Molecular Cloning and Sequencing of a cDNA Encoding 3-Hydroxy -3-Methylglutaryl Coenzyme A Synthase from *Hevea brasiliensis* (HBK) Mull Arg

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Received 19 Mar 2001
Accepted 4 Sep 2001

ABSTRACT A 1.8 kb cDNA clone encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase was isolated from a cDNA library prepared from the C-serum of rubber latex from *Hevea brasiliensis* (H.B.K.) Mull. Arg. using an *Arabidopsis thaliana* cDNA encoding HMG-CoA synthase as a probe. The nucleotide sequence analysis revealed an open reading frame of 1,392 nucleotides which encoded a protein of 464 amino acids with a predicted molecular mass of 51.28 kDa. The deduced amino acid sequence exhibits strong similarities with plant and mammalian HMG-CoA synthase sequences. Analysis of RNA samples from different tissues of rubber tree seedlings showed a higher mRNA level in stem and petiole than in leaves. In mature rubber trees there were higher mRNA levels in latex and petiole than in leaves. This suggests that the expression of the HMG-CoA synthase gene is higher in laticifer cells than in leaves. Genomic Southern blot analysis using the full-length cDNA as a probe indicates the presence of up to three HMG-CoA synthase genes in *Hevea brasiliensis*.

KEYWORDS: 3-hydroxy-3methylglutaryl coenzyme A (HMG-CoA) synthase, cDNA, *Hevea brasiliensis*, cloning, sequencing.

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase (EC 4.1.3.5) catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-CoA. Two forms of HMG-CoA synthase (HMGS) have been reported in mammals, a mitochondrial form and a cytoplasmic form.[1, 2] The mitochondrial enzyme produces HMG-CoA that is converted to ketone bodies. The HMG-CoA produced by the cytoplasmic enzyme is converted by HMG-CoA reductase (HMGR) to mevalonate, which is a precursor for the synthesis of sterols and isoprenoids for various functions. In rats and humans, these iso-enzymes are encoded by two different genes.[3, 4] The HMG-CoA produced by this enzyme in plants acts as a substrate for HMGR to form mevalonate which is further converted to isoprenoid compounds, such as growth regulators (gibberellins, cytokinins and abscisic acid), dolichol, chlorophyll, phytoalexins, carotenoids and natural rubber. HMG-CoA is also an intermediate in the degradation of the branched-chain amino acid leucine.

Hevea brasiliensis, a perenial tropical tree originating from South America, is widely cultivated in South America, Africa and Asia for the production of rubber. The unique isoprenoid compound in natural rubber is cis-1,4 polyisoprene, which is present in latex. Both the HMGS and the HMGR have been shown to be involved in early steps of rubber biosynthesis.[5] The two enzymes possibly function in concert in response to the supply of substrates for rubber biosynthesis, similar to the synthesis of cholesterol in animals.[6, 7]

The HMG-CoA synthase in *H. brasiliensis* has been reported to be present in both the C-serum and the bottom fraction when rubber latex is fractionated by centrifugation.[8] The enzyme was mainly found in the C-serum. Total activity of HMGS and dried rubber content from each tapping were shown to correlated reasonably well. The finding is similar to those observed in the case of HMGR activity and rubber content.[9] It is possible that the rubber biosynthetic pathway is coordinately regulated by the activity of both HMGS and HMGR.

It is interesting to know about the regulation and expression of the genes involved in the biosynthesis of natural rubber. Three genes are known to encode HMGR in *H. brasiliensis*, namely *hmg*1, *hmg*2 and *hmg*3. These have been investigated in some detail. The *hmg*1 gene is likely to be involved in the rubber biosynthesis, whereas the *hmg*3 is possibly involved in isoprenoid biosynthesis of another nature.[10] There is hardly any information about genes encoding for HMGS in *H. brasiliensis*. Limited information concerning HMGS genes in other plants is available. Isolation and characterization of cDNA encoding for HMGS in *Arabidopsis thaliana* and in *Pinus Sylvestris* has recently been reported.[11, 12] However, little is known about HMGS gene in *H. brasiliensis.*

In the present paper, we report about the cloning and characterization of an HMGS cDNA and its gene expression in various parts of the *H. brasiliensis* tree.

Materials and Methods

Plant Material

Approximately 20 year-old *H. brasiliensis* (HBK) Mull. Arg. clone RRIM 600 trees at the Songkla Rubber Research Centre were tapped every second day. One year old *H. brasiliensis* seedlings of the same clone were grown in pots under natural conditions (12 h light/12 h dark cycle at 23-35°C) at Thaksin University, Songkla, Thailand. Leaves, petioles and latex were collected from the mature trees, and leaves, petiole, stem and root were collected from seedlings.

