Research Article

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Expression analysis of rubber biosynthetic pathway genes in *Hevea brasiliensis*

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Abstract

*Hevea brasiliensis*is, the primary commercial source of natural rubber (*cis*-1, 4-polyisoprene), is a fundamental raw material used for manufacturing a wide range of industrial and domestic rubber products in automobile, medical and defense industries. In *Hevea*, biosynthesis of rubber takes place through mevalonate pathway. Clonal variations in the productivity of rubber may be the result of variations in the activities of the enzymes involved in rubber biosynthesis in different *Hevea* clones. In this study, expression of 14 genes corresponding to enzymes/regulatory proteins involved in rubber biosynthesis was analyzed in high and low latex yielding clones of *Hevea brasiliensis*. The level of expression of *HbSUT3*, a sucrose transporter and enzymes related to the synthesis of rubber such as hydroxymethyl glutaryl-CoA synthase (*hmgs*), HMG-CoA reductase (*hmgr*) and mevalonate diphosphate decarboxylase (*MVD*) were found to be significantly higher in high rubber yielding clones compared to the low rubber yielding clones. The higher expression of these genes might result in an increased supply of IPP, the isoprenoid monomer, required for rubber biosynthesis. Expression of genes in the downstream rubber biosynthetic pathway such as *FPPS*, *RuT* and *REF2* were also found to be significantly higher in high rubber yielding clones than low yielders. The results suggest that high rubber yield is associated with high expression of these genes and these genes can be used as markers for high yield potential in *Hevea*.

Keywords: Gene expression, *Hevea brasiliensis*, HMG-CoA synthase, HMG-CoA reductase, rubber biosynthesis, sucrose transporter

Introduction

Hevea brasiliensis is the primary source of natural rubber, a key raw material widely used in several medical and industrial/engineering products development. Hence, the demand of the raw material is increasing worldwide. As a result of recent crop improvement research and development programmes emphasizing the development of rubber clones with high yield potential, several high yielding *Hevea* clones have been developed and widely cultivated in India.

Rubber yield in *Hevea* is a complex trait governed by a large number of major and minor components (Jayasekara *et al*., 1997). Latex harvesting process results in the depletion of cell components from the latex vessels. Regeneration of latex requires an intense metabolic activity involving reconstitution of all the sub-cellular elements with their enzymatic functions (Jacob *et al*., 1989). The precursor for the synthesis of rubber in the laticiferous tissue is sucrose (Sando *et al*., 2008), available from photosynthesis, which is actively transported in to laticiferous cells through the plasma membrane. In *Hevea*, the symplasmic character of the laticifer from its neighbouring cells suggests that sucrose transporters (SUTs) may possibly take part in the trans-membrane uptake of sucrose (Tang *et al*., 2010). Glycolysis is the major catabolic process of sugar in latex which produces acetyl-CoA molecules, as well as ATP and NADPH, which are required for polyisoprene synthesis. Condensation of three molecules of acetyl-CoA produces mevalonic acid which is converted into isopentenyl pyrophosphate (IPP). Polymerization of thousands of IPP molecules leads to the formation of high molecular weight rubber.

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The biosynthesis of rubber from sucrose involves more than 20 enzymatic reactions (Sando *et al*., 2008). Several regulatory proteins other than enzymes are reported to be engaged in the biosynthetic process (Kang *et al*., 2000). The expression of genes corresponding to enzymes involved in rubber biosynthesis were reported in a few *H. brasiliensis* clones (Priya *et al*., 2007; Venketachalam *et al*., 2009; Towaranonte *et al*., 2010; Chow *et al*., 2012).

Hybridization coupled with vegetative propagation and clonal selection of *H. brasiliensis* resulted in a yield increase from 250 kg ha^{-1} $annum^{-1}$ to more than 3000 kg ha^{-1} $annum^{-1}$ (Mydin, 2014). Although earlier workers have identified and characterized few genes drawn in rubber biosynthesis in *H. brasiliensis* (Tang *et al*., 2010; Towaranonte *et al*., 2010; Ruderman *et al*., 2012; Sumanmanee *et al*., 2013), the specific genes associated with high rubber yield potential have not been thoroughly explored. Improving the rubber yield through an understanding of the factors involved in rubber biosynthesis is a major challenge. Hence, a study was conducted to determine the expression of genes corresponding to key enzymes involved in rubber biosynthesis in relation to latex yield trait in *Hevea* clones.

