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EXPRESSION OF RUBBER ELONGATION FACTOR (*REF*) AND SMALL RUBBER PARTICLE PROTEIN (*SRPP*) RELATES TO DRY RUBBER YIELD OF CLONAL VARIETIES

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ABSTRACT:

REF and *SRPP* are necessary for a functional role in rubber polymerization. It involved in the final polymerization step of natural rubber biosynthesis in *Hevea.* The aims of this research were to relate the *REF* and *SRPP* gene expressions and latex yield, in order to use these genes as markers in selection criteria of rubber tree breeding programs. Dry rubber yield and expressions of the two genes were determined for four selected clones (SK1, SK3, NK1 and T2) and the by location paired controls (RRIM 600 clone). Results from cDNA sequencing indicated the *REF* amino acid sequence of lower yield clone present one amino acid, which is different from Proline (CCC) as Serine at site 113 (CCT)**.** The *SRPP* amino acid sequences, Glutamic acid are absence at site 6 of RRIM 600. One amino acid at site 67 of RRIM 600 clone was different from other clone resulting in Threonine (ACT) instead of Alanine (GCT). In NK1 clone, at site 178, different in one protein was recorded different code protein from Threonine (ACT) to Valine (GTT). The annual dry rubber yields of the four clones exceeded that of RRIM 600. Quantitative RT-PCR showed a positive relationship between latex yield and expression levels of *REF* and *SRPP* genes. In seedling leaves, *REF* and *SRPP* genes were expressed at low level, but both genes were abundantly expressed in bark. These results suggest good potential in the early selection of rubber trees, in breeding programs for yield improvement.

Keywords: Hevea brasiliensis, Rubber Elongation Factor, Small Rubber Particle Protein, Gene Expression, molecular marker

INTRODUCTION

Rubber trees are the main source of natural rubber, an important raw material for many industries; for example gloves and airplane tires require natural rubber (Han *et al*., 2000). Thailand is the world leading natural rubber producer. The majority of rubber trees cultivated in Thailand are RRIM 600 clones, a popular clone grown for more than 50 years in this country that contributes approximately 75% of the total plantation rubber (Nakkanong *et al*., 2008). In a Para rubber breeding program, phenotypic evaluation is a tedious process due mainly to long juvenile phase (6-7 years) (Venkatachalam *et al*., 2007). Conventional breeding methods may take at least 20-25 years to develop new variety. Early selection using molecular markers would certain -nly shorten the breeding cycles, and among the agronomic traits of rubber, high yield has the top priority. Molecular markers associated with yield would allow breeders to select high yielding clones during the 2rd or 3rd years of growth, in a breeding program (Priya *et al*., 2007).

Identifying genes involved in rubber biosynthesis pathway of a rubber tree is important for understanding the molecular mechanisms of rubber biosynthesis. It occurs in the cytoplasm of highly specialized latex cells called laticifers (Priya *et al*., 2006). It has been reported that *REF*, a major protein located on the surface of large rubber particles in latex, is highly expressed in laticifer tissue (Oh *et al*., 1999). *REF* encodes an enzyme involved in rubber biosynthesis via its functional role in rubber (*cis*-1,4-polyisoprene) polymerization (Dennis *et al*.,1986; Dennis and Light, 1989). Additionally, *HbREF* promotes latex biosynthesis in vitro, and its expression level is correlated with latex yield (Dennis and Light, 1989; Priya *et al*., 2006). Priya *et al.,* (2007) reported that the expression level of *REF* was higher in a high yielding rubber clone than in a low yielding clone. In addition, Oh *et al*. (1999) reported a novel cDNA of a *Hevea* protein, called small rubber particle protein (*SRPP*), associated with small rubber particles. The sequence analysis revealed that this protein is highly homologous to the *REF* gene and also involved in rubber biosynthesis (Laibach *et al*., 2015; Dennis and Light, 1989), and this REF family is represented

in several other stress-related proteins (Laibach *et al*., 2015; Dennis and Light, 1989). Their expressions are stimulated by stress such as tapping and can induce the wounding or tapping panel dryness syndrome (Berthelot *et al*., 2014). Rubber elongation factors (*REF*) and the small rubber particle (*SRPP*) have been reported as the key genes involved in rubber biosynthesis. Accordingly, the abundances of *REF* and *SRPP* mRNA transcripts could then be used as indicators of high yield rubber clones (Priya *et al*., 2007). According to prior studies these two proteins are directly involved in rubber biosynthesis (Chotigeat *et al*., 2010; Dennis and Light, 1989; Yeang *et al*., 1996). Especially the *SRPP* is confirmed as being involved in rubber biosynthesis *in vitro* (Oh *et al*., 1998).

