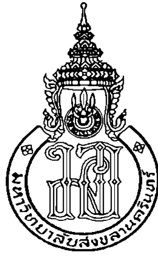

Selection of High Latex Yield Rubber Clones by Rubber Elongation Factor (*REF*), Small Rubber Particle Protein (*SRPP*) and Sucrose Transporter (*SUT*) Genes

Auksorn Klaewklad

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Plant Science

Prince of Songkla University

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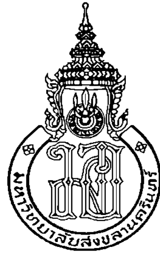

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Major Program Plant Science

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Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

………………………………………. Signature

(Miss Auksorn Klaewklad)

Candidate

ชื่อวิทยานิพนธ์ การคัดเลือกพันธุ์ยางพาราที่ให้ผลผลิตน้ำยางสูงโดยใช้ยีน Rubber Elongation Factor (*REF*) ยีน Small Rubber Particle Protein (*SRPP*) และยีน Sucrose Transporter (*SUT*)

ผู้เขียน นางสาวอักษร แคล้วคลาด

สาขาวิชา พืชศาสตร์

ปีการศึกษา 2558

บทคัดย่อ

การใช้เครื่องหมายโมเลกุลในการคาดคะเนปริมาณผลผลิตจะช่วยลดระยะเวลาในการคัดเลือกพันธุ์ยางพารา ดังนั้นการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์น้ำยาง สามารถ นำมาใช้ เป็นเครื่องมือใน การคัดเลือก พันธุ์ใน โปรแกรมการปรับปรุงพันธุ์ยาง การศึกษาพันธุ์ยางพาราที่มีศักยภาพให้ผลผลิตน้ำยางสูงจำนวน 4 สายพันธุ์ คือ SK1 SK3 NK1 และ T2 โดยมีพันธุ์ RRIM 600 เป็นพันธุ์เปรียบเทียบ ทำการศึกษาใน 4 อำเภอคือ อ.นาทวี (SK1) อ.หาดใหญ่ (SK3) จังหวัดสงขลา อ.นาบอน (NK1) จังหวัดนครศรีธรรมราช และ อ.ปะเหลียน (T2) จังหวัดตรัง เก็บข้อมูลผลผลิตน้ำยางเป็นระยะเวลาสองปีตั้งแต่เดือนมิถุนายน พ.ศ. 2554 ถึงมีนาคม พ.ศ. 2556 พบว่ายางพาราทั้งสี่สายพันธุ์มีค่าเฉลี่ยของ ผลผลิตยางสูงกว่ายางพาราพันธุ์ RRIM 600 คิดเป็น 27.20, 14.65, 32.70 และ 18.62 % ตามลำดับ จากการศึกษาแสดงออกของยีน *REF* และ *SRPP* โดยเทคนิค RT-PCR เริ่มจากการหาลำดับเบสของยีน พบว่ายีน *REF* และ *SRPP* มีความยาวของลำดับเบส 417 และ 615 bp และมีลำดับอะมิโนแอซิด 138 และ 204 อะมิโน แอซิด ตามลำดับ ส่วนการตรวจสอบลำดับเบสของยีนในกลุ่ม Sucrose transporter กับฐานข้อมูลใน NCBI พบว่ามีเพียงการแสดงออกของยีน *SUT3* เท่านั้น ซึ่งมีลำดับเบส 1,373 bp และมีลำดับอะมิโนแอซิด 457 อะมิโน แอซิด จากการทดลองครั้งนี้ พบว่าทั้งสี่สายพันธุ์คัดเลือกให้ผลผลิตและการแสดงออกของยีน *REF* และ *SRPP* สูงกว่ายางพาราพัน ธุ์ RRIM 600 ที่เป็นคู่เปรียบเทียบ ทำการยืนยันผลโดยศึกษาการแสดงออกของยีนโดยใช้เทคนิค qRT-PCR พบว่ายีนมีการแสดงออกสูงที่สุดในช่วงฤดูฝนซึ่งสอดคล้องกับปริมาณผลผลิต และก ารแสดงออกของยีน *REF* *SRPP* และ *SUT3* มีความสัมพันธ์เชิงบวก กับ ผลผลิตน้ำ ยาง นอกจากนี้ยังได้มีการศึกษาการแสดงออกของยีนในต้นกล้ายางโดยสกัดส่วนอาร์เอ็นเอจากส่วนเปลือกและใบ ของต้นกล้าอายุ 1 ปี พบว่าเปลือกมีระดับการแสดงออกของยีนทั้งสามยีนสูงกว่าการแสดงออก

ในใบ จากผลการทดลองแสดงให้เห็นว่าสามารถใช้การแสดงออกของ ยีน *REF SRPP* และ *SUT3* เป็นเครื่องหมายในการคัดเลือกพันธุ์ยางที่ให้ผลผลิตสูง ซึ่งสามารถทำได้ตั้งแต่ในระยะต้นกล้า

Thesis Title Selection of High Latex Yield Rubber Clones by Rubber Elongation Factor (*REF*), Small Rubber Particle Protein (*SRPP*) and Sucrose Transporter (*SUT*) Genes

Author Miss Auksorn Klaewklad

Major Program Plant Science

Academic Year 2015

ABSTRACT

Applying molecular marker for estimating the amount of production yield will help to reduce time for selecting rubber clones. Then the study of gene expressions related to rubber synthesis will be used as a clone-selecting tool for rubber breeding programs. For this study, four rubber clones with the potential of high latex were selected. Those were SK1, SK3, NK1, and T2 which RRIM 600 was used as a comparing clone. The study was carried out in 4 districts of Natawee district (SK1) and Hat Yai district (SK3) in Songkhla province, Nabon district (NK1) in Nakorn Si Thammarat province, and Paliean district (T2) in Trang province. Data were collected for 2 years from June 2011 to March 2013. This study found that all four studied clones had higher average values of dry rubber yields than RRIM 600 at 27.20, 14.65, 32.70, and 18.62%, respectively. For the study of gene expressions of *REF* and *SRPP* by RT-PCR technique with the beginning of finding cDNA sequencing, this study found that *REF* and *SRPP* had cDNA sequencing at 417 and 615 bp in length with encoding for 138 and 204 amino acids, respectively. For finding cDNA sequencing in the group of sucrose transporters available in NCBI database, the study found that there was only the expression of *SUT3* for cDNA sequencing at 1,373 bp with encoding for 457 amino acids. This study revealed that all four selected clones gave higher production yield and exhibited superior gene expressions than paired-control RRIM 600. These results were also confirmed by qRT-PCR technique and found that the highest gene expressions were recorded in raining season that agreed with the amount of production yield. Gene expressions of *REF*, *SRPP*, and *SUT3* had positive relationships

with latex yield. Furthermore, this study also investigated gene expressions by extracting RNA from barks and leaves of one-year old rubber seedling. The study indicated that barks had higher gene expressions of *REF*, *SRPP*, and *SUT3* than leaves. This study mentioned that gene expressions of *REF*, *SRPP*, and *SUT3* were molecular markers that could be used for selecting rubber clones that would give high latex yield since the seedling stage.

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Auksorn Klaewklad

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CHAPTER I

INTRODUCTION

1. Background

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is a major source of natural rubber production for more than 40,000 products such as airplane tires and groves that require natural rubber (Han *et al*., 2000; Priyadarshan *et al.,* 2009). Rubber is an economic plant important for smallholders in Thailand and Southeast Asia. Currently, Thailand is the world leader of natural rubber production and produces natural rubber approximately 3.573 million tons (Rubber Research Institute of Thailand, 2011). In 2010, total area for rubber production in Thailand was approximately 2.88 million ha (Charoenjiratrakoon, 2001). From the past until now, RRIM 600 has been the most popular rubber clone and the major cultivated variety of rubber plantations in Thailand (70-75%) and also accepted to be moderate to high latex yield clone (Nakkanong *et al.,* 2008). However, RRIM 600 is susceptible to phytophthora leaf fall disease and the white root disease (Thanseem *et al*., 2005; Keawchai and Soythong, 2010; Wattanasilakorn *et al*., 2012). Breeding new clones of rubber tree is needed to be carried out. High latex yielding is a primarily aim of rubber breeding program. Other desirable characteristics such as high initial vigor, smooth and thick bark with good latex vessel system, good bark renewal, and tolerance/resistance to major diseases are also the targets of breeding program (Venkatachalam *et al.,* 2013).

Nualsri *et al*. (2009) selected the high latex yield rubber clones from smallholders' rubber plantations in 8 areas of Southern Thailand. Selection focused on recombinant clones derived from natural out crossing. From 21 clones, four clones (SK1, SK3, NK1, and T2) were selected based on their performance evaluations compared to RRIM 600. Data obtained from 10 months of experiment with all selected clones produced higher latex yields than RRIM 600. From physiological parameters such as stomata conductance and photosynthesis rate, all four clones showed higher

performance than RRIM 600. Yield performance, latex biochemical parameters, and anatomical characteristics of those four clones were recorded. From data recorded, yield performances of four clones were superior to the controlled RRIM 600 (Pethin *et al*., 2015). However, in rubber breeding program, data collection will need 8-10 years before clones are recommended.

The conventional breeding program of rubber trees requires 20 to 30 years for one testing cycle (Venkatachalam *et al.,* 2007). Since most rubber breeding programs has been focused on high latex yields. Then the development of molecular markers of gene related to latex biosynthesis can be used as tools for determination of yields in the selecting process. If suitable molecular tools are available, then breeder can easily select elite clones during the 2rd or the 3th year of breeding programs (Priya *et al.,* 2007).

2. Literature Review

2.1 *Hevea brasilensis*

Natural rubber is produced by over 2,000 plant species confined to 300 genera of seven families such as Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae, and Sapotaceae (Cornish et al., 1993; Venkatachalam *et al.,* 2013). *Hevea* belongs to Euphorbiaceae family which has high rubber content, high quality, large volume of latex flow upon tapping, and harvesting easement (Kang *et al.,* 2000; Oh *et al*., 1999). However, *Hevea braziliensis* is the only species being used to produce the commercial product (Ko *et al.,* 2003). *H. brasiliensis* Muell. Arg. is a native rubber to Amazon River basin of South America (Priya, 2005). This native rubber is an important crop for smallholders in Southeast Asia, Over 90% of natural rubber latex is produced from Southeast Asia such as Thailand, Indonesia, and Malaysia (Tang *et al*., 2013).

Thailand is the world leading of natural rubber production. International Rubber Study Group (2004) reported that more than 64% of the world production came from the following countries of Thailand for 33%, Indonesia for 22%, and India for 9%. In 2010, total area for rubber production was approximately 2.88 million ha (Charoenjiratrakoon, 2011) with over one million smallholders in the country and most areas are in the southern part of Thailand (1.76 million ha).

2.2 Botany of *Hevea brasiliensis*

There are 10 species of *Hevea*. *Hevea brasiliensis* is the most important species. *Hevea* is a perennial tree crop which will grow best at temperatures of 20-28°C with a well-distributed annual rainfall of 1,800-2,000 mm. The rubber tree is quick growing, that can reach a height up to 25-30 meters (Rantala, 2006) with fairy straight trunk and bark which are usually grey and fairly smooth. The tree has a well developed taproot. The roots absorb water and nutrients from the soil underneath, and root will provide a base or anchor so that the tree is secure in the ground. The rubber tree has flowers and round shaped leaves. Young leaves are dark red in color, while other green on top and grayish underneath. The leaves collect sunlight to make energy through photosynthesis. Flowers are small, scented, unisexual, and short-stalked with large bell shaped female flowers at the terminal end of main and more numerous smaller male flower. *Hevea* produces nuts which are like seeds that come in a pod. The mature fruit is a large, compressed, 3-lubbed and 3-seeded ellipsoidal capsule wuth weighing 2-4 grams each (Figure 1). Rubber trees start yielding latex at 5 to 7 years after tapping and have a productive lifespan of 30-35 years. After that, replanting is necessary (Rantala, 2006).

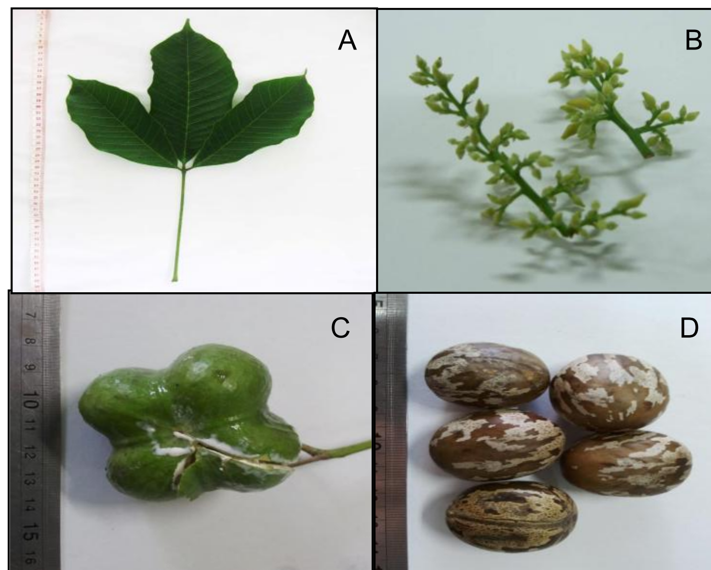

Figure 1. Morphological characteristics of *H. brasiliensis* or rubber tree (A: Leaf; B: Inflorescence; C: Fruits; D: Seeds).

2.3 Rubber introduction in Thailand

Rubber planting materials in Thailand were first introduced by Chao Phraya Ratsadanupradit who introduced some seed from Malaysia and planted at Trang province in Southern Thailand in 1899. After high yield rubber clones were developed in Malaysia, some clones were again introduced and commercial rubber planting was established. Early introduced clones of high yield rubber tree were Tjir1, GT1, RRIM 623, etc. Since 1967, RRIM 600 clone has been accepted as class 1 clone in Malaysia (RRIM, 1967) and was introduced to Thailand. RRIM 600 has been recommended for farmer and is popular continuously in Thailand until now. In the present, there are few clones varieties of rubber tree breeds planted in a large scale in Thailand. Even though RRIM 600 is well adapted to wide range of environment, RRIM 600 is quite sensitive to fungal diseases such as phytophthora leaf fall and the white root rot. Breeding for new clones of rubber tree needs to be done. Crop improvement mainly aims on higher yield and resistance to pathogens. Selection for high latex yields in rubber may need to keep

records on latex yields at least for 10-15 years after planting (Ong *et al.,* 2014). Searching of molecular markers related to latex yield potential will be beneficial to high yield selection and shorten of breeding program.

2.4 *Hevea* latex

Hevea latex is an economic product in the world market (Daengkanit, 2005). Latex from rubber tree has high elasticity, flexibility impact resistance, and efficient heat dispersion (Mooibroek and Cornish, 2000). The latex from *H. brasiliensis* is the specialized cytoplasm of laticiferous tissue or latex vessel in the inner phloem of the tree, which contains 30-40% (W/W) rubber (Sookmark *el al.,* 2002; Chow *el al.,* 2007). Laticiferous tissue is the major location of rubber biosynthesis (Chow *el al.,* 2007). The rubber latex (laticifers cytoplasm) is harvested by bark wounding or tapping (Ruderman *et al.,* 2012). The latex vessels run encounter clockwise 2-1/2° to the vertical. Thus, tapping will be in clockwise direction. The lutoid particles play an important role in the stability and latex flow in *Hevea* (Priya, 2005). The natural rubber latex from *Hevea* consists of 94% cis -1,4-polyisoprene and 6% protein and fatty acids (Berthelot *et al*., 2012). The chemical of natural rubber is 1-4 cis-polyisopren produced from the isoprenoid mevalonate pathway (Chow *el al.,* 2012; Chappell, 1995; Ruderman *et al.,* 2012) as shown in Figure 2.

2.5 Rubber biosynthesis

Natural rubber biosynthesis is a side-branch of the ubiquitous isoprenoid mevalonate pathway (Priya, 2005). Rubber biosynthesis involves fixation of carbon in the leaf specialized metabolic processes driving the precursors for biosynthesis and storage of polyisoprenes in the laticiferous cell. Sucrose is the carbon sources and energy supplies for rubber biosynthesis.

The rubber biosynthesis consists of two following steps: 1) changing the acetate as active isoprene and 2) the polymerization of isopentenyl pyrophosphate

(IPP) and DMAPP for initiation of the rubber chains and then of IPP into long *cis*-1,4-polyisoprene chains (rubber) (Ruderman *et al.,* 2012; Nuntanuwat, 2006).

