Pak. J. Biotechnol. Vol. 14 (1) 115-120 (2017)
www.pjbt.org

ISSN Print: 1812-1837
ISSN Online: 2312-7791

RUBBER ELONGATION FACTOR (*REF*) AND SMALL RUBBER PARTICLE PROTEIN (*SRPP*) GENE EXPRESSION RESPONSES TO VARIATION OF SEASONAL CHANGE IN FOUR SELECTED RUBBER CLONES

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Article Received 18.12.2016; Revised 22.3.2017; Accepted 26.3.2017

ABSTRACT

Applying molecular marker for estimating the amount of production yield will help to reduce time for selecting rubber clones. In this study, gene expression analyses under different seasons were performed in rubber tree since seasonal change is one of the most important factors to latex yield. Four rubber clones (SK1, NK1, T2 and SK3) that have high latex yield potential were used in this study. The latex yields and gene expression levels of *REF* and *SRPP* genes were compared over two year, using RRIM 600 clones in the same fields as their paired controls. Moreover, the seasonal effects on gene expression were analyzed. The average yields and gene expression were obtained from 13 years old mature trees in the early rainy season, the late rainy season and the summer season**.** This study revealed that all four selected clones gave higher production yield and exhibited superior *REF* and *SRPP* gene expressions than compared-control RRIM 600. Gene expressions of *REF* and *SRPP* had positive relationships with latex yield. Moreover, we found that the highest *REF* and *SRPP* gene expressions were recorded in late rainy season that agreed with the amount of production yield. The late rainy season was a good period to investigate differential expression of the candidate molecular marker genes. The results corroborate the two genes' expression as surrogate selection criteria for high yield rubber clones.

Keywords: *Rubber biosynthesis gene, Rubber tree improvement, Seasonal variations,Molecular marker*

INTRODUCTION

In general, the conventional breeding of rubber trees requires 25 to 30 years for one testing cycle. This method has other problems especially in the selection process, which requires large areas with many clones and long sampling time to reliably and accurately determine the yield. 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). Rubber elongation factors (*REF*) and small rubber particle protein (*SRPP*) are the two-major components of *Hevea* latex, so these proteins contribute to rubber biosynthesis (Dennis and Light 1989; Chotigeat *et al*., 2010). *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). Both protein expressions are highly correlated with latex yield (Priya *et al*., 2007, Klaewklad *et al.,* 2016). 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. 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*. However, an extensive literature is available on the relation of latex yield to *REF* and *SRPP* genes' expression, but much less is known about how both genes respond to changing environmental conditions. Additionally, the yield of rubber latex is influenced by the climate and the soil moisture, especially the cumulative rainfall (Sdoodee *et al*., 2010). Therefore, the relation of gene express ion to yield must be assessed under various environmental conditions, to make it more accurate in *Hevea* selective breeding.

The main goal of this study was to assess the expression of *REF* and *SRPP* genes in the latex of four selected clones, and the RRIM 600 clone as baseline control, as well as to identify the best sampling time (season) for differential gene expression relevant to latex yield. The study therefore included the main seasons characterized by rainfall regime. The results obtained from this study are beneficial for a comprehensive understanding of the main genes directly involved in rubber biosynthesis, and for the practical selection of a suitable time to assess rubber clones, based on these genes as predictive molecular markers related to yield.

MATERIALS AND METHODS

Plant materials: The four clones selected that had high latex yield were sampled from rubber plantations in Songkhla (SK1 and SK3), Nakhon Si Thammarat (NK1), and Trang province (T2). The RRIM 600 clones at each location were used as a control. Fresh latex was collected from the trunks

of 13 years old mature rubber trees in the early rainy season (June and August), in the late rainy season (October and December), and in the summer season (January and March) in 2012-2014. The harvesting system was a third spiral tapping (s/3), with a tapping frequency of 2 days (d2), without Ethrel stimulation.

Dry rubber yield: Fresh latex was collected and weighed at each tapping. DRC was measured by the cup coagulation method. Three replications of 10g of latex were weighted and 6.0% acetic acid solution was added and mixed well. The coagulated rubber was dried in a hot air oven at 65 °C for 24 hrs. The obtained dry weight after drying was taken as the rubber content, since rubber is the major nonvolatile component in rubber latex. The DRC was calculated from:

% DRC = (dry rubber weight/fresh latex weight) × 100 (1)

Dry rubber yield (g/ tree/tapping) = fresh latex weight × % DRC (2)

Total RNA extraction: Latex was collected (5 ml of fresh latex) and mixed in 5 ml of cool 5X RNA extractions. The supernatant was extracted with phenol:chloroform (1:1, v/v) and then extracted with chloroform according to Suwanmanee *et al.*, (2002). The cDNA synthesis was done according to manufacturer's protocol with the Super Spercript® Vilo™ cDNA Synthesis kit (Invitrogen, USA).