Nualsri *et* al., (2009) selected high latex yield rubber clones from smallholders' rubber plantations in Southern Thailand. The selected clones were new recombination clones caused by natural crossing, from eight areas in southern Thailand by using RAPD and SSR technique, which are difference with recommend' clones of Rubber Institution of Thailand. This four selected clones were chosen similar to RRIT 251 clone, which was found from natural crossing in rubber plantation in Songkhla, Thailand by Rubber Research Institute of Thailand. RRIT 251 clone was accepted as high yielding clones for latex in Thailand (Susewee, 2001). Next, four clones (SK1, SK3, NK1 and T2) were selected to a performance evaluation that included baseline RRIM 600. Results obtained over 5 months indicated that all the selected clones were superior to RRIM 600, as regards latex yield. In addition, various physiological parameters such as stomata conductance and photosynthesis rate were higher for the four selected clones than for RRIM 600. Recently, Pethin *et al*., (2015) reported that these four clones (SK1, SK3,NK1, and T2) were superior to RRIM 600 in yield performance, latex biochemical parameters and anatomical characteristics of the bark, when compared within the same field. This indicates the high potential of these clones as new preferred rubber clones in the future. So, they are of high interest when yield and gene expression are studied, for potential benefits in further breeding programs.

DNA sequence differences are a basic tool in the study of molecular genetics. However, there has been no report on cloning or expression profiles of *REF* and *SRPP* genes across several clonal varieties of rubber trees. In order to confirm the nucleotide sequences, the full-length cDNA sequences of *REF* and *SRPP* were isolated and characterized for several clones. Additionally, we cloned the full-length *REF* and *SRPP* genes from the four selected rubber clones that are high latex yielding varieties, as well as from an indigenous commonly farmed clone. This allows to compare for differences in the amino acid sequenced genes, and to study the nucleotide sequence relationships of the clones.

The main objective of this work was to investigate the correlation between latex production and the expression levels of *REF* and *SRPP,* in the four selected clones and the baseline RRIM 600. It was expected that the four selected clones may over express the genes of interest relative to RRIM 600, while the RRIM 600 clone has both good adaptability to varied growth conditions and also good yield performance in comparison to other previously recommended clones (Vinod *et al*., 2000). To determine the gene expression levels in seedlings, real-time PCR was used with the four selected clones' seedlings. Previously, Priya *et al.,* (2006) studied tissue specific gene expression in rubber seedlings that had the highest *REF* transcription levels in latex and bark. However, the one-year-old seedlings in our study had low latex activity, so bark and leaves were sampled for gene expression, hoping to find molecular biomarkers supporting future tree breeding programs.

MATERIALS AND METHODS

Plant materials: Four selected clones having high latex yield were sampled from four rubber plantations in three provinces of southern Thailand. Each clone was labeled by the sampling location. The private farm locations were in Natawee and Hat Yai districts of Songkhla province (labels SK1 and SK3), Nabon district in Nakhon Si Thammarat province (NK1), and Palian district in Trang province (T2). Based on information from the holders of the rubber plantations, the sampled clones originated from seedlings of open pollinated progenies. The experimental design was a randomized complete block with three replications. In each individual location, 15 plants each from of a selected rubber tree clone and from RRIM 600 were chosen**.** The selection of trees from a rubber plantation was made on the basis of daily yield pattern and girth at 100-150 cm height. The tapping system for all clones was third spiral downward cut, at two days tapped by one day rest (1/3S 2d/3), without stimulants to enhance latex flow. Latex of each clone was collected

and used for latex yield calculation and RNA isolation.

For gene expression levels, bark and leaves of SK1, SK3, NK1, T2,RIM 600 and indigenous clones were extracted from 1-year-old seedlings cultivated in an experimental plantation of the Department of Plant Science, Faculty of Natural resources, Prince of Songkla University, Songkhla, Thailand. All the samples were immediately frozen in liquid nitrogen at the time of collection, and then stored at -80°C or used immediately for RNA extraction.

Latex yield and rubber content: The dry rubber content (DRC) of latex and the fresh weight of latex yield were recorded for 15 trees along with RRIM 600 at each location, and the monthly dry rubber production was calculated about 1 year (2011-2012). The total latex was collected in the morning and weighed for fresh latex yield. The DRC was measured by the cup coagulation method: 10g of latex was weighed and 6.0% acetic acid solution was added directly into the latex cup and mixed well. The coagulated rubber was dried in a hot air oven at 65˚C for 24 hrs. The total solids after drying were considered to be dry rubber, which is the major component of rubber latex. The DRC percentage and dry rubber yield were calculated using the following equations:

$$\% \text{ DRC} = (\text{dry rubber weight/fresh latex weight}) \times 100 \quad (1)$$

$$\text{Dry rubber yield (g/tree/tapping)} = \text{fresh latex weight} \times \% \text{ DRC} \quad (2)$$

RNA extraction and cDNA preparation: Fresh latex of mature rubber trees was tapped from the four selected clones and RRIM 600 clones, and collected for RNA extraction and cDNA preparation. The latex sample was added into 15 ml tube containing 5 ml of RNA extraction buffer (0.1m Tris-HCL, 0.1m EDTA, 10% SDS, pH 9.5). The RNA was extracted from latex using the method described by Suwanmanee *et al*., 2002. Seedling samples were immediately frozen in liquid nitrogen at the time of collection and then stored at -80°C for RNA extraction. The extraction from seedlings was used an RNA extraction buffer (100mM Tris-HCL, 10mM EDTA 100 mM LiCl, 2% SDS, 100mM Tris base, 100mM HCl). The blended sample was extracted twice with an equal volume of phenol:chloroform (1:1, v/v). The RNA was precipitated in 2M LiCl at 4°C overnight, followed by a 10,000g centrifugation at 4°C for 20 min, and re-precipitation with 300 mM sodium acetate, at pH 5.2 with 2.5 volumes of ethanol at 20°C for 1 h. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated water. The RNA was then treated with RNase-free RQ1 DNase (promega) in order to eliminate DNA. The RNA quantity and quality of the total RNA extract was determined using a spectrophotometer. The integrity of the RNA was checked by electrophoresis in 1% agarose gel. The first strand cDNA synthesis reaction was performed for a 20 ul volume using random primers supplied with the Super Script ® Vilo ™ cDNA Synthesis kit (Invitrogen, USA), as described by manufacturer and the samples were then stored at -70 ˚C until use.

