Study toward the Development of Relationship between *Ref* Gene Expression and Yield Potential of *Hevea* Clones

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ABSTRACT

Rubber is the third largest plantation crop in Sri Lanka. In 2018, Rubber production was declined from 83.1 million kilograms to 82.6 million kilograms. Development of high yielding clones to the rubber industry is a prime objective of *Hevea* breeding and selection. Rubber Elongation Factor (*Ref*) protein mainly helps for the biosynthesis of natural rubber in *Hevea brasiliensis*. Rubber particles are tightly bound to this *Ref* protein. Genomic sequence of *Ref* gene is 1367 bp long. Previous studies found that the positive correlation between *Ref* gene expression pattern and latex yield. The clones in the group III clone recommendation are needed to be upgraded in to group II gradually with their performance. Selected three clones in the group III as RRISL 2006, RRISL Centennial 3 and RRISL Centennial 4 were undertaken to study their *Ref* gene expression and promoter analysis with latex yield to verify their performance. Quantitative gene expression of *Ref* gene was done to identify the expression pattern of *Ref* gene and promoter. Livak method (2⁻ ΔΔCT) used to analyze the quantitative gene expression and paired t test was used to identify significant difference between control and treatments. Compared to the control clone RRISL 203, the yields of all other three clones were significantly higher as well as their *Ref* gene expression was up-regulated differently with 2 to 7 fold difference. Up-regulation of *Ref* gene in the clone RRISL 2006 is significantly higher with seven fold compared to the control clone RRISL 203. The average yield of RRISL 2006 in the year 2019 was 27.3 g/t/t and average yield of RRISL Centennial 3 was 28.3 g/t/t. But the difference was not significant. In January 2020, where samples were collected, the RRISL 2006 recorded highest 55.77 g/t/t. RRISL Centennial 4 was showed around four fold up-regulation compared to the clone RRISL 203 as well as yield also higher to RRISL 203. The clone RRISL Centennial 3 also followed the same pattern but the fold difference was around 2.5 over the control clone RRISL 203. The results we re proved the positive correlation between *Ref* gene expression and the high yield. However this relationship was not observed between RRISL Centennial 3 and RRISL Centennial 4, as the clone RRISL Centennial 4 showed higher fold difference over to RRISL Centennial 3 but yield was low. Therefore this relationship is needed to be further analyzed. The copy number of *Ref* promoter also important to understand the clear relationship between *Ref* gene expression and yield potential of *Hevea* clones.

Keywords- *Hevea brasiliensis*, *Ref* gene, *Ref* promoter, Real Time PCR

I. INTRODUCTION

The para rubber tree (*Hevea brasiliensis*) (Wild. ex A. Juss.) Mull.-Arg belong to family Euphorbiaceae and genus *hevea* (Tang et al., 2016) which is native to the Amazon Basin, started as commercial cultivation in Malaysia in 1896. It is a diploid perennial and cross pollinated tree species (Mantello et al., 2012).

Around 2000 plant species produced rubber in the world, but *Hevea brasiliensis* is the commercial natural rubber producer. Out of other species *Hevea brasiliensis* produces high quality rubber in large quantities (Han et al., 2000).

*Hevea brasiliensis* introduced to Sri Lanka by Sir Henri Wickam in 1876. It has expanded to traditional rubber growing areas in Sri Lanka such as Gampaha, Colombo, Kandy, Matale, Galle, Kalutara, Matara, Badulla, Kurunegala, Kegalle and Ratnapura districts (Ministry of Plantation Industries, 2014).

Rubber (*Hevea brasiliensis*) is the third largest plantation crop in Sri Lanka based on agricultural export (Central Bank Report, 2017). Rubber productivity in Sri Lanka is 82.6 million kilograms in 2018 (Central Bank Report, 2018).

