Proceedings of 15th Agricultural Research Symposium (2016) 144-148

Cloning and Characterization of the Proximal Promoter Sequence of Rubber Elongation Factor (*ref*) Gene of *Hevea brasiliensis* for Expression Studies

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ABSTRACT

Rubber elongation factor (REF) protein involves in biosynthesis of rubber. Main function of this protein is the elongation of the rubber molecule by the interaction with prenyl transferase and rubber particles. This protein is the major constituent of rubber particles and highly expressed in laticiferous tissues. This study was undertaken to prepare the proximal promoter region of the gene encoding rubber elongation factor to be used in eukaryotic expression vector pCAMBIA1391Z for the analysis of promoter activity. Primers were designed to amplify the proximal promoter region from *Hevea brasiliensis* containing restriction sites compatible with the pCAMBIA1391Z GUS expression vector for directional cloning. The nucleotide sequence of the amplified fragment confirmed the presence of proximal promoter elements having required restriction sites. It shows 98% of homology with the *Hevea* sequences available in the GenBank database under accession number AY712939.1 and accession number X56535. The promoter region was cloned into an intermediate vector pGEM-T Easy vector using TA cloning procedure. Rapid screening and colony PCR show the presence of the insert in the recombinant colonies. The yield of the plasmid DNA extracted was not sufficient for further studies.

KEYWORDS: *Hevea brasiliensis*, Natural rubber, Proximal promoter, Rubber elongation factor

INTRODUCTION

Hevea brasiliensis (wild) Muell-Arg. commonly known as Para rubber tree is a member of family Euphorbiaceae, genus *Hevea*. Rubber plant, *Hevea brasiliensis* is an economically important plantation crop in Sri Lanka; which produce natural rubber. It is chemically known as *cis*-1, 4-polyisoprene (Vinod, 2002).

Rubber is the second largest plantation crop in Sri Lanka based on earning through the export. In 2014, total rubber production was 98,573 Mt and export quantity in 2014 was 16,300 Mt (Rubber Research Institute of Sri Lanka-Statistics, 2014). In 2014 rubber productivity was 889 kilograms per hectare and 134,100 hectares were under cultivation out of which 111,000 hectares are under tapping (Central Bank Report 2014). In year 2014 contribution of rubber industry to the GDP was 0.1% (Central Bank Report, 2014).

Rubber (*cis*-1, 4-polyisoprene) is an important raw material for many industrial uses. It is synthesized in the latex of *H. brasiliensis*. In *H. brasiliensis* rubber is synthesized on the surface of particles suspended in latex a constituent in the cytoplasm of laticifers. Laticifers are ducts found in the phloem of the rubber tree. The rubber elongation factor (REF) is a protein which is tightly bound to rubber particles. Rubber elongation factor (*ref*) gene involves in biosynthesis of rubber. Main function of this protein is the elongation of the rubber molecules by the interaction with Prenyl transferase and rubber particles (Denis *et al.*, 1989).

Rubber elongation factor has 137 amino acids long sequence and molecular weight of this protein is 14 600 Daltons. *ref* gene consists with three exons and two introns (Attanayaka *et al.*, 1995). Differential expressions of the *ref* gene within different cultivars of *H. brasliensis* has been reported. High yielding clones has high expression and low yielding clones show low expression (Suganthan *et al.*, 2011) which would be related with variations of sequence of promoter region, introns and enhancers. *ref* gene is highly expressed in laticifers.

The *ref* gene promoter is considered as a strong plant promoter and has the potential to be developed as a eukaryotic promoter that could be used in gene expression studies.

The objective of this study is to clone the proximal promoter region of the *ref* gene from the high yielding RRIC 121 clone of the *Hevea brailiensis*, in order to be used in a GUS reporter gene construct of pCAMBIA1391Z.

MATERIALS AND METHODS

This 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 December 2015 to May 2016.

Collection of Plant Materials

Immature leaves at apple green stage of *H. brasiliensis* clones RRIC 121 were collected from the nurseries of Faculty of Agriculture and Plantation Management of Wayamba University of Sri Lanka.

Genomic DNA Extraction

Genomic DNA was extracted from the leaves of *H. brasiliensis* clone RRIC 121 using rapid DNA extraction method (Herath *et al.*, 1996). The extraction buffer 0.1 M TrisCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl and 2% CTAB. Quality and quantity of extracted DNA were determined by 0.8% agarose gel electrophoresis.

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

The forward and reverse primers were designed using the genomic sequences deposited in the GenBank database under the accession number AY712939.1 and X56535 respectively. The reverse primer contained a part of the coding sequence of the *ref* gene (Anon, 2016a). The Primer 3 Plus program was used in designing the primers (Anon 2016b). In order to facilitate the directional cloning in pCAMBIA1391Z, a *Sal*1 site and *Eco*R1 site were included in the forward and reverse primer sequences respectively.

