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Differential expression of mitochondrial pyruvate dehydrogenase gene correlates with latex yield and tapping in rubber tree

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ABSTRACT: Natural rubber (*cis*-1,4-polyisoprene) is a product of the isoprenoid biosynthesis pathway which requires an allylic pyrophosphate and isopentenyl pyrophosphate (IPP) to initiate and elongate the rubber molecule. The biosynthesis of IPP occurs via two distinct routes: the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways. In this study, the expression of 34 genes related to rubber biosynthesis were compared between high and low latex yielding trees of two rubber tree clones, PB 217 and PB 260. Almost all tested genes revealed no significantly differential expression related to latex yield. Only mitochondrial pyruvate dehydrogenase (*PDCE1*) showed specific up-regulation in the high latex yielding trees of both tested clones. Interestingly, the expression of *PDCE1* involving in the production of acetyl-CoA in mitochondria was also significantly induced by latex loss upon tapping. The increasing of acetyl-CoA and energy production may favor rubber tree to produce more latex. The in silico analysis showed that *HbPDCE1* promoter contained ethylene and copper-responsive elements. Ethylene is worldwide used rubber stimulant while copper sulfate was also reported to be able to stimulate the latex yield. This suggested that *HbPDCE1* may be transcriptionally regulated by these two compounds however the *in vivo* regulation of this gene should be further investigated.

KEY WORDS: Glycolysis, *Hevea brasiliensis*, Latex, Pyruvate dehydrogenase, Rubber biosynthesis, Rubber tree.

INTRODUCTION

Rubber tree (*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.) is the most important commercial source of natural rubber (NR) or *cis*-1,4-polyisoprene which cannot be replaced by the synthetic ones due to its unique properties (Cornish, 2001; van Beilen and Poirier, 2007). The demand of NR is still increasing every year. The ways to increase NR production, to be suitable with the worldwide policy for environmental preservation and no more forest cleaning, are to select the high yielding rubber clones and optimize the exploitation system as well as improve genetic of rubber clones for rubber replanting.

The latex is harvested by tapping which involves periodically cutting the bark on the trunk of rubber tree in order to severe the latex cells for exudation of latex. In many rubber-producing countries including Thailand, the widely used exploitation system is the half-spiral, alternate-day tapping (tapping every 2 days). Three main factors limiting latex yield are the duration of latex flow after tapping limited by coagulation processes (d'Auzac, 1989a), the capability of latex cells to regenerate their lost cytoplasm (latex) (Jacob *et al.*, 1989) and the bark diseases. All these intrinsic factors are clonal characteristics (Jacob *et al.*, 1989). Therefore, the selection of rubber tree clones with long duration of latex flow, high capability to regenerate their lost latex and resistant to bark diseases, might increase latex yield. In addition, the duration of latex flow and flow rate could be transiently increased by ethephon or ethylene stimulation (Coupé and Chrestin, 1989). Ethylene markedly increases latex yield in parallel with decrease of latex total solid content resulting in latex dilution, then latex flow prolongation and consequently increased volume of latex collected.

It is firmly established that rubber is produced in latex, the cytoplasm of specialized cells called laticifers, by the process of rubber biosynthesis pathway with the help of proteins related to rubber biosynthesis. Isopentenyl pyrophosphate (IPP), the precursor of rubber biosynthesis, occurred both in cytosol, by mevalonate (MVA) pathway (Keckwick, 1989), and in plastid, by methylerythritol phosphate (MEP) pathway (Ko *et al.*, 2003). MVA pathway has shown more established evidence for rubber biosynthesis in latex compared to MEP pathway. In comparison, evidence for role of MEP pathway is more recent although less

supported experimentally, nonetheless it is looking promising (Sando *et al.*, 2008; Chow *et al.*, 2012). The MVA pathway precursor is acetyl coenzyme A (acetyl-CoA), which is the product of glycolysis and Kreb's or TCA cycle in mitochondria, while the MEP pathway precursors are pyruvate and glyceraldehyde-3-phosphate, which are also the product and intermediate of glycolysis. Since IPP is the essential building blocks of rubber polymer in rubber producing plants, efficient supply of IPP is one of the most important factors in synthesis of rubber (Cornish, 2001). Hence, the expression analysis of genes in pathways involving in IPP supply, glycolysis and the continuation pathway, along to rubber biosynthesis pathway in latex may reveal the rate-limiting step that might control the latex yield production.

All of these data insist the importance of the enzymes in latex of rubber tree where the rubber biosynthesis and cell metabolism occurred. Moreover, latex is the place that almost of transcripts located in. It means that the trees producing different latex yield might express some genes differentially therefore it is possible to detect the differential accumulating transcripts in the latex.

The first two enzymes, invertase and sucrose synthase, involving in degradation of sucrose (Sturm and Tang, 1999) are major importance in the metabolism and rubber production of laticifers, as they are responsible in feeding glucose to glycolysis (Fig. S1). Glycolysis is the metabolic pathway that converts glucose into pyruvate. Pyruvate is reduced to acetyl-CoA by mitochondrial pyruvate dehydrogenase complex. Acetyl-CoA is then used as carbon source for TCA cycle in mitochondria for energy production and for IPP synthesis via MVA pathway (Fig. S2). Some acetyl-CoA can be transported out in the form of citrate for using in generating cytosolic acetyl-CoA by ATP-citrate lyase (Fatland *et al.*, 2005). After that, three molecules of cytosolic acetyl-CoA are condensed to IPP, the precursor of rubber biosynthesis (Fig. S3). Accordingly, IPP will be polymerized into long *cis*-1,4-polyisoprene chain by *cis*-prenyltransferase, assisted by rubber elongation factor (Dennis and Light, 1989) and small rubber particle protein (Oh *et al.*, 1999).

The possible role of each enzyme in the pathways may relate with (1) different level of rubber production, and (2) different ability on latex regeneration of trees. In this study, the expression of each related gene was compared between high latex yielding trees and low latex yielding ones to find the differentially expressed genes related to only latex yield but not the genetic background. First, quantitative real-time RT-PCR (qRT-PCR) was performed to determine the expression profiles of genes involved in sucrose degradation, glycolysis, pyruvate dehydrogenase complex, IPP synthesis, via MVA/MEP pathway, and rubber biosynthesis pathway in latex of high and low latex yielding trees of two rubber clones, PB 217 and PB 260. Then, the promoter and full-length cDNA encoding the selected interesting gene was cloned, sequenced and characterized. The basic knowledge obtained from this study leads to a better understanding of genes in metabolic and rubber biosynthesis pathway that may be the rate-limiting step of latex yield production.

MATERIALS and METHODS

Plant materials

The field experiments were performed with rubber trees of the PB 217 and PB 260 clones in the SAPH/Bongo rubber Estate, Ivory Coast, West Africa. In the experiment set up to compare gene expression related to latex yield, the latex samples were collected from selected (homogeneous girth) high and low latex yielding trees of the PB 217 and PB 260 clones which were tapped for about 3 years. The latex was collected from 3 individual trees for each group. The latex about 6 ml per tree after discarding the first 20 drops of latex after tapping, was mixed with 6 ml of 2X fixation/extraction buffer (50 mM Tris-HCl, 300 mM LiCl, 10 mM EDTA, 10% SDS, pH 9.0), immediately frozen in liquid nitrogen and stored at -80 ºC. Average yield of rubber trees in this study was shown in Table S1. For qRT-PCR and determination of physiological parameters, latex was individually collected from another batch of three trees for each group.

In the experiment set up to compare gene expression related to tree exploitation, the latex was collected from the first and the second tappings of two virgin (never tapped) and two tapped (after 4-month regularly tapping) trees of the PB 217 clone. The tapping of regularly tapped trees was stopped for 7 days before starting the first tapping in this experiment to avoid the effect of previous tapping. The second tapping was done 2 days after the first tapping. Average yield of virgin and tapped trees was shown in Table S2.