Gene expression analysis by Quantitative Real-Time PCR (qRT-PCR): The transcription levels of genes were determined by quantitative real time PCR with ABI system. Primer designed was based on *Hevea* data from NCBI (Table 1). The reactions were performed using the SYBR® Green Real-Time PCR master mixes (Invitrogen, USA) following the procedure described by the manufacturer, to form 20µl PCR mixture with 25ng cDNA. The PCR cycle program consisted of initial denaturation at 95°C for 10 min, followed by 35 two-step cycles of denaturation at 95°C for 15sec, and annealing and polymerization at 59°C for 1 min. The normalized expression ratios were calculated using the comparative ΔΔCt method and normalized Ct data for each target gene, with Ct values for 18S rRNA gene as the internal control (Ruderman *et al*., 2012).

Table-1: The primer sequences used for qRT-PCR

Gene	Sequence of Primers	Ta (°C)
REF	Forward; 5'- CGGCAACTTATGCTGTGACT -3'	57.3
	Reverse, 5'- AGGTACAGCCACGTTCTTCA -3'	57.3
SRPP	Forward; 5'-GCCAACCGCTGTTTATTTCT -3'	55.2
	Reverse, 5'-TTTCTCAGTGGGCAACAAAG -3'	55.2
18s	Forward; 5'- AAGCCTACGCTCTGGATACATT -3'	58.4
rRNA	Reverse, 5'- CCCGACTGTCCCTGTTAATC -3'	59.3

Statistical analysis: The data on latex yield and gene expression were analyzed by using Student's t-test and their controls. Difference was accepted as highly significant $P \leq 0.01$ and significant at $P \leq 0.05$. The correlation was 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).

RESULTS AND DISCUSSION

Seasonal variations in dry rubber yield: Rubber latex yield was influenced by variations in climate and soil moisture, especially the annual cumulative rainfall. Table 2 presented the monthly dry rubber yield during specific seasonal periods; the early rainy season, the late rainy season and the summer season. The four selected clones had higher monthly latex yields than the control (RRIM 600) at every location (Table 2). The latex yield increased from June (when tapping restarts), attaining its maximum in October, and holding steady till December, after which it decreased to a minimum in March when tapping stops. This pattern was the same regardless of clonal variety, and the dry rubber yield was highest in the late rainy season (Table 2). It is known that cumulative rainfall and relative humidity tend to increase latex yield (Rao *et al*., 1998; Njukeng *et al*., 2011).

In the dry season rubber production is lower than in the rainy season (Mak *et al*., 2008; Kunjet *et al.,* 2013), because both growth rate and leaf count are low in the summer season (Ruangsri *et al*. 2015). From the middle of March the rubber trees exhibit leaf-flushing with rainfall that continues in April and May (Tongsawang and Sdoodee 2008; Ruangsri *et al*., 2015). Clearly latex yield is affected by annual seasonal variations, and such effects must be accounted for in an analysis that looks for predictive molecular markers of yield. Our results are like 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 is related to the cumulative rainfall (Kunjet *et al.*, 2013). In a similar manner, the current study showed significant positive correlation of atmospheric relative humidity and latex yield, for most of the clones studied. This positive correlation is probably due reduced water loss by transpiration at high relative humidity.

Table-2: 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 2014.

Clone	Dry rubber yield (g/t/t/)		
	Early rainy	**Late rainy**	**Summer**
SK1	62.88±10.18	131.82±9.11	93.50±3.53
RRIM 600	59.49±1.73	90.92±2.89	76.27±1.03
T-test	ns	*	*
CV	8.63	4.74	19.71
NK1	56.33±0.93	70.26±4.13	58.02±9.42
RRIM 600	52.44±0.935	56.25±2.24	47.54±8.91
T-test	ns	*	*
CV	10.30	9.90	9.92
T2	40.51±7.14	83.32±9.27	47.92±11.16
RRIM 600	35.19±3.85	67.65±7.15	40.99±9.46
T-test	ns	*	ns
CV	22.90	11.25	12.87
SK3	37.94±1.93	52.25±3.89	42.20±1.24
RRIM 600	37.86±2.62	40.88±4.88	32.53±4.44
T-test	ns	*	*
CV	8.53	9.53	12.67

