Chapter 2

MATERIALS AND METHODS

I. Materials

1. Plant material

Fresh latex (half-spiral cut), leaf and petiole were collected from mature rubber trees and kept on ice. The rubber plants, belonging to an approximately 20-year old, clone RRIM 600, were grown at the Songkla Rubber Research Center and tapped every second day.

2. Bacterial strains

Escherichia coli strain XL1-Blue MRF' *Δ(mcrA)*183 Δ*(mcrCB-hsdSMR-mrr)*173 *endA*1 *supE*44 *thi-1 recA*1 *gyrA*96 *relA*1 *lac[F' proAB lacI*^{q} ZΔM15 Tn10(tet^r)].

Escherichia coli strain SOLR^{TM} e14$^-$ (McrA$^-$) Δ*(mcrCB-hsdSMR-mrr)*171 *sbcC recB recJ uvrC umuC*::Tn5 (Kan^r) *lac gyrA*96 *relA*1 *relA*1 *th*-1 *endA*1 *λR/* [F' *proAB lacI*^{q} ZΔM15]^c Su$^-$ (nonsuppressing).

Escherichia coli strain M15 [pREP4] MRF' Δ*(mcrA)* 183 Δ*(mcrCB-hsdSMR-mrr)*173 *endA*1 *supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac[F' proAB lacI*^{q} ZΔM15 Tn10(tet^r)].

3. Vectors

The pBluescript SK (+/-) vector (Strategene), the pGEM®-T Easy vector (Promega), and the expression vector, pQE-31 (Qiagen) were used in this study.

4. Primers

The nucleotide primers for 5' RACE, RT-PCR, site-directed mutation (Table 5) were purchased from Qiagen, Germany and Life Technologies, USA.

5. Chemicals

Acetyl-CoA Lithium salt, Acetoacetyl CoA Sodium salt, and [1-^{14}C] acetyl-CoA (1.89 GBq m/mole) were purchased from Sigma, (St. Louis, Mo, USA) and Amersham, (Little Chalfont, UK), respectively. Other chemicals and reagents (analytical grade) were purchased from Life Technologies (USA), Sigma; (St. Louis, MO, USA), Fluka (Switzerland), PIERCE (USA), Amersham Biosciences (UK), Stratagene (La Jolla, CA, USA), Promega (USA), and Qiagen (USA).

6. Enzymes

The enzymes were purchased from New England Biolabs (USA), Promega (USA), Stratagene (USA), and Sigma (USA).

7. cDNA library

The latex UniZAP-XR phage cDNA library was provided by Pluang Suwanmanee. The library was prepared as described by Suwanmanee et al. 2002.

8. Helper phage

ExAssit^{TM} interference-resistant helper phage was from Stratagene.

Table 5. The sequences of the primers for 5' RACE, RT-PCR, and site-directed mutagenesis.

Primer	Sequence (5' to 3')	Tm (°C)
5' RACE		
5' Nested Primer	GGACACTGACATGGACTGAAGGAGTA	78
Oligo dT Primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{18}$	-
GSP1	TTAGAGTACCATCTTAAGGGGAACTCATA	78
Full-length *hmgs2*		
ForHS2	CAAGCTCACAATGCCTGCTTT	62
RevHS2	TGATAAAAGTAAAGAGACGTT	54
RT-PCR		
GSP2	CCCATGAGCTGCTCTCTACCCAGTTGAC	88
18SFor	CAAAGCAAGCCTACGCTCTG	62
18SRev	CGCTCCACCAACTAAGAA	62
Mutagenesis		
5' Flanking primer	CGC**GGATCC**GATGGCAAAGAATGTGGGAAT	60
3' Flanking primer	CGC**GTCGAC**CTATCAATGAACATTAGCCAG	60
RevC117A	AGTCCCCCCATA***TGC***TGCATTTGTTGA	66
ForN326A	AAGCAAGTTGGC***GCA***ATGTACACTGCAT	66

Note. The nucleotides 5' **GGATCC** 3' and 5' **GTCGAC** 3' are *Bam*HI and *Sal*I restriction sites, respectively.

Table 6. Instruments used in this study.

Instrument	Model	Company
Analytical balance	Precisa 300A	Precisa balance
Autoclave	LS-2D	Switzerland
Automated DNA sequencer	ABI PRISM 377	ABI PRISM
Document gel	VisiDoc-It system	Rexall Industries, Taiwan
Heat box	Multi-Block	LAB-Line
High speed refrigerated centrifuge	Centrifuge 5804 *R*	Eppendorf, USA
Horizontal gel electrophoresis	AE6450	ATTO
Hybridization incubator	Shack in Stack	Hybaid
Bacteria incubator	Model 70	Enkab Limited, Croydon
Liquid scintillation counter	LS 5000	Beckman, USA
Microcentrifuge	Spectrafuge *16M*	Labnet, USA
Refrigerated microcentrifuge	TJ-6	Eppendorf, USA
Spectrophotometer	UV 160A	Shimadzu, Japan
Thermo cycler (PCR)	OmniGene	Hybaid, USA
UV transluminator	-	Vilber Lourmat
Vertical gel electrophoresis	*i-M*upid	Cosmo Bio Co.LTD

II. Methods

1. Screening for a new *H. brasiliensis hmgs* gene from the latex cDNA library

1.1 Preparation of the plating bacteria (Sambrook et al. 1989).

The XL1-Blue MRF' used as host cells in the cDNA library screening were grown on Luria-Bertani (LB; 5 g tryptone, 10 g yeast extract and 10 g NaCl in 1 L water) medium plate containing 12.5 µg/ml tetracyclin antibiotic and incubated at 37°C overnight. A single colony was cultured in 50 ml NZCYM (10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acid, and 2 g $MgSO_4 \cdot 7H_2O$ in 1 L water) medium broth supplemented with 0.2% maltose and 1 M $MgSO_4$ at 37 °C for 16-18 h with continuous shaking at 200 rpm. The cells were obtained by centrifuging at 10,000 rpm for 10 min at room temperature, and resuspended in 20 ml of 0.1 M $MgSO_4$. The cell suspension was incubated for 1 h at 37 °C with moderate agitation and kept at 4°C up to 1 week for using. The cell suspension was diluted with 0.1 M $MgSO_4$ to an $OD_{600} = 0.1$ before use.

1.2 Titration of the latex cDNA library (Sambrook et al. 1989).

The concentration of the cDNA phage library stored at -70°C was determined by titration as follows. Ten-fold serial dilution of the cDNA phage was prepared in SM buffer (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml of 1 M Tris-HCl, and 5 ml of 2% gelatin; and water in 1 L). Plating bacteria 0.1 ml (from method 1.1) and 0.1 ml of each dilution phage tube were mixed well and then incubated for 20 min at 37°C to allow absorption of bacteriphage particles to the bacteria. Warm top agar (0.7% agar in NZCYM medium) 3 ml was added to each tube, mixed well and immediately poured onto the NZCYM plates (130 mm diameter plate). These plates were then

incubated at 37°C overnight. The number of plaques on the plate was counted and calculated. For example, 350 plaques on the 10^3 dilution plate represent a titer of 3.5 x 10^5 pfu/ml.

1.3 Preparation of the *H. brasiliensis hmgs1* gene as a probe

1.3.1 Isolation of plasmid DNA using QIAprep Spin Miniprep kit (Qiagen)

The single colony of XL1-Blue MRF' containing the pBluescript SK-*hmgs1* (Suwanmanee et al. 2002) was grown in LB medium containing 100 μg/ml ampicillin antibiotic at 37°C with shaking at 200 rpm overnight. The pBluescript SK-*hmgs1* cDNA plasmid, containing the 1.8 kb full-length *hmgs1* cDNA was isolated by using alkaline lysis method with the QIAprep Spin Miniprep kit, according to the manufacturer's instruction. The plasmid DNA was diluted 1:50 with steriled water, DNA concentration was determined by spectrophotometer using an OD_{260} (1 OD_{260} DNA = 50 μg/ml) and the purity of isolated DNA was judged by OD_{260}/OD_{280} (pure DNA solution have an OD_{260}/OD_{280} ratio of 1.9-2.0).