Optimization of PCR Condition

Gradient PCR was conducted with Bio-Rad Thermo cycler to determine the optimum annealing temperature for the primers. Five annealing temperatures were used (45, 46.7, 50.4, 52.1 and 54 °C) with designed primers.

Amplification of the ref Gene Promoter Region by PCR

Polymerase chain reaction amplification of genomic DNA of RRIC 121 of *H. brasiliensis* was done to amplify the *ref* gene promoter region by using designed specific primers. Polymerase chain reaction mixture contained 30 ng of genomic DNA with 1X PCR buffer, 200 µM dNTPs, 1 µL of 10 µM designed primers and 5 units of Taq DNA polymerase (sigma) in 25 µL volume. Polymerase chain reaction program used was 96 °C for 5 min of initial denaturation step, 96 °C for 45 sec of denaturation, 50.4 °C of annealing temperature for 45 sec, and an extension step of 72 °C for 1 min and final extension step at 72 °C for 5 min followed with 30 cycles. Polymerase chain reaction products were analyzed by using 0.8% agarose gel and visualized using gel documentation unit.

Column Purification of PCR Products

Amplified DNA products were purified by using PureLink quick Gel Extraction and PCR Purification Combo Kit (Invitrogen). Purified DNA was quantified by using lambda DNA standard on 0.8% agarose gel and checked by UV Trans-Illuminator.

Sequencing of ref Gene Promoter

Amplified *ref* gene proximal promoter region of RRIC 121 was sequenced at Microgen DNA sequencing, Korea.

Cloning of PCR Products

The Ligation reaction was set-up using 1:3 vector to insert DNA ratio. The amount of vector and insert DNA to be used in the ligation reaction was determined by using NEB ligation calculator. Ligation reaction was carried out in 10 µL volume, which contained 50 ng pGEM-T Easy Vector, 60 ng of purified PCR product, 1 unit of T4 DNA ligase and 1X rapid ligation buffer. Reaction was mixed and incubated overnight at 15 °C.

Preparation of Competent Cells

Fresh LB media (10 mL) was inoculated with 100 µL of an overnight culture of JM109 *E. coli* strain. Cultures were incubated at 37 °C with shaking until the Optimum Density (OD) 0.3-0.6 at 650 nm reached. Cells were harvested by centrifugation at 10 000 rpm after washing in 4 mL of cold 10 mM NaCl. Cells were gently re-suspended in 4 mL of 100 mM $CaCl_2$ and incubated on ice for at least 20 min. The competent cells were collected by centrifugation at 5 000 rpm. Supernatant was discarded. The pellet was resuspended in one milliliter of cold 100 mM $CaCl_2$ stored at -20 °C. Competent cells were check by culturing on LB agar plates with and without 60 µg/mL ampicillin.

Transformation of Ligation Mixture

Hundred microliters of competent cells were transformed with 2 µL of ligation mixture using heat shock method. Positive control was performed using the insert DNA provided with the Invitrogen kit. SOC medium was added into each tube. Finally reaction mixtures were incubated for 1.5 h at 37 °C with shaking. Transformed cultures were plated onto LB plates with 60 µg/mL of ampicillin, 5 µL of IPTG (0.84 M), and 16 µL of X Gal (100 mg/mL). Randomly selected white colonies

were transferred into grid plate which containing ampicillin 60 µg/ml, 5 µL of IPTG (0.84 M) and 16 µL of X Gal (60 mg/mL).

Rapid Screening of Transformation

Randomly selected colonies were transferred into the 50 µL of lysis buffer (20% Sucrose, 5 M NaOH, 3 M KCl, 0.5 mM EDTA, 20% SDS, H_2O) and incubated at 37 °C for 5 min. They were placed on ice for 5min, spun for 1 min at 15 000 rpm. Supernatant was loaded onto 0.8% agarose gel. The recombinant plasmids were identified by the difference of the size of the plasmids (Law and Crickmore, 1997).

Colony PCR Amplification

Randomly selected transformed colonies were introduced to 30 µL PBS (Phosphate Buffer Saline) separately by touching the colony with a toothpick. The mixture was centrifuged using a, microfuge. Five micro liters of supernatant was taken to the PCR in 20 µL volume with 1 µL of each forward and reverse promoter specific primers.