Isolation of Poly(A)$^+$RNA

Total RNA was extracted from rubber latex by a modified method of Kush et al.[13] Fresh latex was kept on ice before fractionation in a prechilled rotor at 30,000 *g* for 30 min. Four fractions were obtained: the top rubber layer; the Frey wyssling particles, the clear C-serum or cytosolic fraction, and the pellet (bottom fraction) containing lutoids and other particles. C-serum was removed with a syringe. The C-serum (cytosolic fraction) was deproteinized by extraction two times with phenol : chloroform (1:1, v/v) and one time with chloroform. RNA was precipitated in 2 M LiCl at 4°C overnight, and followed by centrifugation at 10,000 *g* for 30 min at 4°C. The RNA pellet was dissolved in water and reprecipitated with 300 mM sodium acetate, pH 5.2 and 2.5 volumes of ethanol. Poly(A)$^+$RNA was isolated from total RNA by using the PolyA Tract mRNA isolation system (Promega Corp, Madison, WI, USA), according to the instructions provided by the manufacturer. The undegraded mRNA obtained was used as template for cDNA synthesis.

cDNA Library Construction and Screening.

A cDNA library was prepared from poly(A)$^+$RNA, isolated from C-serum of *H. brasiliensis* latex using the ZAP-cDNA synthesis kit (Stratagene Corp, La Jolla, CA, USA), according to the manufacturer's instructions. First strand cDNA was synthesized with Moloney Murine leukemia virus reverse transcriptase (MMLV-RT, Stratagene). This step was followed by RNase H and DNA polymerase I reaction for second-strand synthesis. The double-strand cDNA was made blunt-ended with T4 DNA polymerase (Stratagene) and ligated to *Eco*RI adapter. After being digested with *Xho*I, the cDNA containing an *Eco*RI site at its 5' end and a *Xho*I site at its 3' end was selected on a Sepharose CL-4B column. The cDNA longer than 500 bp was directly cloned into the *Eco*RI/*Xho*I digested UniZAP XR vector (Stratagene). The ligated DNA was packaged with Gigapack II gold packaging extracts (Stratagene). The cDNA library was screened by preparing plaque lifts on Hybond-N$^+$ (Amersham) nylon membranes after which the lifts were hybridized with ^{32}P-labelled single-strand *Arabidopsis* HMGS cDNA probe (a gift from Dr T Desprez, INRA, Biol Cellulaire Lab Versailes, France). Plaques which showed strong hybridization with the probe were plated at low density and subjected to further rounds of screening. Positive clones were excised *in vivo* and maintained in pBluescript SK(-) using a Exassist helper phage with SOLR strain from Stratagene.

DNA Sequence Analysis

The cDNA insert of HMGS clone was sequenced in both direction by the dideoxynucleotide chain termination method[14] with Sequenase™, version 2.0, DNA sequencing kit (United States Biochemical) and [(^{35}S)dATP], following the manufacturer's instructions. Automated nucleotide sequencing was also performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA Polymerase, FS. (Perkin-Elmer). DNA sequences were analysed, translated to protein sequences and compared with known sequences using the PRETTY-BOX program and the Ghostview solftware.

DNA Isolation and Southern Analysis

Genomic DNA was extracted from young *H. brasiliensis* leaves using the procedure of Roger and Bendich.[15] Approximately 5 g fresh weight of young leaves was ground to powder in liquid nitrogen and mixed with 20 ml of preheated (65 °C) 100 mM Tris-HCl buffer (pH 8.0) containing 1.4 M NaCl and 2% CTAB, 20 mM EDTA and 1% PVP Mr 40,000, before the extraction of DNA using chloroform : isoamyl

alcohol (24:1, v/v). The supernatant was saved and 1:5 volume of 5% CTAB, 0.35 M NaCl was added. The DNA was re-extracted with chloroform : isoamyl alcohol (24:1, v/v) as before. DNA in the extraction mixture was precipitated with 50 mM Tris-HCl buffer (pH 8.0) containing 1% CTAB and 10 mM EDTA. The DNA pellet was redissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 1 mM NaCl. The DNA was reprecipitated with absolute ethanol, washed with 70% ethanol and redissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA].

The genomic DNA (20mg) was digested with restriction endonucleases *Eco*RI, *Hin*dIII, *Xba*I and *Eco*RV. The completely digested DNA was separated on 1% agarose gel before transfering onto a nylon membrane. The membrane was hybridized under stringent conditions with full-length *H. brasiliensis* HMGS DNA probe, which was labeled with Fluoresceine 11-dUTP using Gene Images random prime labeling module (Amersham, Little Chalfont, UK) according to the manufacturer's instruction.