1.3.2 Restriction endonuclease digestion

The *hmgs1* cDNA was liberated using the restriction endonuclease enzymes *Eco*RI and *Xho*I. *Eco*RI and *Xho*I specifically bind and cleave the double stranded DNA at the specific sequences at G$^{\downarrow}$AATTC and C$^{\downarrow}$TCGAG, respectively. The digestion reaction was composed of 1 μg of plasmid DNA, 2 units of enzymes, 1x reaction buffer, and water in a total volume of 20 μl, and was incubated at 37°C

overnight to ensure complete digestion. The digested products were analyzed by agarose gel electrophoresis.

1.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualize the extracted DNA of interest. Agarose 1 % (w/v) was melted in 0.5x TAE buffer (20 mM Tris-acetate and 0.5 mM EDTA), then 1 µl of 10 mg/ml ethidium bromide (EtBr) solution was added into the melted gel before pouring on the plastic tray with a comb placed in the gel. After the agarose gel was completely set, the comb was removed and the gel was placed in an electrophoresis chamber containing 0.5x TAE buffer. Five microliters of the DNA samples were mixed with 1 µl of 6x loading buffer [0.25% (w/v) Bromophenol Blue, 4% (w/v) sucrose] and loaded into the slots of the gel. Gel electrophoresis was carried out at a constant 50-100 V for 60-120 min. The expected DNA fragtment was visualized on a ultraviolet (UV) light box.

1.3.4 Purification of DNA fragment using the QIAquick Gel Extraction kit (Qiagen)

The expected DNA fragment was excised from the agarose gel in as small a size as possible and weighed. The excised gel was transferred into a 1.5 ml microcentrifuge tube and dissolved with 3 volumes of QG buffer (Guanidine thaiocyanate, pH $\leq$ 7.5) to 1 volume of gel. The tube was incubated at 50°C for 15 min mixed by vortexing every 3 min during incubation and then the incubation was continued for 10 min. If the gel slice contains <500 bp and >4 kb DNA fragment, 1 gel volume of isopropanol was added to the mixture. To bind the DNA fragment to silica membrane, the mixture was applied into a QIAquick column placed in a 2 ml

collection tube and centrifuged for 1 min at 14,000 rpm. The residual agarose gel was removed from the DNA fragment by washing buffer (ethanol-containing PE buffer) and centrifuged for 1 min at 14,000 rpm. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube and the DNA fragment was eluted with 30 ml of EB buffer (10 mM Tris-HCl, pH 8.5) by centrifugation at 14,000 rpm for 2 min. The concentration of eluted DNA was estimated by the agarose gel electrophoresis (Method 1.3.3).

1.3.5 Labelling the *hmgs*1 cDNA probe with Gene Images Random Prime Labelling Module (Amersham, RPN3540)

The purified *H. brasiliensis* 1.8 kb full-length *hmgs1* cDNA was used as a template for labelling with a fluorescein-11-dUTP random primer labelling kit (Gene Images Random Prime Labelling Module, Amersham) according to the labelling protocol. The cDNA template (34 µl of 2 µg) was denatured by boiling for 10 min and rapidly chilled on ice for 5 min. The labelling reaction containing 34 µl template, 10 µl nucleotide mix, 5µl of random primer, and 1 µl of 5 units/µl Klenow fragment, was gently mixed and incubated at 37°C for 2 h. The tube was wrapped with aluminium foil and the labelled cDNA probe was monitored, as described below.

1.3.6 Monitoring the incorporation of a fluorescein-11-dUTP in the labelled DNA using the rapid labelling assay

The negative control and reference strips were prepared by dotting 5 µl of 1/5 and 1/10, 1/25, 1/50, 1/100 of nucleotide mix diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) onto a Hybond-N^+ strip. The labelled cDNA probe (5

μl was dotted on to another Hybond-N^+ strip. The reference and sample strips were washed in excess pre-warmed 2x SSC at 60°C for 15 min with gentle agitation. Both strips were placed on pieces of Whatman 3MM paper lightly moistened with TE buffer and then visualized to estimate the labelled cDNA probe with each dilution of nucleotide mix on the reference strip using a UV transluminator.

1.4 Primary screening for a new *hmgs* gene by plaque hybridization with Gene Images detection kit (Amersham, RPN3510)

The screening was performed with approximately 150-200 plaques/plate (method 1.2). The plates containing plaques were placed at 4°C for 2-3 h, then moved to room temperature before plaque lifts. The disc membranes were placed on the surface of the plate, in contact with the plaques. The membranes and the agar were marked using a sterile needle and suitable indelible marker to ensure correct orientation of plaques until the membrane was completely wetted, about 2 min. The membranes were gently removed from the agar plate and the membrane, plaque side up was placed on the sheet of Whatman paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min. The membrane was then removed and placed on a sheet of Whatman paper which was saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 5 min. After that, it was placed in the washing solution [2x SSC (2x Standard Saline Citrate); 0.3 M NaCl and 0.03 M CH_3COONa, pH 7] for 2 x 5 min. The cellular debris was removed to prevent non-specific binding. The membranes were placed DNA side up, on Whatman 3MM paper to allow air drying at room temperature for 1 h. The membranes were then sandwiched between fresh 3MM paper and baked dry to fix the DNA at 80°C for 2 h in a vacuum oven. The membranes

were hybridized with the labeled *hmgs1* probe and candidate clones detected according to the manufacturer's instructions (Figure 11).

The prehybridization buffer (5x SSC, 0.1% SDS, 5% dextran sulphate, and 1x of liquid block) was pre-warmed at 60°C for 30 min, and then the membranes were placed in prehybridization buffer (0.75-1 ml/cm^2 of membrane) at 55°C with gentle shaking for 1 h. The labelled *H. brasiliensis hmgs1* cDNA probe in 20 µl was denatured by boiling at 100°C for 5-10 min and immediately chilled on ice for 15 min. The probes were then added to the prehybridization buffer, avoiding placing probe solution directly on the membrane, and gently mixed into the solution. The membranes were hybridized with labelled *hmgs1* cDNA at the 55°C overnight with gentle agitation. The membranes were transfered into preheated (55°C) primary wash buffer (1x SSC, 0.1% SDS), 2-5 ml/cm^2 of membrane for 15 min with gentle agitation, then washed in the secondary buffer (0.5x SSC, 0.1% SDS). These membranes were transferred to the blocking buffer (5x liquid block in the buffer A; 100 mM Tris, 300 mM NaCl) in 0.75-1 ml/cm^2 then incubated for 1-2 h at room temperature with gentle agitation.

The anti-fluorescein alkaline phosphatase-conjugate solution was diluted in buffer A containing 0.5% BSA, and the membranes were then incubated in the conjugate solution (0.75-1 ml/cm^2 of membrane) with gentle agitation at room temperature for 1 h. Unbound conjugates were removed by washing with 0.3% Tween-20 in buffer A, 2-5 m/cm^2 of membrane at room temperature for 3 x 10 min with vigorously shaking.