Mini Preparation of Plasmid DNA

Three milliliters of overnight culture was centrifuged to collect the cell pellet. Pellet was resuspended in buffer 1 (1 M Tris-HCl, EDTA, RNAse, sterile distilled water). Then buffer 2 (5 M NaOH, 10% SDS, water) was added and mixed gently. The mixture was incubated at room temperature for 5 min and buffer 3 (5 M Potassium acetate, Acetic acid, Water) was added. It was gently mixed and centrifuged at 12 000 rpm for 20 min. Supernatant containing plasmid DNA was precipitated by adding 3 M sodium acetate and absolute ethanol. DNA was washed with 70% ethanol. Pellet was air dried and dissolved in 50 µL TE.

Digestion of Plasmid DNA

Approximately 50 ng plasmid DNA (25 µL) was digested with *Eco*R1 restriction enzyme. Five microliter of restriction 10X buffers, 2 µL of *Eco*R1 enzyme and 0.5 µL of BSA in 50 µL of reaction volume. Then reaction mixture was incubated for 3 h at 37 °C. The digestion mixture was electrophoresed and observed by using Gel Documentation Unit.

RESULTS AND DISCUSSION

Genomic DNA Extraction and Quantification

DNA yield obtained from one milligram of leaf samples of RRIC 121 was 500 ng (Figure 1). The quality and quantity of DNA extracted by this method was comparable to the results obtained by previous workers Erandika *et al., (2014)*.

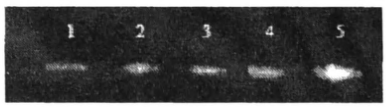

Figure 1. Agarose gel electrophoresis of genomic DNA. *1-5 - RRIC 121 genomic DNA*

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

ref promoter specific primers were designed to amplify the 670 bp sequence which include the proximal promoter region at the 5' and part of the *ref* coding sequence at its 3' end. The sequence of the designed primers are given below,

*Sal*1
Forward; 5'ACG CGT CGA CGG AGG AAA AAC AAA GAC TA 3'

*Eco*R1
Reverse; 5'ACG CGA ATT CTT GCG AGG AAC TTG GTT TGC ATC 3'

The PCR products amplified using these two primers facilitate the directional cloning of the proximal promoter fragment in pCAMBIA1391Z vector to drive the expression of the GUS gene. Melting temperature of forward and reverse primers were 72.9 °C and 78.4 °C respectively.

Optimization of PCR Condition

The optimum annealing temperature determined by gradient temperature experiment for the amplification of *ref* proximal promoter was 50.4 °C (Figure 2).

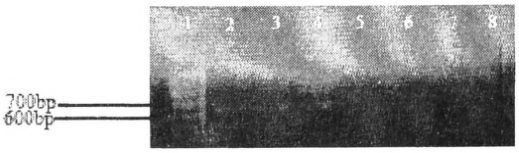

Figure 2. Optimization of annealing temperature for primers. *1- 100 bp Ladder, 2- RRIC 121 at 45 °C, 3- RRIC 121 at 46.7 °C , 4- RRIC 121 at 50.4 °C, 5- RRIC 121 at 52.1 °C, 6- RRIC 121 at 54 °C, 7- RRIC 121 amplicon at 54 °C, 8- RRIC 121 control at 52.1 °C*

Amplification of the ref Gene Promoter Region by PCR

Genomic DNA of RRIC 121 clone amplified 670 bp of proximal promoter region by designed primers at the annealing temperature of 50.4 °C (Figure 3).

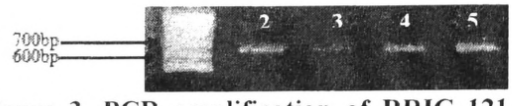

Figure 3. PCR amplification of RRIC 121 genomic DNA at 50.4 °C with *ref* primers. *1- 100 bp Ladder, 2-5 – PCR products of RRIC 121 clone*

Sequence Analysis of the Amplified ref Promoter

The sequence obtained contained the respective reverse and forward primer sequences with *Sal*1 and *Eco*R1 sites in the correct orientation compatible for directional cloning in pCAMBIA1391Z vector. From the sequence obtained by the reverse and forward reaction an unambiguous 300 bp sequence was selected for further analysis. It showed 98% homology with the sequence deposited under accession numbers AY712939.1 and X56535 of the *H. brasiliensis* promoter sequences available in the GenBank database. Putative TATA box and CAAT box were located respectively at position 227 bp and 128 bp (Figure 7). PlantCARE analysis showed different potential regulatory elements in the 5' upstream, i.e. ARE, skn-1 motif and TGACG motif. The presence of the identified promoter elements confirmed the amplified sequence to be a part of proximal promoter region of *H. brasiliensis*.