RNA Isolation and Northern Blot Analysis

Total RNA from C-serum of rubber latex was prepared by modified method of Kush[13] described previously. Total RNA from various tissues of seedling and mature *H. brasiliensis* trees was isolated by using the procedure of Vries et al.[16] Five grams of each plant tissue were ground to powder in liquid nitrogen and mixed well with 10 ml of hot (90 °C) RNA extraction buffer [100 mM Tris-HCl (pH 9.0), 100 mM LiCl, 10 mM EDTA and 1 % SDS] : phenol (1:1, v/v). Then 5 ml of chloroform was added into the homogenate and the mixture was centrifuged at 20,000 *g* for 30 min. The aqueous upper phase was re-extracted with phenol : chloroform (1:1, v/v) and then extracted with chloroform as described above. RNA was precipitated twice with 2 M LiCl. The concentration of total RNA extracted was measured from the absorbance at 260 nm. RNA extracted from various tissues was denatured at 65 °C in the presence of 2.2 M formaldehyde, and then separated by electrophoresis in a MOPS-formaldehyde 1 % agarose gel. The separated RNA, was transferred onto a nylon membrane. The membrane was then probed with Fluorescein 11-dUTP-labeled full-length *H. brasiliensis* HMGS cDNA and detected using the Gene Image CDP-star detection module (Amersham).

RESULTS

Isolation of cDNA for *H. brasiliensis* HMG-CoA Synthase

The cDNA library used in our study represented the mRNA population from C-serum of rubber latex clone RRIM 600. The library was screened by using *A. thaliana* cDNA encoding HMGS[11] as a probe. Several positive clones were obtained from primary screening and secondary screening. After *in vivo* excision, the inserted cDNA from 4 positive clones was analyzed by Southern blotting. The positive clone 1 and clone 2 showed inserted fragments of approximately 1.6 kb cDNA, while clone 3 and clone 4 the cDNA were undigestible (Fig 1). After the inserted DNA fragment from clone 1 with the size of 1.6 kb estimated by DNA blotting had been sequenced, the sequence length of the insert was revealed to be 1,804 bp. This sequence had an open reading frame of 1,392 bp encoding for a polypeptide of 464 amino acids (GenBank Accession AF396829), with a predicted molecular mass of 51.28 kDa, and a deduced isoelectric point of 7.3. The coding region is flanked by 5' and 3' untranslated sequences of 216 and 196 bp respectively. The sequence surrounding the ATG initiation codon (AGAAATGGC) partially fits with the plant consensus sequence AACAATGGC.[17] A putative polyadenylation site (AAUAAA) was found at position 1,546 (Fig 2).

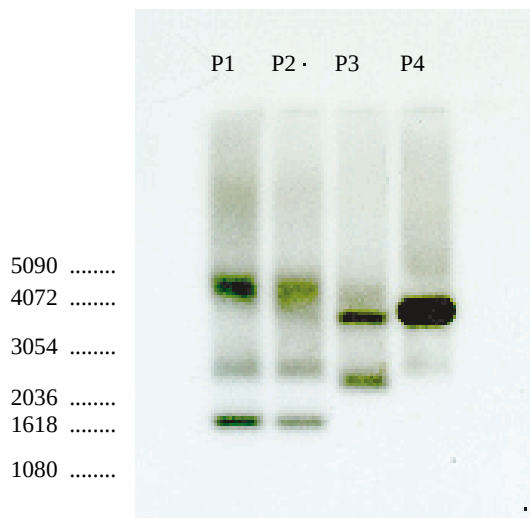

Fig 1. Southern analysis of inserted cDNA fragment from plasmid DNA of positive clones 1 (P1), 2 (P2), 3 (P3) and 4 (P4), digested with unique cloning site restriction enzymes (*Eco*RI/*Xho*I). The digested insert cDNA fragments were separated by electrophoresis on 1 % agarose and probed with the complete *A thaliana* HMGS cDNA. The sizes and positions of marker DNA fragments run in the same gel are shown on the left.

