Investigation on Rubber Elongation Factor Gene (*ref*) Variation among Natural Rubber Producing Plants

H.M.P. HERATH[1], D.P.S.T.G. ATTANAYAKA[1], R.A.C.A. RANATHUNGE[1] and N.V. CHANDRASEKARAN[2]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila. (NWP)*

[2]*Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka.*

ABSTRACT

Rubber Elongation Factor (REF) protein encoded by the *ref* gene is known to participate in *cis*-polyisoprene formation in commercial natural rubber producer, *Hevea brasiliensis*. REF plays an essential role in natural rubber production by changing steriospecificity of the rubber transferase to form *cis*-polyisoprene. Investigation was carried out to find the variation of the *ref* gene encoding REF protein among *cis*- and *trans*- rubber producing species. Genomic DNA from *cis*-rubber producing clones of *Hevea brasiliensis, Hevea spruceana, Ficus elastica* and *trans*-rubber producing *Manilkara zapota* were amplified by *ref* gene specific primers. All clones of *Hevea brasiliensis* produced 1.4 kb bands and *Hevea spruceana* produced a 650 bp band. However *trans*-rubber producing *Manilkara zapota* and *cis*-rubber producing *Ficus elastica* did not produce any bands. *ref* sequence from RRIC 52 showed 97 % homology with that of RRIC 121. Sequence obtained from *Hevea spruceana* showed 25 % homology with *ref* sequence of RRIC 52 and RRIC 121. Dot blot analysis confirms the presence of *ref* gene related sequence in *Hevea spruceana* and clones of *Hevea brasiliensis*. No hybridization was observed in dot blots of *Manilkara zapota* and *Ficus elastica* confirming the absence of *ref* gene related sequence in those species.

KEYWORDS: *Ficus elastica, Hevea* spp, *Manilkara zapota,* Polyisoprene, Rubber Elongation Factor

INTRODUCTION

Natural rubber is an important plantation commodity in the global market. Latex containing Natural Rubber (NR) (*cis*- or *trans*-polyisoprene) is produced by over 2,000 plant species. These isoprenoids are formed by sequential condensation of isopentenyl diphosphate (IDP) units. Numerous classes of isoprenoids are produced from the plant isoprenoid biosynthesis pathway via IDP as a common intermediate (Keckwick *et al.*, 1989). Only a few plant species synthesize polyisoprene in *trans*- configuration. Sapodilla (*Manilkara zapota*) and gutta-percha (*Pallaquium gutta*) are typical representatives of *trans*-polyisoprene synthesizing plants (Morton, 1987).

Multiple IDP units are sequentially added to the growing rubber molecule in *cis*-configuration to form high molecular weight *cis*- polyisoprene in *Hevea* latex. Evidence for the involvement of *Hevea cis*-prenyltransferase in generating high molecular weight rubber molecule has been reported (Asawatreratnakul *et al.*, 2003). Number of other proteins has been also shown to participate in *cis*-polyisoprene formation. Most attention was directed to the major membrane protein of the rubber particle identified as Rubber Elongation Factor (REF) which has a molecular weight of 14.6 kD (Dennis and Light, 1989). *Ficus elastica* also produces natural rubber which has *cis*-configuration (Kang *et al.*, 2000).

REF is one of the major proteins found in *Hevea* latex which constitutes about 10 – 60 % of the total latex proteins and it was reported to play a significant role in rubber biosynthesis. To this protein, it was ascribed the ability to switch the stereospecificity of rubber transferase (Dennis and Light, 1989). However, information on the possible role of REF in rubber biosynthesis and its action as a major allergen is scarce.

The cDNA of the gene coding for REF has been cloned (Attanayaka, 1991) and previous studies have been conducted to find the inter- and intra- specific variation of *ref* gene in *Hevea* species and NR producing plants using PCR based techniques (Kumara *et al.*, 2010).

The objective of the present study is to find the *ref* gene variation among natural rubber producing plant species including inter- and intra- *Hevea* species, predominantly *trans*-polyisoprene producing *Manilkara zapota* and *cis*-polyisoprene producing *Ficus elastica* using PCR, Dot blots, Gene sequencing, and Bioinformatic tools. The information derived from this study would be useful in future studies on biosynthesis of natural rubber in plants.

MATERIALS AND METHODS

This study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka and Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka.

Plant Materials

Immature leaf samples at apple green stage from *Hevea spruceana* and clones of *Hevea brasiliensis* (RRIC 52, RRIC 121, RRISL 203) were collected from the nurseries of the Genetics and Plant Breeding Department of the Rubber Research Institute of Sri Lanka. Immature leaf samples from *Manilkara zapota* (sapodilla) and *Ficus elastica* were collected from the premises of Faculty of Agriculture and Plantation Management of Wayamba University of Sri Lanka.