Rubber particles are produced and stored in the laticiferous cells or latex vessels found in the outer bark of the tree (Mahendra et al., 2013). Rubber particles are tightly bound to a protein called Rubber Elongation Factor (*REF*) and it is 137 amino acid long and Molecular weight of this protein is 14600 Daltons (Dennis et al., 1989). *Ref* gene involves normally biosynthesis of natural rubber. Previous studies found that there is a positive relationship between *Ref* gene expression and latex yield (Priya et al., 2007).

Normally *Hevea* breeding cycle takes 20-25 years to complete. In ECT (Estate Collaborative Trials), mainly evaluated the selected genotypes under commercial scale and established in different estate in different areas (Kalpani et al., 2020). In this level mainly
consider several characteristics including yield, girth and disease resistant. This carried out 10-15 years until the recommendation made for group III (Withanage et al., 2014). RRISL 203 is high yielding clone and already recommended clone in group I, but RRISL 2006, RRISL Centennial 3, RRISL Centennial 4 are in group III (Rubber Research Institute of Sri Lanka, 2013) and already evaluated performances of these clones nearly 20-25 years. Those clones are already established in the field and have limited field trials.

RRISL 203 shows good properties of latex and raw rubber compared to other clones. The average commercial yield of RRISL 203 clone is approximately 2500 kg/ha/yr. PRI (plasticity retention index) value also higher than 60%. It means this clone provide good quality rubber for rubber industry. And also RRISL 203 resistant to Corynespora leaf fall disease. RRISL 203 is a highly recommended clone to maintain the correct clone balance (Liyanage, 2018). According to Ref gene expression, can verify and upgrade RRISL 2006, RRISL Centennial 3 and Centennial 4 with the known clone of RRISL 203.

Smallholders own nearly 65% of rubber, most of which belong to low income groups. The only way to increase the low income is to increase output per unit of land area (Ranasinghe et al., 2020). So use of genetically improved, high yielding clones to farmers is the best solution to solve this problem. For the further upgrade new cultivars, need to confirm and precise new clones.

The objective of this study was to verify the yield performances and upgrade the clone RRISL 2006, RRISL Centennial 3 and Centennial 4. The results obtained from this study are useful to develop a relationship between Ref gene expression and yield potential of Hevea clones which can be used as a parameter of early selection.

II. MATERIALS AND METHODS

The study was conducted at Eladuwa Estate and Genetics and Plant Breeding Department, Rubber Research Institute of Sri Lanka from September 2019 to March 2020.

Collection of Planting Materials

Latex of 11 years old, Hevea brasiliensis such as RRISL 203, RRISL 2006, Centennial 3 Centennial 4 were collected from Eladuwa estate Namunukula plantation, at Kalutara district.

Total RNA Extraction

RNA extraction was done by using TRizol™ obtained from UC Biotech, University of Colombo. 250 µL latex was taken in to 500 µLTRizol™ was centrifuged at 14,500 rpm for 20 min at 4 °C. Then the light pink color aqueous layer was transferred into a new eppendorf tube. It was left 10 min for incubation at room temperature. Then 200 µL of chloroform (CHCl₃) was added and sample was shaking vigorously to mix both solutions. It was incubate 2-3 min at room temperature. Next it was centrifuged at 14,500 rpm at 4 °C for 20 minutes. 400 µL cold isopropanol was added to the upper aqueous layer and incubated at 4 °C for 10 min. Then it was centrifuged at 14,500 rpm for 10 min. The supernatant was discarded and the pellet was washed using 200 µL 95% cold ethanol and centrifuged at 6,500 rpm for 5 min at 4 °C. Again the supernatant was discarded and 200 µL 75% cold ethanol was added. The sample was centrifuged at 6,500 rpm for 5 minutes at 4 °C. The supernatant of the sample was removed and air dried the pellet and Re-suspended the pellet in 25 µL Autoclaved DEPC (diethyl pyrocarbonate) treated water.

cDNA Synthesis

The first strand cDNA was synthesized by using Prime Script™ RT Reagent Kit (Perfect Real Time). Prime Script™ RT is capable of synthesizing full length of cDNA molecules and it is high specificity.

