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Analysis of the Promoter Region of the Rubber Elongation Factor Gene (*ref*) of *Hevea brasiliensis*

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ABSTRACT

Rubber Elongation Factor (REF) protein is a major protein found in the latex of the *Hevea brasiliensis*, and it is very important for the biosynthesis of natural rubber (*cis*-1, 4-polyisoprene). Previous studies have shown differential expression of the *ref* gene among clones of *Hevea brasilienisis*. Primers were designed to amplify the promoter region and a part of the structural gene of the *ref* gene. PCR amplification of the genomic DNA of low yielding clone RRIC 52 and high yielding clone RRIC 121 resulted in approximately a 650 bp band and a 700 bp band respectively. The difference between the size of the amplified bands suggests the possible variations of the nucleotide sequences of the promoter region of the two different rubber clones.

An attempt was taken to construct a genomic library of the clone RRIC 121 to further analyse the upstream of the *ref* gene, by using probe hybridization and sequencing. Genomic DNA of RRIC 121 was partially digested with *Sau*3A I and ligated into (pBluescript) pBS vector. Ligated vectors were transformed into competent *Escherichia coli* (DH5α) cells. The number of colony forming units of the transformed cells was not sufficient enough for the further steps of genomic library screening. Further analysis of the promoter region of the *ref* gene by sequence analysis, bioinformatics and functional analysis will explain the differences of the organisation of the promoter sequence, which can cause variations in *ref* gene expression among clones of *Hevea brasiliensis*.

KEYWORDS: Genomic Library, *Hevea brasiliensis*, *ref* Gene Promoter, RRIC 121, RRIC 52

INTRODUCTION

Rubber *(Hevea brasiliensis)* is the third largest plantation crop in Sri Lanka based on the export earnings. Rubber industry contributes 0.2% to Gross Domestic Production and it has earned 206.4 million US dollars; 2% of the total exports in year 2011 (Central Bank Annual Report, 2011). Over 2000 plant species produce rubber (*cis*-1, 4-polyisoprene) but only *H. brasiliensis* is extensively used for commercial natural rubber production and it satisfies 40% of the global demand for elastomers (Priya *et al.*, 2006).

Rubber particles (RP) are produced and stored within the cytoplasm of specialized laticiferous cells which are present in the bark of the tree (Goyvaerts *et al.*, 1991). RP are tightly bound to a protein; Rubber Elongation Factor (REF), which is 137 amino acids long and has a molecular mass of 14.6 kD (Dennis *et al.*, 1989). REF protein interacts with *trans*-prenyltransferase to form *cis*-prenyltransferase which adds multiple *cis*-isoprene units to growing rubber molecules (Dennis and Light., 1989; Dennis *et al.*, 1989; Light *et al.*, 1989). REF protein accounts for 10-60% of the total protein in whole rubber latex (Dennis and Light., 1989). However Cornish (1993) has concluded that REF protein has no direct role in rubber biosynthesis.

ref gene is 1367 bp long and it has three exons interrupted by two introns (Priya *et al.*, 2006). RRIC 52 is a low yielding *H. brasiliensis* clone in Sri Lanka having a five year mean yield of 21 g/t/t; RRIC 121 is a high yielding clone which has a five year mean yield of 75 g/t/t (Attanayaka, 2001) (g/t/t = grams per tree per tapping). The *ref* gene expression of RRIC 121 clone (high yielder) is 400 fold higher than the *ref* gene expression of RRIC 52 clone (low yielder) (Suganthan *et al.*, 2011). This difference of *ref* gene could be linked with the promoter, enhancer, intron and exon sequences. Promoter region of the *ref* gene could be directly involved in regulating the gene expression, thus analysis on the promoter region of the *ref* gene of low and high yielding *H. brasiliensis* clones is important.

The objective of this study is to analyse the promoter region of the *ref* gene of *H. brasiliensis* and to characterize the variations in the promoter region among different *H. brasiliensis* clones. The information from this study could be useful in breeding of *H. brasiliensis* and development of a strong tissue

specific promoter which can be used to develop a eukaryotic expression vector for plant transformation.

MATERIALS AND METHODS

The study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka and the Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka from January to April 2013.