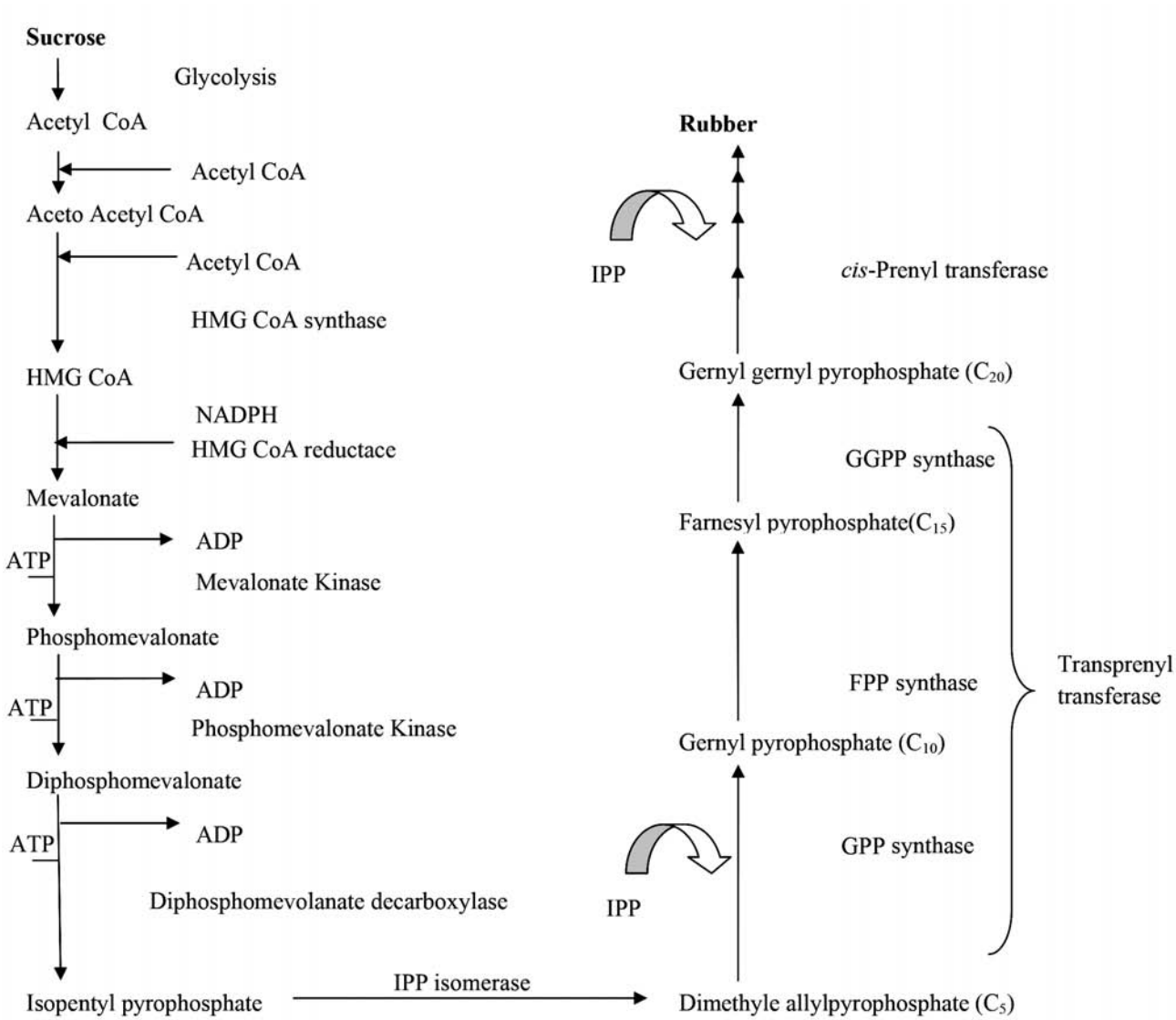

Fig. 1. Schematic representation of rubber biosynthetic pathway in *Hevea*

Materials and methods

Plant material

Healthy trees (3 nos. each) of high yielding (RRII 105, RRIM 600 and PB 217) and low yielding (Tjir 1, RRII 33 and RRII 38) *Hevea* clones planted at Central Experiment Station of Rubber Research Institute of India, Chethackal, Pathanamthitta district, Kerala were selected for the study. The trees were regularly tapped for latex yield in every three days under the S/2 d3 6d/7 system of tapping and were in the 2nd year of tapping. The rubber yield from each tree was recorded as reported earlier (Thomas *et al*., 2000).