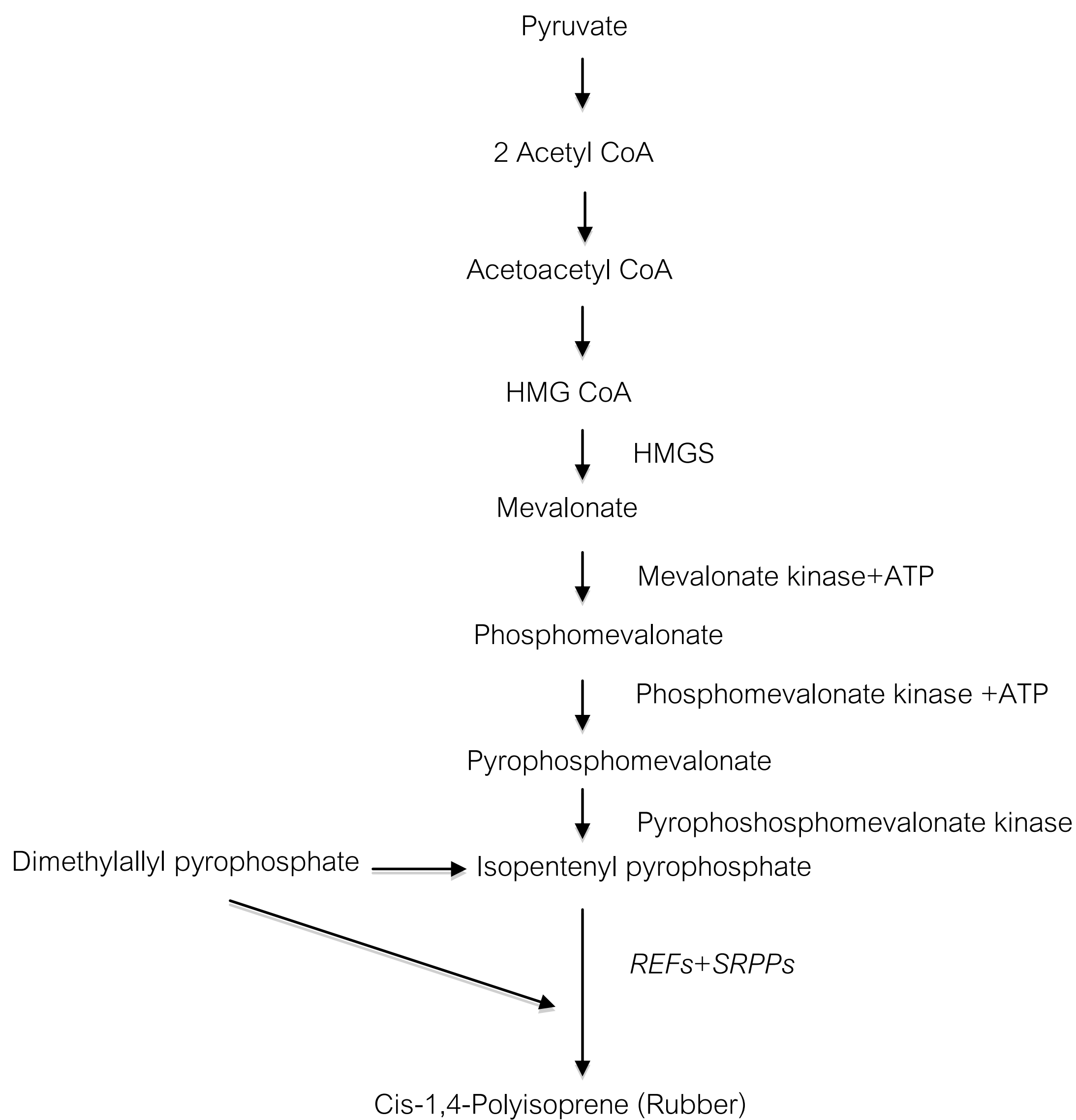

Figure 2 The mevalonate and rubber biosynthesis pathways

Source: Ruderman *et al.* (2012)

2.5.1 Changing the acetate as active isoprene

This step reaction starting from acetate to acetyl-CoA is catalyzed by the enzyme acetyl-CoA synthase from acetyl-CoA (Nuntanuwat, 2006). Mevalonate pathway, arising from the sequential condensation of three acetyl-CoA units to generate 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA). HMG-CoA is changed to MVA in an irreversible reaction catalyzed by HMG-CoA reductase (Suwanmanee *et al.* 2002, 2004; Sirinupong *et al.* 2005). MVA is converted into IPP via the enzymes mevalonate kinase, mevalonate 5-phosphate (MVAP) kinase, and mevalonate 5-diphosphate (MVAPP) decarboxylase such as adenosine triphosphate in *Hevea* latex (Nuntanuwat, 2006).

2.5.2 The Mevalonate Pathway

At this stage, the isomerisation of IPP to DMAPP (dimethylallyl diphosphate) by IPP isomerase and then of IPP into long *cis*-1,4-polyisoprene chains (rubber) by PT, assisted by the *REF* (Dennis and Light, 1989) and *SRPP* (Oh *et al*., 1999), which are located on the surface of the rubber particles (Ruderman *et al.,* 2012).

2.6 Gene related to biosynthesis of latex in rubber tree

By conventional breeding for using new clones of more rubber trees will need at least 25 years. Identification molecular markers with positive related to latex yield will benefit and speed up rubber breeding program (Venkatachalam *et al*., 2007). Expression of genes related to latex biosynthesis can be used as tools in early selection process. Early selection of high yield can be done in 2 or 3 years of breeding and will shorten breeding program (Priya *et al.,* 2007).

Kush *et al*. (1990) displayed the differential expressions of many rubber biosynthesis related genes in *Hevea* latex. The expressions of those genes in

laticifer were 20-30 folds enriched in transcripts encoding enzymes related in rubber biosynthesis and plant defense. The rubber elongation factor (*REF*), a key gene involved in the rubber biosynthesis (Dennis and Light, 1989), is over expressed in laticifers (Goyvaerts *et al*., 1991). However, other rubber synthesis-related genes, such as HMG-CoA reductase (Nuntanuwat, 2006), small rubber particle protein (*SRPP*) (Oh *et al*., 1999), *cis*- prenyltransferase (CIS), geranylgeranyl diphosphate synthase (GGPP), and isopentenyl diphosphate isomerase (IPP) (Venkatachalam *et al.,* 2007) are also highly expressed in the latex (Ko *et al.,* 2003). Ko *et al.* (2003) concluded that *REF* and *SRPP* were the most abundantly expressed genes in the latex from usually tapped trees of the RRIM 600 rubber clone. The over expression of *REF* and *SRPP* mRNA transcript levels could then be compared with rubber yield (Priya *et al.,* 2007) and be used as molecular markers for high yield collection. Both protein expressions are highly correlated with latex yield (Berthelot *et al*., 2012; Priya *et al*., 2007). *REF* and *SRPP* genes have played important role in the latex coagulation in rubber biosynthesis and plants protection by wound sealing (Wititsuwannakul *et al*., 2008). *SRPP* is specifically found in the laticifer layer of the conducting phloem while *REF* is localized in all laticifer layers (Berthelot *et al*., 2012).

Sucrose is an important precursor of natural rubber, and its transport into latex cells may be a key to latex generation (Dusotoit-coucaud *et al.,* 2010). Sucrose transporter has been reported to be a key role in many physiological processes (Dusotoit-coucaud *et al.,* 2009). Sucrose is important into the laticifers cell and limiting factor in latex production, including latex flow (Dusotoit-coucaud *et al.,* 2009). Metabolic pathways of laticifers need sucrose as a precursor for rubber biosynthesis. Thus, the over expression of sucrose transporter (SUT) genes involved in latex cells can be essential in latex regeneration, especially the expression of *SUT3* (*SUT1B*) (Tang *et al.,* 2013) shown to be significantly stimulated in the latex by ethylene treatment and correlated with yield stimulation (Tang *et al.,* 2013).

2.6.1 Rubber elongation factor

Rubber elongation factor (*REF*) is a key gene involved in rubber biosynthesis located on the surface of rubber particles in latex. *REF* is necessary for a functional role in rubber polymerization (Goyvaerts *et al.,* 1991). *REF* involved in the final polymerization step of natural rubber biosynthesis in *Hevea* (Dennis and Light 1989; Dennis *et* al., 1989). Recently, this protein was identified and characterized of genomic DNA from *Hevea* encoded a 138 amino acid peptide (Priya *et al.,* 2007). This protein has a molecular mass of 14 kDa (Dennis and Light, 1989). The presence of this protein on rubber particle is required for the elongation rubber (Hong *et al*., 2014). *REF* gene has 3 exons and interrupted by 2 introns. *REF* was induced by wounding and ethylene responses and was highly expressed in laticifers (Han *et al.,* 2000; Dennis *et al.,* 1989; Kush *et al*., 1990). Previously, Priya *et al*. (2007) reported that the *REF* transcripts were abundant in latex of high yielding rubber clones. Priya *et al*. (2007) indicated that a high level of *REF* transcript accumulation in high yielding clones correlated with latex yield in *Hevea.*

2.6.2 Small rubber particle protein

Small rubber particle protein (*SRPP*) is a α-helical protein which share 72% proteins similarity with REF protein whereas REF is amyloid form (Oh *et al.,* 1999; Berthelot *et al*., 2012). Both *REF* and *SRPP* gene were contained REF super family protein consisted of the highly related rubber elongation factor (REF), Small rubber particle protein (SRPP), and stress-related protein (SRP) sequences. The exact functions of this family (*REF* and *SRPP* genes) are still unclear (Hillebrand *et al.,* 2012). However, there is some evidence revealing a potential role in rubber biosynthesis. *In vitro* revealed that anti-*REF* and *SRPP* antibodies were inhibited IPP incorporation (Hillebrand *et al.,* 2012; Dennis and light, 1989; Oh *et al*., 1999). *SRPP* is involved in rubber biosynthesis (Sookmak *et al*., 2002) and plays role in latex coagulation. This protein is more highly expressed in latex than leaves (Oh *et al.,* 1999). SRPP protein has

encoded 204 amino acids peptide which is a molecular mass of 22 kDa (Chow *et al.,* 2007).

2.6.3 Sucrose transporter gene

Sucrose is the main carbohydrate and abundant carbon form in plant (Reinders *et al*., 2012; Shiratake, 2007). Plants synthesize sucrose as a major photosynthetic product and use sucrose for long distance carbon transport in plant vascular tissue (Shiratake, 2007). Sucrose transporters (SUT), are also called sucrose carries (SUC) gene, belong to the major facilitator superfamily (MFS) of transport proteins (Sauer, 2007) which are highly hydrophobic proteins (Rattanakitti, 2006).

Genes encoding SUT proteins have been identified from both monocot and dicot plant species such as *Arabidopsis*, rubber tree, rice, potato, and maize (Rattanakitti, 2006). This protein is a H^+/sucrose sympoter (Rattanakitti, 2006). SUT gene sequences in dicot plants could be separated into 3 groups such as two isoforms (*SUT1A* and *SUT1B*) in the SUT1 group, three isoforms (*SUT2A, SUT2B* and *SUT2C*) in the SUT2 group, and two (*SUT4* and *SUT5*) in the SUT4 group (Dusotoit-coucaud *et al.,* 2009; Barker *et al*., 2000).

Sucrose transporter 1 (SUT1) has been reported that SUT1 is a proton coupled sucrose transporter and plays an important role in sucrose uptake from mature leaves into phloem (Worawut *et al*., 2007). SUT1 serves as a high-affinity transporter for sucrose and has an important role in retrieving of sucrose along transport path (Worawut *et al*., 2007; Barker *et al.,* 2000). SUT1 was highly expressed all along the transport pathway and essential for maintaining the sucrose gradient. Thereby, SUT1 controlled the rate of phloem translocation into sieve elements (Chincinska *et al*., 2008; Barker *et al.,* 2000; Weise *et al*., 2000). SUT1 consists of sucrose transporters involved in phloem loading and sucrose import into different sink organs (Tang *et al*., 2010).

Sucrose transporter 2 (SUT2) has been reported to have more highly expression in sink than in sucrose leaves and is inducible by sucrose. This group was contained more amino acids (Tang *et al*., 2010). SUT2 may play as a role in controlling tuber starch content and accumulation in developing potato tubes (Rattanakitti, 2006).

SUT4 corresponds to the low-affinity/high-capacity component of sucrose uptake found in leaves and specific localization in either the plasma membrane (Barker *et al.,* 2000; Weise *et al*., 2000; Dusotoit-coucaud *et al.,* 2009). The expression of this gene is very low. In potato and tomato, SUT4 was immunolocalized specifically to enucleate sieve elements with indicating of similarity to SUT1 (Weise *et al*., 2000). Additionally, Chincinska *et al*. (2008) revealed that SUT4 transcripts in *Solanum tuberosum* were accumulated during flower and tuber development.

After tapping, the new latex must be regenerated before the next tapping (Xiao *et al.,* 2013). Sucrose is an important precursor molecule of rubber biosynthesis and latex regeneration, and therefore the laticifers cell in the trunk bark of regularly tapped rubber trees represent a strong sucrose sink (Xiao *et al.,* 2013). Understanding the mechanisms of sucrose transport and metabolism in the laticifers is important for improving rubber productivity (Xiao *et al.,* 2013). In *Hevea*, rubber biosynthesis occurs in laticifer which the sucrose will be the primary substrate, which sugar transported into sink latex cell. SUT genes were expressed in sucrose and sink organs, including latex cells in *Hevea* (Dusotoit-coucaud *et al.,* 2010). SUT1 gene was used for latex generation, rubber synthesis (90% of total metabolism), including with storaging and signaling (Dusotoit-coucaud *et al.,* 2010). However, the transcript expressions showed that the SUT1 group (*SUT1A* and *SUT1B*) isoform was greater expressed in latex. Two isoforms of the SUT2 group (*SUT2A* and *SUT2B*) displayed very low expression while expression of one SUT2 (*SUT2C*) and SUT4 (*SUT4* and *SUT5*) isoforms could barely be detected in *Hevea* (Dusotoit-coucaud *et al.,* 2010). *SUT1A* was highly expressed in sink organ (xylem and bark) than in leaves. *SUT1B* was mostly

expressed in leave and bark but weakly in xylem (Tang *et* al., 2010). On the basis of SUT transcript accumulation pattern, *SUT1B* (*SUT3*) has been reported that the expression of this gene has significance higher than any other SUT genes (Tang *et al*., 2010) in laticifers, whereas *SUT1B* was weakly expressed in the inner bark compared with latex. *SUT3* has been identified as the key protein responsible for sucrose loading into laticifers (Xiao *et al.,* 2013). Additionally, Tang *et al.* (2010) had cloned six distinct full-length cDNAs of SUTs. They found that the sequence of *SUT1B* (Dusotoit-coucaud *et al.,* 2010) and *SUT3* in *Hevea* was showed 99.2% similarity nucleotide sequence. *SUT1B* could be important in assembling the sustained sucrose demand of the latex cells necessary for ethylene-stimulated latex production (Dusotoit-coucaud *et al.,* 2010). Maybe this gene is involved in sucrose importation into the laticifers (Tang *et* al., 2010).

3. Aims of study

Rubber tree is an economic important crop cultivated for rubber production in the world. The knowledge of genes that involved in rubber biosynthesis pathway is necessary to be learned and will be used as molecular markers for selection of high yield rubber clones. Both *REF* and *SRPP* genes play a functional role in rubber (*cis*-1,4-polyisoprene) polymerization (Dennis and Light 1989; Dennis *et al*., 1989), related to high latex yielding in *Hevea brasiliensis*. The expressions of sucrose transporter gene has an important role in loading sucrose into laticifers (Li *et al.,* 2011).

The objectives of this study are:

1. To clone and sequence the *REF*, *SRPP* and *SUT3* genes in the latex of four selected clones (SK1, SK3, T2, and NK1), and RRIM 600.
2. To monitor the expressions of *REF, SRPP*, and *SUT3* genes in four selected clones (RRIM 600 was included) by RT-PCR and qRT-PCR and compare gene expressions to the latex yield production.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Plant materials

Four selected clones from four rubber plantations in three provinces of Southern Thailand were used in this study (Nualsri *et al.,* 2009). Each clone was designated according to their locations (Table 1). Based on information from the holders of rubber plantations, those clones came from open pollinated progenies of unknown clones. The experimental design was a completely randomized design (CRD) with 10 replications. Each of clones was selected from 10 plants of individual location. The selection of trees from a vast rubber plantation was made on the basis of the uniform diameter of tree at 100-150 meter height.