RNA isolation and cDNA synthesis

Total RNA extraction was performed according to the method adapted from Pujade-Renaud *et al.* (1994). RNA samples were then treated with DNase I by DNA-free™ (Ambion) to eliminate trace contaminating DNA. First-strand cDNA synthesis was synthesized with 1.5 µg of total RNA using SuperScript™ III First-Strand Synthesis System (Invitrogen) with 3 replicates for each tree. The generated cDNA was diluted 50-fold for using as the template in relative quantification real-time RT-PCR (qRT-PCR) and diluted 10, 25, 50, 100, 200, 300, and 400-fold for using in percentage amplification efficiency (PE) analysis.

Primers design and verification

The specific primers (Table S3) were designed from

either cDNA sequences available in the NCBI database or our *Hevea* ESTs sequences (unpublished data) using Primer 3 program (http://frodo.wi.mit.edu/primer3/) with the following criteria: primer lengths of 22-24 bp, Tm range of 62-64 °C, and amplicon lengths of 150-250 bp. The percentage amplification efficiency (PE) of each primer pair was verified by qRT-PCR with a serial dilution of the cDNA templates. Standard curves for PE calculation were performed as previously described (Yuan *et al.*, 2006). All the primers pairs used in this study showed the acceptable PE in the range of 0.8-1.2. In addition, the amplification products were cloned, sequenced and validated by homology search against database (data not shown).

Gene expression analysis by qRT-PCR

The qRT-PCR analysis was performed with the ABI-7500 real-time PCR machine as described by Tungngoen *et al.* (2009). Twenty µl of PCR reactions containing 2 µl of 50-fold cDNA dilution, 0.4 µM of each forward and reverse primers, 0.25 mM dNTP mix, 2 mM $MgCl_2$, 1 U of Platinum *Taq* polymerase (Invitrogen) and 1000-fold dilution of SYBR green I (Sigma) in 1X PCR buffer. The C_t (cycle threshold) data were determined using default threshold settings. The relative expression was calculated as $2^{-\Delta Ct}$ where ΔC_t = (C_t of target gene– C_t of internal control gene). The qRT-PCR amplifications were performed in triplicate for each cDNA replicate. SE calculation and One-Way ANOVA with Tukey's test were used for statistical and significance analyses, respectively, taking into account all three-by-three replicates. A value of $P < 0.05$ indicated statistical significance. Four housekeeping genes (40S ribosomal protein gene (*40S*), ubiquitin (*UBI*), actin (*ACTIN*) and elongation factor 1 (*EF1*)) were selected to evaluate the stability by the software package NormFinder (version 0.953, http://moma.dk/normfinder-software) (Andersen *et al.*, 2004). All four genes were suitable as reference genes in the present study due to their stability tested on latex samples of low and high latex yielding trees of PB 217 and PB 260 clones (Table S3, Fig. S4, S5). Expression values were normalized for differences in cDNA input against a reference gene *ACTIN* due to its highest amplification efficiency of primer and its highest expression level to ensure its expression level is above background. The correlation coefficient of gene expression and latex yield was calculating using Microsoft Excel 2007.

Screening of pyruvate dehydrogenase (HbPDCE1) full-length cDNA

Hevea latex cDNA library was constructed in ZAP express vector (Stratagene). Approximately 2.0×10^5 plaque-forming units were screened with the digoxigenin-11-2-deoxy-uridine-5–triphosphate (DIG-11-dUTP)–labeled *HbPDCE1* EST probe (Roche). Immunological detection was performed using DIG luminescent detection kit (Roche) according to the manufacture's instruction manual. The positive plaques were collected and converted to a pBK-CMV phagemid vector and sequenced. The nucleotide sequences were analyzed against GenBank database.

Cloning and characterization of promoter regions

Genomic DNA was extracted from leaves of the rubber tree PB 217 and PB 260 clones as described by Tungngoen *et al.* (2009). The proximal promoter region upstream from the 5'-untranslated region of *HbPDCE1* gene was cloned by PCR amplification of the genomic DNA using the GenomeWalker Universal kit (Clontech) according to the manufacturer's recommendation. The primary PCR was performed with the outer adapter primer (AP1) and the outer gene-specific primer *HbPDCE1*-R1 (5′-CTGCTACTTCAGCATGGAGAGGTTATTCA-3′) using Advantage 2 polymerase mix (Clontech). The secondary PCR was performed with the nested adapter primer (AP2) and the nested gene-specific primer *HbPDCE1*-R2 (5′-GGTTCTTTGGATCGCCATTTTAACATC-3′).
The PCR products were cloned into the pGEM®-T Easy vector (Promega), sequenced and subsequently analyzed for putative promoter *cis*-acting elements using PLACE software (www.dna.affrc.go.jp/htdocs/PLACE/; (Higo *et al.*, 1999)).

RESULTS AND DISCUSSION

PDCE1 differentially expressed corresponding to low and high latex yield

After screening the expression of 34 genes in sucrose degradation, glycolysis, pyruvate dehydrogenase complex, IPP synthesis, via MVA/MEP pathway, and rubber biosynthesis pathway by qRT-PCR in latex of high and low latex yielding trees of two rubber clones, PB 217 and PB 260, only *PDCE1* showed significantly differential expression in moderate correlation with latex yield (Fig. 1, Table S4, S5).

The expression of two genes, *neutral invertase* (*NINV*) and *sucrose synthase* (*SUSY*), involving in sucrose degradation were analyzed by qRT-PCR. NINV is an irreversible enzyme which degrades sucrose to fructose and glucose. SUSY is a reversible enzyme which (1) breakdowns sucrose to UDP-glucose and fructose and (2) synthesizes sucrose and releases UDP from glucose (Fig. S1). The expressions of *NINV* and *SUSY* in latex were not significantly different between low and high latex yielding trees of PB 217 and PB 260 clones even their correlation coefficient show highly correlate with latex yield (Fig. 1A, Table S4). As the optimum pH for NINV activity was about 6.5-7.0 (Vargas and Salerno, 2010), therefore the optimal condition for its activity may have direct effect on sucrose degradation. The degradation of sucrose to generate the precursors of IPP biosynthesis may be regulated at post-translational level of *NINV* rather than