Cloning of cDNA encoding *REF* and *SRPP* genes: The full-length cDNA of *HbREF* and *HbSRPP* were cloned from the latex as the template. Amplification of the *REF* and *SRPP* genes was performed by Reverse Transcription-PCR (RT-PCR) using two sets of constructed oligonucleotide primers designed from the *REF* and *SRPP* sequences of the *Hevea brasiliensis*: *HbREF* forward primer 5'-CGATTATGGCTGAAGAC GAA-3'; *HbREF* reverse primer 5'-GGGCTCA ATTCTCTC CATAA-3'; *HbSRPP* forward primer 5'TTCAATTATGGCTGAAGAGGT-3'; and *HbSRPP* reverse primer 5'-TGCAGATTA TGATGCCTCAT-3'. Amplified fragment of sizes 417 and 615 bp were cloned and sequenced.

Sequencing and bioinformatics analysis: Sequence homology was determined using the automated sequencing facility at BigDye® Terminator v3.1 cycle sequencing kit at the First Base DNA Sequencing Services, Malaysia. Comparison of DNA and the predicted amino acid sequences in a non-redundant database was performed by BLAST analysis (http://www. ncbi. nlm.nih.gov). Multiple sequence alignments were generated using the ClustalX program (Thompson *et al*., 1997).

Gene expression analysis by Quantitative Real-Time PCR (qRT-PCR): For the qRT-PCR analysis, total RNA extracts from latex, bark and leaves were treated with RNase-free RQ1 DNase (promega) and used as templates for *REF* and *SRPP* cDNA synthesis. After the reverse transcription step, the total cDNA served as the template in the PCR implication. Each reaction was performed with a 20 ul reaction volume, composed of 10X PCR buffer, 50 mM $MgCl_2$,

10 uM dNTP mixture, 1 unit Taq polymerase, 1 ul of the first strand cDNA mixture and 10 mM of each primer. The 18s rRNA gene fragments were used in amplification as an endogenous control. The transcription levels of both genes in samples from mature trees and rubber seedlings were analyzed using quantitative real time PCR with ABI 7300 system. To select primers the PCR was performed in order to test the specificity of each primer at Tm 60°C. The reactions were performed using the SYBR® Green Real-Time PCR master mixes (Invitrogen, USA) following the procedures described by the manufacturer; each 20µl PCR mixture had 25ng cDNA. The PCR cycle program consisted of initial denaturation at 95°C for 10 min, followed by 35 two-step cycles of PCR with denaturation at 95°C for 15 sec, and annealing and polymerization at 59°C for 1 min. The normalized expression ratios were calculated using the comparative Ct method, with the formula: Qr = $2^{-\Delta\Delta Ct}$, where $\Delta Ct = (Ct_{\text{of target gene}} - Ct_{\text{of reference gene}})$ (Ruderman *et al*., 2012; Livak *et al*., 2011).

Statistical analysis: The data on latex yield and gene expression were analyzed using Student's t-test and its controls. The term "significantly" is used to indicate statistical significance at a conventional threshold level ($P \leq 0.05$). The correlation of dry rubber yield and gene expression was analyzed using Pearson's correlation. All the analyses were performed using the algorithms within R: a language and environment for statistical computing (version 2.15.2).

RESULTS AND DISCUSSION

Latex yields and Dry rubber content: Table 1 shows the average of dry rubber yields (g/t/t) per tapping. Dry rubber yield was calculated from fresh latex weight and %DRC (equation 2). Faster latex flow rate for a longer duration provides a higher latex yield (Tungngoen *et al*., 2009). These two factors are related to dry rubber content (DRC) or total solid content (TSC), and to latex coagulation efficiency (Kongsaw adworakul and Chrestin, 2003; Tungngoen *et al*., 2009). However, the DRC is positively linked to latex viscosity and thus inversely related to latex fluidity and yield (Tungngoen *et al*., 2009). During the experimental period, there were significant differences in dry rubber yield (g/t/t) between the cases. In the current study, the SK1 clones had the highest yields per tree. However, environmental effects are significant for *Hevea* growth and latex production (Rao *et al*., 1998). The latex regeneration of *Hevea* depends on soil moisture, nutrient status, weather, tapping season and tapping system (Rao *et al*., 1998). These effects on a rubber plantation are difficult to isolate and control (Rao *et al*., 1998; Vinod *et al*., 2000). Therefore, we used baseline RRIM 600 clones grown at the same locations as paired controls. The RRIM 600 clones have not only a good adaptability to environmental conditions, but also have good yield performance in comparison with other recommended clones (Vinod *et al*., 2000). In Table 1, the dry rubber yields of the four selected clones were higher than of the controls paired by location.