Genomic DNA Extraction and Quantification

DNA was extracted from the *Hevea* leaves according to the rapid DNA extraction method developed by Rubber Research Institute of Sri Lanka (Kumara *et al.*, 2010).

DNA was extracted from *Manilkara zapota* and *Ficus elastica* using DNA extraction method adopted for cashew (Ranathunge *et al.*, 2010).

Three µl of extracted DNA was electrophoresed on 0.8 % agarose gel and observed using gel documentation system (Quantum ST 4). Lambda DNA (50 ng/µl) was used as the standard to determine the quality and the quantity of the DNA.

PCR Amplification

PCR amplification of the *ref* gene from genomic DNA of *Hevea brasiliensis* clones, *Hevea spruceana, Manilkara zapota* and *Ficus elastica* were performed by using *ref* gene specific primers; (forward) 5' ACG CGA ATT CGG AGG TTC GAT TAT GGC TGA AGA CG 3' and (reverse) 5' ACG CGT CGA CTT GGG GCT CAA TTC TCT CCA 3' synthesized based on the *ref* gene cDNA sequence (Attanayaka *et al.*, 1991). PCR amplification was done in 25 µl reaction volume with 50 -75 ng of genomic DNA, 200 µM dNTPs, 2.5 mM $MgCl_2$, 1X PCR buffer, 1 µl of 20 pM each of forward and reverse *ref* gene specific primers from previously diluted stocks and 2 units of Taq DNA polymerase (UC Biotec). The PCR program was consisted with an initial denaturation step at 96 ^{0}C for 5 min followed by 30 cycles of 96 ^{0}C for 15 sec, 55 ^{0}C for 30 sec and 72 ^{0}C for 1 min and 30 sec. A final extension step at 72 ^{0}C for 5 min was also included.

Analysis of PCR Products

The PCR products were analyzed on 1 % agarose gel stained with ethidium bromide (0.5 µg/ml). UC Biotec DNA marker 100 bp ladder (100 -1500 bp) was used as DNA size marker. After eletrophoresis, the gels were visualized and photographed using gel documentation apparatus (Quantum ST 4).

Gel Purification of the Amplified DNA Fragments

The amplified *ref* DNA bands were excised from the low melting point agarose gel and purified using an agarose gel purification kit (UC Biotec).

ref Gene Sequencing

Purified *ref* gene DNA bands of RRIC 52, RRIC 121 and *Hevea spruceana* were sequenced by automated sequencing at the Genetics Laboratory, Asiri Surgical Hospital Pvt. Ltd, Colombo.

Analysis of ref Gene Sequence Variation

Nucleotide sequences of RRIC 52, RRIC 121 and *Hevea spruceana* were aligned using the MEGA 4.0.2 (Tamura *et al.*, 2007) tool to find the homology among sequences.

Dot Blot Analysis

Non-Radioactive (Biotin) Probe Labeling and Determination of Labeling Efficiency

The gene (cDNA) coding for REF (Attanayaka, 1991) was used as the probe. The probe labeling was done by PCR on cDNA using *ref* gene specific primers. PCR amplification was done in 25 µl reaction volume with 0.25 µg of cloned cDNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer, 1 µl of 20 pM each of forward and reverse *ref* gene specific primers from previously diluted stocks and 2 units of Taq DNA polymerase (UC Biotec) and 70 µM Biotin-11-dUTP.

The PCR product was analyzed on 1 % agarose gel stained with ethidium bromide (0.5 µg/ml). UC Biotec DNA marker 100 bp ladder (100-1500 bp) was used as DNA size marker and lambda DNA (50 ng/µl) was used as a standard to determine the quality and quantity.

Several dilutions (Tenfold dilution 10^{-1} to 10^{-4}) of the Biotin labeled probe were prepared. Diluted DNA was denatured. The nylon membrane was soaked in 1X SSC for 10 min at room temperature and air dried. One µl of the dilutions were spotted on Nylon membrane using a micropipette, and air dried at room temperature for 20-30 min. The spotted membrane was placed on UV transilluminator for 1-5 min for

immobilization of DNA and developed using Biotin Chromogenic detection kit.

Preparation of Dot Blot

Dot Blot apparatus (BIORAD) set-up was arranged. Appropriate size nylon membrane was soaked in 1X SSC for 10 min at room temperature, air dried and one corner of the membrane was cut to make the orientation. The nylon membrane was placed inside the Dot Blot apparatus. Genomic DNA samples were denatured and an equal amount (8 µg) was blotted on the nylon membrane using Dot Blot apparatus. Human genomic DNA samples of 500 ng and 1000 ng were used as negative control and tenfold dilution series (10^{-1} to 10^{-7}) of unlabeled PCR product of the gene (cDNA) coding for REF was used as the positive control. The blotted membrane was placed on UV transilluminator for 1-5 min for immobilization of DNA.