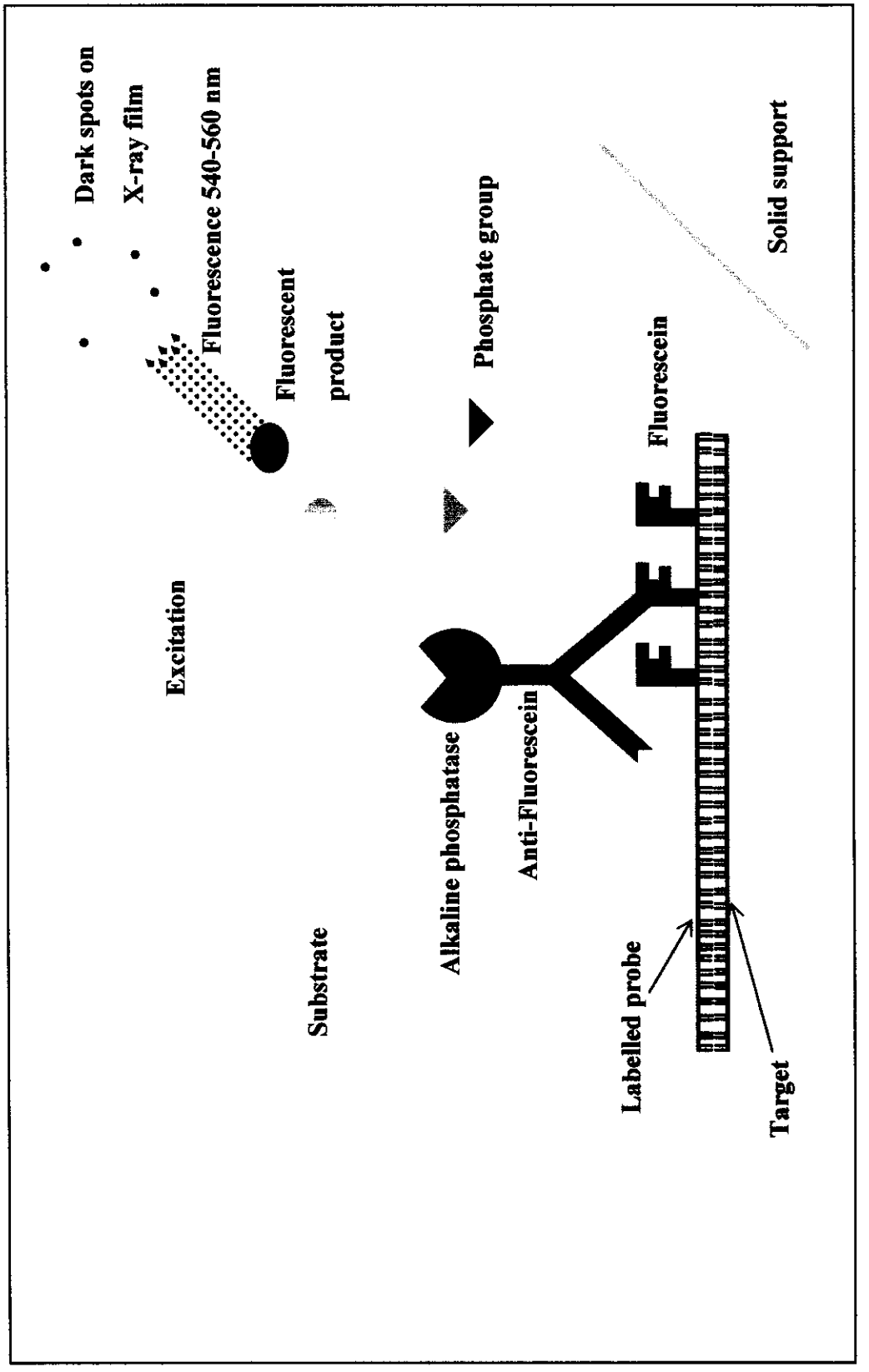

Figure 11. Gene Images-Prime Labelling and Detection System (Amersham).

The membranes were immersed, DNA side up, in the detection reagent (Amersham, RPN3510) and left for 5 min at room temperature. The excess detection reagent was drained off and the membranes were wrapped in Saran Wrap or a plastic bag and air pockets gently smoothed out. The membrane was exposed on the X-ray film for 1-30 min or as long as needed at room temperature.

1.5 Picking bacteriophage λ plaque

The positive plaques from primary screening were picked up for the secondary screening. The SM buffer (1 ml) and 20 µl of chloroform were placed into a 1.5 ml microcentrifuge tube. The chosen plaques on the hard agar were picked up using a micropipette, the plaques together with the underlying agar, were drawn into the pipette, placed a fragment of agar in the SM (1 ml)/chloroform (20 µl) solution and left at room temperature for 2 h to allow the bacteriophage particles to diffuse out of the agar. This phage stock can be kept indefinitely at 4°C.

1.6 Secondary screening of the positive cDNA plaques.

The obtained primary positive plaques were further screened to get rid of the false positive clones as follows. The strongest putative clones which showed up on the film were picked as in the step 1.5 and were then diluted 10^2-10^4 to obtain 150-200 plaques in a plate as previously described in method 1.2. Secondary screening procedure was performed as described in the primary screening (method 1.4). The distinct positive clones with strong dark spot on the X-ray film were picked as in 1.5. The further screening was performed until all clones from the 2^{nd} screening showed the dark spots for all plaque positions.

1.7 *In vivo* excision of the *hmgs2* cDNA phagemid using the ExAssist™ Interference Resistant Helper Phage Kit (Stratagene)

Five positive clones obtained from secondary screening and confirmed by tertiary screening, were excised *in vivo* with helper phage and SOLR cell according to the manufacturer's instruction (Figure 12) and maintained in the pBluescript SK (-), (Figure 13). The XL1-Blue and SOLR cells were grown in LB broth containing 12.5 μg/ml tetracyclin and 25 μg/ml kanamycin, respectively, as described in the method 1.1. The mixture comprising 200 μl of plating XL1-Blue cells, 250 μl of phagestock from secondary screening, and 1μl of ExAssist helper phage in a 50 ml tube, was incubated at 37°C for 15 min, 3 ml of LB broth was added and incubated for 2 h at 37°C with shaking. The mixture was then heated at 65-70°C for 20 min and centrifuged at 4,000 rpm for 15 min. The supernatant containing the excised pBluescript phagemid packaged as filamentous phage was decanted into a new microcentrifuge tube. The solution of excised phagemids (100 μl) was mixed with 200 μl of the fresh plating SOLR cells at 37°C for 15 min. The cell mixture (50, 100, and 200 μl) was spread on the individual LB-ampicillin (100 μg/ml) agar plate and incubated at 37°C overnight.

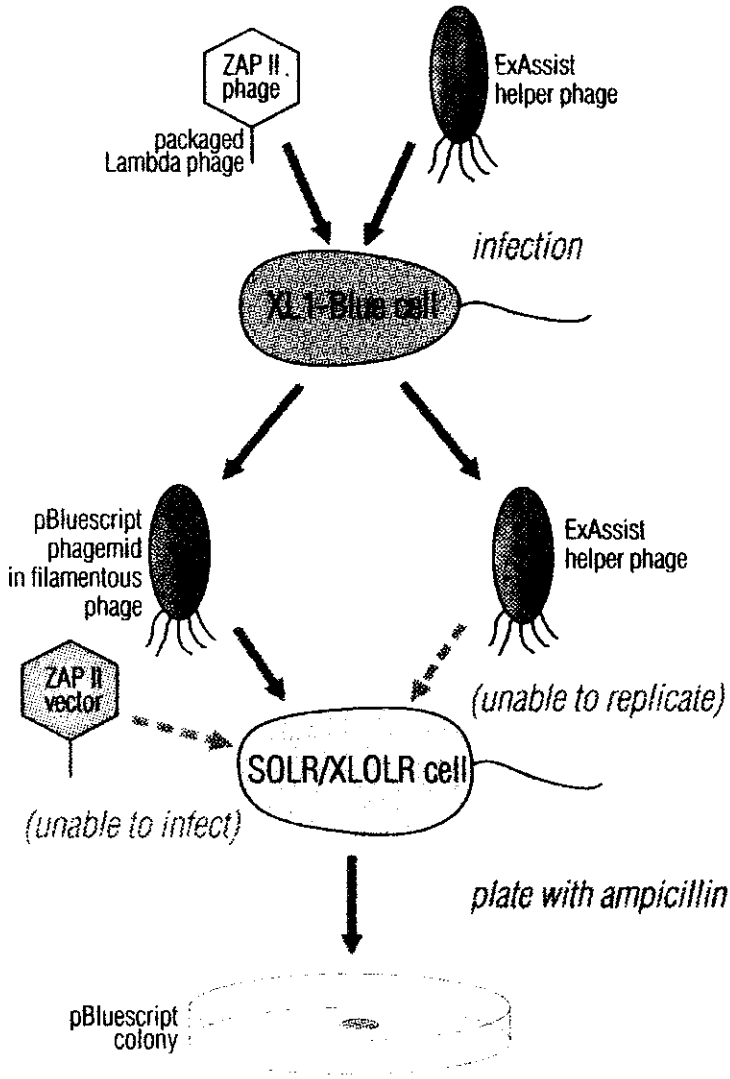

Figure 12. Rapid excision protocol (Strategene).