Comparison with Other HMGS Sequences

Alignment of the deduced amino acid sequence of *H. brasiliensis* HMGS with other known HMGS sequences, including *A. thaliana*[11], *P. sylvestris*[12], human cytosolic[18], human mitochondria[19] and *S. pombe*[20], was done to analyze sequence conservation, as shown in figure 3. The *H. brasiliensis* protein showed 81%, 74%, 51%, 49% and 45% identity with *A. thaliana*, *P. sylvestris*, human cytosolic, human mitochondria and *S. pombe*, respectively. The deduced amino acid sequence of *H. brasiliensis* HMGS showed high similarity within the N-terminal domain where the catalytic site of animal HMGS has been established.[21] The putative active site region of the mitochondrial HMGS from chicken liver demonstrated by Miziorko and Behnke[21] corresponded with the amino acid sequence of *H. brasiliensis* HMGS at position 102-122. The region was shown to be strongly conserved in both plant and animal species. However, higher similarity is observed between plants than between animals (90% and 80% sequence identity, respectively). It also includes a conserved cysteine in position 117 of *H. brasiliensis* HMGS, which is found in all other HMGS enzymes. This cysteine residue has been suggested to be involved in the formation of the acyl-S-enzyme intermediate.[22]

Southern Blot Analysis

H. brasiliensis genomic DNA was digested with the restriction enzyme *Eco*RI, *Hind*III, *Xba*I and *Eco*RV and separated on 1% agarose gel, transfered and UV cross-linked to a nylon membrane. Southern blot of the genomic DNA was analyzed using the full-length *H. brasiliensis* HMGS cDNA as a probe. The hybridization pattern showed 3-4 major hybridizing bands with each DNA restriction digested products, except the *Hind*III (Fig 4). The sequence of *H. brasiliensis* HMGS cDNA has one restriction

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                                                    -216 TTTTTTCTCTCCTTGCTGCTTTCCAGGGACCCCTCT
     CCTTAATTCACAGTTTCTCTTCTCAATTTAGTTTCATTTTTTTCCTGAAACTTTTTTAAATCTCTGTACAAAAGAGAGAATTGCAGCTGC
     TGCTTCCTGCCTGCTTCTGTTTTTTTTCCATTTGATTTCTTTTAGTTTTGCGGCTGTTGTGTCGAGGAGGAATTGGAGAGGCGTAGAGAA
   1 ATGGCAAAGAATGTGGGAATTCTCGCTGTGGACATCTACTTTCCTCCTACCTTTGTTCAGCAGGAAGCACTGGAGGCTCATGATGGTGCA
      M  A  K  N  V  G  I  L  A  V  D  I  Y  F  P  P  T  F  V  Q  Q  E  A  L  E  A  H  D  G  A  30

  91 AGCAAAGGGAAATACACCATTGGACTTGGACAGGATTGCATGGCATTTTGTACTGAGGTGGAAGATGTCATCTCAATGAGTTTGACTGCA
      S  K  G  K  Y  T  I  G  L  G  Q  D  C  M  A  F  C  T  E  V  E  D  V  I  S  M  S  L  T  A  60

 181 GTTACTTCACTCCTCGACAAGTATAATATTGATCCTAAACAAATCGGTCGTCTGGAAGTTGGCAGTGAGACTGTGATCGACAAGAGCAAA
      V  T  S  L  L  D  K  Y  N  I  D  P  K  Q  I  G  R  L  E  V  G  S  E  T  V  I  D  K  S  K  90

 271 TCTATTAAAACCTTCTTGATGCAAATTTTCGAGAAATTCGGAAACACTGACATTGAAGGCGTTGACTCAACAAATGCATGTTATGGGGGG
      S  I  K  T  F  L  M  Q  I  F  E  K  F  G  N  T  D  I  E  G  V  D  S  T  N  A  C  Y  G  G  120

 361 ACTGCAGCTTTATTCAACTGTGTCAATTGGGTTGAGAGCAGTTCATGGGATGGACGCTATGGACTTGTAGTGTGTACTGACAGTGCGGTC
      T  A  A  L  F  N  C  V  N  W  V  E  S  S  S  W  D  G  R  Y  G  L  V  V  C  T  D  S  A  V  150

 451 TATGCAGAGGGTCCAGCCCGACCAACTGGAGGAGCTGCAGCCATTGCGATTTTAGTAGGTCCAGATGCACCTATTGCTTTTGAAAGCAAA
      Y  A  E  G  P  A  R  P  T  G  G  A  A  A  I  A  I  L  V  G  P  D  A  P  I  A  F  E  S  K  180

 541 TTTAGGGGGAGCCATATGTCTCATGCTTATGATTTTTACAAGCCCAACCTGGCTAGTGAATATCCAGTTGTGGATGGCAAGCTTTCCCAA
      F  R  G  S  H  M  S  H  A  Y  D  F  Y  K  P  N  L  A  S  E  Y  P  V  V  D  G  K  L  S  Q  210

