Research Article

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Cloning and expression of *hmgr*1 gene from *Hevea brasiliensis*

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Abstract

Biosynthesis of natural rubber (*cis*-1,4-polyisoprene) takes place through mevalonate pathway in *Hevea*. The enzyme 3-hydroxy-3-methyl glutaryl-CoA reductase (HMGR), which catalyses the synthesis of mevalonate from HMG-CoA is a key regulatory enzyme in this pathway. This study aimed to clone and express *hmgr*1 gene, in order to obtain the HMGR protein *in vitro* and to further use this protein as a marker for yield potential in *Hevea*. For this purpose, mRNA was isolated from the latex of *Hevea* (clone RRII 105). cDNA was synthesized and PCR amplification of coding region of *hmgr*1 was performed using *hmgr*1 specific primers. The PCR amplified product (~1.8 kb) was cloned into an expression vector (pRSET-A) and transformed into *E. coli* (BL21DE3) cells. Protein expression in transformed cells when monitored by SDS-PAGE analysis indicated the presence of HMGR protein (61.6 kDa). The protein would be used for developing specific antibody that could be further utilized for the quantification of HMGR in different *Hevea* clones for screening the yield potential. The details of cloning and expression of *hmgr*1 are presented and discussed.

Keywords: *Hevea brasiliensis*, HMG-CoA reductase, *hmgr*1 gene, rubber biosynthesis

Introduction

Natural rubber (*cis*-1,4-polyisoprene) is synthesized by more than 2,000 plant species. Among these, *Hevea brasiliensis* is the commercial source of natural rubber due to the production of large quantity of good quality rubber as a long chain polymer. Biosynthesis of latex is confined to the latex vessels which exclusively occur in the phloem region. Rubber yield is controlled by several factors related to density of latex vessels, *in situ* regeneration of latex in the laticifers tissues between successive harvesting period, the physiology of latex flow *etc*.

Harvesting of latex results in the depletion of cell constitutes from the latex vessels. Regeneration of latex requires an intense metabolic activity involving reconstitution of all the sub-cellular elements with their enzymatic functions.

Biosynthesis of natural rubber takes place by mevalonate pathway in *Hevea* (Sando *et al*., 2008). Generation of acetyl-coenzyme A and its conversion to isopentenyl pyrophosphate (IPP) *via* mevalonic acid and polymerization of IPP to rubber are the major steps involved in the rubber biosynthetic process. The enzyme 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR), catalyses the synthesis of mevalonate from HMG-CoA, which is the first step in isoprenoid pathway. Wititsuwannakul (1986) and Wititsuwannakul *et al.* (1990) reported a positive correlation between rubber biosynthesis and HMG-CoA reductase activity in *Hevea*. Different members of *hmgr* gene family were cloned and characterized from the *Hevea* clone RRIM 600 (Chye *et al*., 1991, 1992). Ji *et al*. (1993) demonstrated that HMGR activity was positively correlated with rubber biosynthesis as well as rubber yield.

It was observed that *hmgr*1 expressed more in lacticifers than in leaves and is specifically involved in rubber biosynthesis (Chye *et al*., 1991). The increased production of rubber in modern *Hevea*

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clones may be due to enhanced rubber biosynthetic capacity which needs to be investigated. The objective of the present study was to clone *hmgr*1 and to synthesize the HMGR protein *in vitro*. The protein would be used for developing specific antibody that could be further utilized as a biochemical marker for screening yield potential in *Hevea*.

Materials and methods

Latex samples were collected from the *Hevea* clone RRII 105. The trees were grown in the experimental field of Rubber Research Institute of India and they were harvested regularly under S/2 d2 tapping system.

mRNA isolation and cDNA preparation

mRNA was isolated from latex using magnetic beads as per manufacture's instructions (Dyna Beads, Invitrogen, USA). The quantity and quality of mRNA were determined spectrophotometrically (Nanodrop, USA) and by agarose gel electrophoresis respectively. cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen) using 250 ng mRNA as template.

PCR amplification of *hmgr*1 gene

PCR amplification of *hmgr*1 gene from *H. brasiliensis* was performed with gene specific primers designed based on sequences deposited earlier in GenBank database (X54659; Chye *et al*., 1991) (Forward primer: 5'- CTC GAG ATG GAC ACC ACC GGC CGG C 3'; Reverse primer: 5'- GGT ACC ATT GCT GGG ACC AGA TTC CC -3'). cDNA was used as template and PCR amplification was carried out in a thermal cycler (Master gradient, Eppendorff, USA). The amplification profile consisted of a first cycle at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s; 69.2 °C for 30 s; 72 °C for 1 min and finally extension at 72 °C for 10 min. The amplified cDNA fragments were separated by electrophoresis on 1 per cent agarose gel and the amplified cDNA fragment (~1.8 kb fragment) was eluted using gel extraction kit (Sigma).

