PCR amplified genomic and cDNA fragments were gel purified and cloned into pGEM-T vector. The recombinant clones were subjected to colony PCR as well as restriction digestion to confirm the presence of cloned DNA inserts (Fig.). After confirmation, clones containing an insert of approximately 1.8 and 2.4 kb for cDNA and genomic DNA respectively were selected for nucleotide sequencing.

Nucleotide sequence characterization and the deduced amino acid sequence of *HbHMGR*1 protein

The nucleotide sequence analysis showed that the *hmgr*1 cDNA insert was 1838 bp long and contained a 1725 bp open reading frame (ORF excluding stop codon), flanked by a 42 bp 5'-untranslated region (UTR) and a 110 bp 3'-UTR (Fig. 1). The nucleotide sequence of the *hmgr*1 has been deposited in the GenBank database under the accession no. AY706757. The ORF encodes a 575 amino acid polypeptide with a predicted molecular mass of 61.6 kDa and a deduced isoelectric point of 6.6. The genomic DNA fragment of *hmgr*1 was 2440 bp in length and the sequence analysis revealed that it consists of 4 exons with perfect match to the *hmgr*1 cDNA. Three introns (131 bp, 377 bp, 94 bp) were found to interrupt an ORF of 575 amino acids and genomic fragment contains 42 bp of 5'-UTR and 68 bp of 3'-UTR. The exon-intron junctions of the *Hevea hmgr*1 gene obey the rule 5'-AG-GT-3' with some exceptions found for higher plant genes (Breathnach and Chambon 1981), and, as for higher plant genes, the introns are relatively AT-rich in comparison with the coding regions. The genomic *hmgr*1 gene sequence has been registered in the GenBank database under the accession no. AY352338. The *hmgr*1 genomic sequence showed 100 % identity with the *hmgr*1 cDNA sequence after deletion of intron sequences. The multiple alignment of *H. brasiliensis hmgr*1 deduced amino acid sequence showed 99 % identify with *Hevea*, 81-83 % identity to those in *Euphorbia, Litchi* and *Morus* and 70-79 % identity to those in *Cucumis, Corylus, Camptotheca, Picrorhiza, Andrographis, Tilia, Medicago* and *Pisum hmgr*1 sequences (Fig. 2). A computer analysis using the pSORT program (K. Nakai, Osaka University Japan) for protein localization sites suggested that *hmgr*1 consist of 2 transmembrane motifs (LPLYLTNAVFFTLFFSVAYYLLHRWRDKIR; PLHIVTLSEIVAIVSLIASFIYLLGFFGIDFVQ) in the catalytic N terminal site (Fig. 2). As with most plant *hmgr*1 peptides, the majority of the sequence variability is in the N-terminal one-third of the protein, whereas, the carbonyl two-thirds of the protein, containing the catalytic domain, show a high degree of conservation. Moreover, the HMGR in *Hevea* also had motifs crucial to the activity of the C-terminal region that were

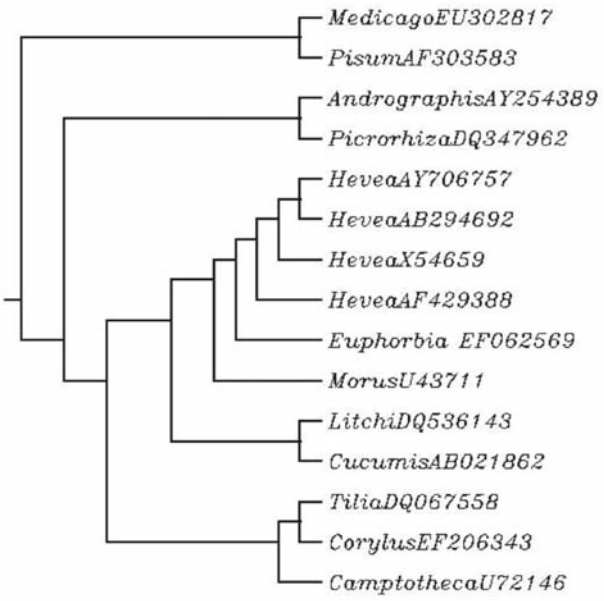

Fig. 3. Phylogenetic tree indicating the relationship of the proteins of the *HMGR* family. The alignments were generated using the Clustal W program (Thompson et al. 1994) and analysed by TreeView program.

highly conserved across plant species. The phylogenetic relationship of *hmgr*1 proteins from various taxa is presented in Fig. 3, which is deduced by the CLUSTAL W programme. The tree showed that the *hmgr*1 genes from *Hevea* formed single cluster while individual clusters were made with other species. Hydropathy analysis of the deduced amino acid sequence indicated that HbHMGR1 was strongly hydrophobic and hydrophobicity plot demonstrate the presence of two hydrophobic regions corresponding to these potential transmembrane motifs (Fig. 4).