Plant Materials

Immature leaves at apple green stage from *H. brasiliensis* clones RRIC 52 and RRIC 121 were collected from the nurseries of the Genetics and Plant Breeding Department of Rubber Research Institute of Sri Lanka.

Genomic DNA Extraction

Genomic DNA was extracted from the leaves of *H. brasiliensis* RRIC 52 and RRIC121 clones according to the miniprep DNA extraction method developed by Rubber Research Institute of Sri Lanka. as described by Suganthan *et al.*, (2011). Quality and the quantity of DNA were determined on 0.8% agarose gel.

Designing of Primers to Amplify the Promoter Region of the ref Gene

Specific primers were designed to amplify the proximal promoter region of the *ref* gene and a part of the *ref* gene, based on GenBank data base (Anon 2013a), accession number AY712939.1 and nucleotide sequence of the *ref* gene published by Attanayaka *et al.*, (1992) using Primer 3 Plus program (Anon 2013b).

Optimization of PCR Conditions

A gradient PCR was done with a Biorad thermocycler. Five annealing temperatures (38°C, 44.2°C, 48.3°C, 51.5°C, and 55°C) were used in order to determine the optimum annealing temperature of the primers. A concentration of 12.5 µM was used as the primer concentration.

PCR Amplification of the Promoter Region of the ref Gene

PCR amplification was done in a 25 µL reaction volume which contained 50 ng of template DNA, 200 µM dNTP, 2.5 mM $MgCl_2$, 1X PCR buffer, 1 unit of Taq polymerase (UC Biotech), and 1 µL each of the designed 12.5 µM forward and reverse primers of the promoter region of the *ref* gene. PCR amplification was done with a Biorad thermocycler with following parameters; 96°C for 5 min followed by 30 cycles of 96°C for 15 sec, 38°C to 48°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min.

Construction of a Genomic Library

Genomic DNA of H. brasiliensis clone RRIC 121 was used to construct a genomic library.

Pilot Digestion using Human Genomic DNA

A small scale test digestion of the human genomic DNA was conducted using different dilutions of the enzyme in order to establish the partial digestion conditions necessary to obtain DNA fragments in the size region above 0.5 kb. An amount of 5 µg of the human genomic DNA was diluted to 99 µL volume with 10 µL of 10x*Sau*3A I reaction buffer and 56 µL of distilled water. This mixture was aliquoted into 5 eppendorf tubes by adding 29 µL to the tube 1, 20 µL each to the tubes 2, 3, and 4, and 10 µL to the tube 5. Tubes were placed on ice. 10 units of *Sau*3A I (Promega.) enzyme was added to the tube 1, mixed and 10 µL transferred to the second tube. This serial dilution was continued until tube 5. The tubes were then incubated at 37°C for 1 hr after which the reactions were stopped by heat inactivating the enzyme at 65°C for 10 min. Then the digested samples were analysed on a 0.8% agarose gel.

Partial Digestion of the H. brasiliensis Genomic DNA

The enzyme concentration (0.006 units/µL) that produced the maximum yield of DNA fragments in the region above 0.5 kb was selected for the partial digestion of genomic DNA of *H. brasiliensis* clone RRIC 121. 500 ng of DNA (15 µL) was digested in 20 µL of reaction volume with 0.12 units (3 µL from 0.04 units/µL diluted enzyme) of the *Sau*3A I and 2 µL of 10x *Sau*3A I reaction buffer at 37°C for 1 hr, followed 65°C for 10 min. Digested products were estimated on a 0.8% agarose gel. 18 µL of the digested product was mixed with 2 µL of 20 mg/µL RNase and incubated at 37°C for 1 hr, followed by 65°C for 10 min.

Digestion of the Cloning Vector

An amount of 3 µg of pBS vector (30 µL of 100 ng/µL pBS) was digested in 35 µL of reaction volume with 15 units (1.5 µL from 10 units/µL enzyme) of the *Bam* HI and 3.5 µL of 10x *Bam* HI reaction buffer at 37°C for 3 hrs, followed by 65°C for 10 min. Digested products were estimated on a 0.8% agarose gel.