Latex sample collection for RNA extraction

Fresh latex was collected from the selected trees after discarding first few drops of latex after tapping. Latex (10 mL) was collected in 10 mL, 2 per cent CTAB extraction buffer (hexadecyl trimethyl ammonium bromide); 2 per cent PVP (polyvinyl pyrrolidone K 30); 100 mMTris-HCl (pH 8.0); 25 mM EDTA; 2.0 M NaCl; 0.05% spermidine; 2 per cent v/v β-mercaptoethanol (added just prior to use), brought to the laboratory in ice and stored at -80 ºC until the extraction of RNA.

Total RNA isolation and cDNA synthesis

Latex RNA was isolated according to the procedure given by Chang *et al.* (1993). The latex samples were centrifuged at 7000 rpm at 4 °C for 20 min. The middle serum layer was collected and mixed with equal volume of chloroform: isoamyl alchohol (24:1). The tubes were spun at 7000 rpm for 10 min at room temperature. The top aqueous phase was transferred into fresh centrifuge tubes and the extraction was repeated using chloroform:isoamyl alcohol mixture. The top aqueous phase was again transferred to another tube and mixed with 0.3 volume of 8 M lithium chloride and kept at 4 °C overnight. The tubes were spun at 7000 rpm for 20 min at 4 °C. The pellet was washed with 2 mL of 2 M lithium chloride and spun at 7000 rpm for 20 min at 4 °C. The pellet was suspended in 500 µL SSTE (1.0 M NaCl; 0.5% SDS; 10 mM Tris-HCl (pH 8.0); 1.0 mM EDTA) and mixed with equal volume of chloroform: isoamyl alcohol (24:1). The tubes were spun at 10,000 rpm for 10 min at 4 °C. The upper phase was transferred to another tube and mixed with double volume of absolute ethanol. The tubes were kept at -20 °C overnight and spun at 10,000 rpm for 20 min to pellet the RNA. The pellet was then dried using vacuum and dissolved in autoclaved DEPC treated water. The RNA samples were subjected to DNase treatment using DeoxyribonucleaseI,AmplificationGrade(AMPD1). cDNA was synthesized using Superscript™ III, Sigma first strand synthesis kit (Invitrogen).

Quantitative real time PCR (RT-qPCR)

Suitable PCR primers were designed and synthesized for 14 rubber biosynthetic pathway genes (Table 1) by availing the services of Eurogentec, Belgium. Quantitative PCR was performed in

Table 1. List of genes and corresponding primers used for RT- qPCR analysis

Gene	Forward primer (5'-3')	Reverse primer(5'-3')
*hmgr*1	CTGTTCTTCTCGGTGGGCGTATTAC	AGAGGCAATGAGGGAGACAATAGC
*hmgr*2	GCGAGGCAATTATCAAGGAAGAGG	GCACCAGCAACAGCGGAAC
*hmgr*3	GGCGTGGGAAGTCTGTTGTATG	AGAGAACCTGCTACGGCTGAG
hmgs	ACACTGACATTGAAGGCGTTGAC	CACTACAAGTCCATAGCGTCCATC
MVD	CTGCTGCTGGATTGGCTTCTTC	GCACTGCCTGAACCTTGTCTTG
FPPS	CGGCACGAGTGATTTCAGAGTTTC	CCAGGCACATTGTAGTCCAACATC
GGPS	GCTGCCATTCCACTCCAAGAACC	TCATTCCCACCAACAAGCTCACAC
RuT	CAGTCAAGACCGCAGCAGATAAG	AGCATACAGCAATGAGAAGCACAC
*REF*1	AGTTTATGCCAGGGCTTCTTTCTC	AGTATTGACGCCAGGCTTGAATG
*REF*2	AGATGCGTCCTTGACAATTGG	GCCCGGTTGTTGCATCTG
*REF*3	GTTTGTAGACAGCACGGTTGTTG	GAAGAAGCCAGAGAACGAGCAG
*HbSUT*3	CACCACAACCACCATCAC	GTGGAAGAGGTTCAGAAGAG
RBSP	ACACCAAGGATGATCTCAGGCTTC	AGACATGACGGTCACCACAAGG
RBIP	TGCTGCGACCAATCCTACACTAC	CACTAGCCTGCTCTTGCTCTCC
18S*rRNA*	CAACCATAAACGATGCCGACCAG	TTCAGCCTTGCGACCATACTCC