 631 ACATGCTACCTCATGGCTCTTGATTCTTGCTACAAACATTTCTGTGCCAAGTATGAGAAATTTGAAGGCAAGCAATTCTCTATTTCTGAT
      T  C  Y  L  M  A  L  D  S  C  Y  K  H  F  C  A  K  Y  E  K  F  E  G  K  Q  F  S  I  S  D  240

 721 GCTGAATATTTTGTATTTCATTCTCCTTACAACAAGCTTGTACAGAAAAGCTTTGCTCGTTTGGTGTTCAATGACTTTGTGAGGAATGCC
      A  E  Y  F  V  F  H  S  P  Y  N  K  L  V  Q  K  S  F  A  R  L  V  F  N  D  F  V  R  N  A  270

 811 AGGTCTATTGATGAGACTGCTAAAGAAAAGCTGGCACCGTTTTCAAATTTATCTGGTGATGAAAGCTACCAAAACCGGGATCTTGAAAAG
      R  S  I  D  E  T  A  K  E  K  L  A  P  F  S  N  L  S  G  D  E  S  Y  Q  N  R  D  L  E  K  300

 901 GTATCCCAACAAGTTGCCAAGCCCCTTTATGATGCGAAAGTGAAACCAACCACTTTGATACCAAAGCAAGTTGGCAATATGTACACTGCA
      V  S  Q  Q  V  A  K  P  L  Y  D  A  K  V  K  P  T  T  L  I  P  K  Q  V  G  N  M  Y  T  A  330

 991 TCTTTGTATGCAGCATTTGCATCCCTCCTTCACAGTAAACATACTGAATTGGCAGGCAAGCGGGTGACACTGTTCTCTTATGGGAGTGGG
      S  L  Y  A  A  F  A  S  L  L  H  S  K  H  T  E  L  A  G  K  R  V  T  L  F  S  Y  G  S  G  360

1081 TTGACAGCCACAATGTTCTCATTGCGACTACATGAAGGCCAACATCCCTTTAGCTTGTCAAACATTGCATCTGTGATGAATGTTGCAGGA
      L  T  A  T  M  F  S  L  R  L  H  E  G  Q  H  P  F  S  L  S  N  I  A  S  V  M  N  V  A  G  390

1171 AAGTTGAAGGCAAGACATGAGCTTCCCCCAGAGAAGTTTGTAAACATCATGAAGCTAATGGAGCACCGGTACGGAGCTAAAGACTTTGTG
      K  L  K  A  R  H  E  L  P  P  E  K  F  V  N  I  M  K  L  M  E  H  R  Y  G  A  K  D  F  V  420

1261 AGAAGCAAGGATTGCAGCCTCTTGGCTTCTGGAACATACTATCTCACAGAAGTTGACAGCTTGTATCGAAGATTCTATGCCCAGAAGGCT
      R  S  K  D  C  S  L  L  A  S  G  T  Y  Y  L  T  E  V  D  S  L  Y  R  R  F  Y  A  Q  K  A  450

1351 GTTGGCAACACAGTTGAGAATGGTTTGCTGGCTAATGGTCATTGATAGCAAATGGAAGTCATGTAGCATGCCAGGAATTTAGCTCGTATG
      V  G  N  T  V  E  N  G  L  L  A  N  G  H                                                464

1441 CTTTTAGATATTCAGTCCTGAGGACAATTGTGTTTCCTTCAAGTTTGTTTTCTACAGCAAATTGTTTGTTCAGCAGCAAGTGTTCCCTGT
1531 ATTTGTTTCCCCTTA ATAAA ATTCCTCTTATTGTCTCTTTAAAAAAAAAAAAAAAAAA
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Fig 2. Nucleotide sequence and deduced amino acid sequence from the cDNA encoding *H. brasiliensis* HMGS, GenBank Accession No. AF396829. The deduced amino acid sequence is shown below the nucleotide sequence. Numbers to the left of the lines indicate nucleotide position. Amino acid residue 1 is the putative initiator methionine. The putative polyadenylation sequence is underlined.