Cloning and Transformation of ref Promoter

The transformed cells using ligation mixture and the control formed only white colonies on both plates. Transformation efficiency of ligation reaction and positive control were 9.2×10^5 c.f.u/µg and 1.14×10^6 c.f.u/µg respectively showing comparatively high transformation efficiency. pGEM-T Easy Vector is linearized with single 3' terminal thymidine at both end. "T" overhang at the insertion site has facilitated the ligation of PCR products which contain the adenosine overhang introduced by Taq DNA polymerase used in this study which does not have proof reading ability. "T" overhangs prevent re-circularization of the vector. Clones containing PCR products were produced white colonies but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene. Insertion inactivation of the α- peptide allows identification of recombinants by white colonies as blue/white screening on IPTG and X Gal plates.

Rapid Screening of Transformation

Lysed cells of controlled blue colonies produced two bands (Figure 4 Lane 1 and 16). This experiment shows the presence of transformed plasmids in the selected colonies. As detected by the gel electrophoresis the size of the plasmid DNA of selected colonies was higher than the vector DNA suggesting the presence of the insert.

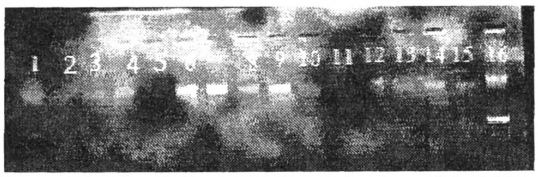

Figure 4. Rapid screening of plasmid DNA of selected colonies. *1 and 16- Plasmid DNA from non-recombinant colonies, 2-15- Plasmid DNA from recombinant colonies*

Colony PCR Amplification

Colony PCR resulted approximately 650 bp band, which confirmed the recombinant colonies to contain the cloned fragment (Figure 5).

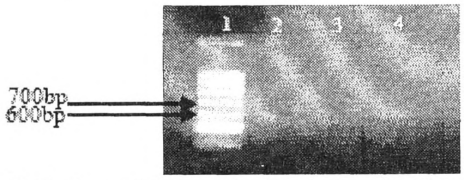

Figure 5. Colony PCR of selected colonies. *L-100 bp Ladder, 1-3 – Colony PCR products*

Mini Preparation of Plasmid DNA

Plasmid DNA extractions by mini preparation procedure repeatedly yielded low concentration of plasmid DNA less than 50 ng/µL. The slow growth of the pGEM-T Easy vector containing JM109 *E. coli* cells could be a reason for low yield obtained in this experiment (Figure 6).

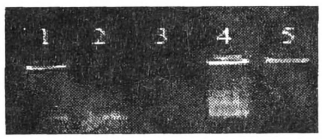

Figure 6. Agarose gel electrophoresis of plasmid DNA. *1-5- Plasmid DNA from recombinant colonies*

Digestion of Plasmid DNA

The plasmid DNA was digested with *Eco*R1 to elute the cloned DNA fragment for confirmation. The digestion reaction was not successful. The low concentration and purity of plasmid DNA and the presence of RNA may have inhibited the digestion reaction.

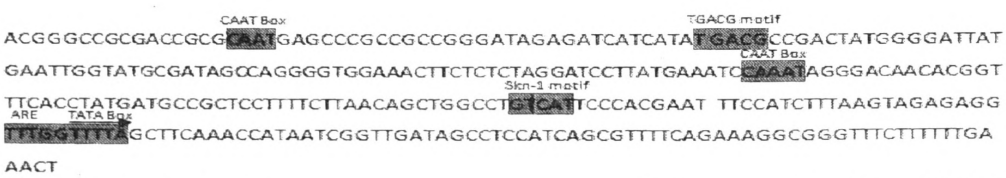

Figure 7. DNA sequence of the proximal promoter region of *H. brasiliensis* clone RRIC 121

CONCLUSIONS

Designed *ref* gene promoter specific primers successfully amplified the target sequence (670 bp). The ends of the promoter sequence of *ref* gene were modified by adding *Sal1* and *Eco*R1 sites for directional cloning in pCAMBIA1391Z vector. The optimum annealing temperature for PCR of *Hevea brasilliensis* (RRIC 121) was 50.4 °C. The nucleotide sequence obtained of the PCR fragment confirmed presence of proximal promoter region of *ref* gene. Column purified DNA was successfully ligated into the pGEM-T Easy Vector and it was transformed into the *E.coli* bacteria competent cells that were prepared. Success of this process was confirmed by rapid screening of transformants and colony PCR amplification. Mini preparation plasmid DNA extractions did not yield DNA with adequate quality and quantity for elution of the fragment to be clone in pCAMBIA1391Z vector.

ACKNOWLEDGEMENTS

The authors wish to thank all staff members of Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka. Sincere gratitude is offered to Mr. M. Wickramanayake, Biotechnology Laboratory, Department of Chemistry, University of Colombo. The authors wish to acknowledge the financial support by CARP through NARP research grant scheme.

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