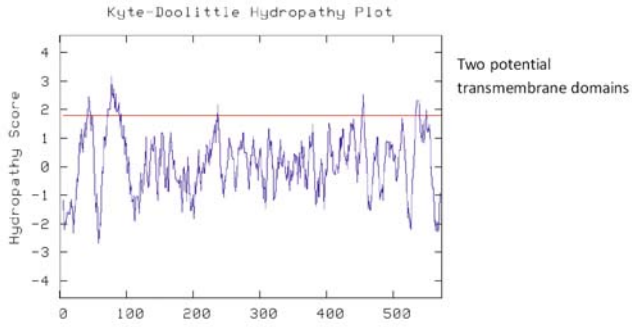

Fig. 4. Hydropathy plot calculated from the deduced amino acid sequence of *HbHMGR1*. The analysis was performed according to Kyte and Doolittle (1982).

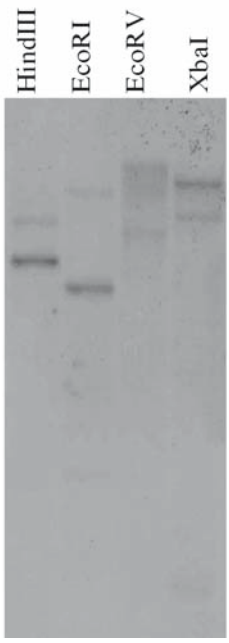

Fig. 5. Southern blot analysis of *H. brasiliensis*. Genomic DNA (15µg) was digested with *Hind*III, *Eco*RI, *Eco*RV and *Xba*I and probed with radiolabelled *HbHMGR*1 cDNA.

Analysis of *Hevea* genomic DNA

To ascertain the relative copy number of *hmgr*1 gene in *H. brasiliensis*, we performed Southern blot analysis using genomic DNA extracted from the leaf tissues digested with enzymes *Hind*III, *Eco*RV, *Eco*RI and *Xba*I using the *hmgr*1 cDNA gene probe. It is important to note that there were no restriction sites for *Hind*III, *Eco*RI, *Eco*RV and *Xba*I enzymes within the *Hevea*

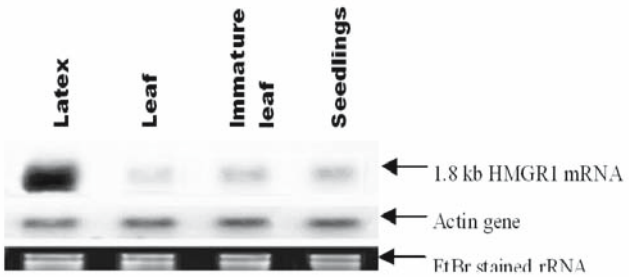

Fig. 6. Northern blot analysis of the *HbHMGR*1 mRNAs. Total RNA (15µg) from *Hevea* latex, leaf, immature leaf and seedlings were separated by agarose gel electrophoresis and transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labelled *HbHMGR*1 cDNA probe. The rRNA bands in EtBr stained gel is shown as loading control.

*hmgr*1 genomic DNA. The *Eco*RI, *Eco*RV and *Xba*I digested DNA revealed 3 hybridizing bands while with *Hind*III, 2 bands were detected in the DNA (Fig. 5). The presence of three bands of *Hevea* genomic DNA suggests that there may be up to three genes for *Hevea brasiliensis hmgr*. The occurrence of additional lighter bands, under high-stringency wash conditions, suggests that the *Hevea* genome contains additional *hmgr* genes. This result suggested that the *hmgr*1 gene is one of the members of a small gene family in *Hevea*.

Gene expression studies by Northern blot analysis

The expression pattern of the *Hevea hmgr*1 gene has been studied. As shown in Fig. 6, the *hmgr*1 mRNA transcripts are 1.8 kb, which suggests that the cloned cDNA is a full-length cDNA. Northern blot analysis indicated that the gene is expressed in latex, leaf, immature leaf and young seedlings. Besides, *hmgr*1 gene exhibits a differential expression pattern among tissues and the maximum level of transcripts of gene was noticed in latex (Fig. 6). This pattern of expression is consistent with an active role of the *hmgr*1 enzyme in the isoprenoid unit formation of laticiferous tissues (latex producing cells) during rubber biosynthesis in *Hevea* tree (Chye *et al*., 1991, 1992).