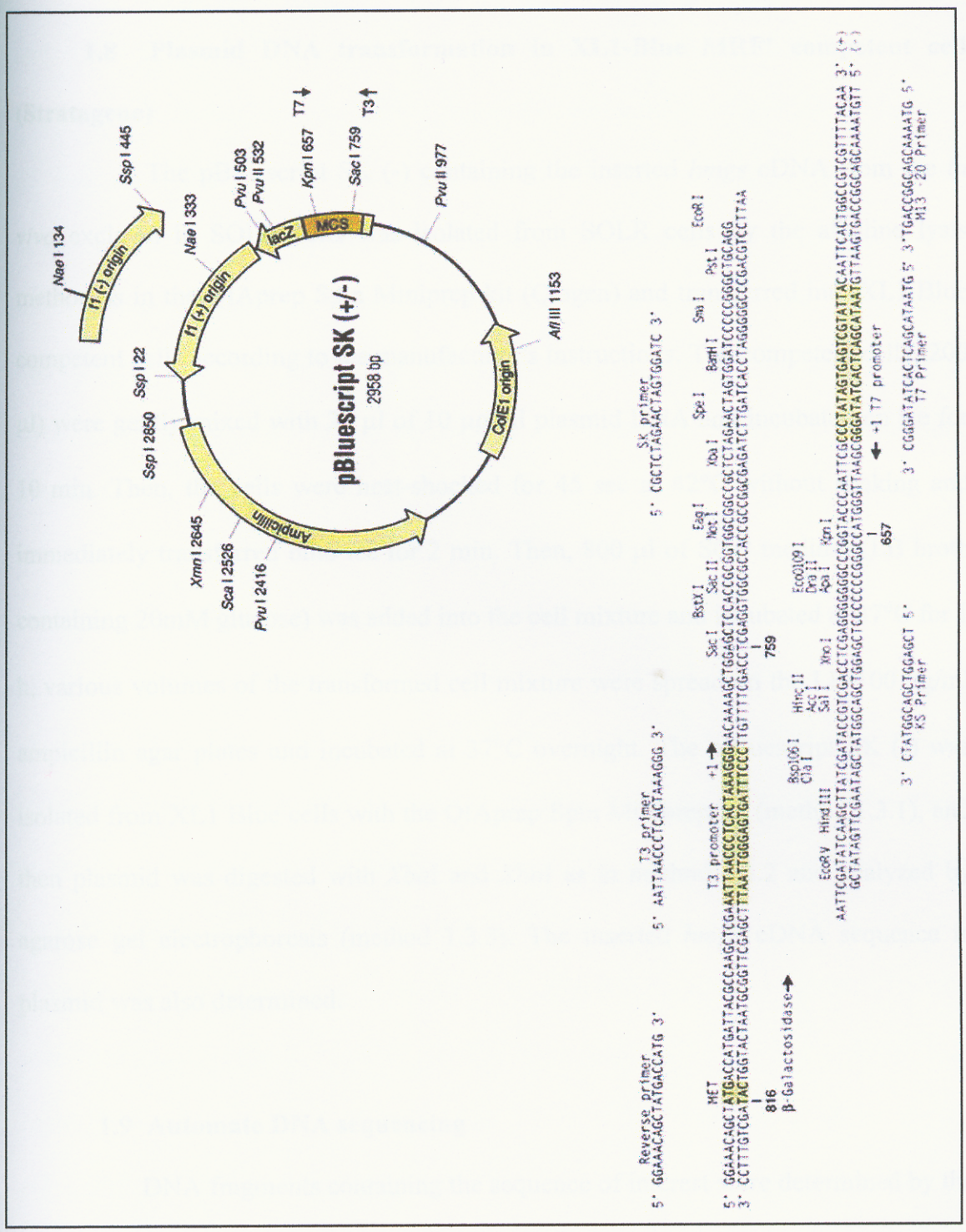

Figure 13. Map of pBluescript SK (+/-), Stratagene.

1.8 Plasmid DNA transformation in XL1-Blue MRF' competent cell (Stratagene)

The pBluescript SK (-) containing the inserted *hmgs* cDNA from the *in vivo* excision in SOLR cells was isolated from SOLR cells by the alkaline lysis method as in the QIAprep Spin Miniprep kit (Qiagen) and transferred into-XL1 Blue competent cells according to the manufacturer's instructions. The competent cells (200 µl) were gently mixed with 20 µl of 10 µg/ml plasmid DNA and incubated on ice for 10 min. Then, the cells were heat-shocked for 45 sec at 42°C without shaking and immediately transferred onto ice for 2 min. Then, 800 µl of SOC medium (LB broth containing 20mM glucose) was added into the cell mixture and incubated at 37°C for 1 h, various volumes of the transformed cell mixture were spread on the LB-100 µg/ml ampicillin agar plates and incubated at 37°C overnight. The pBluescript SK (-) was isolated from XL1 Blue cells with the QIAprep Spin Miniprep kit (method 1.3.1), and then plasmid was digested with *Xba*I and *Xho*I as in method 1.3.2 and analyzed by agarose gel electrophoresis (method 1.3.3). The inserted *hmgs* cDNA sequence in plasmid was also determined.

1.9 Automate DNA sequencing

DNA fragments containing the sequence of interest were determined by the dideoxynucleotide chain termination method Sanger et al. (1977) using the ABI PRISM™ BigDye terminator V1.1 cycle sequencing kit. The PCR cocktail (20 µl) contained 4 µl of the ready reaction mix, 200-500 ng DNA template, 3.2 pmole of a proper primer, and sterile water. The amplification reaction was carried out using the following parameters; initial denaturing at 96°C for 30 sec, 25 cycles of 96°C for 10

min, 55°C for 5 sec, 70°C for 1 min, and final extension at 70°C for 10 min. The amplification product was purified by isopropanol precipitation. The PCR product (20 µl) was added into the tube containing 80 µl of 75% (v/v) isopropanol and vortexed briefly. The tube was left at room temperature for 20 min and then centrifuged for 20 min at 14,000 rpm and the supernatant was immediately removed. The DNA pellet was washed with 75% isopropanol and dried at 90°C for 1 min and the dye-labelled DNA was analyzed by an Applied Biosystems 377 sequencer (Perkin-Elmer, Norwalk, CT, USA).

2. Generating the full-length *hmgs2* cDNA

2.1 Isolation of total RNA from rubber latex of *H. brasiliensis*

Fresh latex was collected from the rubber tree and kept in ice before isolation. Total RNA was isolated from rubber latex by the modified method described by Suwanmanee et al. (2004). The fresh latex was mixed well with an equal volume of extraction buffer pH 9.0 [1 M Tris-HCl, 0.5 M EDTA, 2% (w/v) SDS] and phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) to deproteinize. RNA in cytosolic fraction was precipitated in 1.8 M LiCl at 4°C overnight followed by a 10,000 rpm centrifugation at 4°C for 20 min. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water and reprecipitated with 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol at -20°C for 1h. The precipitated RNA was washed twice with 70% ethanol and air dried for 2 min. Total RNA was dissolved in DEPC treated water and diluted to determine the concentration by spectrophotometer at OD_{260} ($1OD_{260}$ RNA = 40 µg/ml) and the purity of RNA was determined using the OD_{260}/OD_{280} ratio of 1.9-2.0.