Optimization of PCR conditions for the Ref Gene

PCR amplification was carried out in a 20 µL reaction volume which contained 2 µL genomic DNA, 2 µLdNTP, 2.5 µL of 1X PCR green taq buffer, 0.3 µL of Taq polymerase (5u/µL), 12.4 µL nuclease free water and 0.4 µL of each designed forward and reverse primers of ref gene.

Optimum annealing temperature was determined by using gradient PCR and incubating the mixture at 94 °C for 4 min, followed by 45 cycles of 94°C for 30 s, 58°C-68°C for 45 s, 72 °C for 1 min and 72 °C for 4 min. eight annealing temperatures (58°C, 58.7°C, 60°C, 62°C, 64.4°C, 66.4°C, 67.5°C, 68°C) were used to determine the optimum temperature.

Optimization of PCR conditions for the Ref Promoter

Optimum annealing temperature was determined by using gradient PCR. It was performed in a 20 µL mixture, containing 2 µLdNTP, 2 µL genomic DNA, 2.5 µL of 1X green taq buffer, 0.5 µL of each forward and reverse primers, 12.3 µL nuclease free water and 0.2 µL of Taq polymerase.

PCR amplification was carried out at eight annealing temperatures (55°C, 55.7°C, 57°C, 59°C, 61.4°C, 63.3°C, 64.5°C, 65°C) and The mixture was incubated at 94 °C for 4 min, 94°C for 30 s with 40 cycles, 55°C-65°C for 30 s and 72 °C for 1 min and 72 °C for 4 min.

Confirmation of amplified PCR products

Before real time PCR, confirmation of best annealing temperature was done by using agarose gel electrophoresis. 1.5% agarose gel (100 ml of 1X buffer) stained with EtBr was used for gel electrophoresis and 8 µL of PCR products were loaded into agarose gel. Then gel was run under 55V of electric supply. 100 bp ladder was used to determine the band size.

Real time PCR for Ref gene expression

For RT-PCR first strand cDNA, which was synthesized by using Ref gene primers. Forward primer is 5’ ACG CGA ATT CGG AGG TCC GATTT GGC TGA AGA CG 3’ and reverse primer is 5’ AGC CGTCGCA CTT GGG GCT CAA TAAT TTCTCTCCAA3’. SYBR Green 1 used as fluorescence dye and RT-PCR was done by using BIO – RAD CFX96™ system.
RRIC 203 was used as control and RRISL 2006, Centennial 3 (87-370) and Centennial 4 (92-124) were used as treatments. Three replicates from each sample were used. No Template Control (NTC) and one replicate of GAPDH from each clone were used.

Real time PCR for Ref promoter expression

For Ref promoter 5’ ACG CGT CGA CGG AGG AAA AAC AAA GAC TA 3’ was used as forward primer and 5’ ACG CGA ATT CTT GCG AGG AAC TTG GTT TGG ATC 3’ was used as reverse primers. RT-PCR was done to characterize and quantify nucleic acidsusing first strand cDNA.

Three replicates from each RRISL 2006, Centennial 3 (87-370) and Centennial 4 (92-124) were used and three replicates from RRISL 203 were used as control. GAPDH was used as reference gene and No Template Control (NTC) sample was used with Ref promoter and GAPDH separately.

Data Analysis

The livak method (2^{\Delta\Delta CT}) was used to analyze the relative gene expression, for RT-PCR. Paired T-Test used to determine statistically, whether there is a significant between two observations.

III. RESULTS AND DISCUSSION

Total RNA Extraction

Purity and concentration of extracted total RNA was monitored using Nano spectrophotometer. The purity of extracted RNA was measured by A260/A280 and the ratio was more than 2.0. So the RNA purity was higher (figure 1). RNA concentration was high as around 1000-3000 ng/µL. Gel electrophoresis was confirmed this results.