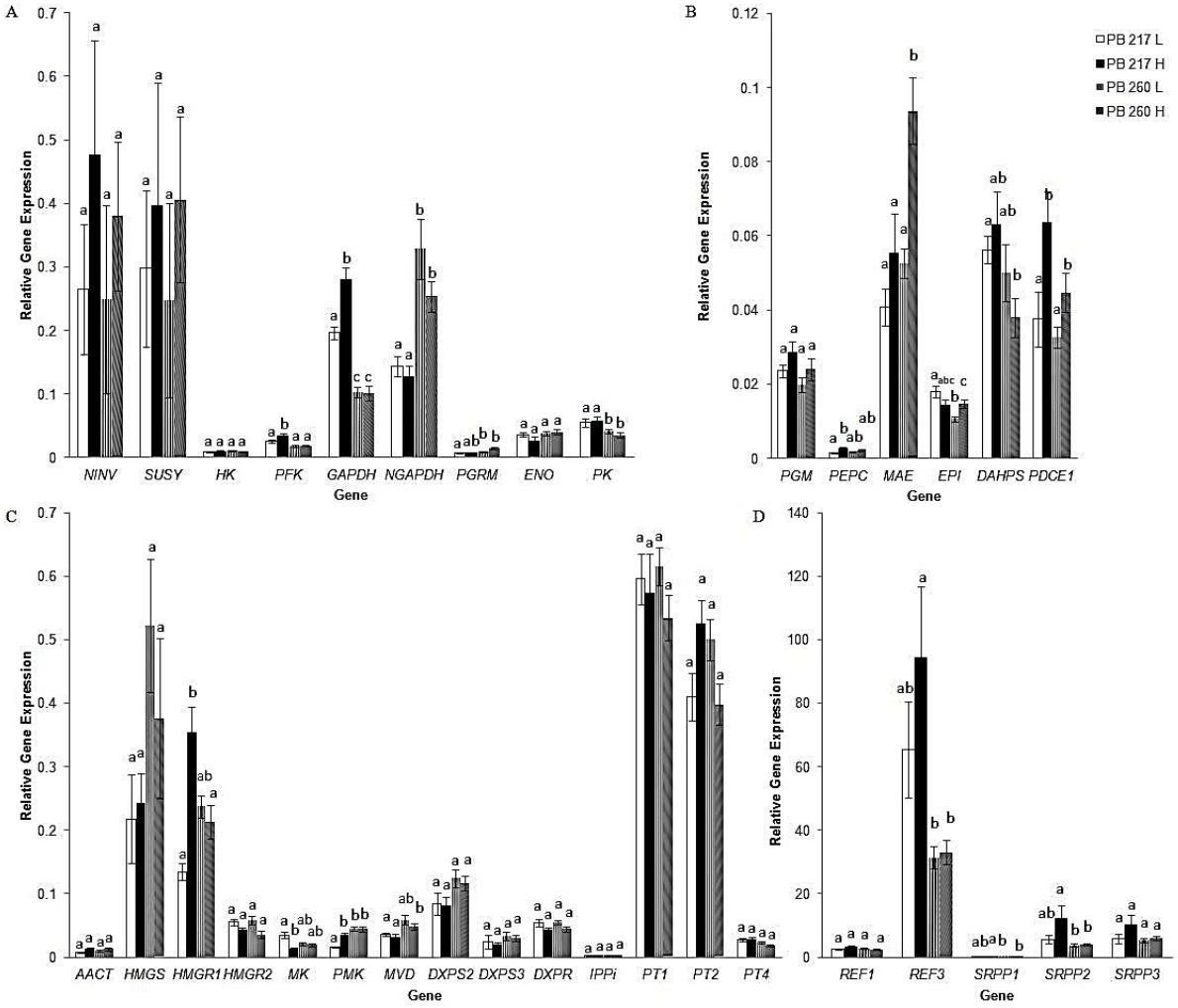

Fig. 1. Expression of the genes in sucrose degradation and glycolysis **(A)**, metabolic pathway **(B)**, rubber biosynthesis pathway **(C, D)** compared between low and high latex yielding trees of PB 217 and PB 260. The transcript levels were analyzed by qRT-PCR, with *ACTIN* as the internal control gene. PB 217 L and PB 217 H are low and high latex yielding trees of PB 217, respectively. PB 260 L and PB 260 H are low and high latex yielding trees of PB 260, respectively. Data are means of three trees, three independent experiments with three replicates (n = 27) and the error bars showed SE. The same letter denotes that there are no significant differences between the samples (One-Way ANOVA with Tukey's test, $P < 0.05$).

its transcriptional level. In case of SUSY, alkaline pH promotes sucrose synthetic activity while acidic pH promotes sucrose breakdown activity (Jacob *et al.*, 1989). When cytosol is more acidic, NINV activity will be low but the breakdown activity of SUSY will be promoted. This evidence may imply that sucrose in the laticifers might be degraded all times and at the same level in low and high latex yielding trees of PB 217 and PB 260 clones, thus sucrose degradation may not be the rate limiting step related to latex yield.

Hexokinase (*HK*) and *phosphofructokinase* (*PFK*) are genes encoding for the enzymes in glycolytic pathway, that play role in preparatory step which involved in modifying hexose (six-carbon compound). Phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH), non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NGAPDH), phosphoglyceratemutase (PGRM), enolase (ENO) and pyruvate kinase (PK) play role in pay off phase which involved in modifying three-carbon compound (Fig. S1). The result of qRT-PCR showed that all of genes encoding enzymes in glycolysis showed no any differential expression according to latex yield (Fig. 1A). It may be because glycolysis is the central metabolic pathway that occurs in every organism. However, *GAPDH* was significantly up-regulated in PB 217 (Fig. 1A) while *NGAPDH* was significantly up-regulated in PB 260. This finding might suggest that the high expression of *GAPDH* in PB 217 and *NGAPDH* in PB 260 were related to the specific clonal characteristics.

The expression of genes encoding glycolysis related enzymes (Fig. S1) including phosphoglucomutase (*PGM*), phosphoenolpyruvate carboxylase (*PEPC*), malate dehydrogenase (*MAE*), aldose-1-epimerase (*EPI*),

phospho-2-dehydro-3-deoxyheptonate aldolase (*DAHPS*), and pyruvate dehydrogenase (*PDCE1*) was analyzed by qRT-PCR (Fig. 1B). *PGM* and *DAHPS* were not differentially expressed between low and high latex yielding trees. Although the differential expression of *PEPC*, *MAE* and *EPI* were observed but not related to latex yield of both PB 217 and PB 260 clones. Only the expression of *PDCE1*, the first enzyme in the pyruvate dehydrogenase complex which generates acetyl-CoA in mitochondria, was up-regulated in high latex yielding trees of both clones. Most of the genes in glycolysis showed no differential expression thus they may not be related to latex yield. However, the enzyme activity of each gene in this pathway should be examined whether there is a relationship between enzyme activity and latex yield.

The expression of genes in isoprenoid biosynthesis (MVA and DPX/MEP) and IPP polymerization (Fig. S3) was investigated. Results showed that the expressions of most genes were not related to the latex yield in both tested clones. The genes known to have an important role in rubber chains elongation; the rubber elongation factors (REF) and small rubber particle protein (SRPP) isoforms were expressed at a higher level compared to the genes in IPP biosynthesis pathway (Fig. 1C-D). It confirmed that *REF3*, and to a lesser extent *REF1*, *SRPP2* and *SRPP3* were the most abundant transcript in the latex of PB 217 and PB 260 clones. This finding is consistent with that reported by Chow *et al.* (2007) in the latex of RRIM 600 clone. The level of *REF* and *SRPP* transcripts was not different between the low and high yielding trees, within the same clone. However, *REF3*, *SRPP1* and *SRPP2* showed clonal specific up-regulation in the latex of PB 217 clone. As PB 217 is a lower latex yielding clone compared to PB 260, the higher transcript level of these genes in PB 217 clone may be related to longer rubber chains and larger rubber particles, leading to higher latex viscosity and impaired latex flow, thus lower latex yield.

Loss of latex induced PDCE1 expression

From the results, only the expression of *PDCE1* was positively correlated with latex yield and the expression were significantly different in latex of low and high latex yielding trees of PB 217 and PB 260**.** The results lead to the hypothesis that *PDCE1* expression may significantly increase in the trees which had loss more latex**.**

The laticifers in the bark of virgin trees are characterized by both low metabolic activity and low latex production**.** With tapping, laticiferous metabolism is activated, and finally, the latex yield increases **(**Pakianathan *et al*.**,** 1992**).** The latex yield will increase progressively with successive tappings at regular intervals, and it will reach a steady state of yield equilibrium after 7–10 tappings **(**Pakianathan *et al*.**,** 1992; Tang *et al*.**,** 2010; Chao *et al*.**,** 2015**).**

In this study, we examined the expression of *PDCE1* in virgin tree in comparison with tapped tree of PB 217 clone**.** Interestingly, the result showed that the expression of *PDCE1* of both first and second tapping of regularly tapped trees were significantly higher than of virgin trees **(**Fig**.** 2**).** This result suggested that the trees losing more latex had higher expression of *PDCE1* gene**.** Acetyl-CoA is not only the precursor of rubber biosynthesis, but also the precursor of mitochondrial Krebs**'** cycle, providing cell energy**.** Both regularly tapped trees, as well as the high latex yielding trees, have lost higher amount of latex, therefore have to regenerate latex more than the virgin trees or the low yielding trees**.** *PDCE1* showed higher expression in the tapped trees than in the virgin tree at both the first and second tapping**.** Accordingly, we proposed that up-regulation of *PDCE1* may be involved in energy production which might favor the tree to produce the constituents in which related to latex production**.**

Previously, Ruderman *et al.* (2012) had studied the expression of the other two enzymes in the pyruvate dehydrogenase complex, PDCE2 and PDCE3, in the other different rubber tree clones and the results showed that their expressions were not correlated with latex yielding clones. It should be noted that their experiment did not compare between high and low yielding trees within the same clone.