2.2 5' Random amplification of cDNA end by 5' RACE kit (Invitrogen)

Generating the 5' end missing region of *hmgs2* cDNA was performed as described in the GeneRacer™ kit (RLM-RACE) protocol (Figure 14). First, 5 μg of total RNA was treated with calf intestinal phosphatase (CIP) to remove the 5' phosphate at 50°C for 1 h. The RNA was extracted using phenol extraction and precipitated by ethanol. The 5'cap structure was removed from intact dephosphorylated mRNA by tobacco acid pyrophosphatase (TAP) at 37°C for 1 h and again phenol extraction and ethanol precipitation was performed. The full-length mRNA was ligated to the GeneRacer RNA oligo sequence at the 5'end with T4 RNA ligase at 37°C for 1 h. The ligated mRNA used as template in first strand cDNA synthesis was reverse-transcribed with GeneRacer oligo (dT) primer and Superscript II Reverse Transcriptase (RT) at 42°C for 1 h. GeneRacer Oligo (dT)-primed cDNA was used as template to amplify the 5' end of the cDNA with the GeneRacer 5'Nested Primer and a gene-specific primer (GSP1, Table 4). The amplification was performed using Platinum *Taq* DNA polymerase High Fidelity (Invitrogen) with the cycling parameters as follows: 94°C for 2 min, 5 cycles of 94°C for 30 sec, 72°C for 2 min, 5 cycles of 94°C for 30 sec, 70°C for 2 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, and 72°C for 10 min. The PCR products were analyzed in agarose gel electrophoresis and gel-purified as described in method 1.3.3 and 1.3.4, respectively. The expected *hmgs2* cDNA fragment was cloned into pGEM-T Easy vector.

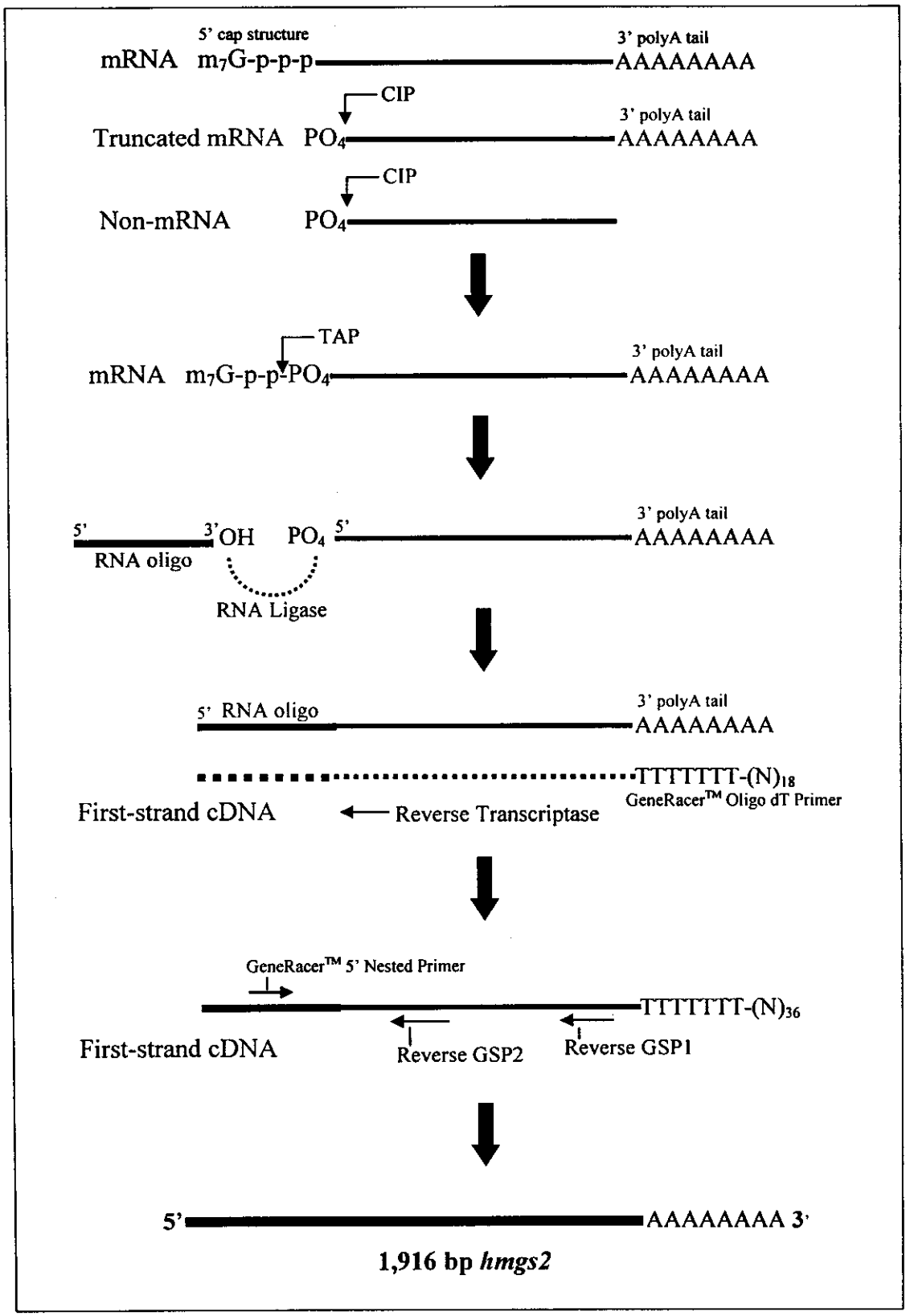

Figure 14. 5' Rapid Amplification cDNA Ends strategy.

The method base on RNA ligase-mediated (RLM-RACE)

2.3 Ligation the DNA fragment into pGEM-T Easy vector (Promega)

The purified interested cDNA (3' adenine (A) overhanged DNA) was ligated with pGEM-T Easy vector (Figure 15) according to the manufacturer's instruction. The reaction mixture (10 µl), containing 5 µl of the 2x Rapid ligation buffer, 1 µl of pGEM-T Easy vector, 1 µl of T4 DNA ligase, and 3 µl of the DNA fragment was incubated at 4°C overnight. The ligated plasmids were then transformed into XL1-Blue competent cells.