Table 1 Details of selected clones investigated in this study

Selected Clones	Location of samples	Plant age (years)
SK1	Natawee district, Songkhla province, Thailand	12
SK3	Hat Yai district, Songkhla province, Thailand	15
NK1	Nabon district, Nakhon Si Thammarat province, Thailand	11
T2	Palian district, Trang province, Thailand	15

2. Laboratory materials

2.1 Chemicals

2.1.1 Analytical grade

slacimehC	seinapmoC
Ethyl ethanol absolute	Macron chemicals
Agarose	Amresco
Acetic acid	J.T. Baker
Boric acid	Ajex Finechem
Chloroform	RCI Labscan
Diethyl pyrocabonate (DEPC)	Applichem
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethidium bromide	Sigma
Glycerol	Amresco
Hydrochloric acid	Sigma
8-hydroxyquinoline	Sigma
Iso-amyl alcohol	J.T. Baker
Isopropyl alcohol	Sigma
Phenol	Loba Chemie
Sodium hydroxide	Merck
Sodium dodecyl sulfate (SDS)	Merck
Sodium chloride (NaCl)	Amresco
Sodium acetated trihydrate	Ajex Finechem
Tryptone	BD. Bioscience
Tris-base	Sigma
Tris hydrochloride	Bio Basic
Yeast extracts	BD. Bioscience

2.1.2 Molecular biology grade

slacimehC	seinapmoC
100bp DNA Ladder	BioLab
Ampicillin	Sigma
Deoxyribonuclease I (DNase I)	Invitrogen
DNTP (dATP, dTTP, dCTP and dGTP)	Promega
E.Z.N.A. Gel Extraction kit	Omega
Kanamycin	Sigma

2.2 Bacteria

Escherichia coli Top 10 F' (Invitrogen, USA)

2.3 Plasmid DNA

T&A cloning vector (RBC Bioscience)

2.4 Laboratory equipment

stnempiuqE	seinapmoC
Autoclave	Amerex Instruments, Inc.
Gel electrophoresis	Cosmo Bio
Gel documentation	Bio-Imaging System
Micro centrifuge	Eppendorf
Micropipette	Eppendorf
PCR Machine (DNA Thermal Cycler)	Biometra
Real-Time PCR Machine (ABI 7300)	Invitrogen
Vortex mixer	-
Water bath	-

Methods

Part 1: Latex dry rubber yield, the girth increments, and latex biochemical parameters

1.1 Latex yield and dry rubber content in over two years

The fresh weight of latex yield and DRC from 10 trees along with RRIM 600 in each location were recorded twice a month during July 2011 to March 2013. The monthly dry rubber yield was collected during three specific periods of early rainy season when tapping restart (June and August 2012), rain period occurred from October and December 2012, and the weak rainfall period (January and March 2013). Latex was obtained from the rubber trunk by tapping, and fresh weight of latex yield was collected and weighted the fresh latex in the morning. The tapping system for all clones was third spiral downward cut at three days tapped by one day rest (1/3S 3d/4) To measure DRC content from each clone, 10 g of latex was weighted, 6.0% of acetic acid solution was added and mixed well, waited for 10-20 min until latex coagulated and dried in hot air oven at 65 °C for 24 hrs. The total solid content in the latex after drying reflects the rubber content because rubber is the major component of rubber latex. The DRC percentage and dry rubber yield were calculated by using the following equations:

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

$$\text{Dry rubber yield } (g^{-1}\ \text{tree}^{-1}\text{tapping}^{-1}) = \text{fresh latex weight} \times \% \text{ DRC}$$

1.2 Girth increment

Rubber tree girth was recorded at 170 cm from the ground with measuring tape once a month.

1.3 Biochemical parameter of latex

To assay the physiological parameters, an aliquot of 10 ml of latex per tree was collected in a 50-ml centrifuge tube placed in ice and transported to the laboratory for immediate analysis. The parameter of TSC, thiols, inorganic phosphate, and sucrose contents were determined according to Eschbach *et al*. (1984).

Part 2: Cloning and sequencing of *REF, SRPP*, and *SUT* genes

2.1 RNA extraction

The latex samples were collected from 4 selected clones and RRIM 600. Samples of fresh latex were collected in a 15 ml tube containing 5 ml of 5X RNA extraction buffer (0.1M Tris-HCL, 0.1M EDTA, 10% SDS, pH 9) according to Suwanmanee *et al,* (2002) and mixed well, stored at –70 °C until receiving RNA isolation. After thawing, an equal volume of buffer saturated phenol was added to the collected latex and mixed thoroughly. The upper aqueous phase was extracted two times with phenol: chloroform (1:1, v/v), mixed well, kept on ice 15 min, and then was centrifuged at 10,000 xg for 20 min at room temp to collect the upper phase. The upper phase was then mixed with one time of an equal volume of phenol: chloroform: iso-amyl alcohol (25: 24: 1, v/v), centrifuged at 10,000 xg for 20 min at room temperature. The upper phase was extracted with chloroform: iso-amyl alcohol (24:1, v/v), centrifuged at 10,000 xg for 20 min at room temperature to collect the upper supernatant, and transferred to a new 1.5 ml centrifuge tube. The total RNA was precipitated by the addition of 1/10 volume of 3M sodium acetate pH 5.2 and added an equal volume of isopropanol. The mixture was incubated at -20 °C for 1 hr, and the RNA pelleted by centrifuging at 10,000 xg for 20 min at 4 °C. RNA pelleted was washed with 70% ethanol, dried, dissolved in DEPC treated water, and kept at -70 °C until use.

2.2 Quantity and quality of RNA

The quantity and quality of RNA were determined by using the spectrophotometer. The integrity of the RNA was checked by 1.5% agarose gel electrophoresis.

2.3 cDNA synthesis

Total RNA extraction from latex was treated with Deoxyribonuclease I (Invitrogen, USA) before using as template of PCR amplification. The first strand cDNA synthesis reaction was performed in 20 μ volume by using random primer supplied with the Super Script ®Vilo ™ cDNA Synthesis kit (Invitrogen, USA) reverse transcriptase at 25°C for 10 min, incubated at 42 °C for 60 min, and terminated the reaction at 85 °C for 5 min.

2.4 Cloning of products of *REF, SRPP* and *SUT* cDNA

The total cDNA served as template in the PCR amplification was performed in a 25 μ reaction volume which was composed of 10X PCR buffer, 50mM $MgCl_2$, 10 μM dNTP mixture, 1 unit *Taq* polymerase, 1 μ of the first strand cDNA mixture, and 10 mM of each primer (Table 2). The thermal profile for PCR was started at 94 °C for 4 min followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72°C for 1 min, and finally 72 °C for 10 min. The amplification was carried out in a thermal cycler (Perkin Elmer, USA). The PCR products were separated on 1.5 % (w/v) agarose gel.

The 5' end of target *SUT* gene was obtained according to the method described by Frohman (1993). The specific primer pairs were designed from partial sequence of target gene for the amplification of the 5' ends as shown in table 1.

Table 2 Specific primer sequences used for cloning and sequencing

Genes	Primers	Ta (°C)
REF	Forward: 5’-CGA TTA TGG CTG AAG ACG AA -3’	59
	Reverse: 5’-GG GCT CAA TTC TCT CCA TAA -3’	57
SRPP	Forward: 5’-TTC AAT TAT GGC TGA AGA GGT -3’	56
	Reverse: 5’- TGC AGA TTA TGA TGC CTC AT -3’	57
SUT	Forward: 5’-ATG GAR RCT GAT CCT ASG AAA GAA-3’	61
	Reverse: 5’-TGT AAC ACT GCT GGT GCT GG-3’	60

2.5 Purify PCR products from agarose gel by E.Z.N.A.®Gel Extraction Kit of Omega

The amplified products were separated on 1.5% (w/v) low melting temperature agarose gel. The slice of agarose containing DNA fragment of interest was excised from gel and transferred to a fresh sterile microcentrifuge tube. Assuming a density of 1 g/ml, this study prepared the volume of derived gel as follows: a gel slice of mass 0.3 g had a volume of 0.3 ml. One volume Binding Buffer (XP2) was added and incubated at 60 °C for 7 min or until the gel was completely melted, vortexed, or shaked the tube every 2-3 min. Insert a HiBind®DNA Mini Column in a 2 ml tube, and added 700 µ DNA/agarose solution to the HiBind®DNA Mini Column, centrifuge at 10,000 xg for 1 min at room temperature, the filtrate was discarded. 300 µ Binding Buffer (XP2) was added and centrifuged at maximum speed (13,000 xg) for 1 min at room temperature. Discard the filtrate and reuse collection tube. 700 µ SPW Wash Buffer was added and centrifuged at maximum speed for 1 min at room temperature. Discard the filtrate and reuse collection tube. Centrifuge the empty HiBind®DNA Mini Column for 2 min at maximum speed to dry the column matrix. The HiBind®DNA Mini Column was transfered to a new 1.5 ml microcentrifuge tube, and 30-50 µ DEPC treated water was added directly to the center of the column membrane, waited for 2 min at room temperature and centrifuged at maximum speed for 1 min and stored at -20 °C until use.

The concentration and purity of the DNA were checked by 1.5% agasose gel electrophoresis.

2.6 Cloning of PCR amplified *REF, SRPP*, and *SUT* genes into plasmid

2.6.1 Ligation of *REF, SRPP* and *SUT* genes into plasmid

Ligation mixture was prepared by TA cloning vector kit (RBC Bioscience crop, Taiwan). The plasmid DNA was performed in a 10 μ reaction volume, which was composed of DNA 300 ng, TA vector 50 ng, T_4 DNA ligation buffer A 1 μ, T_4 DNA ligation buffer B 1 μ, and 3U T4 DNA ligase 1 μ. The mixture was incubated at 4 °C for 16 hr.

2.6.2 Preparation of competent cells

Fresh competent bacterial cells were prepared with calcium chloride. One shot top 10 strain of *Escherichia coli* (Invitrogen, USA) was used as the host. Using a sterile loop, this study took *E. coli* cells directly from a frozen stock and streaked onto the surface of LB agar plate and incubated at 37 °C for 16-18 hr (overnight). Competent cells were prepared as follows:

- A single colony was transferred from the freshly grown plate into 10 ml of LB broth (starter cell) and incubated for 16-18 hr (overnight) at 37 °C with incubator shaker (200 rpm).
- Inoculated 500 μ of the overnight culture (starter cell) into 20 ml of fresh LB broth and incubated at 37 °C incubator shaker until the $O.D._{600}$ reached 0.3-0.5 (about 2-2.5 hours).
- The cells were then transferred to sterile ice-cold polypropylene tubes and stored the tubes on ice for 10 min.
- The cells were harvested by centrifugation for 5 min at 4,500 xg at 4 °C.
- After decanting the media completely, the pellet was resuspended in 10 ml of ice-cold 0.1M $CaCl_2$ solution (freshly diluted from 1M $CaCl_2$

stock solution) and stored the cells on ice for 30 min.

- The cells were harvested by centrifuging at 4,500 xg for 5 min at 4 °C and the $CaCl_2$ solution was decanted completely from the cell pellet.
- The pellet was again resuspended in 5 ml of ice-cold 0.1M $CaCl_2$.
- The cell suspension was dispensed (200 µ) into sterile eppendorf tubes and immediately used for transformation or immediately kept at -70 °C.

2.6.3 Transformation

- The competent cells were thawed and placed on ice immediately.
- The ligation mix (5 µ) was added to 200 µ of competent cells and mixed gently.
- Incubated the reaction mixture on ice for 30 min.
- The transformed cells were heat shocked at 42°C for 90 seconds and returned to ice for 5 min.
- LB broth (800 µ) was added to the heat shocked cells and incubated with incubator shaker at 37 °C for 1-1.5 hr.
- Transformed cells were placed on the LB agar plates with ampicillin (80 µg/ml), incubated at 37 °C overnight for the appearance of colonies.
- Colonies were selected and analyzed by plasmid isolation, PCR, or sequencing.

2.6.4 Confirmation of *REF, SRPP*, and *SUT* genes cloning

2.6.4.1 Amplification of cloned gene by PCR

The colonies that developed on LB agar plate with ampicillin (80 µg/ml) were selected for confirmation of studies. Plasmid DNA without

insert was used as a negative control. The amplification was carried out by using *REF, SRPP,* and *SUT* genes specific primers. The conditions for PCR amplification were similar as described above (section 2.4) and picked the bacteria for template DNA. After amplification, the PCR products were analyzed by 1.5%(wlv) agarose gel electrophoresis. Select colonies (with insert gene) for plasmid purification.

2.6.4.2 Plasmid purification (following by Invitrogen, USA)

Plasmid DNA was isolated from the recombinant colonies (with inserted gene) by PureLink Quick plasmid DNA Miniprep kits (Invitrogen, USA)

- Individual bacterial colonies were picked by using sterile toothpicks and inoculated into 5 ml LB medium containing ampicillin (80 μg/ml) in 15 ml tubes. Incubated with incubator shaker at 37 °C overnight. Removed all medium.
- 250 μ Resuspension Buffer (R3) with RNase A was added to the cell pellet and resuspend the pellet until it was homogeneous.
- 250 μ Lysis Buffer (L7) was added and mixed gently by inverting the capped tube until the mixture was homogeneous. The tube was incubated at room temperature for 5 min.
- 350 μ Precipitation Buffer (N4) was added and mixed immediately by inverting the tube or for large pellets. Vigorously shaking the tube until the mixture was homogeneous. The lysate was centrifuged at 12,000 xg for 10 min.
- The supernatant was loaded from step 4 onto a spin column in a 2 ml wash tube and centrifuged the column at 12,000 xg for 1 min. The flow-through was discarded and placed the column back into the wash tube.
- 500 μ Wash Buffer (W10) with ethanol was added to the column and incubated the column for 1 min at room temperature. The column was centrifuged at 12,000 xg for 1 min, flow was discarded through and placed column back into the wash tube.

- 700 μ Wash Buffer (W9) with ethanol was added to the column. The column was centrifuged at 12,000 xg for 1 min, flow was discarded through, and placed column back into the wash tube. The column was centrifuged at 12,000 xg for 1 min. The flow-through was discarded and placed the column back into the wash tube.

- The Spin Column was placed in a clean 1.5 ml recovery tube. 50 μ of preheated DEPC treated water was added to the center of the column. The column was incubated at room temperature for 1 min.

- The column was centrifuged at 12,000 xg for 2 min. The recovery tube was contained the purified plasmid DNA. Plasmid DNA was stored at 4 °C (short-term) or stored in aliquots at -20 °C (long-term).

- The isolated plasmid DNA was then checked on 1%(wlv) agarose gel. Super coiled, uncut TA vector was also loaded into the gel as control to differentiate from the recombinant plasmid with cloned gene.

- Insert gene was checked again by PCR amplification which was similar as described above (section 2.4). The PCR products were analyzed by 1.5%(wlv) agarose gel electrophoresis. Gene was analyzed by sequencing.

2.6.5 Sequencing and bioinformatics analysis

2.6.5.1 Sequencing analysis

The nucleotide sequence of the cloned DNA fragment was determined by using the automated sequencing facility at BigDye®Terminator v3.1 cycle sequencing kit from First Base DNA Sequencing Services, Malaysia.

2.6.5.2 Bioinformatics analysis

Sequence homology and deduced amino acid sequence comparisons were carried out using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments of *REF, SRPP,* and *SUT* were performed with the ClastalX Multiple

alignment program (http://www.ncbi.nlm.nih.gov), and Multiple Alignment was shown by GENEDOC, version 2.6.001 (Nicholas and Nicholas, 1997).

Part 3: Gene expression

3.1 Expression of *REF, SRPP* and *SUT3* gene in mature rubber and rubber seedling

3.1.1 Gene expression of mature rubber tree

Fresh latex of mature plant was extracted from latex of four selected clones which was grown in the same location with the control RRIM 600. RNA and cDNA of mature plant were collected for analyzing gene expression by RT-PCR and qRT-PCR. The gene expression levels of *REF*, *SRPP,* and *SUT3* genes were compared over for two years using RRIM 600 clones in the same field as their paired controls. The monthly gene expression during three specific periods was collected during 1) early rainy season when tapping was restarted during June - August 2012, 2) light rainfall period occurred during October - December 2012, and 3) the low rainfall period during January - March 2013.

3.1.2 Gene expression of rubber tree seedling

The expression of *REF, SRPP* and *SUT* genes were monitored by qRT-PCR in different organs, stem barks and leaves of 1-year-old seedlings. SK1, SK3, NK1, T2, RRIM 600, and Low yield (indigenous) clones were cultivated in an experimental plantation of the Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand. All samples were immediately frozen in liquid nitrogen at the time of collection and then stored at -80 °C or used immediately for RNA extraction.

3.2 Gene expression analysis by RT-PCR

For RT-PCR analysis, template DNA was used for *REF* and *SRPP* cDNA synthesis. After the reverse transcription step, the total cDNA served as template in the PCR amplification was performed in a 25 µ reaction volume, which was composed of 10X PCR buffer, 50 mM $MgCl_2$, 10 uM dNTP mixture, 1 unit *Taq* polymerase, 1 µ of the first strand cDNA mixture, and 10mM of each primer (Table 3). *18s rRNA* gene fragment was amplified as the positive control. The thermal profile for PCR was started at 94 °C for 4 min followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. After amplification, 5 µ of PCR products were separated by electrophoresis at 100 V for 45 min on 1.5% (w/v) agarose gel by using TBE buffer. The gel was stained with 0.5 µg/ml of ethidium bromide for 10 min, and washed by soaking in double deionized water for 15 min, and photographed by using gel documentation, and then checked a relative mRNA levels by Image J Program. The *REF* and *SRPP* mRNA expression levels were analyzed by using the intensity ratio of the *REF*, *SRPP,* and *18s rRNA* bands.