Probe Hybridization

The blotted membrane was incubated with 25 ml of prehybridization solution (6X SSC/ 5X Denhard's solution) for 3 h at 65 ^{0}C using hybridization oven. Labeled probe was denatured and mixed with 5 ml of hybridization solution (6X SSC/ 0.5X SDS/ 5X Denhardt's solution). Treated membrane was incubated overnight with 5 ml of probe in hybridization solution mixture at 65 ^{0}C using hybridization oven. Hybridized membrane was washed three times using various concentrations of SSC and SDS.

Colour Development

Colour development was done using Biotin Chromogenic detection kit following manufacturer's instruction. The blot was developed separately with negative and positive controls. 10^{-4} dilution of the Biotin labeled probe was used as the control to determine the efficiency of the process.

RESULTS AND DISCUSSION

DNA Extraction from Hevea species

The rapid DNA extraction method (Kumara *et al.*, 2010) was suitable for extraction of DNA from all *Hevea* species (Figure 1). However, the DNA yield from RRIC 52 and *Hevea spruceana* were low when compared with DNA yields obtained from other two clones due to high maturity of the leaves. The DNA yield obtained from RRIC 121 and RRISL 203 was 60 µg/g leaf tissue. RRIC 52 and *Hevea spruceana* produced a DNA yield of 40 µg/g leaf tissue.

DNA Extraction from Manilkara zapota and Ficus elastica

The DNA yields obtained from sapodilla and *Ficus elastica* using cashew DNA extraction protocol (Ranathunge *et al.,* 2010) was 40 µg/g leaf tissue (Figure 1).

Detection of ref Related Sequences from cis- and trans-Rubber Producing Species by PCR

Genomic DNA from both *cis*- and *trans*-rubber producing species were amplified using the *ref* specific primers to detect the variation of *ref* related sequence in these species (Figure 2). *Cis*-rubber producing *Hevea brasiliensis* clones amplified approximately 1.4 kb DNA fragment indicating the presence of *ref* gene related sequence in their genome. These three clones also showed an additional very faint band of 650 bp amplified by the same primers. *Hevea spruceana* amplified approximately 650 bp DNA fragment indicating the presence of a *ref* gene related sequence in its genome. Repeated amplifications in *Manilkara zapota* and *Ficus elastica* did not amplify any bands indicating the absence of *ref* related sequences in their genomes (Figure 2).

ref Gene Variation as Detected by Nucleotide Sequencing

The gel purified *ref* gene from RRIC 52, RRIC 121 and *Hevea spruceana* were subjected to nucleotide sequencing. RRIC 52 and RRIC 121 produced sequences of 1142 nucleotides and 1157 nucleotides respectively. *Hevea spruceana* produced a sequence of 645 nucleotides. These sequences were aligned using MEGA 4.0.2 (Tamura *et al.*, 2007). RRIC 52 and RRIC121 showed very high similarity of 97 %. *Hevea spruceana* showed very little similarity of 25 % with RRIC 52 and RRIC121. These results confirmed the inter-specific variation of *ref* among *Hevea* species.

Dot Blot Analysis

Non-Radioactive (Biotin) Probe Labeling and Determination of Labeling Efficiency

The cDNA template of the *ref* gene amplified approximately 500 bp fragment indicating the efficiency of Biotin PCR reaction. The concentration of the PCR product was determined using lambda DNA (50 ng/µl) and it was 80 ng/µl (Figure 3).

All the labeled probe DNA spots (dilution) were efficiently developed (Figure 4). The labeling efficiency is determined at the lowest detectable concentration of the labeled probe. Labeling efficiency of this probe was 8 pg/µl.

Detection of ref Related Sequences from cis- and trans-Rubber Producing Species

Cis-rubber producing *Hevea brasiliensis* clones and *Hevea spruceana* showed colour development indicating the presence of *ref* gene in their genome. But *cis*-rubber producing *Ficus elastica* and predominantly *trans*-rubber producing *Manilkara zapota* did not show any colour development, suggesting the non-existence of *ref* gene related sequences in their genomes. Human genomic DNA which was used as negative control did not show any hybridization (Figure 5).