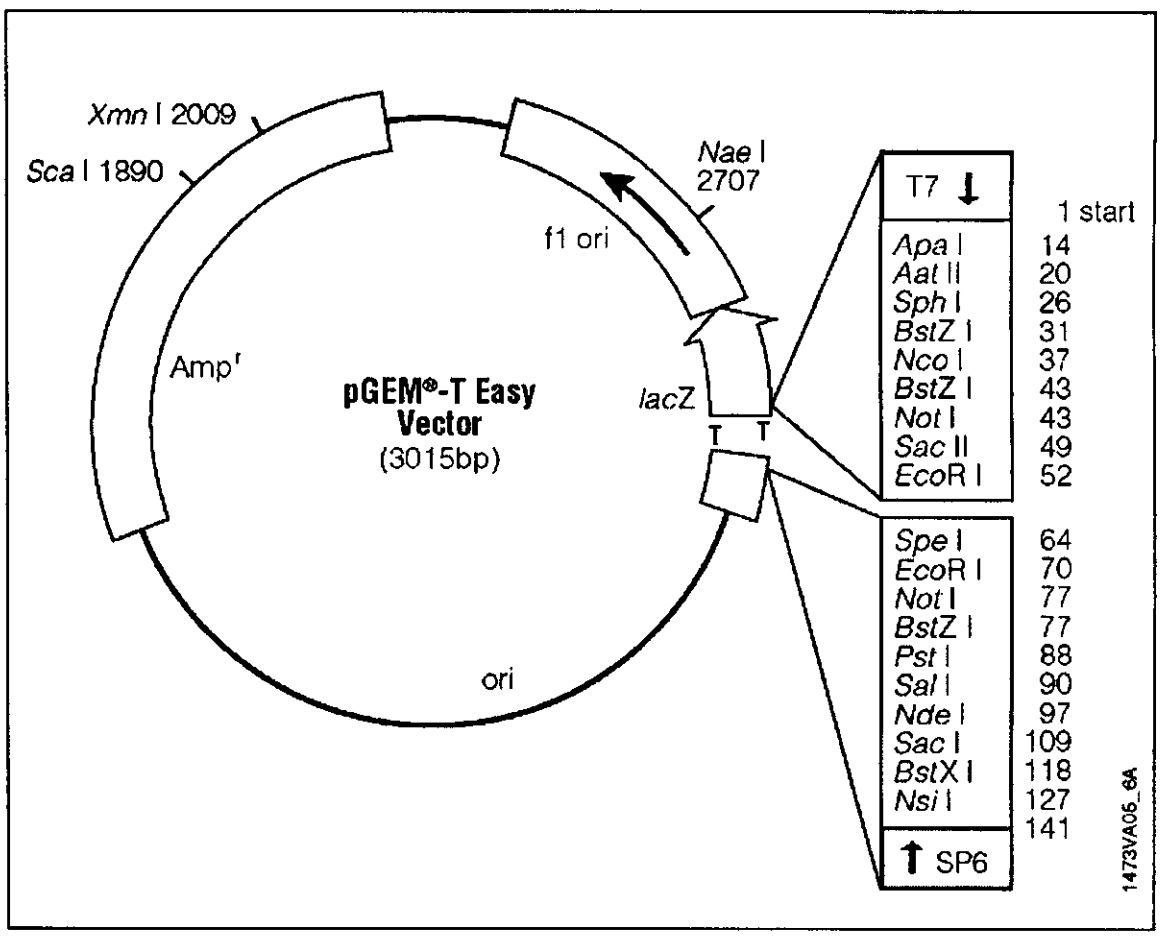

Figure 15. Map of pGEM-T Easy vetor (Promega)

2.4 Plasmid DNA transformation into *E. coli* by $CaCl_2$ method

2.4.1 Preparation of the *E. coli* competent cells (Sambrook et al. 1989).

An isolated single colony of *E. coli* XL1-Blue was inoculated into 1 ml of LB medium containing 12.5 μg/ml tetracycline, grown at 37°C overnight with shaking. The overnight culture was inoculated into 100 ml LB medium (1:100 dilutions) and incubated at 37°C with shaking to an OD_{600} of 0.3-0.5. The culture was placed on ice for 10 min; the cells were centrifuged at 4°C, 4,500 rpm for 10 min. The cells were gently resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and placed on ice for 10 min and then centrifuged at 4,500 rpm for 10 min at 4°C. The pellet was again gently resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$ containing 15% (v/v) glycerol, and the cell suspension was stored in 200 μl aliquots at -80°C.

2.4.2 Transformation of plasmid into *E. coli* competent cells

Frozen competent cells (200 μl) were thawed on ice and gently mixed with 0.1-1.0 μg plasmid DNA (from method 2.3) and placed on ice for 30 min to improve the transformation efficiency. The cells were heat-shocked at 42°C for 90 sec and immediately transferred on ice for 5 min. The transformed cells were gently mixed with warm LB medium (800 μl) and incubated at 37°C for 2 h with moderate shaking. The culture (100-200 μl) was spread on selective LB medium-100 μg ampicillin plate and incubated at 37°C overnight. The selective LB medium plate for pGEM-T Easy vector containing 100 μg/ml ampicillin plate was spread with 100 μl of 100 mM IPTG in water and 20 μl of 40 mg/ml X-gal in dimethylformamide (DMP), incubated at 37°C for 30 min before use.

2.4.3 Analysis of the positive clones from transformation

The positive transformants were directly analyzed by PCR. Five to ten colonies were picked up and resuspended individually in 25 µl steriled water. The cell suspension (15 µl) was heated at 95°C for 10 min to lyze the cell and inactivates nuclease; 10 µl was preserved for future use. The PCR cocktail consisted of 2.5 µl of 10x PCR buffer, 2 µl of 50 mM dNTPs, 3 µl of 25 mM $MgCl_2$, 100-200 ng of each primer 15 µl of heated cell suspension, water to adjust the total volume of the PCR mixture to25 µl, and 0.5 µl of Taq DNA polymerase (2.5 units). The target DNA was amplified as follows: initial denaturation at 95°C for 1 min, 25-30 cycle of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were then visualized by agarose gel electrophoresis. Ten µl of the positive clones were grown in LB broth-ampicillin overnight and the plasmid was isolated and the nucleotide sequence of the insert DNA was determined, as previously described in methods 1.3.1 and 1.9, respectively.

2.5 Amplification of the full-length of *hmgs2* cDNA

This step was performed to obtain the full-length of *H. brasiliensis hmgs2* cDNA. The first strand cDNA was amplified with forward and reverse primers; ForHS2 and RevHS2 (Table 4). The PCR amplification was performed under the same condition as in method 2.2 except that the annealing temperature was lowered to 55°C. The PCR product was ligated with pGEM-T Easy vector (method 2.3) and transformed into XL1-Blue competent cells (method 2.4.2). The positive clones were analyzed by direct PCR (method 2.4.3) and finally the positive clone containing a

recombinant plasmid, designated pGEM-*hmgs2*, was authenticated by DNA sequencing as described in the method 1.9.

3. Expression of *H. brasiliensis hmgs2* mRNA in various tissues

3.1 Isolation of total RNA from various tissues

Total RNA from rubber latex was isolated as described above. RNA from petiole and leaf was extracted by a modified method, as described by Suwanmanee et al. (2004). Five grams of petiole and leaf was ground to powder in liquid N_2 with 0.25% (w/w) polyvinyl polypyrolidone and transferred into the tube containing 10 ml of extraction buffer and 10 ml of the mixture of phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) and vigorously shaken at room temperature for 5 min. The homogenate was centrifuged at 10,000 rpm at 4°C for 30 min. The supernatant was deproteinized with an equal volume of phenol: chloroform: isoamyl alcohol, and followed by extraction twice with chloroform: isoamyl alcohol (24: 1 v/v). The nucleic acid in the supernatant was precipitated with 3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol at -20°C for 1h and centrifuged 14,000 rpm at 4°C for 30 min. The pellet was washed with 70% ethanol twice. The pellet of nucleic acid was resuspended in DEPC treated water. LiCl (8 M) was added to a final concentration of 1.8 M at 4°C overnight. The RNA was precipitated by centrifuging at 14,000 rpm at 4°C for 30 min and washed one time with 1.8 M LiCl, then washed twice with 70% ethanol. The total RNA was air dried for 2 min. and dissolved in DEPC-treated water. The concentration and purity of RNA solution were determined. The pattern of intact RNA was visualized by agarose gel electrophoresis under UV light (method 1.3.3).

3.2 Semiquantitative RT-PCR of *hmgs2* mRNA

3.2.1 First strand cDNA synthesis

Reverse transcription reactions were performed by mixing 5 µg total RNA, 250 ng of random primers, 1 µl of 10 mM dNTP mix, and DEPC treated water in a total volume of 13 µl. The mixture was incubated at 65°C for 5 min and chilled on ice for 5 min to remove the secondary structure of RNA. Then, it was mixed with 4 µl of 5x first-strand buffer [250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$], 1 µl of 0.1 M DTT, 40 units of RNaseOUT, and 200 units of Superscript III RT (Invitrogen), and left at room temperature for 10 min, then incubated at 50°C for 1 h. The reaction was terminated by incubation at 70°C for 15 min. RNA complementary to the cDNA was removed by adding 2 units of RNase H in to the content and incubating at 37°C for 20 min. The first strand cDNA products of latex, petiole, and leaf were kept at -20°C.

3.2.2 Amplification of the target cDNA

The PCR reaction was performed using 2.5 µl of the first strand cDNA mixture as template, 2.5 µl of the 10x PCR buffer, 3µl of 25 mM $MgCl_2$, 2µl of 10 mM dNTP mix, 3 µl of each ForHS2 and GSP2 primers (Table 5), 0.3 µl of *Taq* DNA polymerase (Promega), and sterile water for total 25 µl reaction mixture. The amplification was done using the following PCR program: initial denaturation at 95°C for 1 min after that 94°C for 30 sec, 64°C for 30 sec, and 72°C for 1 min, for 40 cycles and final extension at 72°C for 10 min. The PCR products were resolved by agarose

gel electrophoresis on ethidium bromide-stained gels (method 1.3.3). The intensity of the band was scanned and quantified by Document gel.