Table 3 specific primer sequences used for RT-PCR

Genes	Primers	Ta (°C)
REF	Forward; 5'- CGATTATGGCTGAAGACGAAGACAACC -3'	65
	Reverse, 5'- GGCCAATAATTCAATTGGCCCTTTATTC -3'	61
SRPP	Forward; 5'- CAGCGACTGCGTTTTGAAT -3'	56
	Reverse, 5'- GCCCTTTATTCCACA TCCAA -3'	57
18s rRNA	Forward; 5'- GGTCGCAAGGCTGAAACT -3'	56
	Reverse, 5'- ACGGGCGGTGTGTACAAA -3'	56

3.3 Gene expression analysis by Real-Time PCR (qRT-PCR)

Twenty-five ng of cDNA was used as the template in subsequent qualitative qPCR. The transcription levels of *REF, SRPP* and *SUT3* were analyzed by

using quantitative real time PCR with ABI (7300) system. Primer was designed based on the analogs *Hevea brasiliensis* from NCBI. For primers selection, PCR was performed to test the specificity of each primer with Tm at 60 °C (Nakkanong, 2012). PCR products were run on 2% (W/V) agarose gels by using 0.5% TBE buffer. The gel was stained with 0.5 μg/ml of ethidium bromide for 10 min, washed by soaking in double deionized water for 15 min, and photographed using gel documentation. The chosen primers used for quantitative real time PCR were shown in Table 4.

The reactions were performed by using the SYBR® Green Real-Time PCR master mixes (Invitrogen, USA) following the procedure described by the manufacturer which 20 μ PCR mixtures were composed of 25 ng cDNA. A negative control was included by using distillated water as a template in each reaction. The PCR cycle program was as the following: initial denaturation at 95 °C for 10 min, followed by 35 cycle of two steps of PCR as denaturation at 95 °C for 15 sec, and annealing and polymerization at 59 °C for 1 min. The 18s rRNA was used as internal control gene in this study.

The normalized expression ration was calculated by using the comparative Ct method with the formula: $Qr = 2^{-\Delta\Delta Ct}$ (Pfaffl, 2011), $\Delta\Delta Ct$ was calculated from $\Delta Ct = Ct_{(treated\ sample)} - Ct_{(control\ sample)}$. The Ct values presented are the means of three independent replications and each reaction had three biological repeats (Nakkanong, 2012). The method made these studies possible to visualize the increase ($Qr > 1$) and decrease ($Qr < 1$) of genes (up and down regulation, respectively).

Table 4 Specific primer sequences used for qRT-PCR

Genes	Primers	Ta (°C)
REF	Forward; 5’- CGGCAACTTATGCTGTGACT -3’	57.3
	Reverse, 5’- AGGTACAGCCACGTTCTTCA -3’	57.3
SRPP	Forward; 5’- CACTAGCCTAGATGGCGTTG -3’	55.2
	Reverse, 5’- CTGAAGAAGCCACATCAAGAA -3’	55.2
SUT3	Forward; 5’- CCCATGTGGATCCTTCTTCT -3’	58.94
	Reverse, 5’- TCTCCGCCGTATACCTCTCT -3’	58.91
18s rRNA	Forward; 5’- AAGCCTACGCTCTGGATACATT -3’	58.4
	Reverse, 5’- CCCGACTGTCCCTGTTAATC -3’	59.3

Part 4: Statistical analysis

4.1 Pearson’s correlation coefficient

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

Chapter 3

Results

1. Sequencing analysis

Sequencing of DNA differences is the basic requirement for the study of molecular genetics. In order to confirm the nucleotide sequences and the full-length cDNA sequence of insert gene, *REF*, *SRPP*, and *SUT*, genes were isolated and characterized from four selected clones and RRIM 600 was included. Sequence homology was determined by using the automated sequencing facility at BigDye® Terminator v3.1 cycle sequencing kit from First Base DNA Sequencing Services, Malaysia.

1.1 Amino acid sequencing of *REF* gene

For the nucleotide sequence analysis, the result showed that the present amplified *REF* gene was contained of 417 bp open reading frame encoding for 138 amino acids (Figure 3). The *REF* nucleotide and deduced amino acid sequences submitted to NCBI database were shown in Table 5. Furthermore, different sequencing of individual clone (the four selected clones, RRIM 600, indigenous clone, and *Hevea* in NCBI database) was demonstrated. The study was found that the *REF* amino acid sequence of lower yield clone presented one amino acid different from Proline (CCC) as Serine at site 113 (CCT).

1.2 Amino acid sequencing of *SRPP* gene

For amino acid sequence of *SRPP* gene, the result showed that the *SRPP* gene was contained of 615 bp open reading frame encoding for 204 amino acids (Figure 4). The *SRPP* nucleotide and deduced amino acid sequences submitted to NCBI

database was shown in Table 5. For the *SRPP* amino acid sequences, Glutamic acid was absence at site 6 of RRIM 600. One amino acid at site 67 of RRIM 600 clone was different from other clones resulting in Threonine (ACT) instead of Alanine (GCT). In NK1 clone, at site 178, one amino acid was different from Threonine (ACT) to Valine (GTT). For *SRPP* sequencing, GVV motif was found in position 83.

1.3 Amino acid sequencing of *SUT* gene

To determine amino acid sequence of *SUT* gene, SUT gene was isolated and characterized from four selected clones and RRIM 600. The results showed that the present amplified partial sequence of *SUT* gene contained 1,373 bp open reading frame encoding for 457 amino acids (Figure 5). From this study, 84% of sequences were identical to the isoform of *SUT3* and *SUT6* sequences in the NCBI database.

```
                    *         20         *
EU182586.1  : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
AB074308.1  : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
AY120685.1  : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
HQ640230.1  : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
T2          : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
SK1         : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
Indigenous  : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
SK3         : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
RRIM600     : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
NK1         : MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF :  38
              MAEDEDNQQGQGEGLKYLGFVQDAATYAVTTFSNVYLF

              40         *         60         *
EU182586.1  : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
AB074308.1  : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
AY120685.1  : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
HQ640230.1  : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
T2          : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
SK1         : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
Indigenous  : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
SK3         : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
RRIM600     : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
NK1         : AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL :  76
              AKDKSGPLQPGVDIIEGPVKNVAVPLYNRFSYIPNGAL

                80         *         100         *
EU182586.1  : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
AB074308.1  : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
AY120685.1  : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
HQ640230.1  : KFVDSPVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
T2          : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
SK1         : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
Indigenous  : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAASE : 114
SK3         : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
RRIM600     : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
NK1         : KFVDSTVVASVTIIDRSLPPIVKDASIQVVSAIRAAPE : 114
              KFVDStVVASVTIIDRSLPPIVKDASIQVVSAIRAApE

                 120         *
EU182586.1  : AARSLASSLPGQTKILAKVFYGEN- : 138
AB074308.1  : AARSLASSLPGQTKILAKVFYGEN- : 138
AY120685.1  : AARSLASSLPGQTKILAKVFYGEN- : 138
HQ640230.1  : AARFLAFFLPGQTKILAKVFYGEN- : 138
T2          : AARSLASSLPGQTKILAKVFYGEN- : 138
SK1         : AARSLASSLPGQTKILAKVFYGEN- : 138
Indigenous  : AARSLASSLPGQTKILAKVFYGEN- : 138
SK3         : AARSLASSLPGQTKILAKVFYGEN- : 138
RRIM600     : AARSLASSLPGQTKILAKVFYGEN- : 138
NK1         : AARSLASSLPGQTKILAKVFYGEN- : 138
              AARsLAssLPGQTKILAKVFYGEN
```

Figure 3 Multiple alignment of predicted amino acid sequence of *REF* gene with *H. brasiliensis*; Accession no. HQ640231.1, AY120685.1, AB074308.1, AY430052.1, EU182586.1, KF734663 (RRIM 600 clone), (KF734662SK1 clone), KF734661 (Indigenous clone), SK3 clone, NK1 clone, and T2 clone. Amino acids highlighted in black are 100% homology conserved proteins; grey, 80–90% homology; light grey, 60–70% homology; and white, less than 50% homology. Red asterisk indicated differences of amino acid sequences

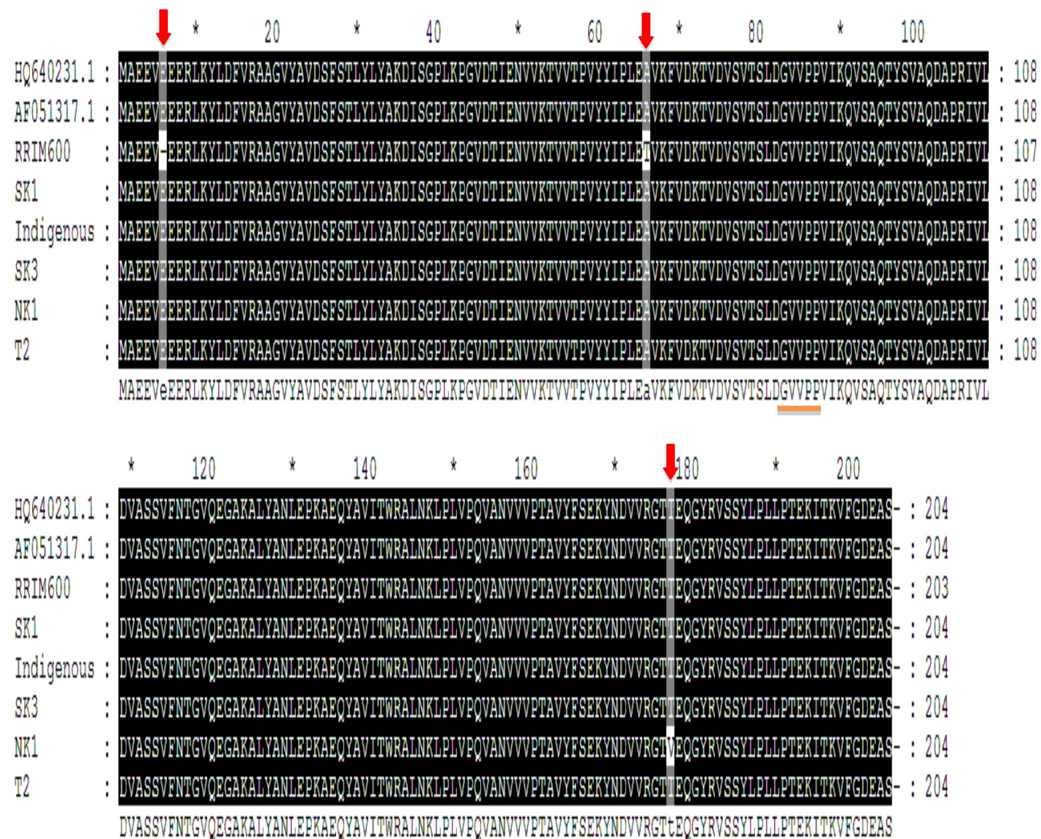

Figure 4 Multiple alignment of predicted amino acid sequence of *SRPP* gene with *H. brasiliensis*; Accession no. AF051317.1, HQ640231.1, KF734667 (RRIM 600 clone), KF734666 (SK1 clone), KF734664 (Indigenous clone), SK3 clone, KF734665 (NK1 clone), and T2 clone. Amino acids highlighted in black are 100% homology conserved proteins; grey, 80–90% homology; light grey, 60–70% homology; and less than 50% homology was indicated as white. Red asterisk indicated differences in amino acid sequences. The underline presented GVV motif

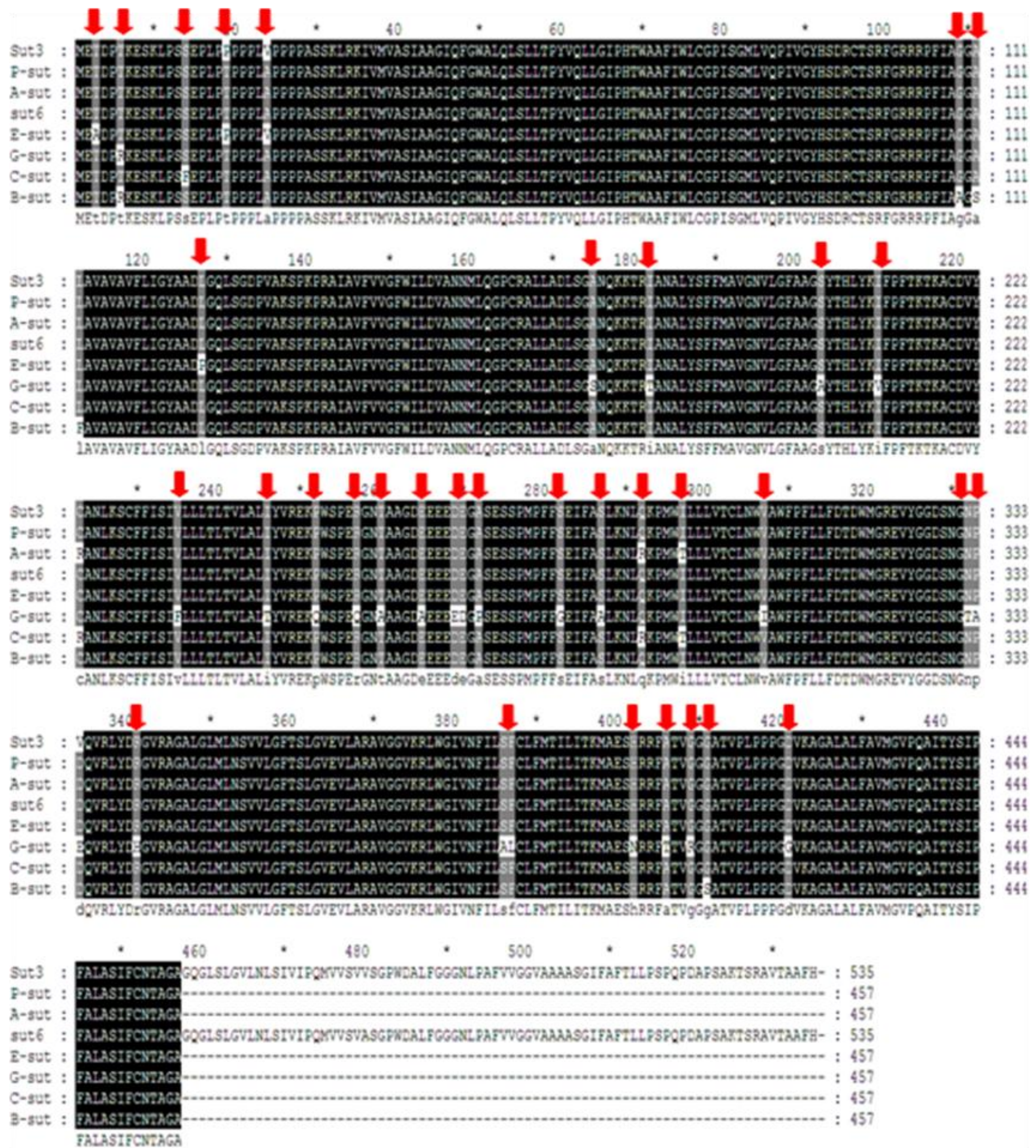

Figure 5 Multiple alignment of predicted amino acid sequence of *SUT* gene with *H. brasiliensis*; Accession no. AM492537.1 (*SUT* 6), EF067334.2 (*SUT* 3), RRIM 600 clone (B-sut), Indigenous clone, SK1 clone (A-sut), SK3 clone (G-sut), NK1 clone (E-sut), and T2 clone (C-sut). Amino acids highlighted in black are 100% homology conserved proteins; grey, 80–90% homology; light grey, 60–70% homology; and white: less than 50% homology

Table 5 Gene encoding rubber biosynthesis isolated from *Hevea brasiliensis*

Genes	GeneBank Accession numbers	Lengths (bp)	Identity with the best homologs
REF (SK1)	KF734662	417(ORF)	*Hevea* (EU182586.1), AY120685.1, AB074308.1 (100%)
REF (RRIM 600)	KF734663	417(ORF)	*Hevea* (EU182586.1), AY120685.1, AB074308.1 (100%)
REF (Ind)	KF734661	417(ORF)	*Hevea* (AY299405.2) (100%)
SRPP (SK1)	KF734666	615(ORF)	*Hevea* (HQ640231.1), AF051317.1(100%)
SRPP (NK1)	KF734665	615(ORF)	*Hevea* (HQ640231.1), KF734667, KF734664.1, KF734666.1, AF051317.1 (99%)
SRPP (RRIM 600)	KF734667	612(ORF)	*Hevea* (HQ640231.1) (99%)
SRPP (Ind)	KF734664	615(ORF)	*Hevea* (HQ640231.1), AF051317.1(100%)
SUT3 (RRIM 600)	KU302356	1,373(ORF)	*Hevea* (EF067334.2, AM492537) (98%)
SUT3 (NK1)	KU302358	1,373(ORF)	*Hevea* (EF067334.2) (99%)
SUT3 (SK3)	KU302359	1,373(ORF)	*Hevea* (DQ985466.1), (EF067334.2) (94%)

2. Latex dry rubber yield, the girth increments, and biochemical parameters of latex

2.1 Average of dry rubber yield (g/tree/tapping) over two years during June 2011 to March 2013

Means of annual fresh latex yield over two years indicated that four selected clones (SK1, SK3, NK1 and T2) produced higher latex yield than paired-RRIM 600 in each location. The means' of dry rubber yields were 129.47, 48.13, 74.19, and 53.92 g/tree/tapping in SK1, T2, NK1, and SK3, respectively, those of dry rubber yield of the four paired-control RRIM 600 were 94.25, 41.08, 49.93, and 43.88 g/tree/tapping, respectively (Figure 6).