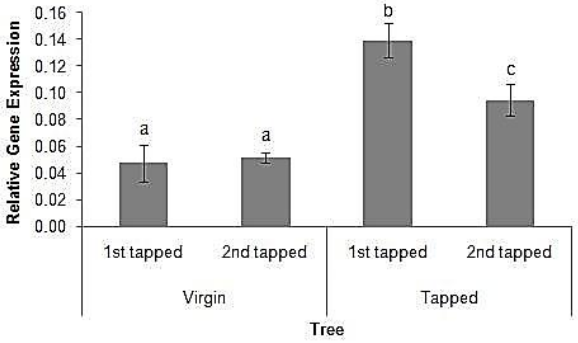

Fig. 2. qRT-PCR analysis of *PDCE1* expression in virgin and regularly tapped trees of PB 217 clone. The 2nd tapped samples were collected 2 days after the 1st tapping. Virgin trees were the tree that had never been tapped before while tapped trees were regularly tapped for 4 months. The relative expression corresponds to the ratio of the transcript abundance of the *PDCE1*/transcript abundance of *ACTIN*. Data are means of three independent experiments with three replicates of two individual trees (n=18) and the error bars showed SE. The same letter denotes that there are no significant differences between the samples (One-Way ANOVA with Tukey's test, $P < 0.05$).

Cloning and characterization of PDCE1 full-length cDNA and its promoter

PDCE1 was differentially expressed by up-regulating in high latex yielding trees (Fig. 1B). Its function was proposed to be involved in latex regeneration. Hence, its promoter and full-length cDNA sequences were cloned and characterized.

PDCE1 full-length cDNA (*HbPDCE1*; GenBank

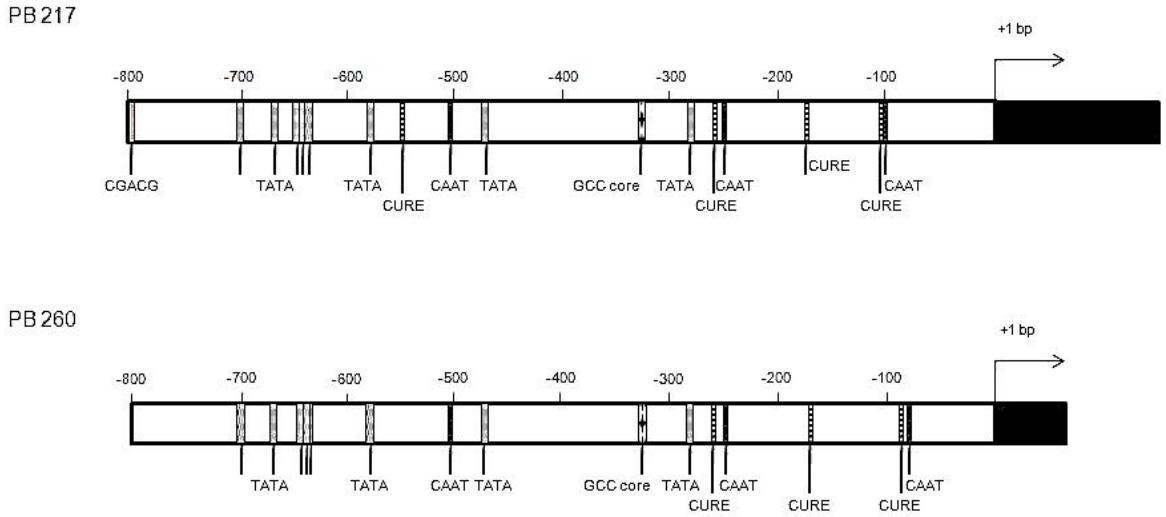

Fig. 3. Diagram of the promoter sequences of *HbPDCE1* gene. The upstream sequences of *HbPDCE1* gene in rubber tree PB 217 and PB 260 were cloned, sequenced, and analyzed for putative *cis*-acting elements using the PLACE software. The CAAT and TATA boxes, as well as the copper responsive (CuRE), GCC-box, and CGACG elements are indicated. The negative numbers represent the upstream ends of the promoter fragments.

accession number KT246293) was successfully cloned and found to be 1,611 bp long and contained a complete open reading frame of 1,098 bp flanked by 148 bp 5'-untranslated region (UTR) and a 365 bp 3'-UTR, including 18 bp long poly(A) tail. The ORF encoded a 365 amino acid polypeptide with a predicted molecular mass of 40 kDa with theoretical pI of 8.20 (Appendix 1). In addition, the putative protein exhibited five predicted conserved serine amino acid residues for phosphorylation sites, Ser_{29}, Ser_{127}, Ser_{165}, Ser_{282}, and Ser_{308} (NetPhosK 1.0) (Appendix 2). Moreover, this HbPDCE1 has the typical features of PDCE1 peptides, including a tyrosine phosphorylation site, cofactor (thiamine pyrophosphate) binding site (http://www.uniprot.org/uniprot/P11177) and four conserved domains of PDCE1 as first pointed out by Wexler *et al.* (1991). After analysis by the pSORT program (http://wolfpsort.org/), the result suggested that the HbPDCE1 protein is targeted to the mitochondria. According to five serine phosphorylable residues, the regulation of PDCE1 via reversible phosphorylation catalyzed by intrinsic kinase and phosphatase components has also been suggested to exist in HbPDCE1 as observed in other plants (Thelen *et al.*, 1999). In pea (*Pisum sativum*), changes in the levels of *PsPDCE1* mRNA and protein were coordinated with changed in PDC activity (Luethy *et al.*, 2001). The highest activities were observed in cells or organs that were rapidly expanding and/or differentiating such as in seedling and youngest leaves. The activities decreased in mature leaves and were nonexistent in senescing leaves. This evidence was related to the earlier analysis of phosphorylation states of the PDC in pea seedling (Miernyk *et al.*, 1985).

In rubber tree, *HbPDCE1* was higher expressed in high latex yielding trees and regularly tapped trees that required more energy for latex regeneration therefore *HbPDCE1* might be used as a marker of the ability in latex regeneration. However, the analysis of transcript and protein levels of all PDC components (PDCE1-3) should be further investigated in more samples of high and low yielding trees and clones. Additionally, their intrinsic kinase and phosphatase components, pyruvate dehydrogenase kinases and pyruvate dehydrogenase phosphatases, should be also investigated.