Analysis of *hmgr*1 mRNA in wild-type and transgenic *Arabidopsis*

Using *Agrobacterium*-mediated transformation, the super promoter : *hmgr*1 fusion gene was introduced into *Arabidopsis* and hygromycin transgenic lines were identified. The super promoter : *hmgr*1 gene introduced into the *Arabidopsis* transformants is a transcriptional fusion between the super promoter and the full-length *Hevea hmgr*1 cDNA and consequently, the super promoter is expected to direct high levels of expression from the *hmgr*1 transgene. In order to compare the accumulation of *hmgr*1 mRNA transcript levels between wild-type and transgenic plants, RNA isolated from transgenic plants was analyzed by Northern blot hybridization using an *hmgr*1 gene specific probe. RNA blot analysis revealed that transgenic lines accumulated much higher levels of the *hmgr*1 mRNA transcripts than wild-type plants (Fig. 8). It is also remarkable to note that the leaf size with 50 % enlargement was detected in transgenic plants relative to control wild-type plants. In addition, plants over expressing *HMGR*1 gene grew more vigorously than their non-transgenic counterparts.

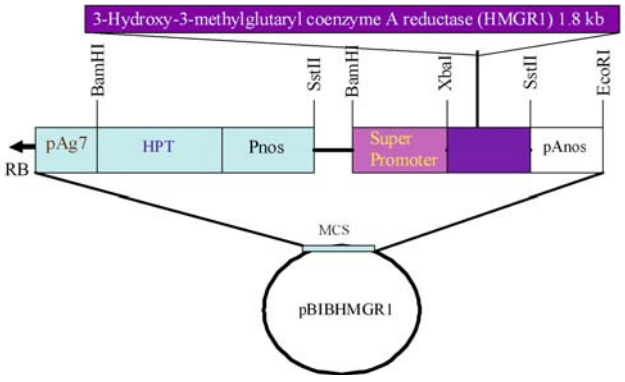

Fig. 7. A schematic representation of plant binary vector HMGR1 gene constructs. The *HMGR*1 cDNA was placed under the transcriptional control of the super promoter and pAnos terminator and the *HMGR*1 gene transferred into wild-type *A. thaliana* by *Agrobacterium*-mediated transformation. RB- Right border, LB-Left border, HPT-Hygromycin, MCS-Multiple cloning site, nos-Nopaline synthase.

DISCUSSION

HMGR is a key enzyme in plants since HMG-CoA acts as a precursor to many vital isoprenoid compounds including natural rubber, it is not surprising that the activity of HMGR in plants is regulated in part by the level of its mRNA. In this study, we isolated genomic and cDNA fragments encoding *hmgr*1 gene of the rubber tree *H. brasiliensis*. The deduced amino acid sequences showed high homology. *Hevea hmgr*1 was found to be highly homologous (81 % nucleotide sequence identity) to *Euphorbia hmgr*1 gene. This similarity is extended to their genomic clones where in each case, all three introns interrupt the coding sequence at the same position as reported earlier by Chye *et al*. (1991). The amino

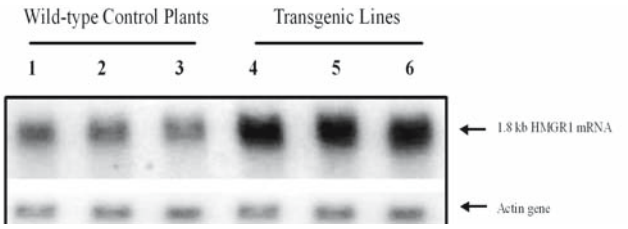

Fig. 8. Northern blot analysis of *HMGR*1 gene expression in wild-type and transgenic *Arabidopsis*. Total RNA was isolated from 14-day-old *Arabidopsis* plants grown under glass house and 15 ìg RNA samples were fractionated by formaldehyde gel electrophoresis and transferred into nylon membrane. Blot hybridization analysis was carried out using ^{32}P-labelled DNA probe derived from *Hevea HMGR*1 cDNA. *Hevea* Actin gene was used as internal control. RNA samples from: Lanes 1-3: wild-type control (non-transgenic) and 4-6: Transgenic lines of *Arabidopsis*.