Rubber latex yield was influenced by variation in climate and soil moisture especially for the annual cumulative rainfall. Table 6 presented the means of dry rubber yield during three specific periods of 1) early rainy season when tapping restarted during June - August 2012, 2) rain period occurred from October - December 2012, and 3) is the weak rainfall period during January - March 2013. The climate variations in productivity of the various rubber tree clones in the current study showed very similar patterns. The four selected clones had higher monthly latex yield than the control (RRIM 600) at every location (Table 6). The latex yield increased from June (when tapping restarted) with attaining its maximum in October, holding steady till December, and gradually decreasing to a minimum in March before falling leaves period.

2.2 The girth increments

The rates of girth increments during tapping period among 5 rubber clones were recorded over 2 years during June 2011 to March 2013. Mean girth increments of three high yield clones; SK1, SK3, and T2 were higher than the paired control (RRIM 600) whereas no significant difference was recorded between NK1 and paired-control RRIM 600 (Table 7).

3.3 Biochemical parameters of latex

Table 8 showed latex biochemical parameters of the selected clones, except for SK1, with significantly higher dry rubber contents than RRIM 600. In all selected clone, the TSC values in latex of all clones were higher than RRIM 600. RSH did not differ between the selected clones and the control. Latex sucrose content showed lower than the control whereas latex inorganic phosphorus content (Pi) was showed higher than the control. These results revealed that the NK1 had a higher and better latex biochemical parameter because this clone exhibited high latex yield together with low Suc and high Pi contents.

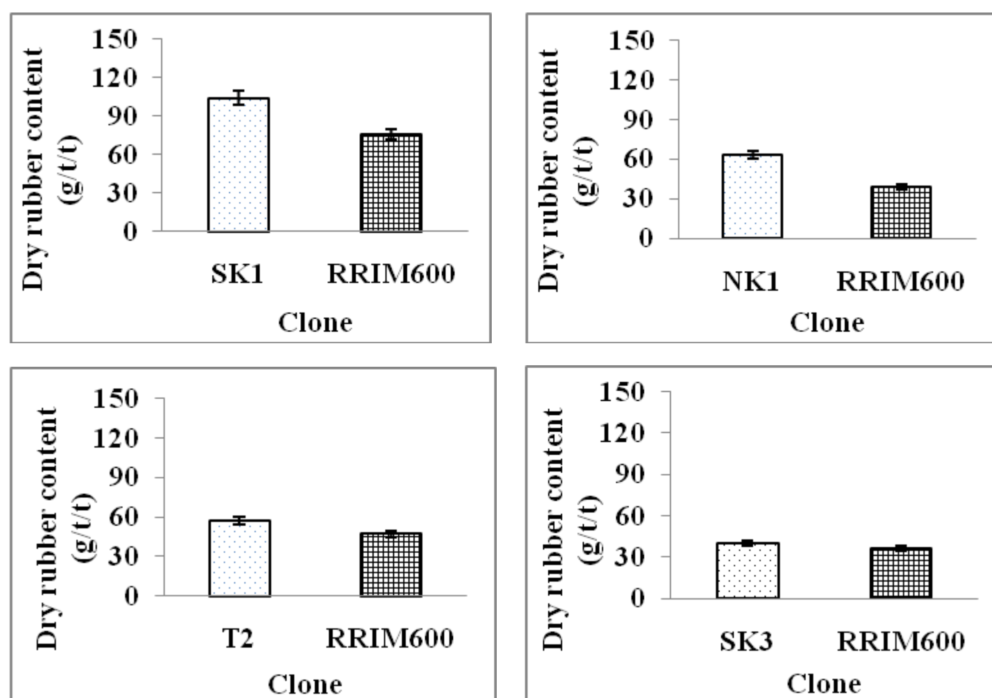

Figure 6 Dry rubber yield (g/tree/tapping/) of SK1, NK1, T2, SK3 clones, and RRIM 600 in each location during June 2011 to March 2013. Each bar represented mean value result from tri-replicate ±SD. Significant difference was indicated by the asterisks above the bars (** $p \leq 0.01$). Significant difference was indicated by the asterisks above the bars (* $p \leq 0.05$), where ns was represented at non significance

Table 6 Dry rubber yield (g/tree/tapping) of SK1, SK3, NK1, T2, and the paired RRIM 600 clones in each location observed from June 2012 to March 2013.

Clones	Dry rubber yield (g/tree/tapping)		
	June - August 2012	October - December 2012	January - March 2013
SK1	78.88	131.82	101.50
RRIM 600	59.49	90.92	76.27
T-test	*	*	*
CV (%)	8.63	4.74	19.71
T2	40.51	83.32	47.92
RRIM 600	33.19	67.65	40.99
T-test	*	*	ns
CV (%)	22.90	11.25	12.87
NK1	61.33	70.26	58.02
RRIM 600	37.44	46.25	32.54
T-test	*	*	*
CV (%)	10.30	9.90	9.92
SK3	37.34	52.25	42.20
RRIM 600	37.86	39.88	32.53
T-test	ns	ns	*
CV (%)	8.53	9.53	12.67

* = Significant difference at $P \leq 0.05$, LSD

ns = Non significance

Table 7 Girth increments of SK1, SK3, NK1, and T2 rubber tree clones compared with RRIM 600 grown in the same location. The data were recorded from 2011 to 2013.

Clones	Girth increment (cm)	t-test	CV (%)
SK1	1.81±0.12 a	*	18.24
RRIM 600	0.71±0.22 b		
NK1	2.86±0.17	ns	25.40
RRIM 600	2.71±0.21		
T2	1.602±0.08 a	*	12.20
RRIM 600	0.76±0.21 b		
SK3	1.76±0.40 a	**	15.63
RRIM 600	0.41±0.15 b		

Mean followed by the different letter indicated significantly differences at 0.05

ns = Non significance; * =Significant at $p \leq 0.05$ (Student's t-test); CV = Coefficient of variation.

Table 8 Evaluation of latex biochemical parameters of SK1, SK3, NK1, and T2 rubber tree clones compared with RRIM 600 grown in the same farming trail.

Rubber tree clones	DRC (%)	Suc (mM)	Pi(mM)	RSH(mM)
SK1	42.63±1.95 a	8.27±1.75	10.46±3.77	0.31±0.15
RRIM 600	39.67 ±2.27 b	8.29±1.75	9.03±4.34	0.26±0.13
	*	ns	ns	ns
SK3	42.03±2.91 a	9.12±2.92	11.74±2.19	0.34±0.09
RRIM 600	37.12±4.89 b	9.13±1.93	10.90±3.05	0.21±0.05
	*	ns	ns	ns
NK1	40.60±1.19 a	6.37±2.24	13.93±7.84 a	0.24 ±0.06
RRIM 600	32.72±1.06 b	6.51±2.82	6.61 ±3.59 b	0.17±0.06
	*	ns	*	ns
T2	44.15±2.22 a	5.50±1.43	9.67±1.66	0.16±0.03
RRIM 600	37.34±3.22 b	8.80±1.99	9.96±1.88	0.17±0.06
	*	ns	ns	ns

Mean followed by the different letter indicated significantly differences at 0.05

ns = Non significance;*Significant at $p \leq 0.05$ (Student's t-test); DRC = Dry rubber content; Suc = sucrose; Pi = inorganic phosphorus, RSH=Thiol.

3. Gene expression analysis

3.1 *REF* and *SRPP* gene expression analysis by RT-PCR

For preliminary test, total RNA was extracted from latex of mature rubber trees and used for gene expression experiments. The relative transcription levels of the gene encoding for rubber biosynthesis were analyzed by RT-PCR. The expression levels of *REF* and *SRPP* genes in four selected clones were compared with the paired RRIM 600 in the same location. The results showed that the transcription levels of *REF* gene were significantly higher than the paired RRIM 600 clone in SK1, SK3, and NK1. No significant difference was noted in T2 and RRIM 600 in the same location. Four selected clones trended to have higher expression in *SRPP* gene. However, significant difference was found in only SK1 and the paired clone RRIM 600 (Figure 7 and 8).

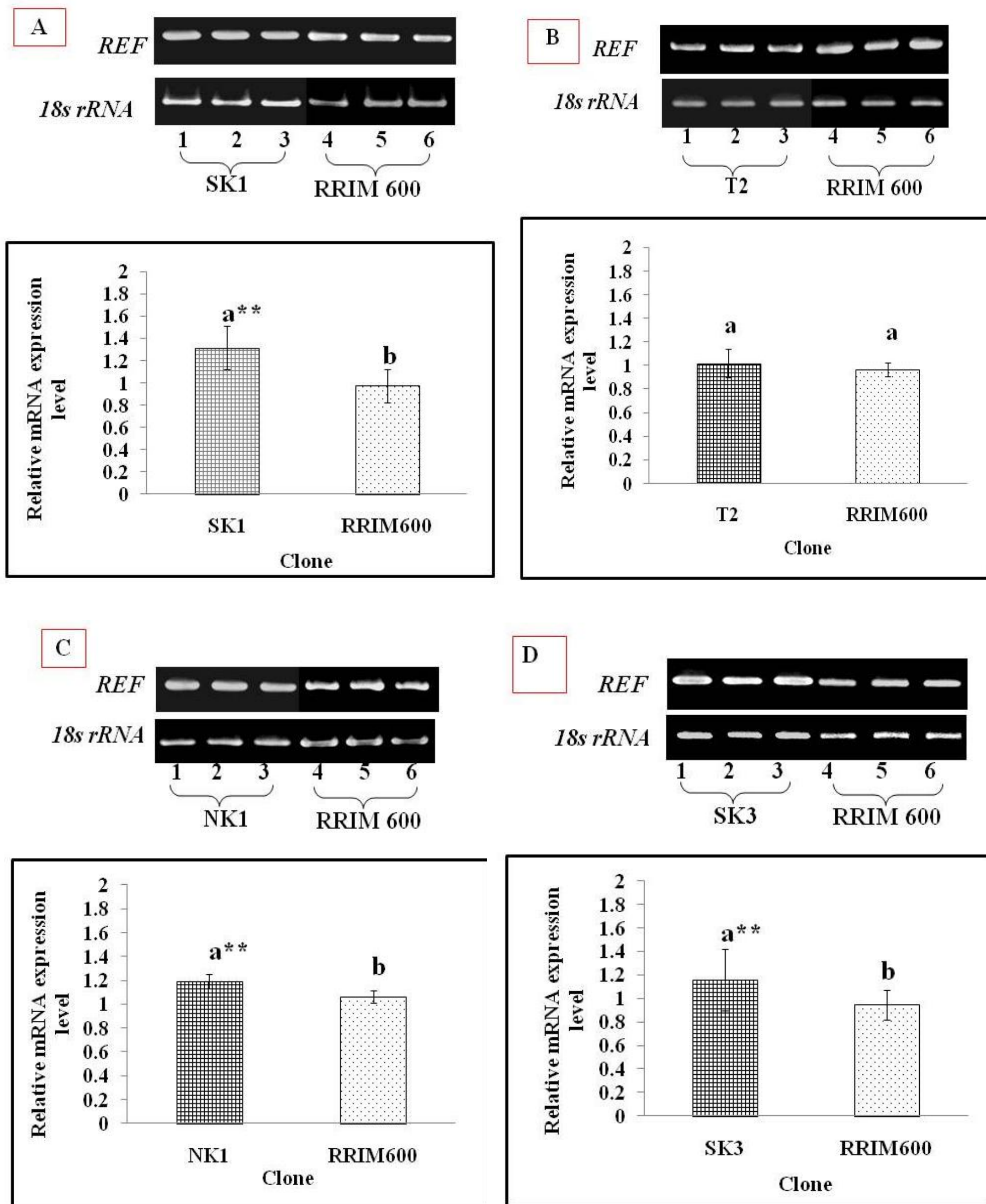

Figure 7 *REF* transcription levels of four selected clones compared with RRIM 600 grown in the same famer trail. a; SK1 clone. b; T2 clone. c; NK1 clone. d; SK3 clone. Each bar represented mean value result from tri-replicate ±SD

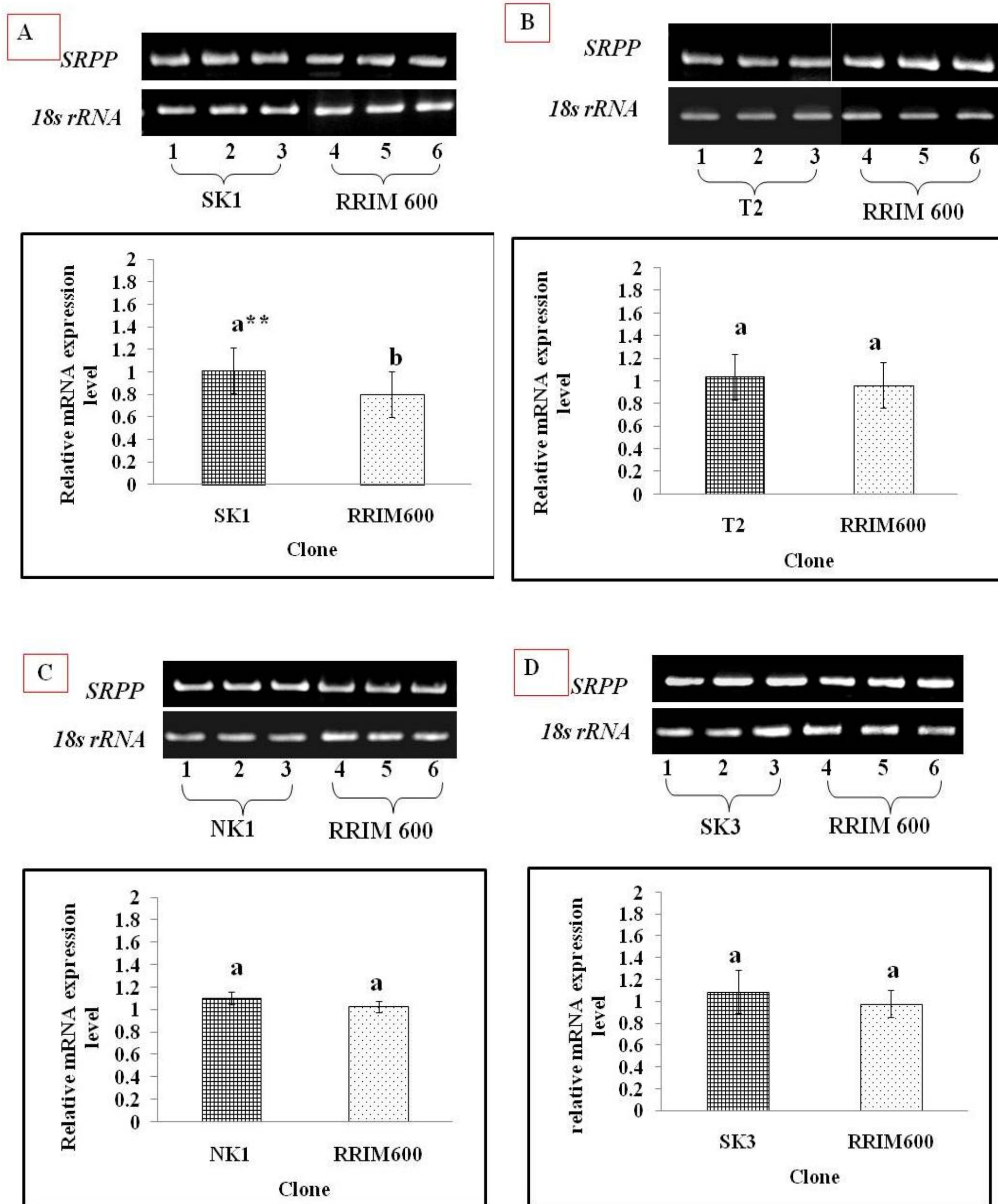

Figure 8 *SRPP* transcription level of four selected clones compared with RRIM600 which grown in the same famer trail. a; SK1 clone. b; T2 clone. c; NK1 clone. d; SK3 clone. Each bar represents mean value result from tri-replicate ±SD

3.2 Gene expression analysis by qRT-PCR

Because of some disadvantages of RT-PCR, qRT-PCR was then used to confirm the results. The qRT-PCR amplification of RNA from *REF*, *SRPP,* and *SUT3* genes were performed with specific oligonucleotide primers for the first strand cDNA. The specific primers were designed on the basis of *REF*, *SRPP* and *SUT3* genes (Table 4). By expecting for the gene expression, the analysis is found that the transcription levels were more expressed in the four selected clones than in the RRIM 600 paired-clone, with matching the high latex yield of the selected clones throughout the year.