* = Significant difference at $P \leq 0.05$, LSD; ns = Non significant

Gene expression results from Quantitative Real-Time PCR (qRT-PCR): The main genes involved in rubber biosynthesis have been characterized. Among the genes identified, *REF* is a key rubber biosynthesis gene involved in the polymerization of isoprene chains (Chotigeat *et al*., 2010). 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, et al., 1989; Laibach *et al*., 2015). Priya *et al*., (2006) reported that among the transcripts expressed in latex, *REF* was the most abundant followed by small rubber particle protein (*SRPP*). Additionally, Klaewkla*d et al.,* (2016) indicated that the mRNA accumulation of both *REF* and *SRPP* was positively correlated with the high latex yield clones. Thus, the expression levels of these two genes, in the clones studied, were of interest as molecular marker candidates predictive of latex yield. The motivation for such molecular markers 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 as shown in Figures 1 and 2. Interestingly, we found that at the start of tapping (June) the dry rubber yield and gene expression were both low. After the late rainy season in October, the gene expression levels increased, and started to decrease in January, reaching their minima in March. These trends are expected, as by February most trees have lost their leaves and photosynthesis activity is minimal, hence latex production is also low. Similarly, when the rubber tree tapping is started after the rest period in April, some lag time is taken before the tree metabolism is fully activated for optimal yield (Njukeng *et al*., 2011; Kunjet *et al.*, 2013). In conclusion, all selected clones showed highest of both genes expression in the late rainy season matching the pattern in latex yield. The results obtained from this study are beneficial for a comprehensive understanding of the main genes directly involved in rubber biosynthesis, and for the practical selection of a suitable time to assess rubber clones.

We investigated the correlation between the gene expression levels and dry rubber yield for each clone. The Pearson's analysis in this study showed positive correlation between the expression of *REF* and *SRPP* genes and the average of dry rubber yields (Table 3 and 4). The results demonstrated that the expression patterns of both genes and their dry rubber yield changed similarly upon seasonal variations. These results suggested that there was a putative linear relationship between the expression of these genes and the clonal rubber yield in seasonal variations. This study supported a previous reported by Sdoodee *et al*., (2010) presented the environmental effects are significant for *Hevea* latex production.

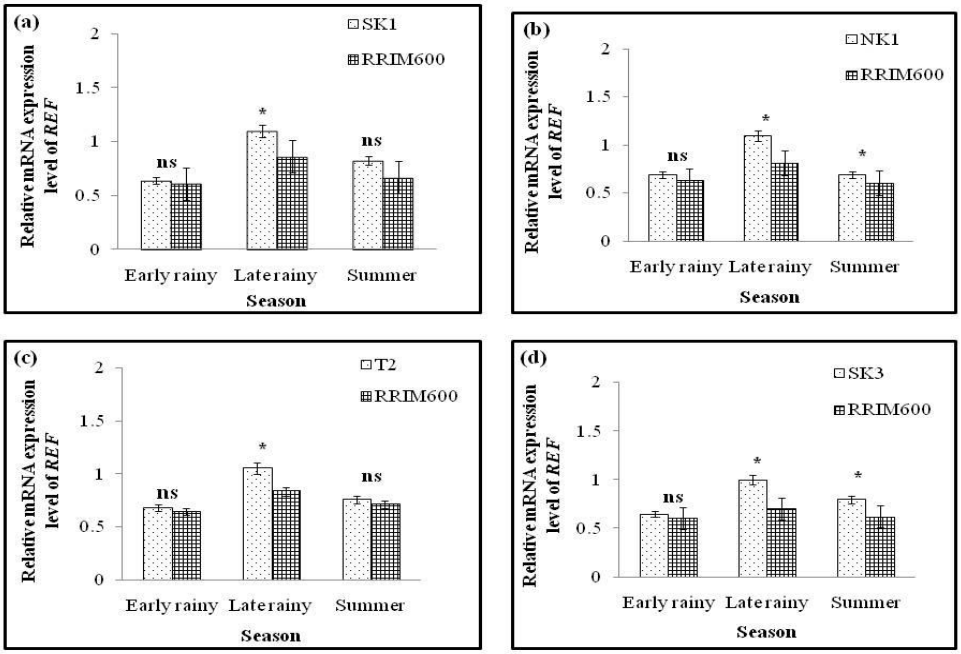

Figure -1: The relative gene expression of *REF* in clone SK1 (a), NK1 (b), T2 (c) and SK3 (d) comparing to RRIM600. The values shown are means from triplicate observations ± SD. *: $P \leq 0.05$