Cloning and DNA sequencing analysis

The PCR amplified *hmgr*1 fragments were cloned into pGEM-T Easy vector. The ligated plasmids were transferred into *E. coli* cells (Gen Hunter) and plated onto LB agar plates containing 50 µg mL^{-1} ampicilin and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 µg mL^{-1} 5-bromo-4-indolyl-β-D-galacto-pyranoside (X-Gal). The presence of insert in the recombinant clone was confirmed by colony PCR and further confirmed by restriction digestion analysis using *Kpn*I and *Xho*I. The nucleotide sequence of the cloned DNA fragment was determined by gene sequencing (Macrogen, Korea). The nucleotide sequence of *hmgr*1 was compared with the NCBI database using BLASTN programme.

Cloning in expression vector

The plasmid (pGEMT/*hmgr*1) was double digested using *Kpn*I and *Xho*I and purified the *hmgr*1 fragment. The expression vector (pRSET-A, Invitrogen) was also double digested using *Kpn*I and *Xho*I and purified. The *hmgr*1 was ligated to pRSET-A using T4 DNA ligase and was used to transform *E. coli* cells (Gen Hunter). The transformed colonies were selected by colony PCR and restriction digestion analyses. The orientation of the insert was confirmed by DNA sequencing (Macrogen, Korea). The sequencing results were analysed and the clone with *hmgr*1 in the right orientation was selected. Plasmid DNA isolated from these colonies were used to transform expression specific BL21 (DE3) pLysS cells and the transformed colonies were selected by colony PCR.

Expression of protein

The clone pRSET-A/*hmgr*1 was cultured in SOB containing ampicillin (50 µg mL^{-1}) and chloramphenicol (35 µg mL^{-1}) at 37 ºC and the overnight culture was inoculated in to SOB medium. The culture was grown at 37 ºC with vigorous shaking to an OD_{600} between 0.4 and 0.6 and culture sample was collected as zero hour sample. IPTG (1 mM) was added to the culture and samples were collected at different time intervals (0, 1.5 and 3 hours after induction). Protein was isolated from these cells by mechanical lysis and quantified (Bradford, 1976). The protein was subjected to SDS polyacrylamide gel electrophoresis (PAGE) analysis and visualized using Coomassie blue stain.

Results and discussion

HMGR is a key enzyme in plants since HMG-CoA acts as a precursor to many vital isoprenoid

compounds including natural rubber. In *Hevea*, HMGR is encoded by a small gene family comprised of five members. *hmgr*1 is expressed predominantly in the lacticifers, the site of rubber biosynthesis, whereas *hmgr*3 expression has been found to be not cell specific (Chye *et al.*, 1992, Venkatachalam *et al*., 2009). The genes *hmgr*4 and *hmgr*5 are expressed in mature leaves and xylem tissues respectively. The *hmgr*1 gene has been reported to be differentially expressed in various tissues. *hmgr*1 mRNA transcript level was found more abundant in latex than in leaf, immature leaf and rubber seedlings (Sando *et al*., 2008). An increased expression of *hmgr*1 mRNA was positively correlated with HMGR activity (Schaller *et al*., 1995). It is assumed that HMGR, the enzyme that is involved in the synthesis of mevalonic acid plays a crucial role in the over all control of the isoprenoid biosynthesis.

In this study, *hmgr*1 coding region was isolated and over-expressed in an expression vector for protein production. Antiserum raised against this protein could be employed for quantification of HMGR in different *Hevea* clones to identify whether the high levels of HMGR is associated with rubber yield. For this purpose, mRNA was isolated from latex of clone RRII 105 (Fig. 1) and cDNA was synthesized. A PCR based approach was employed to isolate *hmgr*1 gene from *Hevea*. Primers were designed and synthesized based on the published sequences of *hmgr*1 from RRIM 600 clone by Chye *et al*. (1991). PCR amplicon with a size of 1.8 kb corresponding to the length of *hmgr*1