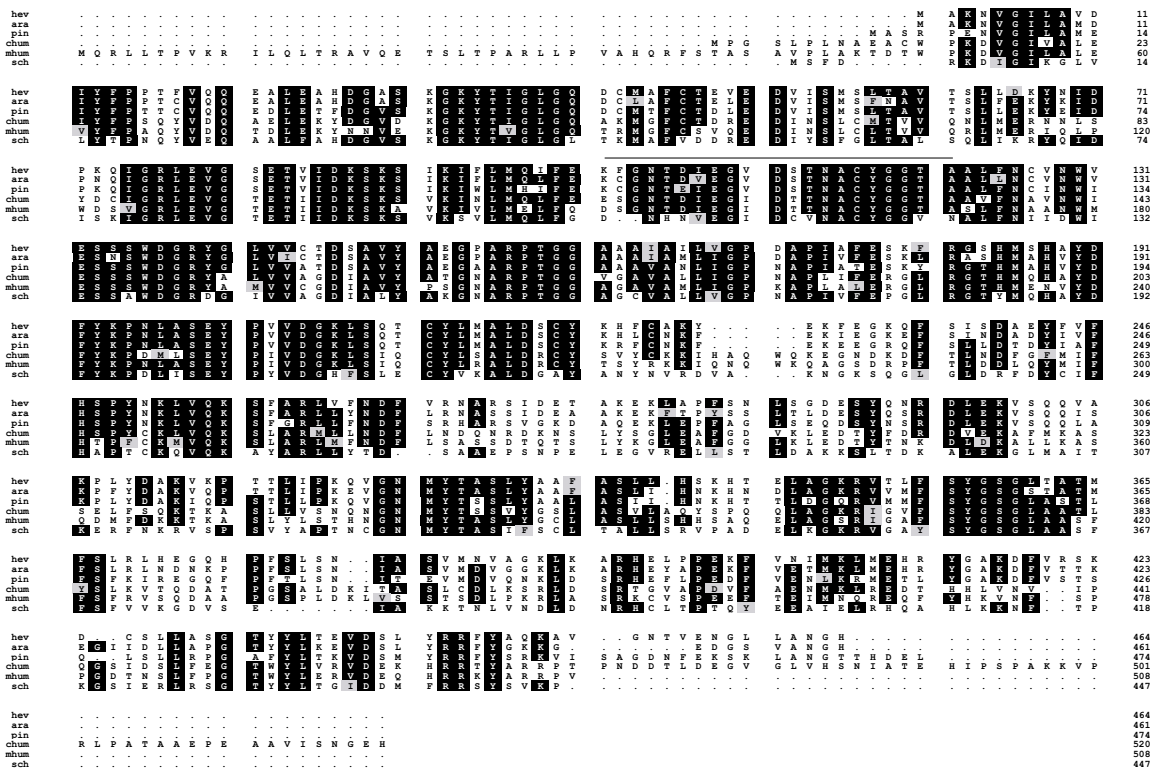

Fig 3. Alignment of HMGS sequences. The translated amino acid sequence of *H. brasiliensis* (hev) was aligned with *A. thaliana* (ara), *P. silvestris* (pin), human cytosolic (chum), human mitochondria (mhum) and *S. pombe* (sch) HMGS protein sequences. The alignment was performed using the PRETTYBOX program. The catalytic region of the enzyme is overlined.

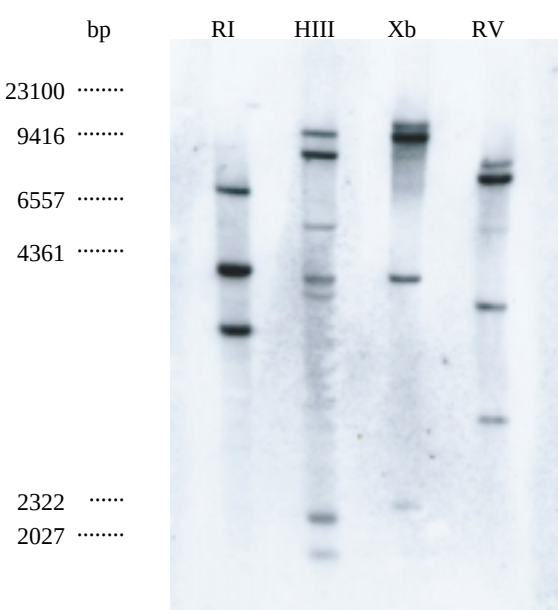

Fig 4. Southern analysis of *H. brasiliensis*. Genomic DNA (20 µg) was digested with *Eco*RI (RI), *Hind*III (HIII), *Xba*l (Xb) and *Eco*RV (RV) and probed with the complete *H. brasiliensis* HMGS cDNA. The sizes of marker DNA fragments run in the same gel are shown on the left.