triplicate, using specific primers and generated cDNA as template in real- time PCR machine (Light cycler 480 II**,** Roche, Germany). RT-qPCR was set in a 20 µL reaction mixture containing 1 µL of 1/10 dilution of cDNA, 125 nM of forward and reverse primer and 10 µL of Light cycler 480 SYBR Green I PCR Master mix (Roche Diagnostics Gmbh, Germany). RT-qPCR was set by incubating the mix at 95 ºC for 7 min, continued by 40 cycles of 95 ºC for 20 sec, 60 ºC for 30 sec and accompanied by a melt curve analysis (95 ºC for 20 sec, 60 ºC for one min, 95 ºC for 5 min). The PCR reaction was carried out in triplicate with no template controls (NTC). The reaction efficiency of the endogenous control and target genes was calculated based on the formula, Efficiency = $10^{(-1/slope)}$ -1). Primers with slope values between -3.2 and -3.5 were only employed for these reactions. The relative quantification (RQ) values were analyzed using the software of Light Cycler 480 (v.1.5.0) and 18S rRNA was used as endogenous control.

Data analyses

The $2^{-\Delta\Delta}$Ct method was adopted to study the comparative changes in gene expression (Livak and Schmittgen, 2001). For each gene, three biological replications were performed. Statistical study was performed in data using ANOVA. The ratio with a P value ≤ 0.05 was adopted as significant.

Results and discussion

Expression of genes associated with rubber biosynthesis was analyzed in high yielding (RRII 105, PB 217 and RRIM 600) and low yielding (Tjir 1, RRII 38 and RRII 33) *Hevea* clones. Among the clones studied, RRII 105 recorded the highest (98.3 g tree^{-1} tap^{-1}) and RRII 33 the lowest (4.5 g tree^{-1} tap^{-1}) dry rubber yields (Fig. 2). Gene expression of one of the low yielding *Hevea* clone (Tjir 1) was used as calibrator.

The relative gene expression of HMG-CoA synthase gene (*hmgs*) was found to be significantly higher in high yielding *Hevea* clones than the low yielders (Fig. 3). HMGS catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-CoA. HMGS activity has been positively related with rubber production in *H. brasiliensis*, suggesting

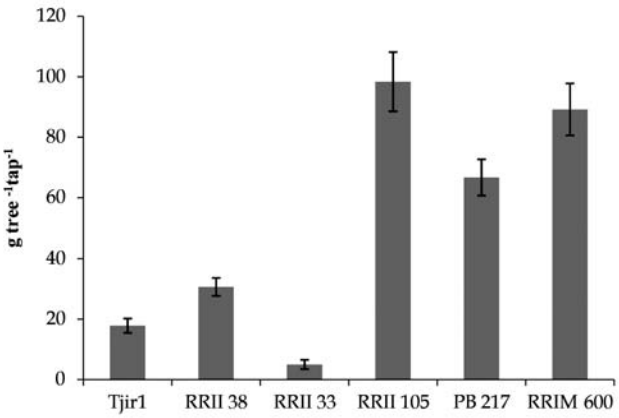

Fig. 2. Dry rubber yield (g tree^{-1} tap^{-1}) of *Hevea* clones

that this enzyme has a regulatory role (Nagegowda *et al*., 2004; Li *et al.*, 2015). In many plants, *hmgs* expression showed an association with rapid cell division and growth (Alex *et al.*, 2000). Wounding and application of chemicals like methyl jasmonate, salicylic acid and ozone are known to induce *hmgs* expression signifying that *hmgs* is implicated in plant defence mechanisms (Wegener *et al.*, 1997). *hmgs* showed up regulation in tapping panel dryness (TPD, situation where partial or complete cessation of latex flow occurs) affected trees (Liu *et al.*, 2015).