The promoter regions obtained from both PB 260 and PB 217 clones (Fig. 3) contain several TATA boxes and CAAT box motifs indicating the minimal elements reported to be necessary for the accurate initiation of basal transcription in rubber tree. Among the known hormonal *cis*-regulatory sequences, the most common GCC box ethylene responsive element (Kitajima *et al.*, 1998) was found located upstream from the *HbPDCE1* gene start codon of both clones. Moreover, typical copper-responsive element was also identified. It is worth noting that before the discovery of ethylene as the best rubber yield stimulant, copper sulfate was utilized as yield stimulant for rubber tree (Tixier, 1951; d'Auzac, 1989b). Therefore, *HbPDCE1* gene may be regulated at the transcriptional level by ethylene and copper leading to increase energy production for rubber biosynthesis. Promoter sequences of *HbPDCE1* from the two rubber clones were basically same in the motif. This could be related to no differential expression of *HbPDCE1* in the PB 260 and PB 217 trees with the same yield potential. With these short sequences of promoter, it could not explain how the *HbPDCE1* expression was significantly higher in trees which loss more latex. This gene regulation should be further analyzed in relation to latex yield.

In summary, almost of the genes in metabolic

pathway related to rubber biosynthesis showed no significantly different expression between trees giving high and low latex yield except *HbPDCE1*. The high expression of *HbPDCE1* in high latex yielding trees might relate to generating acetyl-CoA and energy for latex production. The higher expression in regularly tapped trees compared to virgin trees could confirm its function related to latex regeneration. Moreover, *HbPDCE1* promoter contains the elements response to latex stimulants including ethylene and copper sulfate. Accordingly, we propose that the expression of *HbPDCE1* may favor latex regeneration. To understand how this gene promotes the latex production, in-depth study of all PDC components (HbPDCE1-3) in rubber tree is necessary. This better understanding of genes related to rubber biosynthesis pathway should provide an alternative method for future improvement of rubber tree breeding program.

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Appendix 1 Nucleotide and deduced amino acid sequences of a full-length *PDCE1* cDNA obtained from latex cDNA library. This full-length cDNA contained 5′ and 3′-UTR regions and the ORF was indicated by the start and stop codons which were emphasized with boxes.

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                            M  L  G  I  I  R  Q  K  A  F  A
cagtccctgcagaggattcggcctgctacttcagcatggagaggttattcatccgccgct       241
 Q  S  L  Q  R  I  R  P  A  T  S  A  W  R  G  Y  S  S  A  A
aaagagatgactgttagagaagcactgaactctgctctcgacgaggaaatgtctgctgat       301
 K  E  M  T  V  R  E  A  L  N  S  A  L  D  E  E  M  S  A  D
cctaaagtcttcttgatgggagaagaggttggggaatatcagggcgcatataagatatct       361
 P  K  V  F  L  M  G  E  E  V  G  E  Y  Q  G  A  Y  K  I  S
aaagggcttttggagaagtatggtcctgaaagggttcttgatactccgattactgaggct       421
 K  G  L  L  E  K  Y  G  P  E  R  V  L  D  T  P  I  T  E  A
ggatttactggcattggagttggcgctgcttactatggtctcaagccagttgttgagttt       481
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 M  T  F  N  F  S  M  Q  A  I  D  H  I  I  N  S  A  A  K  S
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 N  Y  M  S  A  G  Q  I  S  V  P  I  V  F  R  G  P  N  G  A
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 A  A  G  V  G  A  Q  H  S  H  C  Y  A  S  W  Y  A  S  C  P
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 G  L  K  V  L  A  P  Y  S  S  E  D  A  R  G  L  L  K  A  A
ataaggcaccctgatcctgttgttttccttgaaatgagttattatatggtgagtcatttc       781
 I  R  H  P  D  P  V  V  F  L  E  M  S  Y  Y  M  V  S  H  F
ctgtttcagctgaagtacttgactccagttttgcctcctataggaaaggccagcatgag       841
 L  F  Q  L  K  Y  L  T  P  V  F  A  S  Y  R  K  G  Q  H  E
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 R  E  G  K  D  V  T  I  T  A  F  S  K  M  V  G  Y  A  L  K
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 A  K  R  A  C  Y  R  S  V  P  M  A  A  V  -
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aaattgtggcttttaaaaaaatttttttggggtcaaatttggctacctgatgtggacca      1441

attatatgccaaagccaaaatttaaatttacttgtggtttctgtatgtgcatttcatttt      1501

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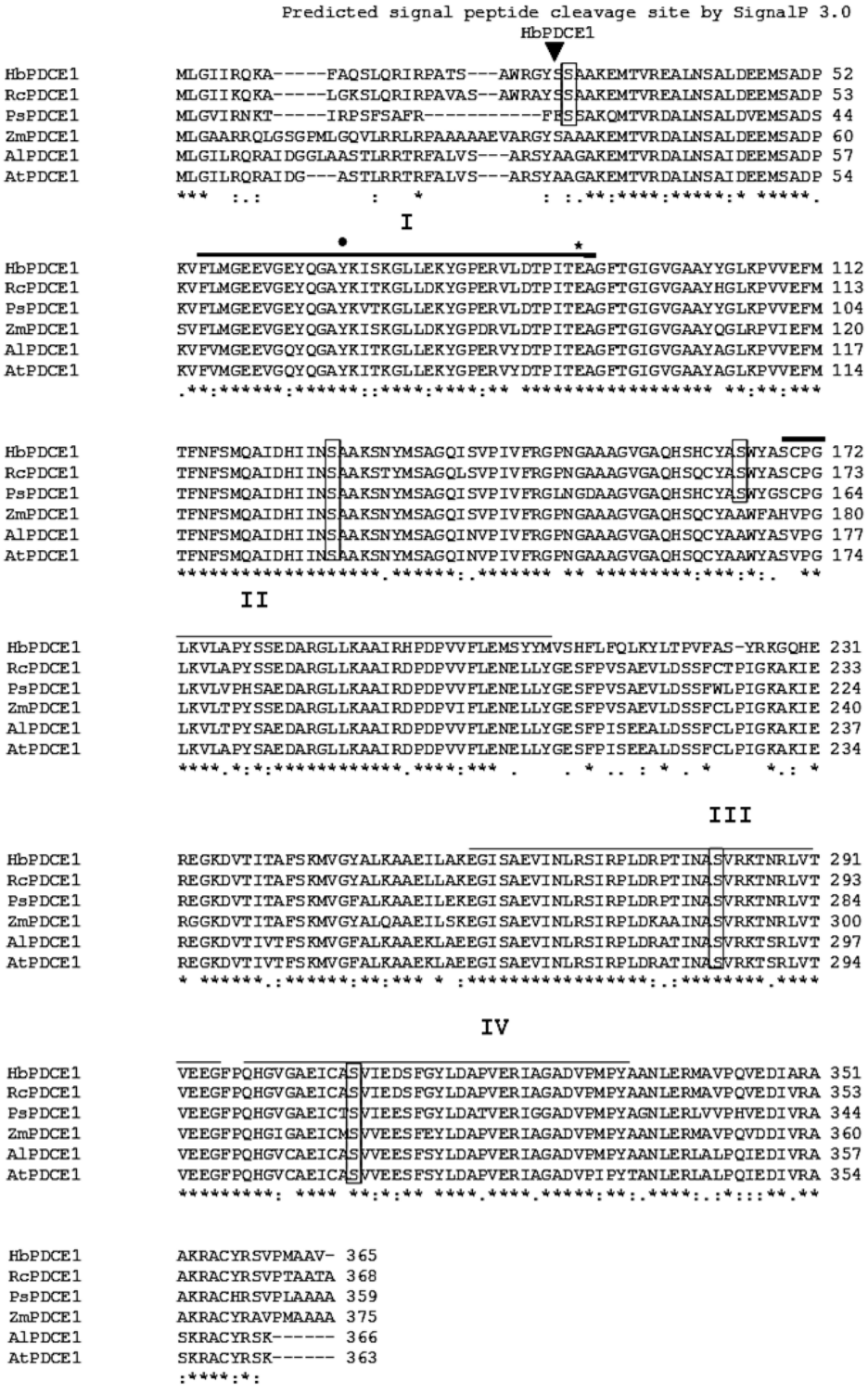
Appendix 2. Amino acid sequence alignment of the pyruvate dehydrogenase (PDCE1) from ***Hevea brasiliensis*** (HbPDCE1[GenBank ID: KT246293]), ***Ricinus communis*** (RcPDCE1 [GenBank ID: XP_002512633]), ***Pisum sativum*** (PsPDCE1 [GenBank ID: P52904]), ***Zea mays*** (ZmPDCE1 [GenBank ID: ACG34647]), ***Arabidopsis lyrata*** (AlPDCE1 [GenBank ID: XP_002864080]), and ***Arabidopsis thaliana*** (AtPDCE1 [GenBank ID: AAA52225]). Locations of conserved motifs found in PDCE1 sequences are identified. *Arrowhead* (topped with the corresponding PDCE1 name): predicted signal peptide cleavage sites, box: predicted serine (S) phosphorylation site, *asterisk*: cofactor (thiamine pyrophosphate) binding site, *dot*: tyrosine (Y) phosphorylation site, *Overlines*: indicated the four conserved domains of PDCE1 as first pointed out by Wexler *et al.* (1991).