Table-1: The average dry rubber yields (g/tree/tapping) of SK1,SK3,NK1,T2 and RRIM 600 clones. The base line RRIM 600 clones were paired to the others by growth location, and the results are tabulated by location. The data were recorded from 2011-2012

Clones	Dry rubber yield	T-test	C.V.
SK1	94.06		
RRIM 600	75.56	*	16
SK3	58.59		
RRIM 600	48.45	*	28
NK1	93.19		
RRIM 600	53.49	**	10
T2	57.25		
RRIM 600	47.27	*	12

* = Significant difference at $P \leq 0.05$ ns = Non significance

DNA sequences analysis: In order to confirm the nucleotide sequences and the full-length cDNA sequences of *REF* and *SRPP*, these genes were isolated and characterized from the four selected clones, RRIM 600 and Indigenous. The nucleotide sequence analysis showed that the amplified *REF* and *SRPP* genes contained 417 and 615 bp open reading frames, encoding for 138 and 204 amino acids, respectively (Figures 1 and 2). The *REF* nucleotide and deduced amino acid sequences were submitted to NCBI database as shown in Table 2. The results from the present study are very similar to those reported earlier by Priya *et al*. (2006), in which the *REF* gene contained an open reading frame of 414 bp with a calculated Mw of 14.7 KDa and encoded a 138 amino acid peptide. In addition, the *REF* gene had 100% sequence identity to the *REF* sequences previously deposited in the database. For the *SRPP* gene, it had 615 bp encoding for 204 amino acids long polypeptide, and was 100% identical with the isoform of *SRPP* sequence in the database. This is similar to Oh *et al*. (1999) who reported that the sequence of *SRPP* cDNA contained a 612 bp open reading frame coding for 204 amino acids long polypeptides. Furthermore, different sequences for the individual clonal varieties (the four selected clones, RRIM 600, indigenous clone, and *Hevea* in NCBI database) were demons-

trated. It was found that the *REF* amino acid sequence of the Indigenous clone (LY) has one amino acid, so that Proline (CCC) is replaced by Serine at site 113 (CCT).

Table- 2: Summary from sequence analysis results of the genes encoding rubber biosynthesis, isolated from *Hevea brasiliensis*.

Gene	GeneBank Accession number	Length (bp)	Identity with the best homologs
REF (SK1)	KF734662	417(ORF)	*H. brasiliensis* (EU182586.1), AY120685.1, AB074308.1 (100%)
REF (RRIM 600)	KF734663	417(ORF)	*H. brasiliensis* (EU182586.1), AY120685.1, AB074308.1 (100%)
REF (LY)	KF734661	417(ORF)	*H. brasiliensis* (AY299405.2) (100%)
SRPP (SK1)	KF734666	615(ORF)	*H. brasiliensis* (HQ640231.1), AF051317.1(100%)
SRPP (NK1)	KF734665	615(ORF)	*H. brasiliensis* (HQ640231.1), KF734667, KF734664.1, KF734666.1, AF051317.1 (99%)
SRPP(RRIM 600)	KF734667	612(ORF)	*H.brasiliensis* (HQ640231.1) (99%)
SRPP (LY)	KF734664	615(ORF)	*H. brasiliensis* (HQ640231.1), AF051317.1(100%)

The *SRPP* amino acid sequence at site 6 had no Glutamic acid present for RRIM 600. At site 67 the RRIM 600 clone had Alanine (GCT) changed to Threonine (ACT). The NK1 clone has one protein at site 178, changed from Threonine (ACT) to Valine (GTT). These SNP site of *REF* and *SRPP* genes from Indigenous clone, RRIM 600 and NK1 clone were revealed through comparison of the different sequences between high and low yield clones (Figures 1 and 2). Therefore, amino acid of individual clone changes may affect the expression, structural and function in gene product toward rubber biosynthesis and latex regeneration. This results similar to report of Mantello *et. al.* (2014) who revealed the MVA pathway of GT1, RRIM 606, RRIM 701 and RRIM 728 contained a deletion polymorphism from positions 161 to 168 bp, whereas, PR 255 which was high yielding clones in Sao Paulo State, was contained a insertion polymorphism from positions 161 to 168 bp. Moreover, Devitt *et al.* (2009) revealed SNP mutation of lycopene beta cyclase (*lyc-β*) gene at position 607 and 881 affected to difference color of fresh papaya fruit between Tainung and Hybrid 1B cultivars. This result has potential application to selection of fruit color in papaya breeding through the development of a molecular marker assay the TT insertion in *lyc-β*. Similarity to Yadav *et al.* (2015) who exhibited a non-synonymous SNP in 12 soybean cultivars of India, which was amino acid sequence of *Mungbean Yellow Mosiac India virus* (*MYMIV*) gene was changed from proline to alanine. This study revealed that non synonymous SNP of *MYMIV* in soybean cultivar was affected to *MYMIV* gene expression and can be use as molecular tools for select between resistance and susceptible soybean. For *SRPP* sequencing, we found GVV motif in position 83 that could play the role of a beta-sheet breaker (Berthelot *et al*., 2012). Berthelot *et al.,* (2012) suggested that REF and SRPP proteins are hydrophobic and *REF* has amyloid properties, contrary to *SRPP* that has an α-helical protein. Moreover, the *REF* is inserted into the membrane of the Large Rubber Particle (LRP) while the *SRPP* is bound to the surface of the Small Rubber Particle (SRP). Wititsuwannakul *et al*., (2008) reported that *SRPP* has an important role in the latex coagulation to protect plants by wound sealing, thus suggesting an important role for *TbSRPPs* in rubber biosynthesis and plant stress. An analysis of the full length for both genes across the different clones revealed 99-100% similarity to previous sequences in the GenBank. The observed differences in the amino acid sequences should not affect transcription levels, as they do not coincide with important motifs or domains.