The quantifications for *hmgs2* mRNA levels were normalized with respect to a 530-bp of 18S ribosomal RNA. The amplification reaction and PCR program for the 18S ribosomal RNA was performed under the same conditions as for *hmgs2* except that the 18SFor and 18SRev (Table 5) were used as forward and reverse primers, respectively.

4. Sequences alignment and computer analysis

The *hmgs2* cDNA sequence was translated to amino acid sequences and used as query for searching all of known HMG-CoA synthase sequences from other species including the HMG-CoA synthase's relative enzymes by the BLAST network server from NCBI (National Center for Biotechnological Information; http://www.ncbi.nim.nig.gov/) database. All of HMG-CoA synthase sequences were aligned by ClustalX multiple sequence alignment program version 1.81 and represented by the GeneDoc program, also then HMG-CoA synthase sequences were aligned by progressive alignment program. Consequently, some of the selected HMG-CoA synthase and its relative, ACP synthase III, amino acid sequences were aligned by the progressive alignment program (Feng and Doolittle, 1990), which was run under UNIX. Alignments were obtained by a binary alignment algorithm (Needleman & Wunsch, 1970); the similarity scale of each amino acid is based on the mutation matrix (PAM 250), Dayhoff et al. (1978).

The computer programs for creating the phylogenetic trees were also run under UNIX. Phylogenetic trees were constructed by the MATRIX method. The MATRIX

method was used to determine branching order and branch lengths from distances calculated in the multiple alignment. The tertiary structure information of ACP synthase III; Protein Data Bank (PDB) file: 1HZP verified by search with BLAST was used as a template to predict the secondary structure of HMG-CoA synthase.

5. Expression of recombinant wild-type and mutant HMG-CoA synthase1 in *E.coli*

5.1 Construction of pBluescript SK (-) containing coding region of *hmgs1*

The pBluescript SK-1.4*hmgs1* containing a 1,413 bp cDNA, an open reading frame (ORF) of *hmgs1* was constructed by PCR using the pBluescript SK-*hmgs1* containing the 1,804 bp full-length of *hmgs1* cDNA (Suwanmanee et al. 2002) as template. The amplification was performed by *Taq* DNA polymerase (Promega) with a primer pair designed to obtain the full-length coding sequence of *hmgs1* with an extended 5'end to incorporate a *Bam*HI restriction site (5'flanking primer) and a complementary region harboring a *Sal*I site at 3'end (3'flanking primer), as shown in Table 5. The PCR reaction was performed as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 10 min for the final extension step. The amplification products were analyzed by agarose gel electrophoresis (method 1.3.3) and were purified as described in the method 1.3.4. After that the 1,413 bp cDNA was ligated to pGEM-T Easy vector (method 2.3) and transformed to propagate in XL1-Blue cells as in the method 2.4.2, and then the positive clones were analyzed as in method 2.4.3.

5.2 Site-directed mutagenesis of *hmgs1* cDNA

Point mutations were engineered with single-mutant primers. Cys^{117} and Asn^{326} of wild type *hmgs1* were substituted with Ala, primers for Cys^{117}-Ala: RevC117A and Asn^{326}-Ala: ForN326A (Table 5). The amplification of the mutant cDNA fragment was achieved with the 20 pmol of 5' flanking primer and RevC117A primer (Table 4), or (20 pmol of 3' flanking primer and ForN326A primer), 1.3 units of proof-reading *Pfu* Turbo-DNA polymerase (Stratagene), 50 ng of pGEM-1.4*hmgs1* as template and 200 μM dNTPs in a total volume of 50 μl. Conditions for PCR reaction were as follows: denaturation at 95°C for 1 min followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The full-length of open reading frame mutant *hmgs1*, was generated by using 20 pmol of the gel-purified mutant cDNA fragments as 5'-'megaprimers' or 3'-'megaprimers' in a second PCR reaction with *Taq* DNA polymerase and the other PCR cocktail components. The PCR program was carried out using the same parameters as in the first PCR, except that the annealing temperature was raised to 58°C. The resulting mutant 1,413 bp cDNA was ligated into a pGEM-T Easy vector as in method 2.3 and used to transform competent XL1-Blue cells (method 2.4.2 and then 2.4.3). The plasmids containing the point mutant *hmgs1* cDNA were isolated from selected transformants as described in the method 1.3.1 and the point mutant cDNA was confirmed by DNA sequencing.

5.3 Ligation of wild-type and mutant *hmgs1* with expression vector

The pQE31 expression vector (Figure 16), (supplied by QIAexpressionist kit, Qiagen) was digested with the *Bam*HI and *Sal*I restriction endonuclease enzymes which cleave the specific sequences G$^{\downarrow}$GATCC and G$^{\downarrow}$TCGAC, respectively. The digestion reaction was done in a 50 µl mixture containing 5 µl of 10x *Bam*HI buffer, 5 µl of 10x BSA, 28 µl water, 10 µl of 2 µg pQE-31 vector, 1 µl of *Bam*HI, and 1 µl of *Sal*I as described in method 1.3.2. The digested vector was purified by the QIAquick PCR purification kit (Qiagen) to remove restriction enzymes and the excised fragment. The 1,413 bp of wild type and mutant *hmgs1* cDNA fragments in the pGEM-1.4*hmgs1* were also isolated by the endonuclease digestion reaction with *BamH*I and *Sal*I to facilitate ligation in-frame in the *Bam*HI and *Sal*I cloning site of the pQE-31 vector, then the cDNA fragments were gel-purified. The ligation was performed using 1 µg of digested plasmid, 3 µg of cDNA fragment, 1 unit of T4 DNA ligase, 1x ligation buffer [50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000], and sterile water in 20 µl and incubated at 4°C for 18-24 h.

5.4 Analysis of the recombinant expression plasmid

The ligated plasmids were transformed into *E. coli* XL1-Blue competent cells (method 2.4.1 and 2.4.2, but without X-gal/IPTG on the selective medium since pQE31 does not allow blue/white screening). The transformants were checked by direct PCR (method 2.4.3). The positive clones were then cultured overnight in LB-100 mg ampicillin medium and the recombinant plasmids were isolated (method 1.3.1) then analyzed by agarose gel electrophoresis (method 1.3.3).