Dephosphorylation of the Vector DNA

Digested pBS vector was dephosphorylated with CIAP enzyme (Promega) according to the manufacturer's instructions (Anon 2013c). Dephosphorylated vector was run in a 0.8% gel to verify the linearization and the gel fragment with the dephosphorylated vector was excised and purified using a column purification kit (UCBiotec).

Ligation of Partially Digested H. brasiliensis DNA with the Vector

From RNase treated partially digested DNA 5 µL was column (UCBiotec) purified and 3 µL (300 ng) of that was ligated with 3.3 µL (99 ng) vector. From RNase treated partially digested DNA 13 µL was run on a 0.8% agarose gel and gel fragment which contains, partially digested DNA fragments of more than 0.5 kb was excised and column purified using a column purification kit (UCBiotec) and 3 µL (300 ng) was ligated with 3.3 µL (99 ng) vector. Ligations were done according to the manufacturer's instructions (Anon, 2013d).

Transformation of the Cloned Vectors

Ligated plasmids were transformed into frozen competent *Escherichia coli* (DH5α) cells prepared by UCBiotec according to the protocols available in Sambrook *et al.*, (1989). 100 µL of the transformed cells were cultured on 90 mL of agar SOB medium containing 20 mM $MgSO_4$ and Ampicillin with a concentration of 20 µg/ml.

RESULTS AND DISCUSSION

Genomic DNA Extraction

Miniprep DNA extraction method produced sufficient DNA for PCR from *H. brasiliensis* clones RRIC 52 and RRIC 121 (Figure 1). Fresh and immature leaves gave higher yield of DNA than mature leaves.

Designing of Primers to Amplify the Promoter Region of the ref Gene

Forward primer 5' ACG CGA ATT CGG AGG AAA AAC AAA GAC TA 3' with *EcoR* I site and reverse primer 5' ACG CGT CGA CTT GCG AGG AAC TTG GTT TGC ATC 3' with *Sal* I site were designed to amplify a 632 bp region which includes the upstream and a part of the structural gene (*ref*). Melting temperatures of forward and reverse primers were accordingly 60.2°C and 67.5°C.

Optimization of PCR Conditions

Based on banding pattern (Figure 2) in RRIC 52, 48.3°C was the optimum annealing temperature for the amplification of *ref* promoter of RRIC 52. At 38°C and 44.2°C two bands of 500bp and 632bp were amplified and in 55°C a single band of 632bp was amplified, the intensity of the band was not enough for further studies. However PCR amplification was absent in the RRIC 121 and it is assumed that the template DNA was contaminated with PCR inhibitors.

PCR Amplification of the Promoter Region of the ref Gene

Genomic DNA of RRIC 52 amplified a target sequence of approximately 650 bp for *ref* promoter specific primers while genomic DNA of RRIC 121 amplified a band of approximately 700 bp (Figure 3). This could be a result of the differences of the nucleotide sequences of the promoter regions between the two clones. Further sequence analysis of the promoter regions of the *ref* gene would be required to identify the sequence elements important for differential expression of *ref* gene between the clones.

Pilot Digestion using Human Genomic DNA

By analysing the digested human genomic DNA on a 0.8% agarose gel (Figure 4), an enzyme concentration of 0.006 units/µL of *Sau3A* I was the suitable concentration to obtain partially digested DNA fragments of more than 0.5 kb.

Partial Digestion of the H. brasiliensis Genomic DNA and Digestion of the Cloning Vector

0.006 units/µL *Sau3A* I enzyme concentration was proven to give partially digested DNA fragments of more than 0.5 kb (Figure 5). Agarose gel analysis showed a single band of the vector confirming the complete digestion at *Bam* HI site.

Transformation of the cloned Vectors

No colonies could be observed in the transformed cells which contained vectors ligated with gel purified partially digested RRIC 121 DNA fragments of more than 0.5 kb. 5 colonies were observed in the transformed cells which contained vectors ligated with column purified partially digested DNA of RRIC 121. The number of colony forming units was not sufficient for conducting further steps of genomic library screening. It is required to construct a representative library to clone the fragments containing the promoter region for further analysis.