3.2.1 *REF* gene expression

Expression of *REF* gene and latex yield were compared in different periods of year. The pattern of *REF* gene expression was identical in all locations (Figure 9). The highest latex yield and gene expression of four selected clones were observed in raining periods (October to December 2012) with showing of positive correlation.

3.2.2 *SRPP* gene expression

The relative *SRPP* expression levels of four selected clones revealed the similar patterns as *REF* gene expression (Figure 10). This result presented the *SRPP* gene of all selected clones at the highest transcription levels in raining periods and downward in the slightly rainfall periods. After that transcription levels were high when the early rainfalls started (June). Gene expression levels in all clones were correlated to the dry rubber yield of individual clone. According to the statistical analysis, the expressions of *REF* and *SRPP* genes were positively correlated to dry rubber yield.

3.2.3 *SUT3* gene expression

In this study, the qPCR amplification of *SUT* gene was performed by specific oligonucleotide primers by using the first strand cDNA preparing from RNA samples. Among eight *Hevea* putative cDNAs sucrose transporter; *SUT1A*, *SUT1B*, *SUT2A, SUT2B*, *SUT2C*, *SUT4*, *SUT5* and *SUT6,* only *SUT1B* were detected and showed the highest expression level in latex. As shown in figure 11, *SUT3* was transcribed significantly higher in four selected clones than the paired-control RRIM 600.

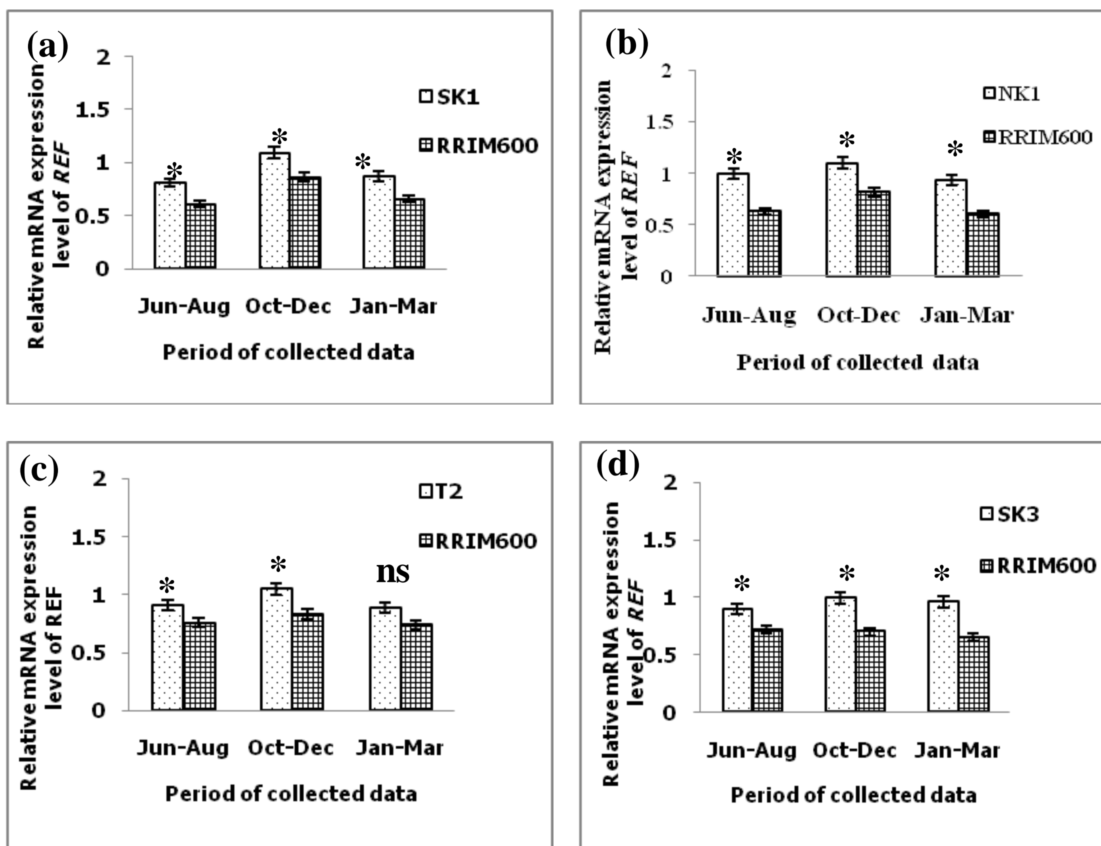

Figure 9 The *REF* gene's transcription observed by qRT-PCR in four selected rubber tree clones along with RRIM 600 baseline clone. Comparisons to baseline shown are paired by location. a; SK1 clone. b; NK1 clone. c; T2 clone. d; SK3 clone. The values shown are means from triplicate observations ± SD. Significant difference was indicated by the asterisks above the bars (*$p \leq 0.05$). ns represented non significant

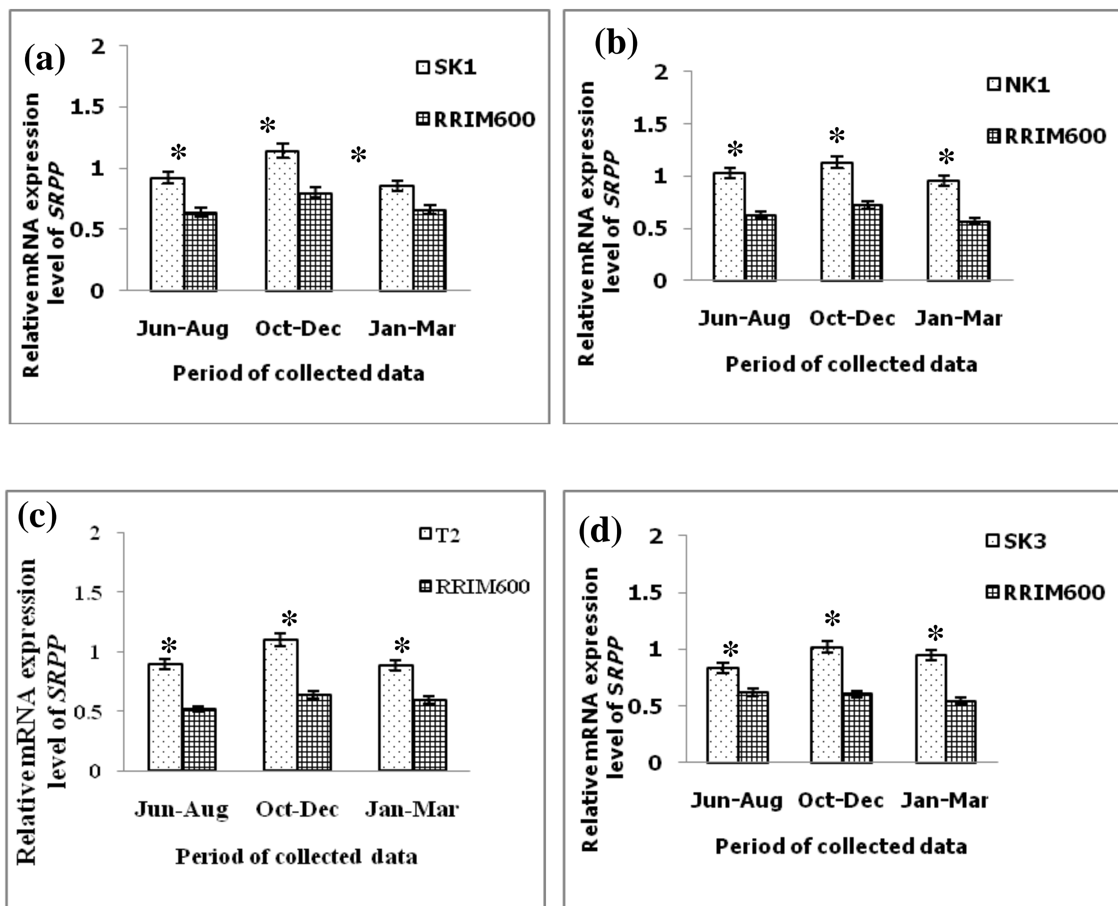

Figure 10 The *SRPP* gene's transcription observed by qRT-PCR in four selected rubber tree clones along with RRIM 600 baseline clone. Comparisons to baseline shown were paired by location. a; SK1 clone. b; NK1 clone. c; T2 clone. d; SK3 clone. The values shown were means from triplicate observations ±SD. Significant difference was indicated by the asterisks above the bars (*$p \leq 0.05$). ns represented non significance

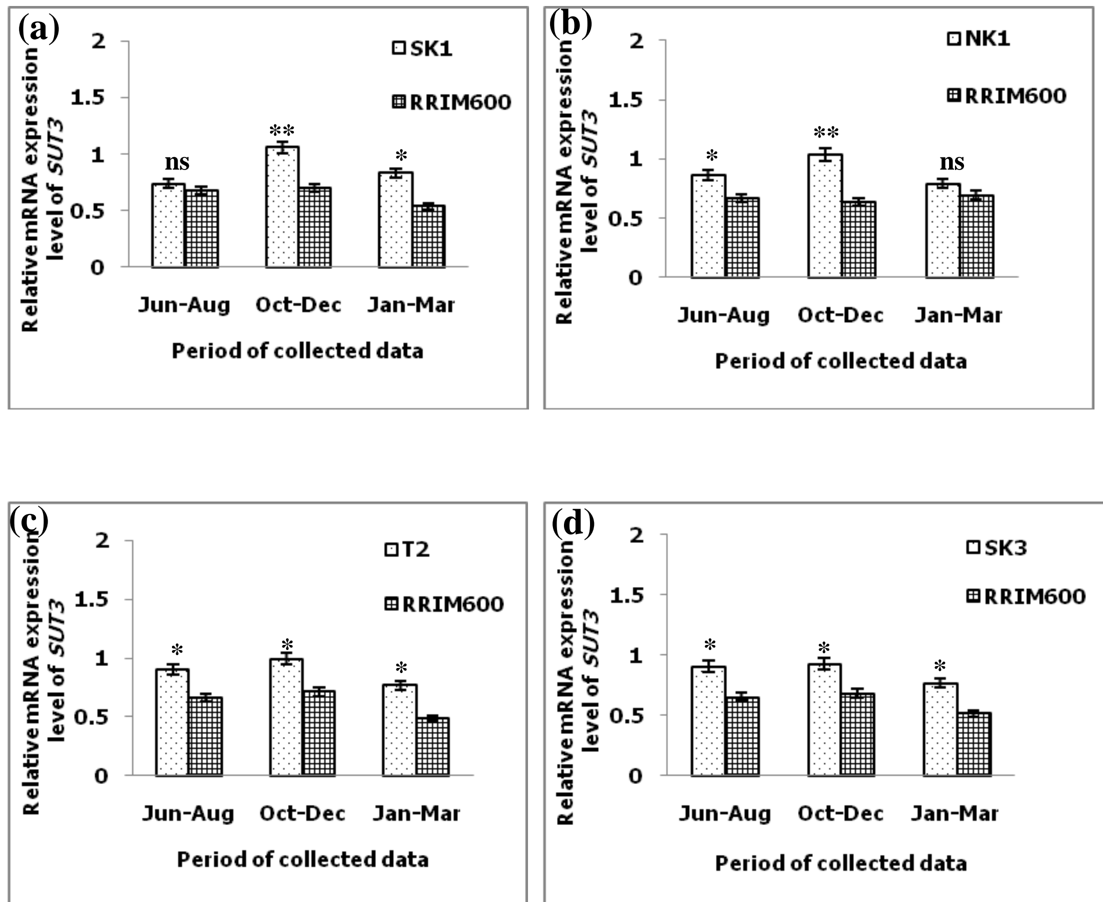

Figure 11 The *SUT3* gene's transcription observed by qRT-PCR in four selected rubber tree clones along with RRIM 600 baseline clone. Comparisons to baseline shown are paired by locations. a; SK1 clone. b; NK1 clone. c; T2 clone. d; SK3 clone. The values shown were means from triplicate observations ±SD. Significant difference was indicated by the asterisks above the bars (*$p \leq 0.05$). ns represented non significance

3.2.4 Gene expressions in various parts of rubber tree seedling

The expressions of *REF, SRPP,* and *SUT3* gene were monitored by real-time RT–PCR in barks and leaves of one year seedling (Figure 12-14). The transcription levels of those genes were used as a molecular tool for detection latex yield of selected clones compared to RRIM 600 and Indigenous clone. This study displayed that transcription levels in barks of three genes were higher than the transcription level in leaves.

For *REF* gene, the highest expression was found in the SK1 clone followed by NK1, T2, and SK3, respectively. The Indigenous clone showed the lowest transcription level. The similar result was obtained for *SRPP*, except SK3 presented higher transcription level than T2 clone. NK1 clone presented high *SUT3* level followed by SK1 clone. These results indicated the optimal perspectives for early selection of rubber tree in breeding program.

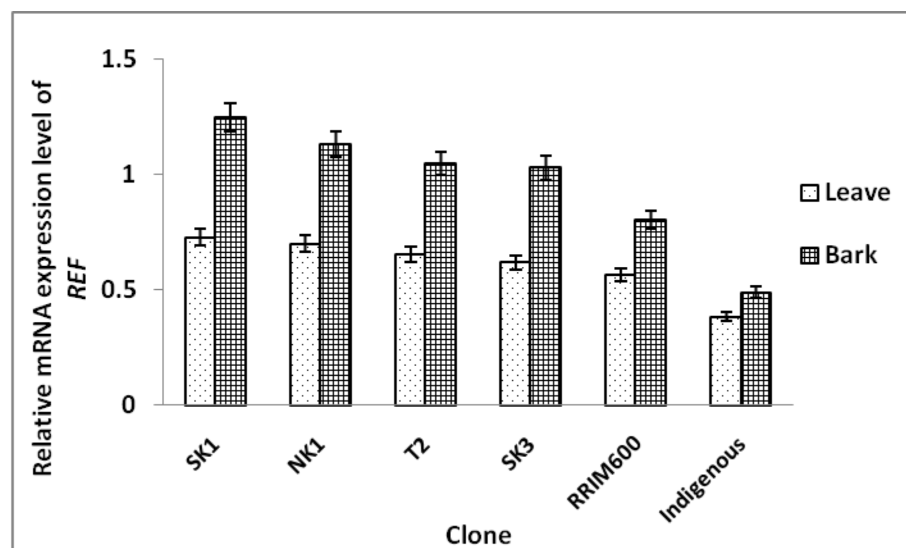

Figure 12 Comparison of the levels of *REF* transcripts in leaves and barks by qRT-PCR analysis in four selected clones, RRIM 600 and Indigenous clones in the same location

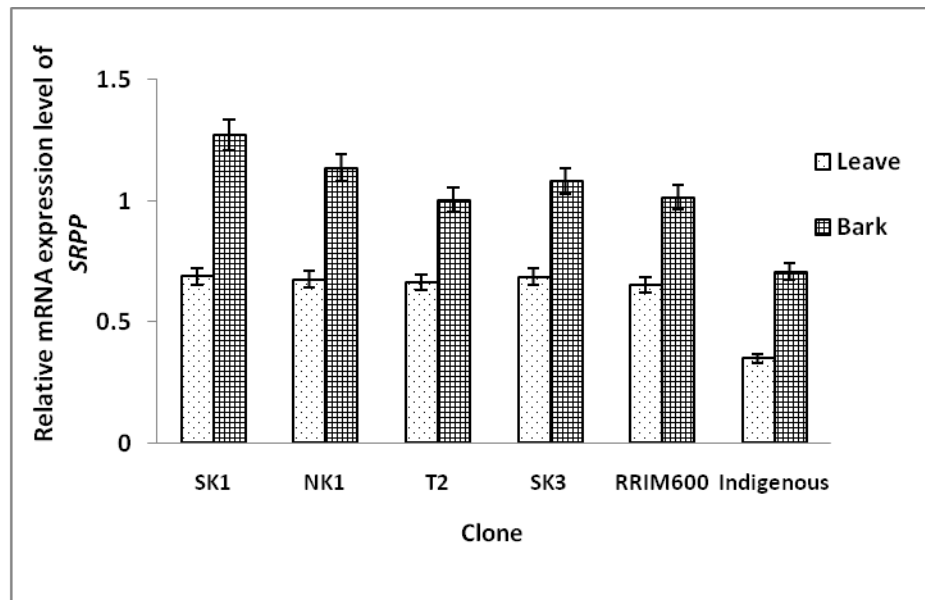

Figure 13 Comparison of the levels of *SRPP* transcripts in leaves and barks by qRT-PCR analysis in four selected clones, RRIM 600 and Indigenous clones in the same location