site for the *Eco*RI (at position 17) and three restric--tion sites for *Hind*III (at position 619, 753 and 768). There are no restriction sites for *Xba*I and *Eco*RV. The presence of three bands of *H. brasiliensis* genomic DNA digested with *Eco*RI and *Xba*I suggested that there may be up to three genes for *H. brasiliensis* HMGS. Four bands were obtained in the case of *Eco*RV digestion although there was no restriction site in the cDNA. The observation may result from the presence of one or more restriction sites in introns in the *H. brasiliensis* HMGS genomic DNA. The higher number of bands shown in the case of *Hind*III digestion corresponds to the fact that there are three restriction sites at positions 619, 753 and 768 for *Hind*III in the cDNA of *H. brasiliensis* HMGS. This result supports the existence of as many as three HMGS genes in *H. brasiliensis.*

Tissue expression of *H. brasiliensis* HMGS

The expression of *H. brasiliensis* HMGS in various tissues of seedling and mature rubber tree was determined using gel blot analysis of total RNA isolated from various tissues. Leaf, petiole and stem from seedlings, and leaves, petiole and latex from

mature rubber trees clone RRIM 600 were analyzed. These RNA blots were probed with labeled *H. brasiliensis* HMGS cDNA. The size of *H. brasiliensis* HMGS transcript was estimated to be 1.9 kb using a 0.28-6.58 kb RNA marker. It was shown that HMGS was expressed differently in various tissues. The expression of HMGS genes in latex and petiole was higher than in leaves of mature rubber trees (Fig 5A). In seedling, similar results were obtained. The HMGS was also more highly expressed in latex and petiole than in leaves (Fig 5B).

Discussion

The full-length cDNA encoding HMGS from *A. thaliana* was used to isolate a cDNA fragment of *H. brasiliensis* HMGS. The cDNA isolated was estimated to be 1.6 kb from southern blot. After the sequence was revealed, the cDNA was found to be 1,804 nucleotides. The discrepancy between the size obtained was possibly due to the fact that there was one restriction site for the enzyme *Eco*RI at position 17. The size of HMGS cDNA isolated (1.8 kb) is close to the size of cDNA reported for *A. thaliana* (1.7 kb) Montamat et al [11] and for *P. sylvestris* (1.9 kb) Wegener et al.[12]

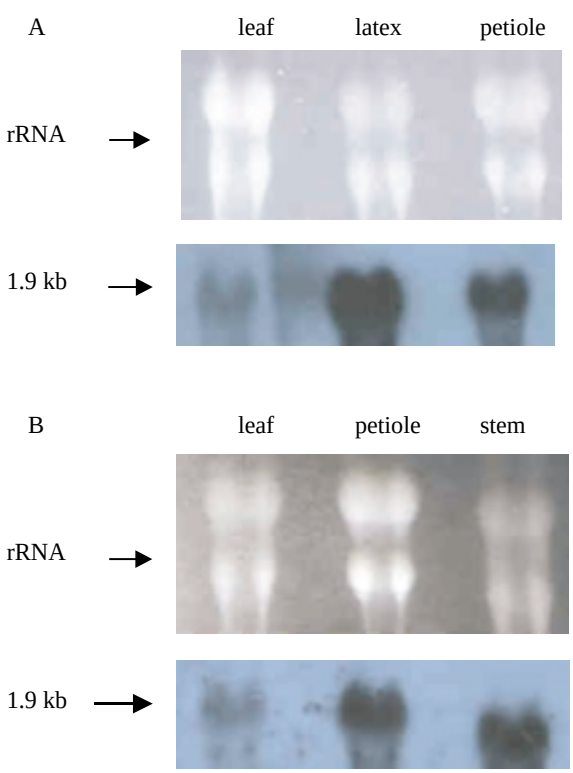

Fig 5. Total RNA (20 μg) isolated from leaf, latex and petiole of mature rubber tree (A; upper panel) and leaf petiole and stem of seedling of rubber trees (B; upper panel) were electrophoresed through agarose gel containing formaldehyde. Northern blot of total RNA were probed with the complete *Hevea brasiliensis* HMGS cDNA (A and B; lower panel).