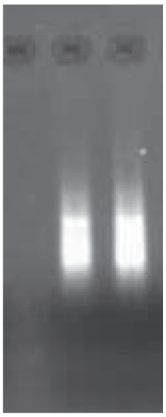

Fig. 1. mRNA isolated from latex of clone RRII 105 on 2 per cent agarose gel

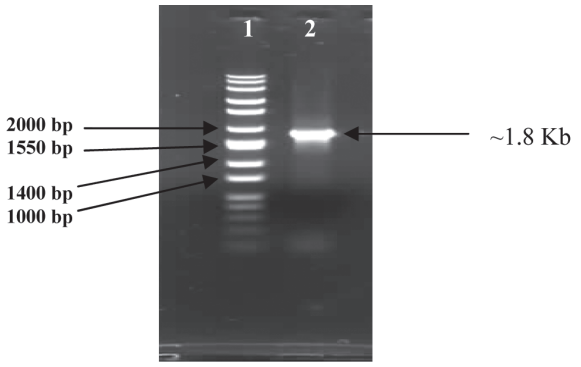

Fig. 2. PCR Amplification of *hmgr*1 from *Hevea*. Lane 1- Wide range DNA Marker; Lane 2- amplicon of *hmgr*1

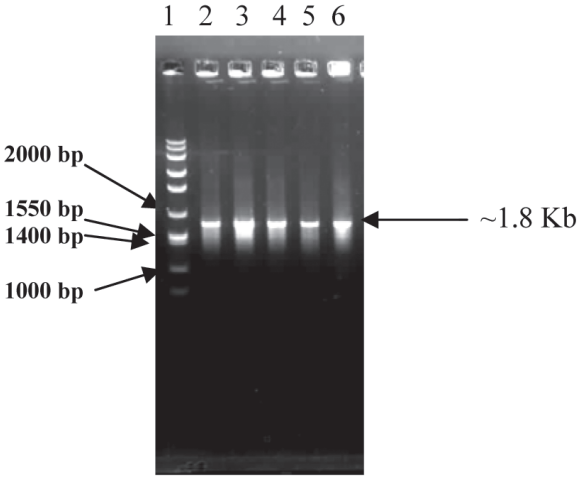

Fig. 3. Colony PCR of pGEMT/ *hmgr*1. Lane 1- Wide range DNA Marker; Lanes 2-6- clones with pGEMT/*hmgr*1

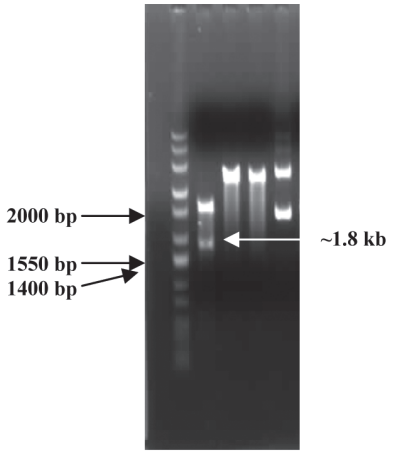

Fig. 4. Restriction digestion of pGEMT /*hmgr* 1. Lane 1- Wide range DNA Marker; Lane 2- Double digestion *Xho*I & *Kpn*I; Lane 3- Single digestion *Xho*I; Lane 4- Single digestion *Kpn*I; Lane 5- Uncut plasmid DNA

(Fig. 2) obtained was later gel purified and cloned in to pGEM-T vector. The cells after transformation with vector were selected on the basis of blue white screening. The recombinant clones when subjected to colony PCR (Fig. 3) as well as restriction digestion (Fig. 4), confirmed the presence of *hmgr*1 coding region. After confirmation, clones containing *hmgr*1 were selected and its nucleotide sequencing was carried out. Clone exhibiting 100 per cent homology to previously submitted sequence (Acc. No. X54659) in the database was selected for further steps. The ORF of *hmgr*1 encoded a 575 amino acid polypeptide with a predicted molecular mass of 61.6 kDa.

The selected clone of pGEM-T vector containing *hmgr*1 was double digested to release the *hmgr*1 insert and the gel purified insert DNA was cloned into double digested pRSET-A expression vector at the *Xho*I and *Kpn*I restriction sites. The ligated products were transformed successfully into *E. coli* cells and the transformants were confirmed based on colony PCR and restriction digestion analyses (Fig. 5 and 6). Orientation of the insert in the expression vector was confirmed by sequence data. The above results indicate the successful cloning of *hmgr*1 gene in pRSET-A expression vector.