The HMG-CoA formed by the HMGS enzyme acts as substrate for HMG-CoA reductase (HMGR) to generate mevalonate which is further converted to isoprenoid compounds. In *Hevea, hmgr* is encoded by a small gene family comprising of five members (*hmgr*1, *hmgr*2, *hmgr*3, *hmgr*4 and *hmgr*5), *hmgr*4 and *hmgr*5 were specifically expressed at high levels in mature leaves and xylem (Sando *et al.*, 2008). In this study, the expressions of *hmgr*1, *hmgr*2 and *hmgr*3 were analyzed. The expression of *hmgr*1 was found higher in high yielding clones than the low yielders (Fig. 3). It was significantly high in RRII 105 followed by RRIM 600 and PB 217. NADP-dependent synthesis of mevalonate from HMG-CoA by HMGR is the most committed step of the mevalonate (MVA) biosynthetic pathway (Caelles, *et al.*, 1989). Mevalonate, a rate limiting enzyme in the rubber biosynthetic pathway which catalyses the universal precursor in isoprenoid compounds, is crucial for regular growth, development and a range of other normal physiological activities in plants. In the study, *hmgr*2 expression showed no

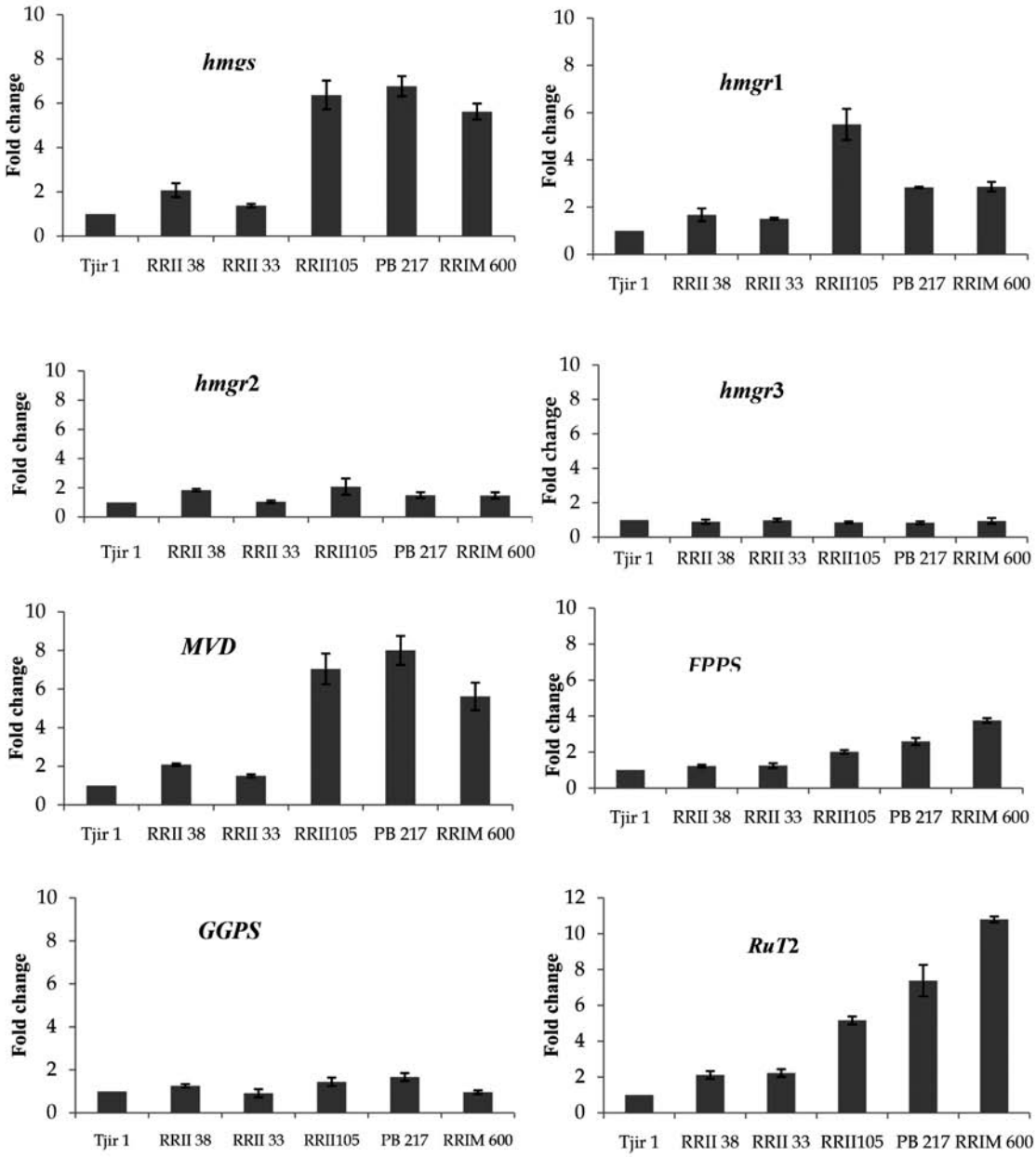

Fig. 3. Quantitative expression analysis of genes in *Hevea* clones with different yield potential
Error bars indicate standard error of three biological replicates