The isolated cDNA from *H. brasiliensis* was identified as a (cytosolic) HMGS based on the following criteria. (i) The amino acid sequence predicted from this cDNA shares an extensive conserved region with HMG-CoA synthase from various organisms such as *A. thaliana*, *P. sylvestris*, human cytosolic, human mitochondria, and *Schizosaccharomyces pombe* (81%, 74%, 51%,49% and 45% amino acid identity, respectively). The cDNA isolated from *H. brasiliensis* is similar to cytosolic HMGS more than mitochondrial HMGS in vertebrates because it lacks the N-terminal mitochondrial leader peptide sequence that makes up the first 37 amino acids of the human mitochondrial HMGS sequence. (ii) The predicted amino acid sequence from the reported cDNA contains a region with a high level of identity to the sequence at the active site of chicken mitochondrial HMGS. (iii) The sequence contained the conserved residues important for HMGS function, including the cysteine that has been suggested to be involved in the formation of the acetyl-S-enzyme intermediate (Cys129 of chicken mitochondrial HMGS)[22], and the histidine thought to act in anchoring the second substrate, acetoacetyl-CoA (His 264 in chicken cytosolic HMGS).[23] These two amino acids corresponded to residues Cys 117 and His 247 of the predicted amino acid sequence from the *H. brasiliensis* cDNA according to the sequence alignment. These observations strongly suggested that the cDNA encoded protein represented *H. brasiliensis* HMGS. Moreover, when the cDNA was used as probe to detect mRNA, it was found that the level of mRNA is closely related to the HMGS activity and total rubber content in rubber latex. Rubber latex with high HMGS activity and high rubber content, showed strong mRNA bands. On the other hand, latex with low HMGS activity, mRNA band was also less intense (details to be published elsewhere).

Southern blot analysis indicated that there are possibly 3 HMGS genes in *H. brasiliensis* genome. There are two genes encoding for HMGS in mammals[3,4] and in insects such as *Blattella germanica.*[24, 25] It has been suggested that in plants, there is more than one gene in *A. thaliana.*[11] The two forms of HMGS in mammals have been detected: a mitochondrial form and a cytosolic form. These are involved in different metabolic pathways. In *Blattella germanica*, the two HMGS genes were expressed differently throughout development.[26] The diversity

of isoprenoid compounds in plants suggests that these compounds occur in multi-branched isoprenoid pathways.[27] The pathway for rubber biosynthesis in *H. brasiliensis* is different from the pathway(s) leading to the biosynthesis of other isoprenoid compounds. Three HMGR genes in *H. brasiliensis* have been shown to differently regulate the isoprenoid biosynthesis.[10] It is possible that three genes encode for HMGS in *H. brasiliensis* and these genes may function in a similar way to the function of HMGR genes in the rubber and other isoprenoid biosynthesis pathways. From its expression pattern, it appears likely that the HMGS gene isolated and characterized is involved in rubber biosynthesis and the expression of this gene could be directly related to the rubber content. The other gene (s) to be isolated and characterized possibly encodes the enzyme involved in biosynthesis of other isoprenoid compounds.

The level of mRNA detected by Northern blot analysis of RNA from several tissues of *H. brasiliensis*, RRIM 600 were different. The HMGS gene is differently expressed in different tissues. HMGS mRNA is more abundant in the stem and petiole than in the leaf of rubber seedlings. When tissues and latex from mature trees were analyzed, latex and petiole also showed higher expression of HMGS gene. In case of rats, mitochondrial HMGS showed tissue specific expression, HMGS mRNA was found in the liver and in the intestine (with a decreasing mRNA gradient from duodenum to colon).[28] Mitochondrial HMGS was not found in the brain, heart, hind limb, muscle, brown and white adipose tissues and placenta. Mitochondrial HMGS mRNA was undetectable in fetal liver but the level increased markedly after birth. Transcripts for the two HMGS genes in *B. germanica* are expressed differently in different developmental stages.[24] Differences in the level of HMGS mRNA from the tissues of seedlings and from mature rubber trees were not observed. The expression of the HMGS gene was correlated to the presence of more laticiferous cells in the corresponding tissues. This finding is similar to the report that for HMGR genes, *hmg*1, *hmg*2, and *hmg*3 are differentially expressed.[10] The *hmg*1 which predominantly expresses in laticifers, and hardly in leaves, is believed to be involved in natural rubber (cis 1-4 polyisoprene) biosynthesis, since laticifers are the sites for rubber biosynthesis. Therefore, it is likely that the HMGS transcript detected in laticiferous cells is involved in forming HMG-CoA as an intermediate in the biosynthesis of rubber.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T Desprez for the *Arabidopsis thaliana* HMGS cDNA clone, Dr. Wojech Griemski and Dr. Maria Hrmova for their help in the cDNA library construction and screening and DNA sequence analysis. We also thank Prof S Hempenius for reading the manuscript. This work was supported by the following organizations:

1) Faculty of Science, Prince of Songkla University, Hat Yai, Thailand.
2) The University Development Council (UDC) program for Thaksin University, Songkla, Thailand.
3) National Science and Technology and Development Agency, Thailand, (GREC 40-01-002).
4) Australian Agency for International Development Program, Australia.

P Suwanmanee would like to thank Thaksin University for granting study leave, which enabled him to complete this work.

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