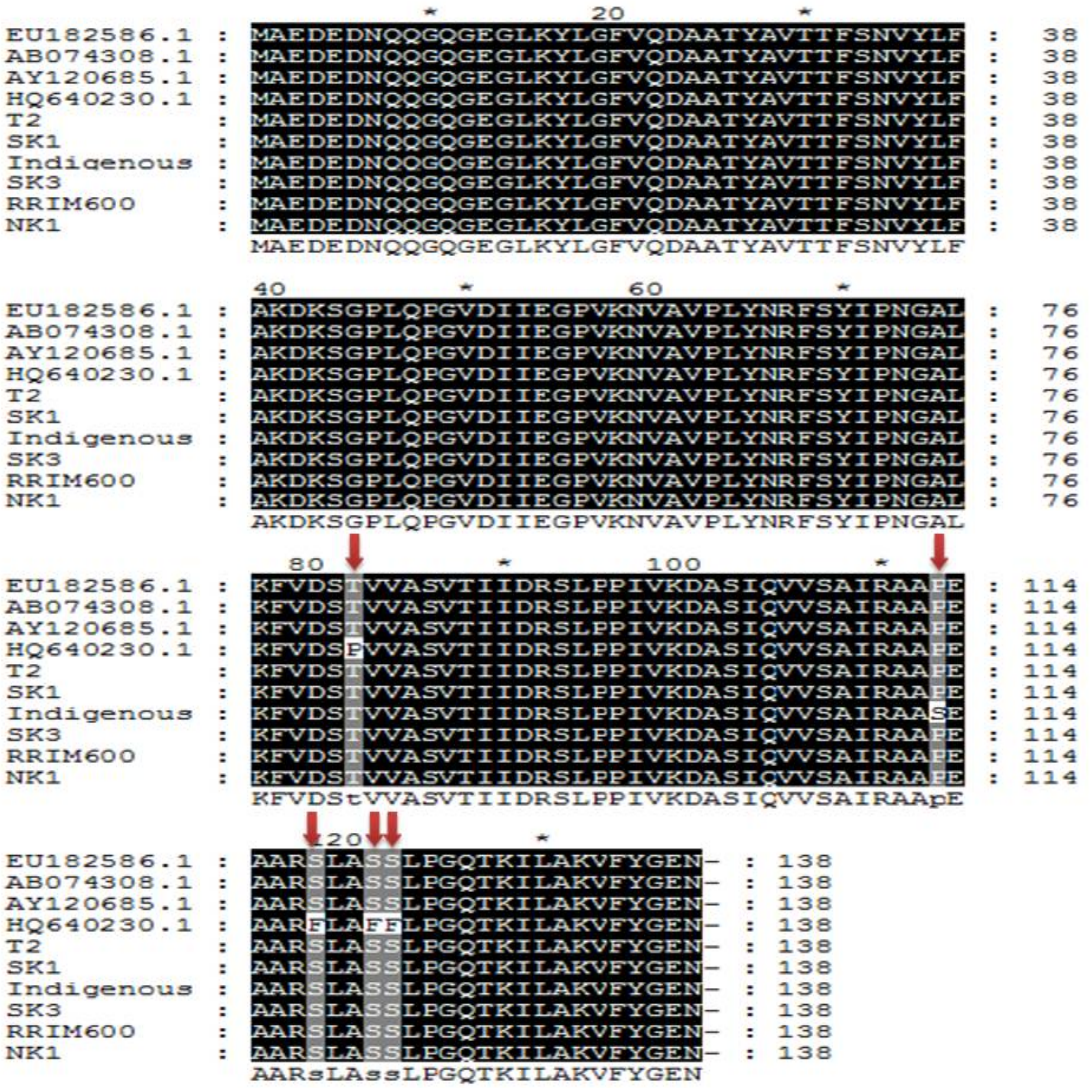

Figure -1: Multiple alignments of the amino acid sequence for *REF* gene in *H. brasiliensis*. Accession numbers: HQ640231.1, AY120685.1, ABO 74308.1, EU182586.1, KF734663 (RIM 600 clone); KF734662 (SK1 clone); KF734661 (Indigenous clone); no accession numbers assigned to SK3 clone, NK1 clone, and T2 clone. The amino acids high lighted in black were 100% homology conserved while minor differences were found in the amino acid sequences.