Taiwania 61(4): S1-S5, 2016

DOI: 10.6165/tai.2016.61.295

Supplementary data

Chuenwarin *et al.* 2016. Differential expression of mitochondrial pyruvate dehydrogenase gene correlates with latex yield and tapping in rubber tree. Taiwania **61**(4): 295–304.

Table S1. Values (mean ± SE) for latex sample of high- and low- yielding trees of PB 217 and PB 260 clones.

Sample	Classification	Girth (cm)	Cut Length (cm)	Average DRC (g/Tapping/tree)	Average Yield/ cm cut (g/cm)	Average Yield/ cm girth (g/cm)
PB 217 L	LY	66.33 ± 0.51	38.00 ± 0.67	51.57 ± 1.18	1.40 ± 0.03	0.80 ± 0.00
PB 217 H	HY	68.33 ± 0.69	41.67 ± 0.38	151.27 ± 26.46	3.60 ± 0.19	2.07 ± 0.15
PB 260 L	LY	69.00 ± 0.58	46.33 ± 1.07	68.93 ± 10.61	1.50 ± 0.09	1.03 ± 0.05
PB 260 H	HY	65.67 ± 0.38	41.67 ± 2.31	182.13 ± 0.77	4.40 ± 0.21	2.80 ± 0.10

HY: high-yielding trees; LY: low-yielding trees; DRC: dry rubber content

Table S2. Average latex yield of virgin and regularly tapped tree.

Tree	Virgin Tree		Regular Tapped Tree	
Tapping	1st Tapped	2nd Tapped	1st Tapped	2nd Tapped
Average Fresh Weight (g)*	6.6	17.1	71.4	78.9

*** Average latex fresh weight was calculated from two rubber trees.**

Table S3. Primer sequences and amplicon lengths characteristics.

Gene	Forward primer sequence (5′-3′)	Reward primer sequence (5′-3′)	Percentage amplification efficiency (PE)	Amplicon length (bp)
40S	ACAGGCTCATCACCTCCAAG	CAACCACAAAAGTGCAATGG	0.90	283
UBI	TTATCCAATGCGATCCAACC	CAAGGTCAAGGCTCACCAAT	0.84	271
ACTIN	AGTGTGATGTGGATATCAGG	GGGATGCAAGGATAGATC	0.96	196
EF1	TGACTGTGCTGTCCTCATCATT	AGTGGTGGCATCCATCTTGT	0.81	151
NINV	AAATTGCTGCCAGAGCTGTT	CATGCAAGAGAAGGCATTGA	0.90	230
SUSY	ACAATAAGAATGCGAGGGGTGA	TCATGGATACTTTGCCCAGGAC	0.89	153
HK	TTCGGCAAACATCAATAGCATC	ACAGCAGCAATAACATCCTGGTT	1.00	208
PFK	CGAAGATCGTGAACGGTGATTT	CGTCGGAAATGTATCCCTCTTG	0.98	208
GAPDH	GGCTTCGCGTTACTCTCTGT	TGAAAGGATCGTTAACAGCTACG	0.74	200
NGAPDH	GGACCGGATCATTTTCCTTT	TCGATGAAGAGAATGCACGA	0.82	197
PGRM	AAGGTTTTGACGCACAGATTGC	CGAGGTACTGGTCATTGGCTTT	0.89	195
ENO	ATTATTATTGGCCCTGCATTGG	GCAAACTGCAAGAGACACTGCT	0.86	159
PK	CAGCAACATCTTTCCATCACATTC	CAGGATCTGCTAGGGCTTCTCA	0.85	233
PGM	TTGTTTCTGGTGATGGTCGCTAT	AAATGCTCCTGTTGCTTTCGAT	0.84	182
PEPC	AACCAAAAAGAGGACGCTTGC	TTGACATCCGACAGGAATCTGA	0.84	150
MAE	TTCGACAAGGGACTCATATACCC	ACAGCTTTCAGCATACTTGACCA	0.79	150
EPI	GTTGACTGCGGGGAAGAGAAAT	ACGACCACTGAAGGGAAGTTTG	0.85	239
DAHPS	CCTGCCATTCTTCCCACCTT	CATTTCCACCCATTGTTTTCG	0.81	226
PDCE1	ATTTGCGTTCAATTAGGCCACT	AGATTAGCTGCATAGGGCATGG	0.84	211
AACT	CCATTATTGATGGCATGCTG	CCCCAGAAACTTCAACAGGA	1.20	207
HMGS	TTCTATGCCCAGAAGGCTGT	GGAACACTTGCTGCTGAACA	1.23	194
HMGR1	AGGGTGCAAACAAAGAGTCG	ACATTGCTGGGACCAGATTC	0.77	199
HMGR2	AGTGGCGGATACTGTGTGTG	TGCTCACAGGTCAATTGGAG	0.60	249
MK	CTGACTCGCATCAAGTCCAA	TCATCTGGAGCAGGTGACTG	0.90	197
PMK	GGCAAAAGCTTGGAGTTCAC	CAGATTGCATTTCCCACCTT	0.82	201
MVD	GCGGAGTCATGGGTGATAAT	CATTAAGCCACATCCGATCC	0.87	199
DXPS2	TGCCATTTTGGGATATGGTT	CATGGGAAGAAAAACCTCCA	0.78	200
DXPS3	TTCTGGATCAACCCTCTTCG	ACCCCATGGTACTTGTCTGC	0.76	198
DXPR	CCCGATATGCGTTTACCAAT	TCATTCGCAGCACTAAGCAC	0.71	209
IPPI	AGCTTGGTATCCCTGCTGAA	CCTTCAACTGATCCCGGTTA	0.92	198
PT1	TGCATGCTGTTGAAGAATCC	CCCCAGAAGTTCGAATCAGA	0.60	201
PT2	GAGCAACGTTGTAGCATGGA	GAAGCAGATGTGCCTGTGAA	0.63	203
PT4	AATGCTGACCTCGACACACA	GAAGCATCGACGAGACGTAA	0.52	200
REF1	AAGCCTGGCGTCAATACTGT	AGCTCCTGGGGCTACTGAAT	0.71	198
REF3	CAGGGGGAGGGGTTAAAATA	TTGAGAGCTCCATTGGGAAT	1.04	200
SRPP1	CAAGCAGGTGTCTGCTCAAG	CGGTGACTGCACATTGTTCT	1.01	200
SRPP2	ATTGAGGGTGTGGCAAAGAC	AAGGTTAGCGAACGCATGTT	0.90	200
SRPP3	GTTCGTGGCACTACTGAGCA	GCCCTTTATTCCACATCCAA	1.12	200

Table S4. Pearson's correlations between expressions of genes involved in metabolic pathway and latex yield of PB 217 and PB 260.