![Figure 1: Agarose gel electrophoresis of RNA for hevea latex. 1-4 RNA samples. Two bands of RNA were visualized by EtBr staining.](image)

Optimization of PCR conditions for Ref gene

The optimum annealing temperature determined by gradient temperature for the amplification of Ref gene was 64.4°C (figure 2). In this temperature clear and less intensity banding pattern was observed between 1000 and 1500 bp. Normal banding pattern of Ref gene is 1367bp. After the 64.4°C, no banding patterns were visible.

![Figure 2: Agarose gel electrophoresis of Gradient PCR products with Ref gene specific primer. L is 100bp ladder. L 1-8 are 58°C, 58.7°C, 60°C, 62°C, 64.4°C, 66.4°C, 67.5°C, 68°C respectively. Optimum temperature was 64.4°C.](image)
Optimization of PCR conditions for Ref promoter

Correct 670 bp size band was observed between 55-57°C gradient temperatures and 55°C was selected due to low primer dimers (lane number 01…) (figure 3).

Figure 3. Gel electrophoresis of Gradient PCR products with Ref promoter specific primer. L is 100 bp ladder. L 1-8 are 55°C, 55.7°C, 57°C, 59°C, 61.4°C, 63.3°C, 64.5°C, 65°C respectively. Optimum temperature was 55°C.

Yield in Eladuwa estate

Average annual yield in 2019

Figure 4: Average annual yield of RRISL 203, RRISL Centennial 3, RRISL Centennial 4 in Eladuwa estate in 2019 year.

Average yield of RRISL 2006, Centennial 3 (87HP-370), Centennial 4 (92HP-124) and RRISL 203 were considered. According to the Figure 4, the average yield of clones RRISL 203, RRISL 2006, Centennial 3 (87-370) and Centennial 4 (92-124) in 2019 were around 15.66 g/t/t, 27.33 g/t/t, 28.34 g/t/t, 17.60 g/t/t respectively. All the three clones showed higher yield over control clone RRISL 203. Highest yield of 28.34 g/t/t showed in RRISL Centennial 3 and 27.33 g/t/t showed in RRISL 2006 compared to RRISL 203. According to the Paired T-
Test, P value is greater than 0.05. So there is no significant difference in yield between RRISL Centennial 3 and RRISL 2006. The lowest yield as 17.60 g/t was observed in RRISL Centennial 4 compared to the control clone of RRISL 203.

According to the yield data collected during the month of January 2020, average of RRISL 203, RRISL 2006, Centennial 3 and Centennial 4 clones were 33.61 g/t, 55.77 g/t, 46.40 g/t, 41.75 g/t respectively. Compared to RRISL 203, the yields of all three clones were higher (Figure 5). With respect to average yield during the month of January 2020, the highest yield as 55.77 g/t was observed in RRISL 2006 and lowest yield was 41.75 g/t observed in RRISL Centennial 4 but higher to the control clone RRISL 203.

**Quantitative Real Time PCR for Ref Gene and Ref promoter expression**

Quantitative gene expression of *Ref* gene and *Ref* promoter were analyzed for the RRISL 203, RRISL 2006, Centennial 3 (87-370) and Centennial 4 (92-124). RRISL 203 was used as a control clone. The threshold cycle (CT) was analyzed to determine the expression levels of *Ref* Gene and *Ref* promoter. In this study GAPDH used as reference gene and no non-specific products were amplified.

According to the result (Table 1) *Ref* gene expression was up-regulated in all three clones compared to RRISL 203, showing higher gene expression over to the clone RRISL 203. According to paired t-test, p value of RRISL 2006, RRISL Centennial 3, and RRISL Centennial 4 was lower than 0.05. So the *Ref* gene expression was significantly difference with each clones compared to RRISL 203.