***REF* and *SRPP* genes expression by qRT-PCR analysis:** The latex of rubber trees is produced by specialized cells known as laticifers (Oh, *et al*., 1999). We studied the expression profiles of two genes to assess their potential roles in rubber biosynthesis. We used quantitative RT-PCR to compare the expression profiles of the two genes in latex of mature trees, and in two tissues of immature seedlings: bark and leaves. The qRT-PCR has several advantages over other PCR based quantification approaches, including its broad quantification range (Exposito-Rodriguez *et al.,* 2008)**.** Thus, qRT-PCR is an effective method for initial checking of gene expression in the clonal rubber tree varieties of our study. The *REF* and *SRPP* genes were used as a candidate molecular marker in the four selected clones, along with RRIM 600 and Indigenous clone (LY). Interestingly, both these genes are predominantly expressed in the latex followed the bark of *Hevea,* and correlate with the dry rubber yield (Oh *et al.,* 1999; Priya *et al.,* 2007, Berthelot *et al*., 2012).

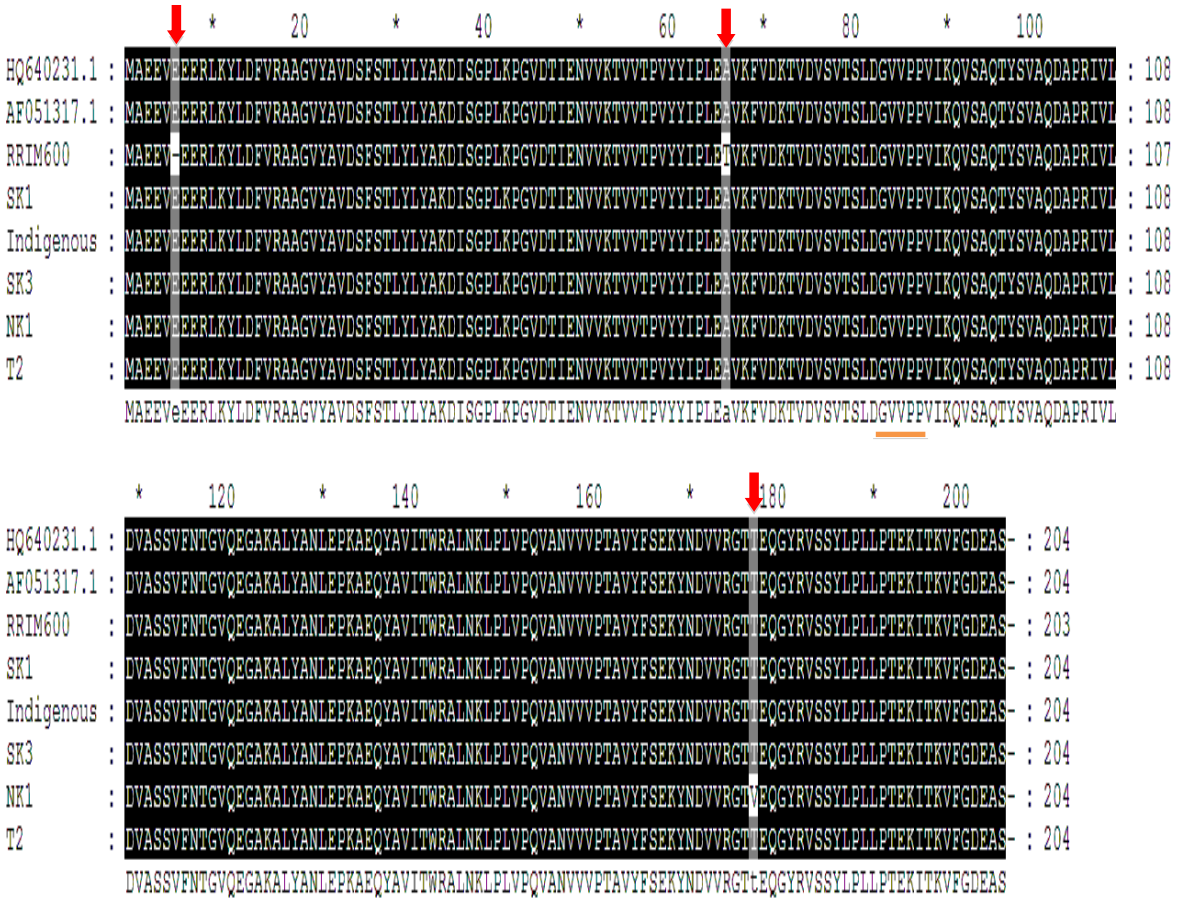

Figure -2: Multiple alignment of the amino acid sequences for *SRPP* gene in *H. brasiliensis*. Accession numbers: AF051317.1, HQ640231.1, KF734667 (RIM 600 clone); KF734666 (SK1 clone); KF734664 (Indigenous clone); KF734665 (NK1 clone); and without accession numbers T2 and SK3 clones. Amino acids highlighted in black were 100% homology conserved while minor differences were found in the amino acid sequences. The underline indicates the GVV motif.

Gene expression in latex of mature rubber tree: The genes related to rubber biosynthesis are highly expressed in latex (Oh *et al.,* 1999). The expression levels in latex of *REF* and *SRPP* genes were compared between the selected clones and RRIM 600, paired by growth site (Figures 3and 4), and the transcription levels were higher in the selected clones. Previously, Priya *et al*., (2007) reported that *REF* transcription level can distinguish between high yielding clones (RRII 105, PB 235 and PB260) and low yielding clones (KRS 25, KRS 128 and KRS 163). Besides, in *Taraxacum kok-saghyz*, *SRPP* is involved in controlling rubber content and molecular weight (Collins *et al*., 2009). These findings are supported by Wititsuwannakul *et al*. (2008), reporting that the *SRPP* binds to a *Hevea* latex lectin protein and induces latex coagulation. The SRPP family has a broader role in the rubber biosynthesis, not only influencing rubber polymerization and rubber quality but also influencing latex coagulation (Collins *et al*., 2009).