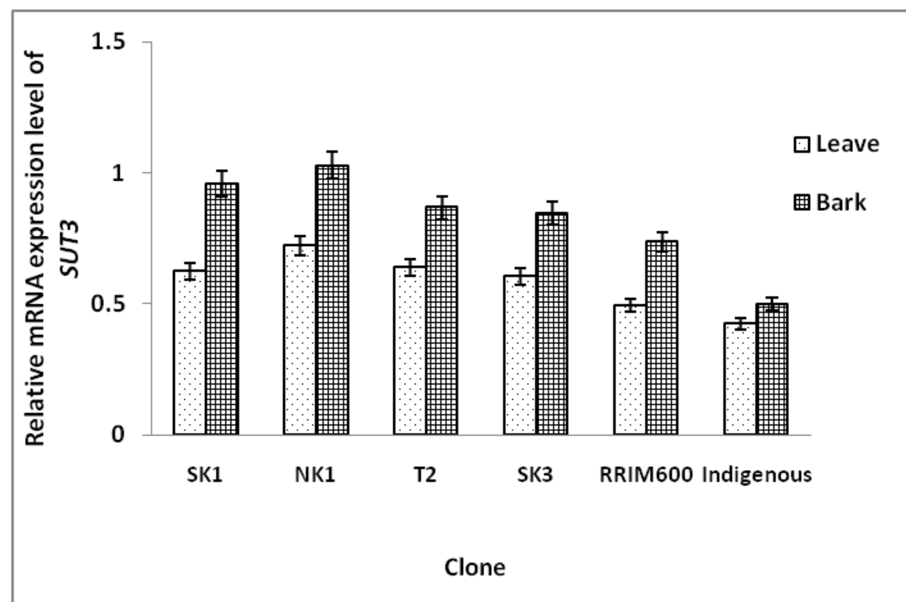

Figure 14 Comparison of the levels of *SUT*3 transcripts in leaves and barks by qRT-PCR analysis in four selected clones, RRIM 600 and Indigenous clones in the same location

4. Correlations among latex yield and *REF*, *SRPP* and *SUT3* gene expression

The Pearson's analysis in this study showed significant positive correlation among the expression of *REF*, *SRPP*, and *SUT3* genes and dry rubber yield. The correlation coefficient among yield and *REF*, *SRPP*, and *SUT3* expression were 0.7769-0.9521, 0.6220-0.9681, and 0.7110-0.8840, respectively (Table 9).

Table 9 Pearson's correlations among expressions of *REF*, *SRPP*, and *SUT3* gene and dry rubber yield (g/tree/tapping) of SK1, SK3, NK1, T2 clones, and RRIM 600 in each location.

Clones	Correlation coefficients (r) between dry rubber yield and *REF* gene expressions	Correlation coefficients (r) between dry rubber yield and *SRPP* gene expressions	Correlation coefficients (r) between dry rubber yield and *SUT3* gene expressions
SK1	0.9130**	0.6220*	0.8840*
RRIM 600	0.8150*	0.8610*	0.7150*
SK3	0.7769*	0.7060*	0.7323*
RRIM 600	0.8110*	0.8451*	0.7110*
NK1	0.8320*	0.9500**	0.8187*
RRIM 600	0.9190**	0.9681**	0.8190*
T2	0.9521**	0.7589*	0.8167*
RRIM 600	0.8700*	0.7839*	0.8280*

* = significant difference at $p \leq 0.05$, LSD

** = significant difference at $p \leq 0.01$, LSD

Chapter 4

Discussion

Selection of elite mother rubber tree from existing polyclonal seedling population and development of new clones ones of the most important methods for evolving new clones (Marattukalam *et al*., 1990). Polycross mother seedlings offer possibilities in the new clone development with desirable traits such as high yield, rapid growth, and tolerant to diverse environments. In the process of plant breeding, tree is still slow regarding to its long juvenile period in particular. The development of molecular markers related to the trait of interest provides efficient tools for selection the target genotypes. In rubber tree, selection for high latex yield may need at least 18-20 years. Priya *et al* (2007) reported a positive correlation between *REF* gene expression and latex yield. Expression of gene related to rubber biosynthesis such as *REF*, *SRPP*, and *SUT* genes can be possibly used for selection of high latex yield. The goal of this study was to identify and to clone a full-length of *REF* and *SRPP* genes that were associated with the latex biosynthesis and expression of these genes.

In order to confirm the nucleotide sequences and the full-length cDNA sequence of *REF*, *SRPP,* and *SUT3*, all genes were isolated and characterized from four selected clones and paired-control RRIM 600. Results from this study was similar to those reported earlier by Priya *et al*. (2006) which presented that the *REF* gene contained an open reading frame of 414 bp with a calculated Mw of 14.7 KDa and encode 138 amino acid peptides. *REF* gene revealed 100% sequence identity to *REF* sequences with previously reported cDNAs in the database. *SRPP* gene was also showed 100% identical with the isoform of *SRPP* sequences in the database. This report was similar to Oh *et al*. (1999) which contained a 612 bp open reading frame coding for 204 amino acid polypeptides. Furthermore, this study found GVV motif in position 83 in *SRRP* gene that could play the role of beta-sheet breaker (Berthelot *et al*., 2012). Berthelot *et al.* (2012) suggested that REF and SRPP proteins were hydrophobic and

REF had amyloid properties in contrast that had to SRPP α-helical protein. Berthelot *et al.* (2012) displayed that REF was inserted into the membrane of the Large Rubber Particle (LRP) while SRPP was bound to the surface of the Small Rubber Particle (SRP). Wititsuwannakul *et al*. (2008) reported that SRPP had an important role in the latex coagulation to protect plants by wound sealing with suggesting an important role for *TbSRPPs* in rubber biosynthesis and plant stress. Analysis of the full-length of both genes in different clone was revealed that it was shown 99-100% similarity to amino acid from previously submitted to the GenBank. This study revealed that the different sequencing amino acid had no effect to transcription levels of both genes because the different sequencing was not important motif or domain.

A full-length of *SUT* gene was identified. For the nucleotide sequence analysis, the results showed that the amplified partial sequence of *SUT* gene contained 1,373 bp open reading frame encodes a protein of 457 amino acids. From this study only 84% of the cloned *SUT* was identical to the isoform of *SUT3* and *SUT6* sequence in *Hevea* according to the NCBI database. Dusotoit-coucaud *et al*. (2009) reported that *SUT3* gene was more response to the latex biosynthesis than those of the other *SUT* genes.

Latex dry rubber yield, girth increments and biochemical parameters of latex

1. Average of dry rubber yield and girth increment of selected controlled clones

Yield figure presented that the selected clones (SK1, NK1, T2 and SK3) were superior to the paired-control RRIM 600. The highest yield was recorded on SK1. This data were confirmed from a previous reported by Pethin *et al.* (2015) who presented that the average yields of the 3 clones including SK1, NK1, and SK3 were surpassing the paired RRIM 600 controls. Not only the latex superior performance, but also girth increment was differed upwards from the paired-controls.

The girth increment of rubber tree is one of the important factors used for selective high latex yield. The positive correlation between girth increment and latex

yield rubber trees had been reported by Karunaratne *et al.* (2005). The study showed that one unit of girth increment resulted for the increasing in GTT clone and volume of latex. This study had similarity with the previous one that had found the higher of girth increment in high latex yielding clones than RRIM 600 (Table 7).

Rubber latex yield was influenced by variations in climate and soil moisture, especially the annual cumulative rainfall. Table 6 presented the monthly dry rubber yield during 3 periods of the year 2012-2013: June - August 2012, October - December 2012, and January - March 2013. Sdoodee and Chiarawipa (2005) reported that February to March was a dry period with low rainfall. The rainfall increased again from May to July and tended to decrease from August to September (Sdoodee *et al*., 2010). Variations in the productivity of various rubber tree clones in this study showed very similar patterns. The four selected clones had higher monthly latex yield than the paired control (RRIM 600) at all locations. The latex yield increased from June (when tapping restarted), attaining its maximum in October, and holding steady till December, after that yield decreased to a minimum in March when tapping stopped. This pattern was the same regardless of clonal, and the dry rubber yield was highest during October to December. Cumulative rainfall and relative humidity tended to increase latex yield (Rao *et al*., 1998; Mak *et al*., 2008; Suwannmanee *et al*., 2002; Tungngoen *et al*., 2009). Latex yield during January to March 2013 was lower when compare to other periods. Mak *et al*. (2008) also reported that latex yield of rubber tree in dry season was lower than those in rainy season. This would be explained by low growth rate and the number of leaves in these seasons (Tongsawang and Sdoodee, 2008). From the middle of March, rubber trees started to exhibit leaf-flushing. Clearly latex yield was affected by annual seasonal variations, and such effects had to be accounted in an analysis looking for predictive biomarkers of yield. Results of this study were similar to Mak *et al*. (2008), who reported that the fresh latex yield increased to its maximum from September to December, a rainy season, and the latex volume was related to the cumulative rainfall (Rao *et al*., 1998; Tungngoen *et al*., 2009). In a similar manner, this study showed significant positive correlation of atmospheric relative humidity and latex yield, the most

clones studied. This positive correlation was probably due to reducing of water loss by transpiration at high relative humidity.

2. Biochemical parameters of latex

Latex diagnosis was carried out during June 2012 to March 2014. Biochemical such as dry rubber content (DRC), sucrose, organic phosphorus, and thiol were evaluated. In all selected clones, the DRC values in latex were higher than those of RRIM 600. DRC had been reported of its effect to viscosity of latex so that a high DRC might limit latex flow whereas low TSC was presented weak latex regeneration (Pethin *et al*., 2015; Tang *et al*., 2013). In this study, RSH or thiol did not differ between the selected clones and the control. Thiols played roles to activate the key enzymes in the laticiferous system and to maintain the normal functions of the laticifers (Pethin *et al*., 2015; Tang *et al*., 2013). Thiol indicated the level of lutoid protection and stability of latex (Traove *et al*., 2011). In all selected clones showed latex sucrose content lower than the control whereas latex inorganic phosphorus content (Pi) was higher than the control. This result was confirmed by Chantuma *et al.* (2006) who reported that latex sucrose and Pi content were negatively correlated. The low sucrose content reflected active sucrose catabolism in the lactiferous cells. Moreover, the high Pi content presented the level of available energy in metabolism of latex regeneration process that had correlated with rubber production (Traore *et* al., 2011; Tang *et al*., 2013; Pethin *et al*., 2015). These results revealed that the NK1 had a better latex biochemical parameter because this clone exhibited high latex yield together with low Suc and high Pi contents.

Gene expression analysis

1. *REF* and *SRPP* gene expression RT-PCR technique

Total RNA was extracted from latex of mature rubber trees and used for gene expression experiments. In this study, the relative transcription levels of the gene

encoding for rubber biosynthesis were analyzed by RT-PCR. Results showed that transcription levels of *REF* and *SRPP* genes in the four clones were higher than those of RRIM 600 in the same field (Figure 7 and 8). Previously, Priya *et al*. (2007) revealed that the *REF* transcription level could be separated for high yielding clones (RRII 105, PB 235, and PB260) from low yielding clones (KRS 25, KRS 128 and KRS 163). Research in *Taraxacum kok-saghyz* by Collins-silva *et al*. (2012) reported *that SRPP* was involved in controlling rubber content and molecular weight. These findings were supported by Wititsuwannakul *et al*. (2008) reported that the SRPP bound to a *Hevea* latex lectin protein and induced latex coagulation. SRPP family had a broader role in the rubber biosynthesis, not only influencing rubber polymerization and rubber quality, but also influencing to latex coagulum (Collins-silva *et al*., 2012).

2. Gene expression analysis by qRT-PCR

Gene expression analysis is very useful for understanding signaling and metabolic pathways (Hu *et al.,* 2009). RT-PCR has been used for gene expression analysis, however several disadvantages are found in this technique such as its complexity, low sensitivity associated problems, reproducibility, and specificity (Bleve *et al.,* 2003). The qRT-PCR technique is popular in the quantitative analysis of gene expression. qRT-PCR has better sensitivity and specificity along with a broader quantification range in comparison to prior molecular techniques such as Northern blot analysis and RT-PCR (Exposito-Rodriguez *et al.,* 2008). The qRT-PCR has been shown to be a robust of highly reproducible and sensitive method to quantitatively track functional gene changes across temporal and spatial scales with under varying environmental or experimental conditions (Exposito-Rodriguez *et al.,* 2008). Thus, qRT-PCR is effective method for early checking the gene expression in selection new rubber clones of this study.

2.1. *REF* and *SRPP* gene expressions

The main genes involved in rubber biosynthesis had been characterized. Among the genes identified, *REF* was a key rubber biosynthesis gene involved in the polymerization of isoprene chains (Venkatachalam *et al.,* 2007). Reverse northern blot analysis had shown that the product of *REF* gene was accumulated more than 10-fold in the latex (Ko *et al*., 2003) while an SRPP protein is closely associated with rubber particles and might be directly involved in rubber biosynthesis (Dennis and Light 1989; Yeang *et al*., 1996). Priya *et al*. (2006) reported that among the transcripts expressed in latex, *REF* was the most abundant followed by small rubber particle protein (*SRPP*). Thus, the expression levels of these two genes, in the clones studied, were of interest as biomarker candidates predictive of latex yield. The motivation for such biomarkers was rapid early surrogate selection criteria in breeding high yielding rubber tree clones in breeding programs.

The four high-yield clones exhibited the strongest differential expression of *REF* and *SRPP* relative to the baseline clone in the late rainy period (October to December) as shown in Figures 9 and 10. Results obtained from Pearson's analysis in this study showed significant positive correlations between the expression of *REF* and *SRPP* genes and dry rubber yield (Table 6). These results suggested that both genes would be used for selection of high yield clone. This study supported a previous reported by Priya *et al*. (2006) presented the positive correlation of *REF* gene expression between high and low yield clones. In contrast, Ruderman *et al*. (2012) reported no significantly correlation between *REF* expression and latex yield. However, its real role of this gene was rather controversial (Ruderman *et al*., 2012). Ruderman *et al* (2012) also reported that *SRPP* expression had suggested negative correlation between *SRPP* expression and latex yield which had contrary to this study. *SRPP* had been reported of involving in rubber biosynthesis in several plants such as *Tarakxacum kok*, and *Parthenium argentatum* and played a role in synthesis rubber particle of bacterial in *in vitro* (Kim *et al.,* 2004). Therefore, using *SRPP* gene expression as a marker for high

yield rubber selection was interesting. This result corroborated the association of latex yield to the expression of these two genes.

The study found that when tapping started in June, the dry rubber yield and gene expression were both low. After the rainy season in October, the gene expression levels increased, and started to decrease in January and reached their minimum yield recorded in March. By expecting, for the expression, the study indicated that the low yield was obtained in February because most trees had lost their leaves and photosynthesis activity was slow down. Similarly, when the rubber tree tapping was started after the rest period in April, some lag time was taken before the tree metabolism was fully activated for optimum yield (Njukeng *et al*., 2011).

Results of this study showed that the transcription levels of *REF* and *SRPP* appeared to have potential to be predictive molecular markers to be used in selection criteria for high-yield rubber tree improvement. The late rainy season was a good period to investigate differential expression of the candidate molecular marker genes. The yearly seasons greatly affected differential gene expression with restricting the latex sampling for gene expression in case for using in a selective breeding program.

2.2 *SUT3* gene expression

The sucrose transporter had been reported of involving in many physiological processes of both sink and source organs (Dusotoit-Coucaud *et al*., 2009). For example, Baud *et al.* (2005) presented that *AtSUC5* was expressed in the endosperm and played for the supply of sucrose to seeds during early stages of development. In *Juglans regia*, sucrose transporter *(JrSUT1*) was isolated from xylem parenchyma cells. Not only sucrose retrieved from the xylem vessel after freezing and thawing (Decourteix *et al*., 2006) but also provided sucrose to bursting vegetative buds (Decourteix *et al*., 2008). In *Hevea*, sucrose was the raw material particularly for rubber biosynthesis metabolism (Tupy, 1985). Relationship between latex yield and latex

biochemical parameters was needed to be used for selection the best-adapted clone suited for the local conditions.