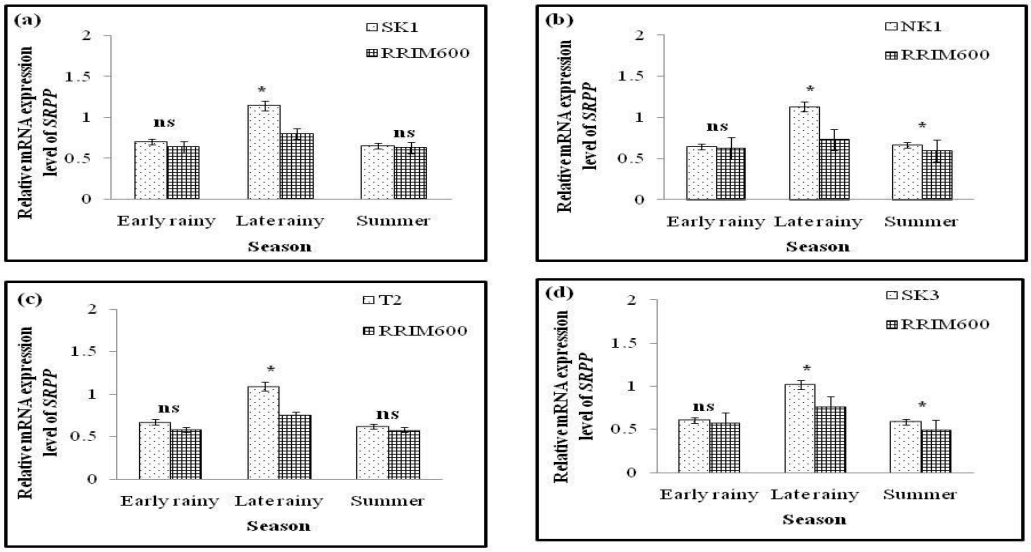

Figure- 2: The relative gene expression of *SRPP* in clone SK1 (a), NK1 (b), T2 (c) and SK3 (d) comparing to RRIM600. The values shown are means from triplicate observations ± SD. *: $P \leq 0.05$

Table -3: Pearson's correlations among expressions of *REF* gene and dry rubber yield (g/tree/tapping) of SK1, SK3, NK1, T2 clones and RRIM 600 in each location in June 2012 to March 2014.

Clone	Correlation coefficient (r) between Dry rubber yield and *REF* gene expression		
	Early rainy	Late rainy	Summer
SK1	0.77*	0.91**	0.72*
RRIM 600	0.71*	0.82*	0.76*
NK1	0.73*	0.83*	0.71*
RRIM 600	0.82*	0.92*	0.81*
T2	0.81*	0.95*	0.71*
RRIM 600	0.72*	0.87**	0.80*
SK3	0.76*	0.78**	0.73*
RRIM 600	0.78*	0.81**	0.75*

* = significant difference at $P \leq 0.05$, LSD; ** = significant difference at $P \leq 0.01$, LSD

Table -4: Pearson's correlations among expressions of *SRPP* gene and dry rubber yield (g/tree/tapping) of SK1, SK3, NK1, T2 clones and RRIM 600 in each location in June 2012 to March 2014.

Clone	Correlation coefficient (r) between Dry rubber yield and *SRPP* gene expression		
	Early rainy	Late rainy	Summer
SK1	0.81*	0.82*	0.92**
RRIM 600	0.82*	0.86**	0.85**
NK1	0.83*	0.95*	0.91*
RRIM 600	0.76*	0.97*	0.98**
T2	0.81*	0.86**	0.79**
RRIM 600	0.76*	0.78**	0.74**
SK3	0.77*	0.76**	0.76*
RRIM 600	0.80*	0.85*	0.85*

* = significant difference at $P \leq 0.05$, LSD; ** = significant difference at $P \leq 0.01$, LSD

CONCLUSION

The expression levels of both *REF* and *SRPP* genes correlated with environmental parameters, and were induced from October to December when monthly latex yields were high. Our results showed that the late rainy season was a good period to investigate differential expression of the candidate molecular marker. 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.

ACKNOWLEDGEMENTS

This research was supported by the National Research University Project of Office of the Higher Education Commission, Prince of Songkla University, Thailand and the Graduate School, Prince of Songkla University. Author is thankful to Associate Professor Seppo Karrila and the Publication Clinic, Research and Development Office, Prince of Songkla University for technical comments and manuscript correction.

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