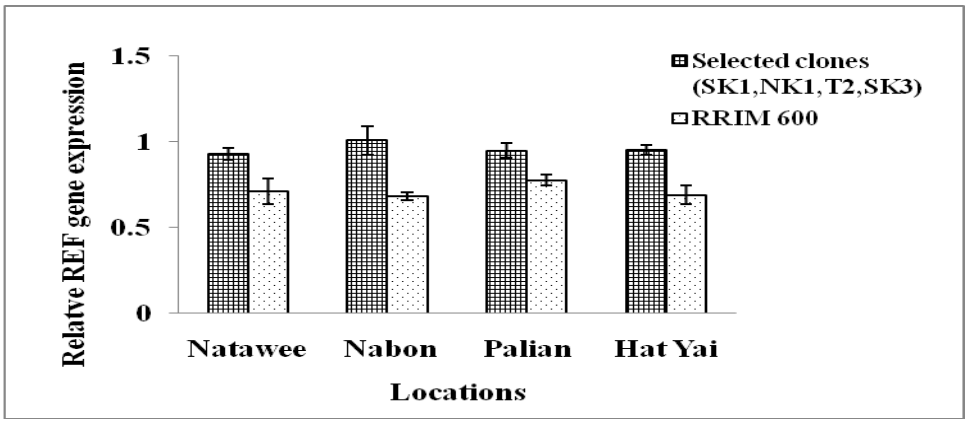

Figure -3: *REF* transcription levels measured by qRT-PCR, in the four selected clones shown with paired RRIM 600 baseline clones grown in the same location. The bars represent mean ± SD from triplicate samples. The clone labels in the legend match the order of locations from left to right.

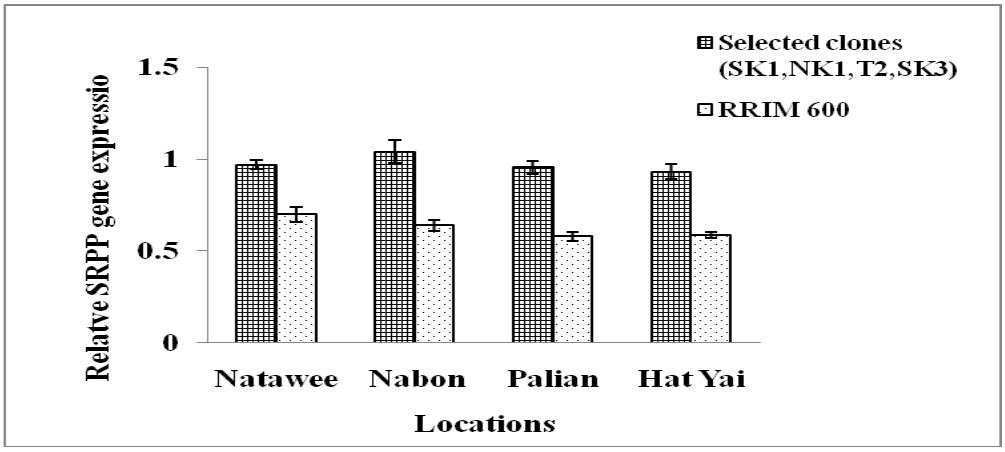

Figure -4: *SRPP* transcription levels measured by qRT-PCR, in the four selected clones shown with paired RRIM600 baseline clones grown in the same location. The bars represent mean ± SD from triplicate samples. The clone labels in the legend match the order of locations from left to right.

Gene expression in rubber tree seedlings: We assessed the expression of *REF* and *SRPP* genes in the bark and the leaves of one-year-old plants (Figure 5 and 6). Both genes had higher transcription levels in the bark than in the leaves, similar to the report of Priya *et al*. (2007) in which the *REF* transcription level of seedling rubber trees was predominant in the bark higher than in the leaves. Surprisingly, both these genes' transcription levels appear able to distinguish between high and low yielding clonal varieties, already in the tissues of rubber tree seedlings. Especially the bark of rubber seed lings is suitable for an early check of the gene expression, which can be used for selection in rubber tree breeding. The high level of gene expression in bark may be due to its high number of laticifers, while the leaves of seed lings have less of these (Priya *et al.,* 2007). Furthermore, the SK1 clone had the highest *REF* and *SRPP* transcription levels, followed by NK1 (Figure 5 and 6). The SK1 and NK1 clones are superior to RRIM 600, and on comparing seedling plants grown at the same location these two clones also displayed higher transcription levels. These data corroborate a previous investigation by Pethin *et al*., (2015), with results indicating potential biomarkers for the early selection of rubber trees in breeding programs.