significant difference between high and low yielding *Hevea* clones. Its expression was high in RRII 105 and RRII 38 and was similar in PB 217 and RRIM 600. The expression pattern of *hmgr*3 was almost similar in all the *Hevea* clones studied (Fig. 3). Chye *et al*. (1992) reported that *hmgr*1 was inducible by ethylene, while *hmgr3* gene was constitutively expressed and *hmgr*2 could be linked to the defense reactions against wounding and pathogens. *hmgr*1 was more abundant in latex than in other tissues like leaf, flower and seedlings (Venketachalam *et al.*, 2009). Schaller *et al.* (1995) reported a positive correlation with enzymatic activity and increased *hmgr*1expression level. In *Arabidopsis*, loss of function of *hmgr*1 leads to male sterility, dwarfing, reduced sterol levels and early senescence (Suzuki *et al*., 2004).

Chappell *et al*. (1995) reported 3 to 6 fold increase in total HMGR enzyme activity and an

overall increase in the sterol accumulation in tobacco plants (*Nicotiana tabacum*) that may be due to the constitutively expressed Hamster HMGR cDNA. The HMGR activity showed correlation with the amount of sterol production in developing seeds of tobacco and rape (Harker *et al*., 2003). HMGR activity and transcript levels were huge in vigorously dividing *Arabidopsis,* tobacco culture cells and tomato (Hemmerlin *et al.,* 2003). Yang *et al.* (1991) reported that some solanaceous species, challenged with elicitor treatment or pathogen, induced HMGR activity and transcript levels that provided the increase of phytoalexin (sesquiterpenoid). Thus, the regulatory role of HMGR in mevalonate pathway seems to be imperative for acclimatization of plant to the challenging environmental stress and growth and development. Ji *et al*. (1993) positively correlated HMGR mRNA, HMGR activity with rubber biosynthesis as well as latex yield in *Parthenium argentatum*. Li *et al*. (2015) reported that the expression of *hmgr*1 was more in a high yielding clone compared to a low yielding clone. *hmgr*1 was significantly up regulated in CATAS8-79 (high yielding) than PR 107 (low yielding) suggesting that *hmgr*1 is critical for providing IP (Chao *et al*., 2015). Elevated levels of *hmgr*1 gene expression in high yielding clones might be credited to high yield potential.

The mevalonate diphosphate decarboxylase gene (*MVD*) was found significantly upregulated in high yielding than low yielding *Hevea* clones (Fig. 3). Among the high yielding clones, PB 217 showed higher *MVD* expression followed by RRII 105 and RRIM 600. The MVD catalyzes the irreversible decarboxylation of 6-carbon compound mevalonate-5-pyrophosphate (MVAPP) into 5-carbon IPP. Cordier *et al.* (1999) reported that in *Arabidopsis thaliana*, MVD was invented to be transcribed at a low level in whole plant or in a tissue specific pattern. The greatly expressed *HbMVD* in latex, play a central role in rubber biosynthesis (Sando *et al.,* 2008). The up regulation of *MVD* gene might have resulted in an increased IPP supply.

Farnesyl diphosphate (FPP) synthase expression was significantly upregulated in high yielding than the low yielding clones (Fig. 3). FPP is a branch point and FPPS is treated as a regulatory enzyme in the pathway. Geranyl geranyl diphosphate (GGPP) synthase expression was not significantly different among the clones irrespective of yield traits. The expression of *GPPS* and *FPPS* was reported to be significantly upregulated in high yielding clones (Li *et al.,* 2015). FPP synthase and GGPP synthase were variously expressed in different tissues of *H. brasiliensis*, which reflects their specific functions and roles in each tissue (Takaya *et al.,* 2003). It has been reported that GGPP synthase expression is higher in leaf, flower and young leaf than those in petioles and latex. On the contrary, the expression of FPP synthase was high in latex as well as in flower. Okada *et al*. (2000) reported that in *Hevea,* the GGPP synthase showed the similar functions as that of *A. thaliana GGPS,* which is located in the chloroplast and engaged in the biosynthesis of chlorophylls, gibberellins and carotenoids.