Gene	Correlation coefficient: Expression and Yield/cm girth (g/cm)
NINV	0.749
SUSY	0.893
HK	-0.115
PFK	0.060
GAPDH	-0.053
NGAPDH	-0.008
PGRM	0.832
ENO	0.003
PK	-0.420
PGM	0.486
PEPC	0.735
MAE	0.901
EPI	-0.037
DAHPS	-0.464
PDCE1	0.567
AACT	0.922
HMGS	-0.020
HMGR1	0.426
HMGR2	-0.974
MK	-0.676
PMK	0.540
MVD	-0.075
DXPS2	0.164
DXPS3	-0.052
DXPR	-0.900
IPPi	0.865
PT1	-0.929
PT2	-0.147
PT4	-0.652
REF1	0.169
REF3	-0.036
SRPP1	-0.015
SRPP2	0.186
SRPP3	0.354

Table S5. Pearson's correlations between expressions of *PDCE1* and latex yield of PB 217 virgin and tapped trees.

Gene	Correlation coefficient: Expression and Yield/cm girth (g/cm)
PDCE1	0.866

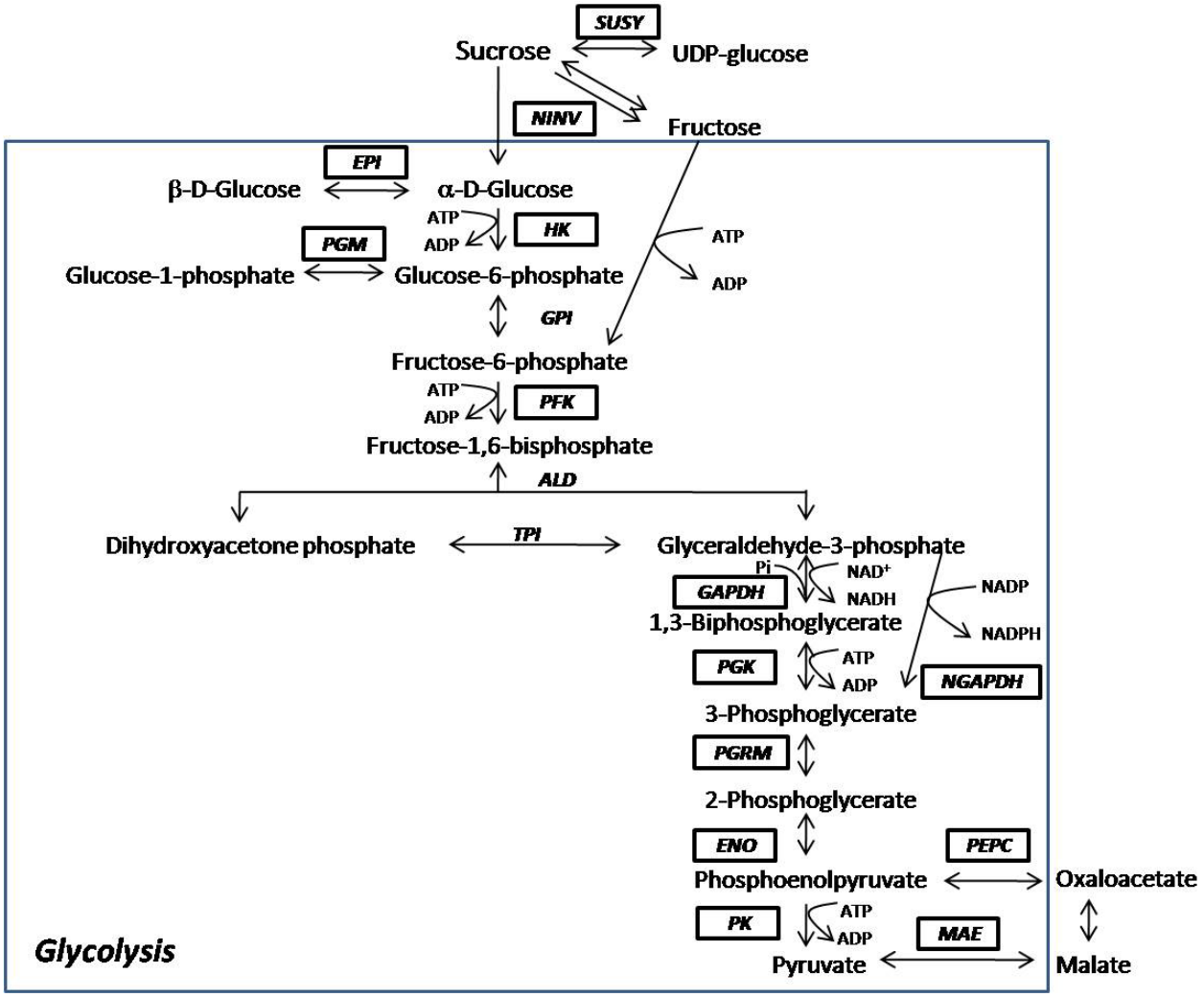


Fig. S1. Glycolysis and continuation pathway (adapted from Jacob et al., 1989). ALD: fructose phosphate aldolase, ENO: enolase, EPI: aldose-1-epimerase, GAPDH: phosphorylating glyceraldehyde-3-phosphate dehydrogenase, GPI: glucose-6-phosphate isomerase, HK: hexokinase, MAE: malate dehydrogenase, NGAPDH: non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase, NINV: neutral invertase, PEPC: phosphoenolpyruvate carboxylase, PFK: phosphofructokinase, PGK: phosphoglycerate kinase, PGM: phosphoglucomutase, PGRM: phosphoglycerate mutase, PK: pyruvate kinase. SUSY: sucrose synthase, TPI: Triose-phosphate isomerse.

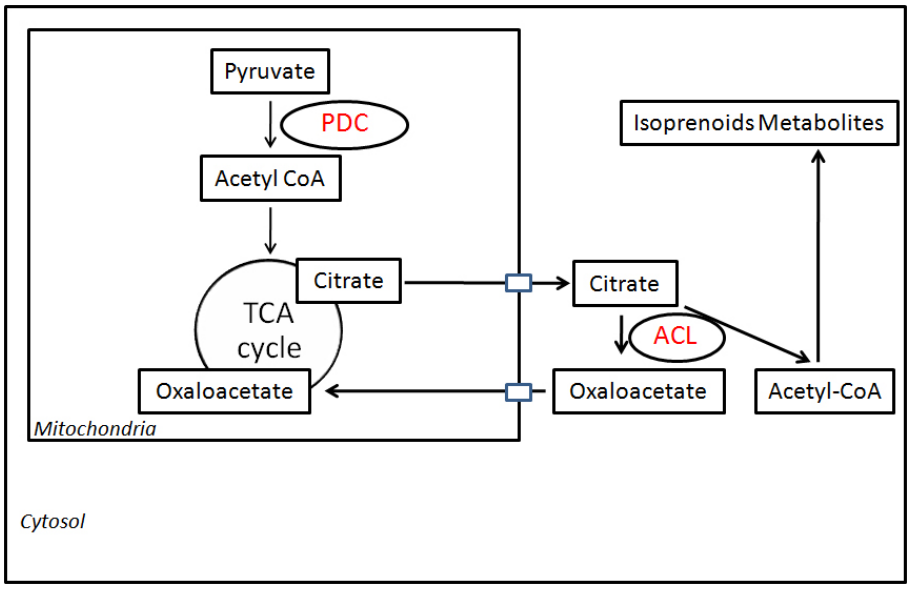


Fig. S2. Compartmentalization of acetyl-CoA metabolism. Acetyl-CoA is produced by pyruvate dehydrogenase complex (PDC) in mitochondria and plastid and consumed in their compartments. Acetyl-CoA lacks of its transporter, and then it has to be transported in the form of citrate. Citrate is transported to cytosol and changed to acetyl-CoA by ATP-citrate lyase (ACL). Precursor of isoprenoids, which produces in cytosol, is acetyl-CoA. It might come from these three compartments.