Figure 1. Agarose Gel Electrophoresis of Genomic DNA; *Lanes M- λ DNA, 1- RRIC 52, 2- RRIC 121, 3- RRISL 203, 4- Hevea spruceana, 5- Manilkara zapota, 6- Ficus elastica*

Figure 2. PCR profile of Genomic DNA; *Lanes M- 100bp ladder, 1- RRIC 52, 2- RRIC 121, 3- RRISL 203, 4- Hevea spruceana, 5- Manilkara zapota, 6- Ficus elastica*

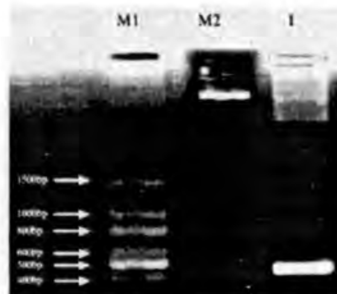

Figure 3. PCR profile of Labeled Probe; *Lanes M1- 100bp ladder, M2- λ DNA, 1- Labeled probe*

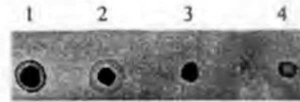

Figure 4. Dilution Series of Biotin Labeled Probe; *Dots 1- 8 ng/μl, 2- 0.8 ng/μl, 3- 0.08 ng/μl, 4- 8 pg/ μl*

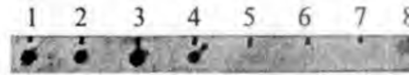

Figure 5. Dot Blot profile of Genomic DNA; *Dots 1- RRIC 52, 2- RRIC 121, 3- RRISL 203, 4- Hevea spruceana, 5- Manilkara zapota, 6- Ficus elastica, 7- 500 ng Human genomic DNA, 8- 1000 ng of Human genomic DNA*

CONCLUSIONS

Presence of *ref* gene related sequences in *cis*-rubber producing *Hevea brasiliensis* and *Hevea spruceana* was confirmed by PCR and Dot Blot analysis. Nonexistence of *ref* homologous sequences in *trans*-rubber producing *Manilkara zapota* and *cis*-rubber producing *Ficus elastica* was observed by above techniques. Absence of *ref* gene related sequence indicates possible non-significant role of *ref* gene in *trans*-rubber biosynthesis. Presence of *ref* gene variation at inter specific level of *Hevea* was confirmed by PCR and nucleotide sequencing.

ACKNOWLEDGEMENTS

The authors wish to thank all the staff members of the Department of Biotechnology. Sincere gratitude is also offered to Mr. M. Wickramanayaka and staff members of the Department of Chemistry, Faculty of Science, University of Colombo. The authors offer their sincere thanks to Dr. S.P. Withanage and staff members of the Genetic and Plant Breeding Department, Rubber Research Institute of Sri Lanka, Agalawaththa for providing leaf samples. Dr N.S. Kottearachchi and Head Department of Genetics and Plant Breeding CRISL are also acknowledged for their support in carrying out this study.

REFERENCES

Asawatreratnakul, K., Zhang, Y.W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayaporn, A. and Koyama, T. (2003). Molecular cloning, expression and characterization

of cDNA encoding *cis*-prenyltransferases from *Hevea brasiliensis*, a key factor participating natural rubber biosynthesis. *European Journal of Biochemistry* **270**,4671-4680.

Attanayaka, D.P.S.T.G., Keckwick, R.G.O. and Franklin, F.C.H. (1991). Molecular cloning nucleotide sequencing of the Rubber Elongation Factor gene from *Hevea brasiliensis , plant. Mol. Biol.* **16,** 1079-1081.

Dennis, M.S. and Light, D.R. (1989). Purification of apenyltransferase that elongates *cis*-isoprene rubber from the latex of *Hevea brasiliensis, The journal of Biological Chemistry.* **264** (5), 18597-18598.

Kang , H., Kang, M.Y and Han, K.H. (2000). Identification of Natural Rubber and Characterization of rubber Biosynthetic Activity in Fig Tree. American Society of Plant Physiologist. Available from: htpp://www.dx.doi.org/10.1104/pp.123.3.1133.

Keckwick, R.G.O., Auzac, J., Jacob., J.L. and Chrestin, L. (1989). *Physiology of rubber tree latex , the formation of isoprenoids in Hevea latex*. 145-164.

Kumara, K.R.W., Attanayaka, D.P.S.T.G. and Samarajeewa, D.A.D.S. (2010). Inter and Intra specific variation of *ref* gene in Natural Rubber producing plants. In Proceedings of 10th Agriculture research Symposium, August 2010, Wayamba University of Sri Lanka.

Morton, J.F. (1987). *Sapodilla in Fruit of warm climates*. 393-398.

Ranathunge, R.A.C.A., Attanayaka, D.P.S.T.G., Samarajeewa, D.A.D.S. and Jayasekara, S.J.B.A. (2010). Genetic diversity of cashew germplasm collected from five districts of Sri Lanka as revealed by Random Amplified polymorphic DNA (RAPD). In Proceedings of 10th Agriculture research Symposium, August 2010, Wayamba University of Sri Lanka.

Tamura, K., Dudley, J., Nei, M. and Kumar,S. (2007). Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599. Available from: http://www.kumarlab.net/publications