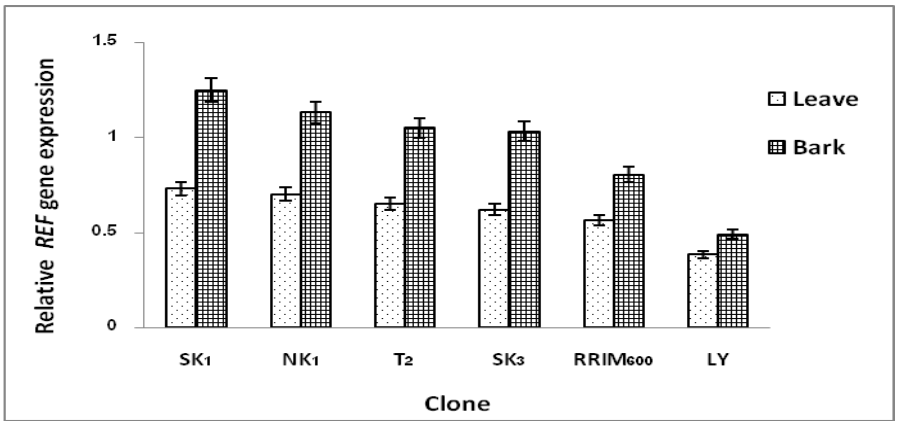

Figure- 5: The levels of *REF* transcripts measured by qRT-PCR, in the leaves and the bark from the four selected clones, baseline RRIM 600, and an indigenous clone, at one-year-old seedling stage, grown at one shared location.

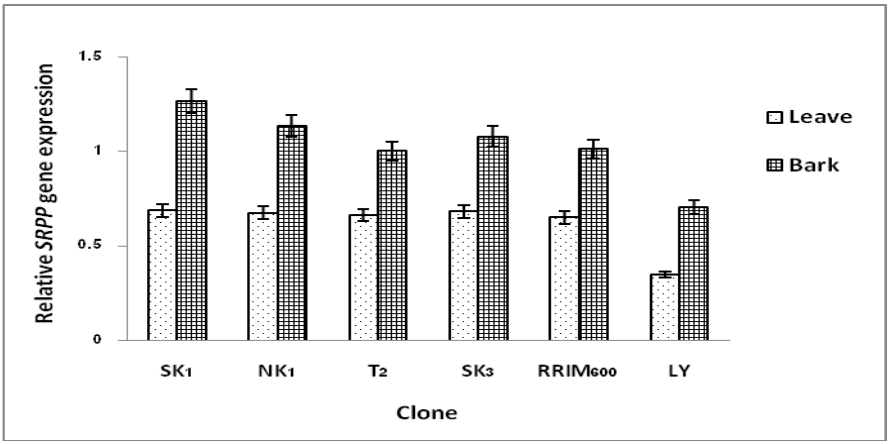

Figure -6: The levels of *SRPP* transcripts measured by qRT-PCR, in the leaves and the bark from the four selected clones, baseline RRIM 600, and an indigenous clone, at one-year-old seedling stage, grown at one shared location.

Statistical analysis: The analysis of Pearson's correlation in this current study gave significant positive association between the expression of *REF* and *SRPP* genes and the dry rubber yield (Table 3). This confirms a previous report (Ko *et al.,* 2003) that had the expression of these genes positively correlate in *Hevea*. However, the current study contradicts a previous report (Ruderman *et al*., 2012), in which the *REF* expression did not significantly correlate with the latex yield, whereas the expression of *SRPP* was negatively correlated to the clonal latex yield. Interestingly, the *SRPP* is involved in rubber biosynthesis in several plants and plays a role in the synthesis of rubber particles by bacteria *in vitro* (Kim *et al.,* 2004). Our results suggest these genes as potential molecular tools for the selection of high latex yielding rubber tree clones.

Table- 3: Pearson's correlations *REF* and *SRPP* expression with dry rubber yield (g/t/t) for SK1, SK3, NK1 and T2 clones, and baseline RRIM 600. Summary by location across observations from 2011-12.

Clone	Correlation coefficient (r) between Dry rubber yield and *REF* gene expression	Correlation coefficient (r) between Dry rubber yield and *SRPP* gene expression
SK1 RRIM 600	0.9690** 0.8340*	0.7822* 0.8610*
SK3 RRIM 600	0.8069* 0.8550*	0.7567* 0.7851*
NK1 RRIM 600	0.8640* 0.7560*	0.8579* 0.7581**
T2 RRIM 600	0.8395* 0.8354*	0.8394* 0.8039*

* = significant difference at P≤0.05, LSD ** = significant difference at P≤0.01, LSD

CONCLUSIONS: The present study covered four new-generation clones, the majority of which originated from seedlings of open pollinated progenies. It confirmed superiority in terms of latex rubber yield of the clones SK1, NK1, T2 and SK3 (named according to sampling locations, see main text for details) over the high yielding baseline RRIM 600 clone, which is common in Thailand. The results lend support to the benefits of these new rubber clones in Thailand. According to qRT-PCR analysis, the mRNA accumulation of both *REF* and *SRPP* was positively correlated with the latex yield, in terms of the dry rubber amounts collected from the high yielding clones. In one year old seedlings that can't be assessed for latex production, we found that the expression of *REF* and *SRPP* genes was higher in bark than in leaves. The bark appears a good choice to sample from seedlings, for determining the transcription levels of rubber biosynthesis genes. In this study the SK1 clone was superior in both dry rubber yield and in expresion levels of the two genes studied, followed by the NK1 clone. This suggests that these two clones should have a high priority in the further breeding of rubber trees to improve the latex yield.

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