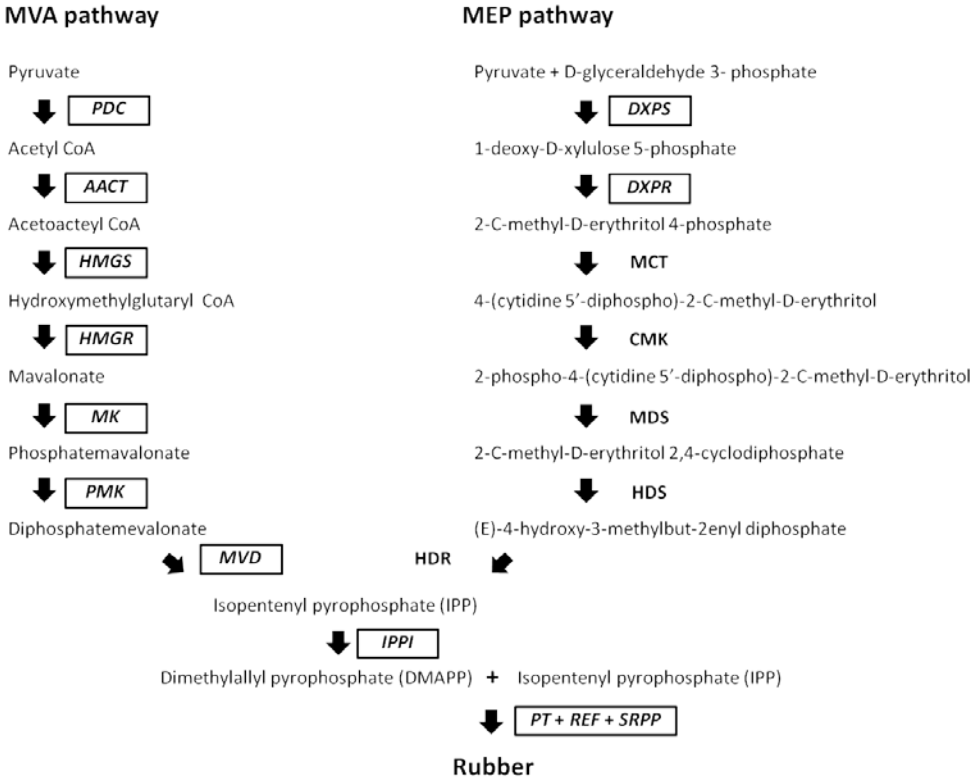


Fig. S3. Rubber biosynthesis pathway. IPP precursor may be contributed by the cytosolic MVA and plastidic MEP pathways (Adapted from Chow et al., 2012). AACT: acetyl-CoA acetyltransferase, CMK: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase, DXPS: 1-deoxy-D-xylulose 5-phosphate synthase, DXPR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase, HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, HMGR: 3-hydroxy-3-methyl-glutaryl-CoA reductase, HMGS: 3-hydroxy-3-methyl-glutaryl-CoA synthase, IPPI: isopentenyl pyrophosphate isomerase, MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MK: mevalonate kinase, MVD: mevalonate diphosphate decarboxylase, PDC: pyruvate dehydrogenase complex, PMK: phosphomevalonate kinase, PT: prenyl transferase, REF: rubber elongation factor, SRPP: small rubber particle protein.

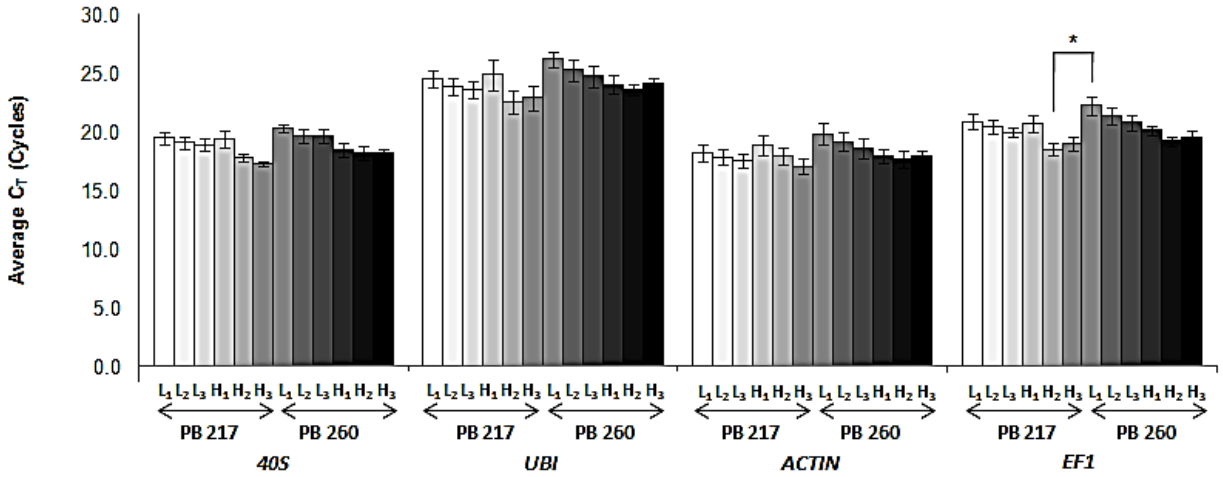


Fig. S4. Validation of four housekeeping genes (*40S*, *UBI*, *ACTIN* and *EF1*) expression by qRT-PCR. PB 217 (L1-L3) and PB 217 (H1-H3) were low and high latex yielding trees of PB 217, respectively. PB 260 (L1-L3) and PB 260 (H1-H3) are low and high latex yielding trees of PB 260, respectively. The error bar showed standard error of three independent experiments with three replicates (n = 9). An *asterisk* indicates transcription level values that are significantly different between PB 217 H2 and PB 260 L1 of *EF1* expression (One-Way ANOVA with Tukey's test, $P < 0.05$).

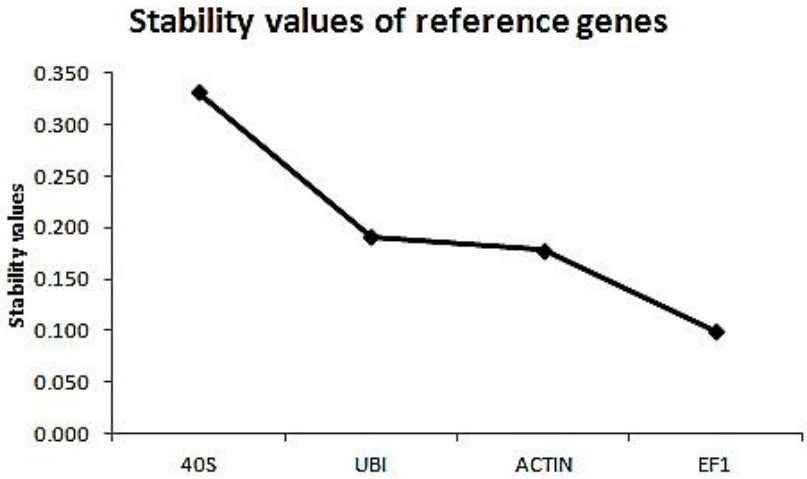


Fig. S5. Expression stability values of control genes.