Up-regulation of *Ref* gene in the clone RRISL 2006 was significantly higher as 6.9 fold compared to the control clone RRISL 203 (Figure 6) and it indicates the highest expression. The yield of RRISL 2006 in the month of January where samples were taken was 55.77 g/t and it was recorded as the highest yield. RRISL Centennial 4 was showed around 4.2 fold up-regulation compared to the clone RRISL 203 as well as the yield also higher to RRISL 2003. RRISL Centennial 3 was showed lowest fold of 2.5 and yield was higher compared to RRISL 203. These three clones showed high yield compared to RRISL 203. The results were confirmed the positive correlation between *Ref* gene expression and the yield performance.

According to previous studies *Ref* gene expression in RRIC 121 is higher and RRIC 52 is lower. RRIC 121 was recommended as high yielding clone and RRIC 52 named as low yielding clone (Suganthan et al., 2011). RRIC 121 included in to group I clone recommendation (Rubber Research Institute of Sri, 2013). Normally high yielding clones shows 3 to 4 folds higher expression of *Ref* gene than low yielding clones (Priya et al., 2007). *Ref* gene expression was down-regulated in RRISL 2005 and RRISL 2100 under water stress (Samindi, 2018).

![Average annual yield in January month](image)

Figure 5: Average annual yield of RRISL 203, RRISL Centennial 3 and RRISL Centennial 4 in Eladuwa estate in January month.
Table 1: Fold difference of Ref gene for the selected clones

| Gene          | Clone            | Treatment  | CT    | ΔCT | ΔΔCT | \(2^{-\Delta\Delta CT}\) |
|---------------|------------------|------------|-------|-----|------|------------------------|
| RRISL 2006    | Control          | 10.58      | -7.47 |     | -2.51 | 6.93                   |
|               | Test             | 13.36      | -4.36 |     |       |                        |
| Ref           | RRISL Centennial 3 | Control   | 10.77 | -5.99 | -1.03 | 2.55                   |
|               | Test             | 13.36      | -4.96 |     |       |                        |
|               | RRISL Centennial 4 | Control   | 10.53 | -6.87 | -1.91 | 4.24                   |
|               | Test             | 13.36      | -4.96 |     |       |                        |

Figure 6: Fold difference of Ref gene expression (log scale) for RRISL 2006, RRISL Centennial 3, and RRISL Centennial 4 clones compared to RRISL 203. Calculated using livack method.

Table 4.2: Fold difference of Ref promoter for the selected clones

| Clone          | Treatment  | CT    | ΔCT | ΔΔCT | \(2^{-\Delta\Delta CT}\) |
|----------------|------------|-------|-----|------|------------------------|
| RRISL 2006     | Treatment  | 23.25 | 3.13|      | 2.69                   |
| Promoter       | Control    | 22.5  | 0.44|      | 0.17                   |
| RRISL Centennial 3 | Treatment   | 23.36 | 5.29|      | 4.85                   |
|                 | Control    | 22.5  | 0.44|      | 0.04                   |
| RRISL Centennial 4 | Treatment   | 22.26 | 3.99|      | 3.99                   |
|                 | Control    | 22.5  | 0.44|      | 0.07                   |

Expression of Ref promoter was down-regulated (Table 2) when compared to RRISL 203. According to the paired t-test p value of Ref promoter in three clones were less than 0.05. It means down-regulation was significantly difference from each clone.
IV. CONCLUSIONS

Ref gene expression was significantly up-regulated in RRISL 2006, RRISL Centennial 3, and RRISL Centennial 4 clones compared to control clone of RRISL 203. Average yield of RRISL 2006, RRISL Centennial 3, and RRISL Centennial 4 in 2019 at Eladuwa estate was higher than the RRISL 203.

All the situations are confirmed that the positive correlation of Ref gene expression to the yield performance. Molecular characteristics and yield performances were high in RRISL 2006, RRISL Centennial 3 and RRISL Centennial 4 with compared to RRISL 203.

The copy number of Ref promoter is emphasized and further studies needed to observe the relationship further.

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