In this study, the qPCR amplification of *SUT* gene was performed by specific oligonucleotide primers by using the first strand cDNA preparing from RNA samples. Among eight *Hevea* putative cDNAs sucrose transporter; *SUT1A*, *SUT1B*, *SUT2A, SUT2B*, *SUT2C*, *SUT4*, *SUT5*, and *SUT6,* only *SUT1B* (*SUT3*) was detected and showed the highest expression level in latex. *SUT3* was significantly higher transcribed in four selected clones than those of the paired control. This finding was confirmed by Tang *et al.* (2013) who reported that *SUT3* was the dominant SUT member expressed at least six folds in latex as compared with other SUT genes with indicating its role in metabolic function of laticifers (Dusotoit-coucaud *et al*., 2009; Dusotoit-coucaud *et al.,* 2010). *SUT3* was expressed at the highest level in NK1 followed by SK1 clone. This study found that *SUT3* exhibited positively correlation to dry rubber yield. Results of this study were similar to the previous report in China by Tang *et al*. (2013). Tang *et al*. (2013) concluded that transcription level of *SUT3* correlated with the rubber yields and was expressed at the highest level in supper high yielding tree (SY107).

This study suggested that latex yield figure observed in one-year had presented the selected clones greater than the RRIM 600 control. The NK1 clone was featured in its high latex yield with good latex biochemical parameters and had the highest *SUT3* transcription level. Thus, the NK1 clone was interesting to be supported as a new latex clone which was confirmed by Pethin *et al*. (2015) dealing in genetic variability of rubber tree clones. Pethin *et al*. (2015) reported that the genetic background of NK1 was closely related to RRIT 250. RRIT 250 was the rubber clone that would give medium to high latex yield (RRIT, 2011).

2.3 Gene expression in various plant parts of rubber tree seedling

From the result of transcription levels in mature rubber tree, the study revealed the good perspective for screening and selection new rubber clone by gene related to rubber biosynthesis such as *REF, SRPP,* and *SUT3*. Thus, this study had

interesting to study expression levels of these genes in rubber seedling for applying to use as molecular tools of detection high yielding clone at seedling stage. Since seedlings had not enough latex to proceed gene expression protocol, leaves and barks were investigated. This study indicated the different transcription level of *REF, SRPP,* and *SUT3* between leaves and barks of selected rubber clones. Gene expression in the barks displayed higher levels than leaves. Both leaves and barks contained latex, but laticifer in barks might reflect the higher accumulated transcription level of genes than in leaves (Aoki *et al.,* 2014).

In this study, SK1 seedling showed the highest *REF* and *SRPP* transcription levels followed by NK1 (Figure 12 and 13), and both transcription levels were significant higher than those of RRIM 600 and Indigenous clones. Results of this study implied that all selected clones showed the different transcription levels of *SUT3* between leaves and barks. The barks was displayed higher accumulation of sucrose gene than leaves (Figure 14). In untapped rubber tree, the highest transcription level of SUT3 was obtained from female flowers, green branch barks, and leaves, respectively (Tang *et al*., 2013). The similar results were reported by Dusotoit-coucaud *et al*. (2009) that the *SUT3* gene was predominantly expressed in leaves and barks of untapped rubber tree. Trunk barks had been reported to be a strong artificially created sucrose sink for rubber producing (Tang *et al*., 2013). Priya *et al*. (2007) reported that a transcription level in barks was higher than leaves with over 10 folds. These results implied that the bark of rubber seedling is suitable for early checking of gene expression in rubber breeding program.

This study indicated that three genes transcription levels in the bark could distinguish between low and high latex yielding clones (Figure 12-14) so that candidate clones for high latex yield could be scored in their seedling stages. Using multiple selection criteria is necessary to speed up effective rubber breeding programs (Venkatachalam *et al*., 2007). On comparing seedling plants grown at the same location these two clones (SK1 and NK1) also displayed higher transcription levels. The clones

of SK1 and NK1 appeared to be the excellent starting points for further rubber tree improvement. These data corroborated a previous investigation by Pethin *et al.* (2015).

Correlation between gene expression and latex yield

The Pearson's analysis in this study showed significant positive correlation between the expression of *REF* and *SRPP* genes and dry rubber yield (Table 9). These results suggested that both genes had relationship between the expression genes and the clonal rubber yield. This confirmed a previous reported by Priya *et al.* (2006) presenting the positive correlation of *REF* gene expression between high and Indigenous clone. However, this study indicated the contrary to the previous report by Ruderman *et al*. (2012) that *REF* expression has no significantly correlation with latex yield. However, its real role of this gene is rather controversial (Ruderman *et al*., 2012). Besides, Ruderman *et al* (2012) reported that *SRPP* expression showed negative correlation with the clonal latex yield contrary to the result of this study. Thus, *SRPP* had been reported with involving in rubber biosynthesis in several plants such as *Tarakxacum kok*, and *Parthenium argentatum* and played a role in synthesis rubber particle of bacterial in in vitro (Kim *et al.,* 2004). Therefore, *REF* and *SRPP* gene were interesting to use as marker for check relationship between gene expression and rubber yield.

In this report, the study found that *SUT3* was positively correlated to dry rubber yield. This was similar to a previous report by Tang *et al*. (2013) in which the transcription level of *SUT3* correlated with the rubber yield and was the most strongly expressed in the super-high yielding rubber tree (SY107) in China. Sucrose is the raw material for rubber biosynthesis and reflects the balance of sucrose loading and utilization in the laticifers (Tang *et al*., 2013). Sucrose utilization had been reported of being limited by the activity of a neutral/alkaline type of invertase and thus constituted one of the limiting factors for latex production in *Hevea* (Tang *et al*., 2013). The low sucrose contents in all selected clones were recorded in this study. The result revealed that the lower sucrose content in selected clone was due to more active sucrose

utilization. Catabolism in the latex was important factor affecting rubber productivity (Tupy, 1985; Tang *et al*., 2013) whereas *SUT3* encoded a functional sucrose transporter and played an active role in sucrose loading into the laticifer (Tang *et al.,* 2010).

Chapter 5

Conclusion

The study covered four new generation clones, the majority of which originated from seedlings of open pollinated progenies. The four clones selected that had high latex yield were sampled from rubber plantations in three southern provinces of Thailand. The private farm locations were in Songkhla province (SK1 and SK3), Nakhon Si Thammarat province (NK1), and Trang province (T2). The data were collected for two years from June 2011 to March 2013. The clones studied presented varying levels of latex yield. The latex yield of four selected clones was significantly higher than RRIM 600 in the same location. Besides, the study showed a superior of girth increments and biochemical parameter. The nucleotide sequences and the full-length cDNA sequences of *REF*, *SRPP*, and *SUT* genes were isolated and characterized from the four selected clones and RRIM 600. Results from cDNA sequencing indicated that the open reading frames of *REF* and *SRPP* genes contained 417 and 615 bp in length encoding for 138 and 204 amino acids, respectively. Among the putative full-length cDNAs of *Hevea* sucrose transporters available in the NCBI database, only *SUT3* was detected. The open reading frames of *SUT3* gene were 1,371 in length encoding for 457 amino acids. According to RT-PCR analysis, the mRNA accumulation of both *REF* and *SRPP* exhibited superior to the paired-control RRIM 600. Results were confirmed by qRT-PCR. *REF, SRPP*, and *SUT3* genes presented significantly higher transcribed in four selected clones than the paired-control RRIM 600. According to qRT-PCR analysis, the mRNA accumulation of *REF*, *SRPP* and *SUT3* was positively correlated with the latex yield in terms of the dry rubber amounts collected from the high yielding clones. In various parts of one year old rubber seedling, the study found that the expressions of *REF*, *SRPP,* and *SUT3* genes were presented higher levels in barks than leaves. The barks appeared a good choice to sample from seedlings for determining the transcription levels of rubber biosynthesis genes. These data indicated that the

expressions of *REF, SRPP,* and *SUT3* genes could be used as molecular tools for selection high yield in seedling stage.

In brief, studies on gene related to biosynthesis of latex in rubber tree will be used as the information in breeding program. In this study, three genes could be used as molecular tools for selection high yield clones. Especially, *SUT3* was interesting for investigation of gene expression which was related to latex or rubber biosynthesis and used as molecular makers to determine high yield rubber tree. From preliminary results, the performance of SK1, SK3, NK1, and T2 clones along with RRIM 600 over a experimental period proved that all 4 clones had high potential to be developed as new rubber materials. In addition, other characteristic should be studied such as resistance to diseases, pests, or adaptability to different environments. Collecting data for several years will probably be useful in confirming the outstanding *Hevea* clones for yield improvement.

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Appendix

Appendix A

Chemical for RNA extraction

1) TE buffer, 500 ml

1.0 M Tris-HCl (pH 7.5)	500	ul
0.25M Na_2EDTA (pH 7.0)	200	ul

Deionizer water was added to make the final volume to 500 ml and sterilize using an autoclave. Kept the buffer solution at 4 °C.

2) 5X RNA extraction buffer, 200 ml

0.1m Tris-HCL	12.1 g
0.1m EDTA	4.0 g
10% SDS	40.0 g

Deionizer water was added to make the final volume to 200 ml and sterilize using an autoclave. Kept the buffer solution at 4 °C.

3) 5X TBE, 1L

Tris Base	54 g
Boric acid	27.5 g
0.5M Na_2EDTA (pH 8.0)	20 mL

Deionizer water was added to make the final volume to 1 L and sterilize using autoclave. Kept the buffer solution at room temperature.

4) 0.5M Na_2EDTA (pH 8.0), 500 mL

Na_2EDTA .2H $_20$	93.50 g

Deionizer water was added to make the final volume to 500 mL and slowly adjust the pH with 10N NaOH after that sterilize using autoclave. Keep the buffer solution at 4 °C.

Note: EDTA would not completely dissolve until the pH reaches 8.

5) 5M NaCl, 1L

NaCl	292.20 g

Deionizer water was added to make the final volume to 1 L and sterilize using autoclave. Kept the buffer solution at 4 °C.

6) 10M NaOH, 500mL

NaOH	200 g

Deionizer water was added to make the final volume to 500 mL. Kept the buffer solution at 4 °C.

7) 1M Tris base, 1L

Tris base	121.10 g

Adjusted the pH to 8 with concentrated HCL. Brought up the volume to 1L with deionizer water. Kept the buffer solution at 4 °C.

8) 50x TAE, 1L

Tris base	242.20 g
Glacial acetic acid	57.10 mL
0.5M EDTA	100.0 mL

Adjusted the pH to 8 with glacial acetic acid. Brought up the volume to 1L with deionizer water and sterilize using autoclave. Kept the buffer solution at room temperature.

9) 1% SDS, 100 mL

Sodium dodesyl sulfate (SDS)	1 g

Brought up the volume to 1L with deionizer water and sterilize using autoclave. Kept the buffer solution at 4 °C.

10) 3M sodium acetate (pH 5.2), 1L

Sodium acetate 408.1 g

Adjusted the pH to 5.2 with glacial acetic acid. Brought up the volume to 1L with deionizer water and sterilize using autoclave. Kept the buffer solution at 4 °C.

11) Ethidium bromide (10 mg/ml)

Ethidium bromide 1 g

Brought up the volume to 1 mL with deionizer water. Stirred on a magnetic stirrer for several hour that the dry has dissolved. Wrapped the container in aluminum foil or solution to a dark bottle and stored at room temperature.

12) 0.1 M $CaCl_2$, 1L

$CaCl_2.H_2O$ 14.7 g

Brought up the volume to 1L with sterilizes deionizer water. Keep at 4 °C.

13) Phenol Preparation (Buffering) Procedure

1. Removed the crystalline phenol from the –20°C freezer and thawed it at 65°C
2. Added desired volume of phenol to an appropriate sized bottle. (If you want/need 250-500ml of equilibrated phenol, use a 1000ml bottle.)
3. Added an equal volume of 10X TE to the phenol.
4. Shaked vigorously. Allow the layers to separate. This may take a while.
5. Aspirate off the aqueous (the top) layer. Do this in the fume hood.
6. Repeated with a second equal volume of 10X TE.
7. Added an equal volume of 1X TE to the phenol.
8. Repeated with a second equal volume of 1X TE.
9. Leaved a small layer of 1X TE above the phenol after the final aspiration.
10. Checked the pH of the TE by dropping about 10 μ onto pH paper. It should be about pH 8; if it is still too high, perform additional TE equilibration steps.

Antibiotic stocks

1) Ampicillin (1000 mg/mL)

Ampicillin	100 mg

Dissolved in 1 mL of sterile deionizer water. Stored at -20 °C.

2) Tetracyclin (10 mg/mL)

Tetracyclin	10 mg

Dissolved in 1 mL of absolute ethanol. Stored at -20 °C.

3) Kanamycin (25 mg/mL)

Kanamycin	50 mg

Dissolved in 1 mL of sterile deionizer water. Stored at -20 °C.

LB broth media, 1L

tryptone	10 g
yeast extract	5 g
NaCl	10 g

Brought up the volume to around 900 mL with deionizer water, then adjusted the pH to 7.4. Brought up the volume to 1 L with deionizer water and sterilize using autoclave. Kept the buffer solution at 4 °C.

LB agar media, 1L

tryptone	10 g
yeast extract	5 g
NaCl	10 g
1.8% Agar	18 g

Brought up the volume to around 900 mL with deionizer water, then adjusted the pH to 7.4. Brought up the volume to 1 L with deionizer water and sterilize using autoclave. Kept the buffer solution at 4 °C.

Buffer preparation protocol for biochemical parameter of latex

Sucrose content analysis

1. 2.5% w / v TCA + 0.01% w / v EDTA

Trichloride acetic acid (CCl3COOH) 25 g

Ethylenediaminetetra acetic acid (C10H16N2O8) 0.1 g

Brought up the volume to 1 L with deionizer water

2. anthrone 1000 milligrams per liter

conc. Sulfuric acid: $H\ 2SO_4$ 100 ml

anthrone ($C_{14}H_{10}O$) (molecular weight = 194.23) 0.10 g

Brought up the volume to 1 L with deionizer water

3. 25 mM sucrose standard solution

Sucrose ($C_{12}H_{22}O_{11}$) 0.8557 g

Brought up the volume to 100 mL and preparation 0, 0.25, 0.50, 1.00 and 1.50 mM sucrose standard solution by micro-pipette. Stored at solution at 4 $^{\circ}$C.

Thiol content analysis

1. 2.5% w / v TCA + 0.01% w / v EDTA

Trichloride acetic acid (CCl3COOH) 25 g

Ethylenediaminetetra acetic acid (C10H16N2O8) 0.1 g

Brought up the volume to 1 L with deionizer water.

2. Tris buffer) 0.5 M, 100mL

Tris (hydroxymethyl) -aminomethane (C4H11NO3) 6.06 g

Brought up the volume to 100 mL with deionizer water.

3. DTNB solution

5,5'-dithiobis (2-nitrobenzoic acid) (MW = 396.36) 397 mg

Ethylenediaminetetraacetate (MW= 292.2) 710 mg

Tris buffer 0.5 M 20 mL

Adjusted the pH to 6.5. Brought up the volume to 100 mL with deionizer water. Stored in a dark bottle and kept at 4 °C.

4. 2.5 mM glutathione standard solution

Glutathione (MW = 307.33) 0.0192 g

Glutathione was mixed with TCS + EDTA solution. Then adjusted volume was 25 mL. Preparation 0, 0.02, 0.04, 0.06 and 0.08 mM glutathione standard solution by micro-pipette. Stored at solution at 4 °C.

Inorganic phosphorus content analysis

1. 2.5% w / v TCA + 0.01% w / v EDTA

Trichloride acetic acid (CCl3COOH) 25 g

Ethylenediaminetetra acetic acid (C10H16N2O8) 0.1 g

Brought up the volume to 1 L with deionizer water.

2. 25 mM phosphate standard solution

Potassium dihydrogen phosphate (KH2PO4) 0.3125 g

Brought up the volume to 100 mL with deionizer water. Preparation 0, 0.5, 1.0, 2 and 3 mM phosphate standard solution by micro-pipette. Stored at solution at 4 °C.

4. Reagents solution

1. Ammonium metavanadate (NH4VO3) 1.25 g

Brought up the volume to 250 mL with deionizer water and 250 mLnitric acid was added.

2. Ammonium molybdate ((NH_4) 6MO $_7O_{24}.H_2O$) 25.00 g

Brought up the volume to 400 mL with deionizer water.

3. The solution in 1 and 2 was mixed and adjusted the volume with deionizer water to 1L. The reaction 1:3 of water was prepared to use for phosphate analysis.

Calculation methods

The sucrose, thiol, and inorganic phosphorus content were measured by UV Visible spectrophotometer. The data of absorbance was collected and built the relationship among the concentrations of Sucrose, Thiol, and inorganic phosphorus (y) and the absorbance (x) by Microsoft Excel.

Appendix B

Amino acid

Table 1 Abbreviations and molecular weight for amino acid

Amino acid	Three letter abbreviation	One letter abbreviation	Molecular weight (Da)
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic acid	Glu	E	147
Glycine	Gly	G	75
Histidine	His	H	155
Isoleucine	Ile	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

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