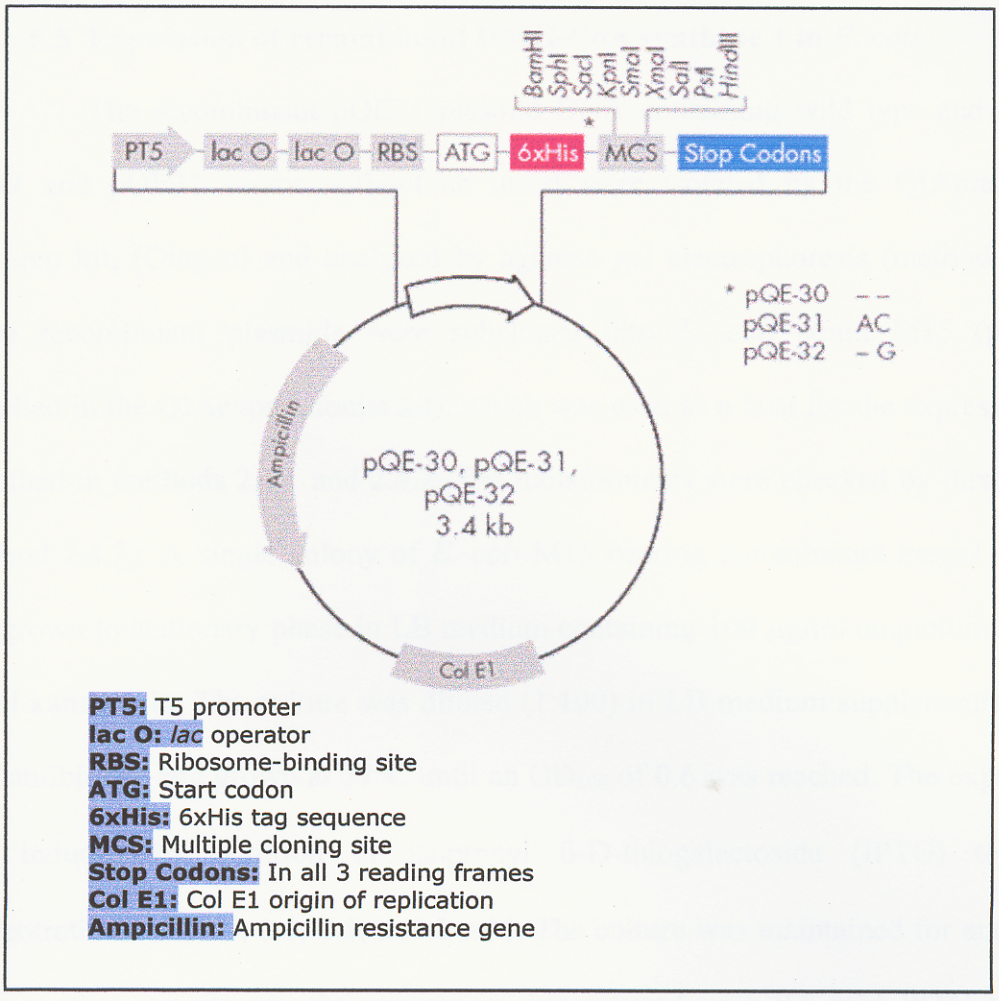

Figure 16. Map of pQE-31 expression vector (Qiagen).

5.5 Expression of recombinant HMG-CoA synthase 1 in *E. coli*

The recombinant pQE31 plasmid DNA containing wild type and mutant *hmgs1* and pQE31 vector without an insert were isolated by the QIAprep Spin Miniprep kit, (Qiagen) and analyzed by agarose gel electrophoresis (method 1.3.3). These recombinant plasmids were subcloned into *E. coli* strain M15 (pREP4) (supplied in the QIAexpressionist kit), which was used as a host for the expression, as described in methods 2.4.1 and 2.4.2. The transformants were checked by direct PCR (method 2.4.3). A single colony of *E. coli* M15 bearing recombinant *hmgs1* pQE31 was grown to stationary phase in LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. The culture was diluted (1:100) in LB medium supplemented with both antibiotics and grown at 37°C until an OD_{600} of 0.6 was reached. The expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to final concentrations of 0.05, 0.1, 0.5, and 1 mM. The culture was maintained for another 5 h. In another experiment, the expression was induced with 0.5 mM IPTG and the bacteria were grown at 37°C for 1, 2, 3, 4, and 5 h. Bacterial cells were harvested by centrifugation (10,000 rpm) for 2 min at 4°C and stored at -80°C.

5.5.1 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The rapid screening of small expression cultures was performed by SDS-PAGE. Bacterial cells harvested from 1 ml of induced culture were resuspended in 250 μl of 5x SDS-PAGE sample buffer [15% (v/v) β-ME, 15% (w/v) SDS, 1.5% (w/v) bromphenol blue, and 50% (v/v) glycerol]. The sample was mixed well by vortexing and boiled for 10 min then briefly centrifuged and the supernatant was saved. Polyacrylamide gels were prepared as shown in Table 7. Twenty microliters of

the supernatant was loaded on the gel and electrophoresed in SDS-running buffer [1M Tris-HCl, pH 8.8, 50 mM EDTA, 0.1 % (w/v) SDS] with a constant current of 24 mA for 90 min. The pattern of proteins was visualized by staining in the Coomassie blue staining solution.

5.5.2 Coomassie blue staining

The gel was stained for at least 5 h at room temperature in Coomassie blue staining solution [1% (w/v) coomassie blue R-250 dissolved in 50% (v/v) methanol, and 7% (v/v) acetic acid, and filtered before use]. Proteins appeared as a dark blue bands, background stain in the gel was removed with destaining solution I [50% (v/v) methanol, 7% (v/v) acetic acid, and water] for 1-2 h with gentle shaking. After that, the gel was again destained and soaked in the destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid, and water] at room temperature.

5.6 Preparation of cleared *E. coli* lysate under native condition

The cell pellet from 10 ml of induced culture was thawed on ice and resuspended in 200 µl lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole) containing 1% (v/v) Tritron X-100 and 0.2 mM PMSF. The cell suspension was mixed with lysozyme and DNase to final concentrations of 1 mg/ml and 10 µg/ml, respectively. The cell mixture was vigorously vortexed 1-2 min during incubation for 5 h on ice. Supernatant was recovered from crude extract by centrifugation at 12,000 rpm at 4°C for 20 min. This protein lysate was then used to determine the protein content and the HMG-CoA synthase activity, and for purification.

Table 7. Composition of SDS-polyacrylamide gel

Solution	Stacking gel (3%)	Running gel (12%)
30% Acrylamide[a] (ml)	0.3	2.4
1.5 M Tris-HCl, pH 8.8 (ml)	-	1.5
0.5 M Tris-HCl, pH 8.8 (ml)	0.75	-
10% Sodium dodecyl sulfate, SDS (µl)	30	60
10% Ammonium persulfate (µl)	30	60
H_2O (ml)	1.86	1.97
TEMED[b] (µl)	3	6
Total volume (ml)	3	6

30% Acrylamide[a] = Acrylamide: N,N'-methylenebisacrylamide, 29: 1

TEMED[b] = N,N,N',N',-tetramethylethylenediamine

5.7 Determination of protein concentration

The concentration of protein in the *E. coli* lysate was determined by the Lowry assay (Lowry et al. 1951) using bovine serum albumin (BSA) as the standard at the concentrations of 12.5, 25, 50, and 100 µg/ml. Protein standards and sample (50 µl) were mixed well with prepared 1.5 ml fresh alkaline copper solution [1% (w/v) $CuSO_4 \cdot 5H_2O$, 2% (w/v) $C_4H_4KNaO_6 \cdot H_2O$ and 0.2 M NaOH, 2% (w/v) Na_2CO_3 in

ratio 1: 50] and left for 10 min at room temperature. Folin-Ciocalteau reagent (150 µl) was then added into the alkaline copper-protein solution and mixed well by vortexing, then left at room temperature for 30 min. The absorbance of standards and samples was measured at 550 nm by spectrophotometer. The protein content of *E. coli* lysate was calculated from the protein standard curve.

5.8 Purification of recombinant HMGS1 in *E. coli*

The cleared *E. coli* lysated prepared under native condition was purified by Ni-NTA agarose (supplied by QIAexpressionist kit, Qiagen). The supernatant was mixed gently with 50% slurry Ni-NTA agarose and shaken at 4°C for 2h. The lysate-Ni-NTA mixture was loaded into a column and the solution flow-through was removed from the column. The column was washed twice with 4 ml of washing buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole) containing 1% (v/v) Tritron X-100 and 0.2 mM PMSF. The histidine tagged fusion protein was eluted with 3 x 500 µl elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole containing 0.2 mM PMSF). The eluted protein was analyzed by SDS-PAGE.

5.9 HMG-CoA synthase assays

HMGS activity was determined by a modified method of Miziorko as described by Suvachittanont and Wittisuwannakul, (1995). The reaction mixture included 100 mM Tris-HCl, pH 8.2, 200 µM acetly-CoA, 20 µM acetoacetyl-CoA, 100 µM EDTA, and 0.2 mg of enzyme sample in a total volume of 100 µl. The reaction was initiated by adding [1-^{14}C] acetyl-CoA, which was diluted with the

unlabelled ester (to 67 dps/nmol) to the pre-warmed mixture at 30°C for 2 min. After the addition, 40 µl aliquots were transferred into glass vials containing 100 µl of 6 M HCl and the mixture was heated to dryness at 95°C for 2 h. Water (500 µl) was added and the acid-stable radioactivity due to [1-^{14}C] HMG-CoA was measured by liquid scintillation counting (6 g PPO, 0.4 g POPOP, 1.5 L xylene, and 0.5 L Titron X-114 in a total volume of 2 L). To assess the amount of [1-^{14}C] acetyl-CoA not removed by the above procedure, a blank incubation was carried out using an assayed sample which had been boiled for 5 min. The enzyme specific activity was calculated as nmole/min/mg protein (see appendix).