Expression of rubber transferase (RuT) was found upregulated in high yielding than the low yielding clones (Fig. 3). Among the clones, RRIM 600 showed the highest level of expression followed by PB 217 and RRII 105. The low yielding clones (RRII 38 and RRII 33) displayed relatively low level of gene expression. Rubber transferase (RuT), belonging to the *cis* prenyl transferase family,catalyses the polymerization of IPP in to *cis*-rubber (Archer and Audley, 1987). The successive addition of IPP was found to take place only on the surface of pre-existing rubber particles (McMullen and McSweeney, 1966) implying that rubber transferase was located on the surface of the rubber particles. RuT was first cloned from *Arabidopsis* (Oh *et al.,* 2000) and in *Arabidopsis* RuT catalyzes the formation of long-chain dolichols (C120).

The gene expression analyses of different isoforms of REF were carried out in the present study (Fig. 4). *REF1* showed a low level of expression in all the selected *Hevea* clones and no significant difference was observed between high and low yielders. The gene expression of *REF2* was found higher in high yielding than low yielding clones. Among them, PB 217 showed the highest expression followed by RRII 105 and RRIM 600. Interestingly *REF3* showed a different pattern of expression, a low (RRII 38) and a high yielder (PB 217) showed high

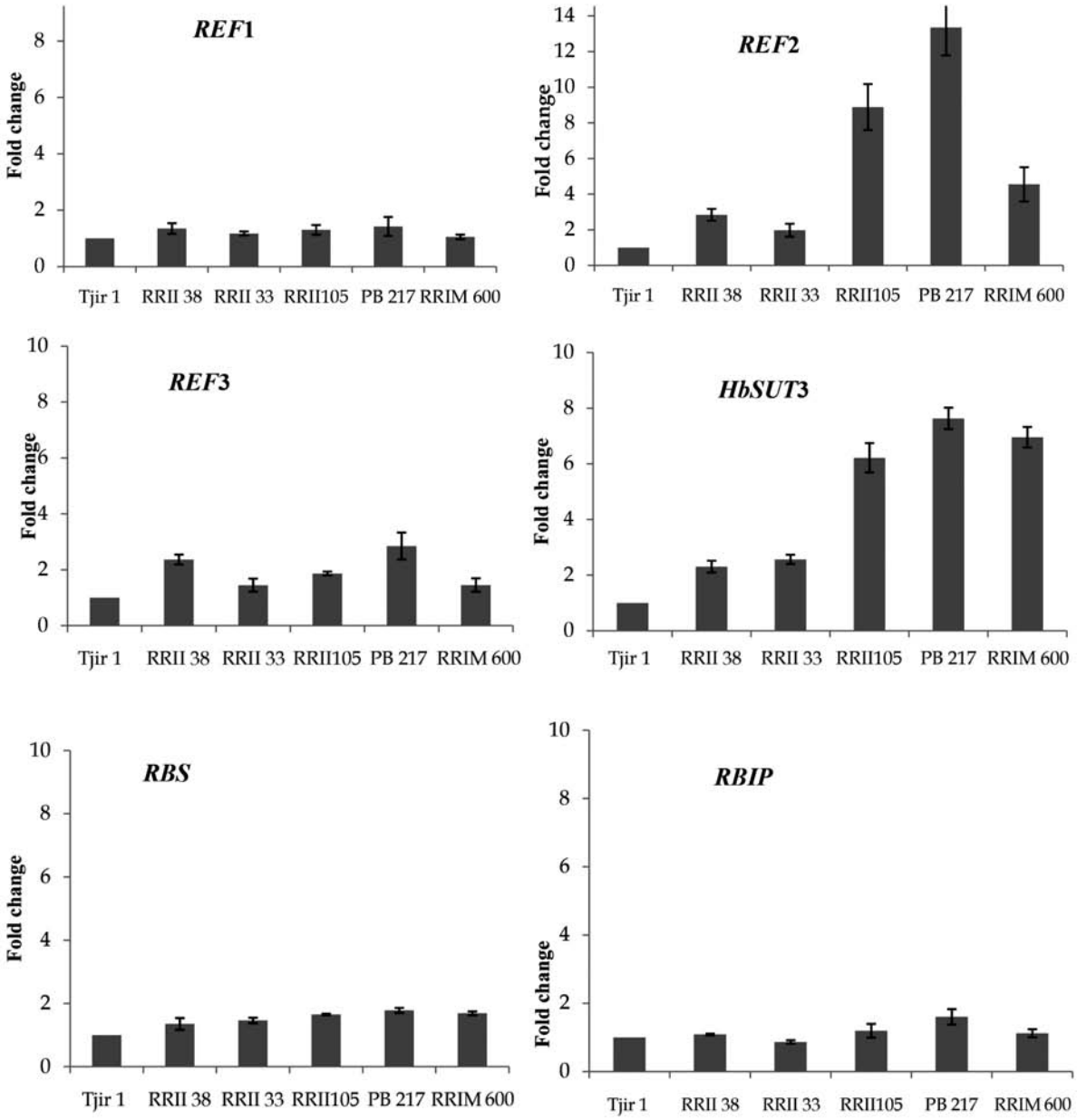

Fig. 4. Quantitative expression analysis of genes in *Hevea* clones with different yield potential
Error bars indicate standard error of three biological replicates

magnitude of expression compared to other clones. Rubber elongation factor (REF) gene is encoded by a small gene family consisting of three members and it plays an important role in the final polymerization of rubber. REF present in large amounts at the surface of the rubber particles is involved in polymerization of rubber (Sando *et al.*, 2009). REF expression showed direct positive correlation with *Hevea* latex yield (Priya *et al*., 2007).

*HbSUT*3 was found significantly up regulated in high yielding *Hevea* clones (RRII 105, PB 217 and RRIM 600) than low yielding clones (RRII 33 and RRII 38) (Fig. 4). Among the high yielders, PB 217 showed the highest fold level change in gene expression (7.63) followed by RRIM 600 (6.95) and RRII 105 (6.21), respectively. The sucrose transporter gene *HbSUT*3 plays an active role in sucrose loading to lacticifer and rubber productivity