acid sequence predicted from this species shares an extensive conserved region with HMGR1 from various organisms. The predicted amino acid sequence from the reported cDNA contains two conserved regions with a high level identity to a sequence at the active site of N-terminal region. The sequence also contained the conserved motifs important for HMGR function that has been suggested to be involved in transmembrane catalytic activity in N-terminal region. Bioinformatic analysis showed that a comparison of HMGRs from plants, yeast and mammals has shown that their carboxyl-termini are highly conserved. Comparison of the amino-termini of *Hevea* and *Euphorbia* HMGRs has shown amino acid conservation at the two hydrophobic regions which correspond to the potential transmembrane domains. It is interesting to note that plant HMGRs have only two such domains while seven are present in HMGRs of other organisms. The apparent molecular mass of *Hevea hmgr*1 was predicated to be 61.6 kDa and this matches quite well to the estimated molecular mass in other species.

To elucidate the evolutionary relationships between *Hevea* and other plant species, a dendrogram was created based on *hmgr*1 gene sequences obtained from GenBank. With regard to those *hmgr*1 genes, the genes in *Hevea* were located in one cluster including two more plant species (*Euphorbia* and *Morus*) and were clearly distinct from other plant species. A total of five clades were obtained in which four individual groups were also formed (Fig. 3). The sequences of *Hevea* were located near those for *Litchi* and *Cucumis*.

Southern result is now evident that in *Hevea*, HMGR is encoded by a small gene family consisting of three members. As evidenced in Southern blot analysis, it is not surprising to detect more than one isoforms of HMGR in plants as it has been previously suggested that subcellular compartmentation of different isoforms of the enzyme occurs (Brooker and Russell, 1975). It has been reported earlier that HMGR is encoded by three genes in *Hevea* (Chye *et al*., 1992) and tomato (Park *et al*., 1992), two distinct genes in yeast (Basson *et al*., 1986, 1988) and *Arabidopsis* (Enjuto *et al*., 1994, 1995), and even large multigene families in maize (Choi *et al*., 1992), potato (Korth *et al*., 1997), mulberry (Jain *et al*., 2000) while only one type of HMGR is known in mammals (Chin *et al*., 1984; Luskey and Stevens, 1985). Based on the appearance of multiple bands in the *Hevea* DNA gel blot, it is likely that *hmgr*1 also belongs to a small family of *hmgr* genes with divergent members. These are all involved in different metabolic pathways. The diversity of isoprenoid compounds in plants suggests that these compounds occur in multi-branched isoprenoid pathways (Bach, 1995). The pathway for rubber biosynthesis in *H. brasiliensis* is different from the pathway(s) leading to biosynthesis of other isoprenoid compounds (Suwanmanee *et al*., 2002; Priya *et al*., 2006).

The level of mRNA detected by Northern blot analysis of RNA from different tissues of *H. brasiliensis* RRII 105 was different. The *hmgr*1 gene is differently expressed in various tissues. *hmgr*1 mRNA transcript level is more abundant in latex than in the leaf, immature leaf and rubber seedlings. We have analyzed three independent transgenic *Arabidopsis* lines expressing *hmgr*1. Northern analysis showed that the accumulation of *hmgr*1 mRNA transcripts was higher in transgenic lines compared to that of control wild-type. Schaller *et al*. (1995) reported that an increased expression level of *hmgr*1 mRNA was positively correlated with higher enzymatic activity for HMGR. The expression of the *hmgr*1 gene was correlated to the presence of more laticiferous cells in the corresponding tissue. This finding is in agreement with the earlier reports for HMGR and HMGS in *Hevea* (Chye *et al*., 1992; Suwanmanee *et al*., 2002). In *Hevea*, a plant used for production of natural rubber, HMGR is encoded by a small gene family comprised of three members, *hmg*1 is expressed predominantly in the laticifers, the cells specific to rubber biosynthesis, but *hmg*3 expression is not cell type specific (Chye *et al*., 1992). It has been demonstrated that the *hmgr*1 which predominantly expresses in laticifers (latex) compared to other tissues, is believed to be involved in natural rubber (cis 1,4-polyisoprene) biosynthesis, since laticifers are the sites for rubber biosynthesis. One of the most challenging aspects of plant isoprenoid biosynthesis is the identification of the enzymes that catalyze the rate-limiting steps in the pathway. It is widely assumed that 3-hydroxy-3-methylglutaryl-CoA reductase, the enzyme that synthesizes mevalonic acid, plays a relevant role in the overall control of plant isoprenoid biosynthesis. The differential expression pattern suggests that this enzyme might be indicative of a specialized function in directing the flux of isoprenoid biosynthetic pathway in rubber tree.

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