Figure 1. Agaraose gel electrophoresis of *H. brasiliensis* clones; RRIC 52 and RRIC 121 genomic DNA

M-50ng of λ genomic DNA marker, 1-RRIC 52, 2-RRIC 121

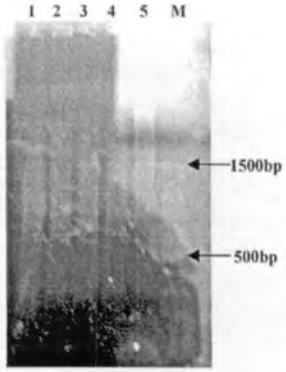

Figure 4. Gel electrophoresis of partially digested Human Genomic DNA with different *Sau*3A I enzyme concentrations

1-0.333 units/μL, 2-0.111 units/μL, 3-0.037, 4-0.012 units/μL, 5- 0.006 units/μL

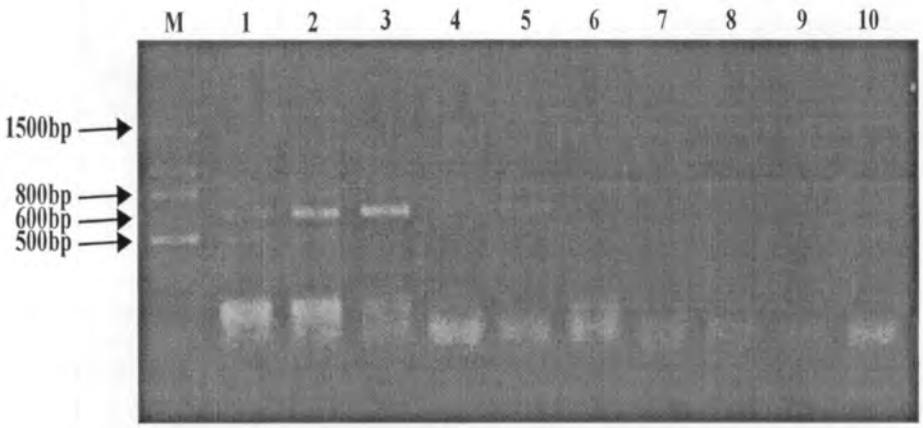

Figure 2. Gel electrophoresis of Gradient PCR products of *H. brasiliensis* with *ref* promoter specific primers

1-RRIC 52 at 38°C, 2-RRIC 52 at 44.2°C, 3-RRIC 52 at 48.3°C, 4-RRIC 52 at 51.5°C, 5-RRIC 52 at 55°C, 6-RRIC 121 at 38°C, 7-RRIC 121 at 44.2°C, 8-RRIC 121 at 48.3°C, 9-RRIC 121 at 51.5°C, 10-RRIC 121 at 55°C

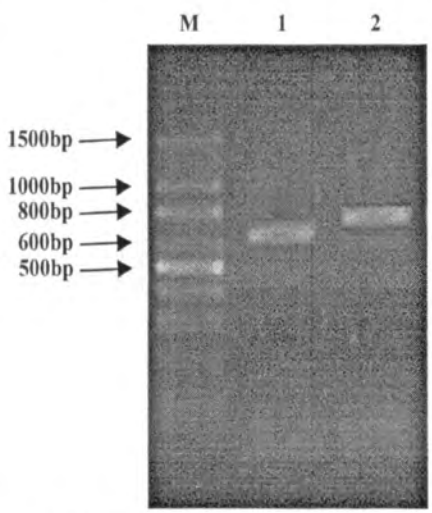

Figure 3. Agarose gel electrophoresis of PCR products amplified with *ref* promoter specific primers.

1-RRIC 52, 2-RRIC 121

Figure 5. Gel electrophoresis of partially digested *H. brasiliensis* genomic DNA

1-undigested DNA, 2-partially digested DNA

CONCLUSIONS

RRIC 52 amplified a 632 bp band that contains the promoter region and a part of the structural gene of the *ref* gene while RRIC 121 amplified a 700 bp band. Variations in the length of the amplified fragments suggest the presence of nucleotide differences between the promoter regions between the two clones. By constructing a genomic library, cloning of the upstream region of the *ref* gene can be done, using the promoter specific PCR fragments derived from this study as probes for further analysis of the *ref* gene promoter.

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