in exploited trees of *H. brasiliensis*. *HbSUT*3 was transcribed in all the tissues, suggesting a functional redundancy of this gene. The preferential expression of this gene in laticiferous system and female flowers suggests its major role in these two tissues (Tang *et al*., 2010). Sucrose transporters belong to the major facilitative superfamily (MFS) and they have 12 typical transmembrane-spanning domains (Sauer, 2007). The expression of *HbSUT*3 in the present study was found to be significantly higher in high yielding clones than low yielding clones. Efficiency of sucrose transportation and related metabolism and rubber biosynthesis are closely associated with the ability of latex regeneration between intervals of successive tappings (Tang *et al*., 2010). Several unigenes associated with carbohydrate metabolism was reported to be over expressed in CATAS8-79 (a high yielding clone) than that in PR107 (a low yielding clone), suggesting a more efficient sucrose transportation and carbohydrate metabolism in CATAS8-79 (Chao *et al*., 2015). The result shows the involvement of *HbSUT*3 in sucrose loading in to lacticifers and further rubber productivity. The up regulation of *HbSUT*3 in high yielding clones suggests an enhanced level of sucrose loading to the lacticifers in these clones. Yusof *et al*. (1998; 2000) had purified a patatin like inhibitor protein (*RBIP*) with a molecular weight of 43.7 kDa and a rubber biosynthesis stimulator protein (*RBSP*) identified as *eIF*-5A by amino acid sequencing from *H. brasiliensis* latex. In the present study, no significant difference was observed for the expression of *RBSP* and *RBIP* between high and low yielding *Hevea* clones (Fig. 4).

Conclusion

Expression of genes involved in rubber biosynthesis was analysed in different *Hevea* clones with varied yield potential. The expression of *HbSUT*3, a sucrose transporter and rubber elongation factor (*REF2*) was found to be significantly higher in high yielding than low yielding clones. The gene expression of rubber biosynthetic enzymes like HMGS, HMGR1, MVD, FPPS and RuT was significantly higher in high yielding than low yielding clones. The present study suggests that high rubber yield is associated with higher rate of expression of specific genes and they are useful markers for screening of *Hevea* clones for rubber yield.

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