The pRSET-A/*hmgr*1 construct was further transformed in to BL21 (DE3) pLysS cells for the expression of HMGR protein. The bacterial culture was induced with 1 mM IPTG at 37 ºC for 4 hours. Protein expression in transformed cells when

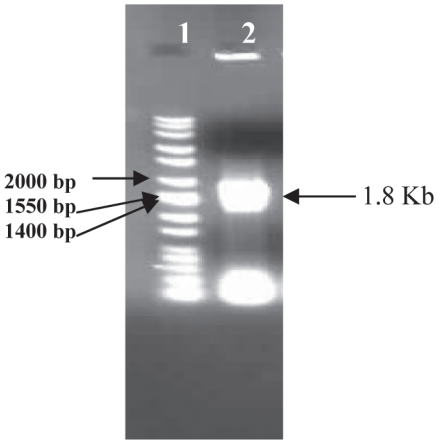

Fig. 5. Colony PCR of pRSET- A/ *hmgr*1. Lane 1- Marker; Lane 2- clone with pRSET-A/*hmgr*1 gene construct

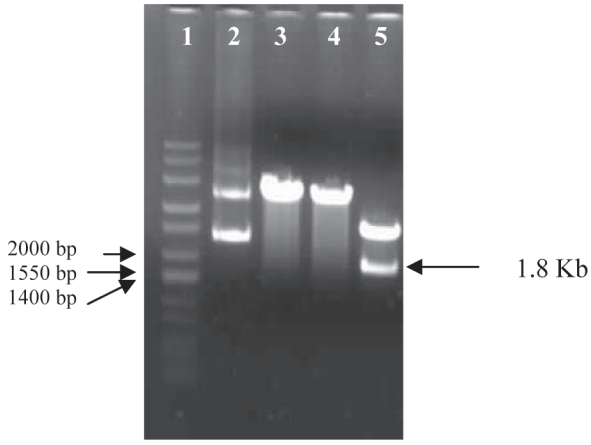

Fig. 6. Restriction digestion of pRSET- A/*hmgr1*. Lane 1- Wide Range DNA Marker; Lane 2- Uncut plasmid DNA; Lane 3- Single digestion *Kpn*I; Lane 4- Single digestion *Xho*I; Lane 5- Double digestion *Kpn*1/*xho* I

monitored by SDS-PAGE analysis indicated the presence of HMGR protein (Fig. 7). An increase in the intensity of protein band with a size of 64.6 kDa (including His Tag) corresponding to HMGR was

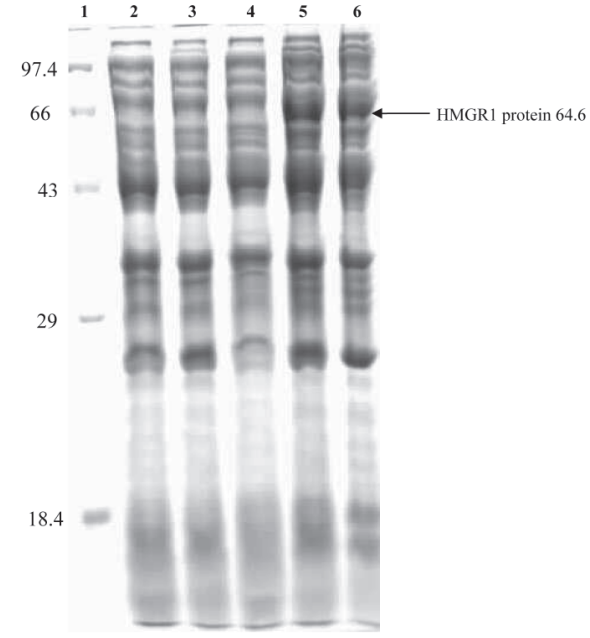

Fig. 7. SDS-PAGE analysis of proteins from transformed cells with pRSETA/*hmgr*1. Lane 1- Marker; Lane 2- Vector only (0 hr); Lane 3-Vector only after induction; Lane 4- Vector+hmgr-1 (0 hr after induction); Lane 5-Vector+hmgr-1 (1.5 hr after induction); Lane 6-Vector+hmgr1 (3 hr after induction)

obtained. The pRSET-A in BL21 (DE3) pLysS was used as negative control. The protein profile of transformed cells (pRSET-A/*hmgr*1) showed a clear band of 64.6 kDa (including His Tag protein) indicating the expression of HMGR1 protein. The maximum quantity of HMGR was observed in culture collected 3.5 hours after induction with IPTG.

One of the most challenging aspects of plant isoprenoid biosynthesis is the identification of enzymes that catalyzes the rate limiting steps in the pathway. Positive correlation between HMGR with rubber biosynthesis and rubber yield was demonstrated by several workers. In this study, the cloning of *hmgr*1 gene in expression vector and *in vitro* synthesis of HMGR protein was successfully carried out. The protein would be used for developing specific antibody and that could be further utilized for the quantification of HMG-CoA reductase in different clones of *Hevea* for developing this enzyme as a marker for yield potential in *Hevea.*

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