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Screening of Chloroplast Promoters for *Hevea brasiliensis* Chloroplast Transformation

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Abstract

In recent years, the growth in the use of recombinant proteins has grown tremendously. With the aid of the advances in DNA recombinant biotechnology, molecular farming in plants has been applied to meet this increasing demand where plants have emerged as one of the most promising general production platforms for recombinant proteins. *Hevea brasiliensis* is one of the main commodities in Malaysia and widely cultivated species for commercial production of latex. This important plant has been used to express recombinant proteins such as a single-chain variable fragment (scFv) antibody against the coat protein of *Streptococcus gordonii* (an oral dental bacterium), human serum albumin and human atrial natriuretic. The genes that encodes for the recombinant proteins were targeted into the nucleus genome of *Hevea* but the proteins were expressed in low concentration. Generating transgenic plant using chloroplast transformation offers many advantages in comparison to nuclear transformation and many researches have been made to apply this strategy to enhance agronomic traits or produce recombinant protein in several plant species. Since chloroplast is highly polyploidy, it allows high-level foreign protein expression. Given the generally very high foreign protein accumulation rates that can be achieved in transgenic chloroplasts, the aim of this study is to screen a number of chosen endogenous *Hevea* chloroplast promoters to drive the expression of the reporter gene, *uidA* for *Hevea* specific chloroplast transformation vector. Three promoters were chosen for this experiment which are; *rbcL*, *psbA* and *rrn16* promoters. The putative regions of these promoters were derived from the chloroplast genome sequence of *Hevea*. Analyses of the three putative promoter regions using multiple sequence alignment with comparable regions from other plant species show significant sequence homology. Further analyses of the putative regions using in-vitro transcription are planned for future study. It is hoped that with the development of an optimized expression vector will allow high expression of valuable recombinant protein in the chloroplast of *Hevea*.

Keywords: Hevea brasiliensis, chloroplast transformation vector, promoter, *rbcL*, *psbA*, *rrn16*

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1.0 INTRODUCTION

Hevea brasiliensis is a commodity crop cultivated mainly for the production of natural rubber. Originating from the Amazon forest, *Hevea* plants have been planted in many countries for economic purposes including Malaysia. Similarly like other cash crops, with the advancement of genetic engineering technology, there has been a steady increase in the interest in implementing genetic modification techniques on *Hevea* to improve the agronomic traits of the plants or to utilize the plant as a platform for the production of recombinant proteins [1]. Several successful attempt on genetic modifications of *Hevea* plants have been recorded, such as the successful transformation with gene coding for superoxide dismutase [2] and the genetic transformation of *Hevea* with gibberellic acid insensitivity (*GAI*) gene to induce dwarfism [3], with all attempts were done using nuclear transformation. The problem with nuclear transformation of *Hevea* is that it consistently produce a transformant with a low expression of the recombinant protein and the main problem that this research is aiming to solve by using chloroplast transformation technique.

Chloroplast transformation offers a few significant advantages over nuclear transformation. This is due to the fact that while a single cell could only possess one nucleus, it could have thousands of chloroplast depending on the cell type [4]. Thus by modifying the genome of the chloroplast, the recombinant gene will be highly represented in the cell and thus translates to higher expression of the gene. The chloroplast genome is also less susceptible to epigenetic gene silencing than the nuclear genome that may cause inconsistency in the expression of the recombinant gene [5]. Other than higher expression, the compartmentalization of the chloroplast genome inside the stroma of chloroplast provides more stability to the expressed recombinant protein, this is because the stroma of the chloroplast have fewer protein degradation pathways than the cytosol of the cell [5].

Nevertheless, using chloroplast transformation technique alone does not ensure a high expression of recombinant protein, a good design of the chloroplast transformation vector is crucial in not only ensuring the successful transformation of the plant but also a good expression of the recombinant gene once it is integrated. In a transformation vector, one of the component that will determine the effectiveness of the expression is the promoter that drives the expression of the recombinant gene [6]. In previous researches in chloroplast transformation vector design, a number of chloroplast specific promoters were commonly used such as the 16S ribosomal RNA promoter (*prrn*) and the *psbA* gene promoter [6]. This research aims to select three promoters, all derived from *Hevea brasiliensis* chloroplast genome, which are 16S ribosomal RNA (*rrn16*), photosystem II preotein D1 (*psbA*) gene, and RuBisCO large subunit (*rbcL*) gene promoters. The predicted region of the promoter sequences were analyzed for any conserved sequence by comparing the sequence with sequences of the same region derived from the chloroplast genome of other plant species.

2.0 EXPERIMENTAL

2.1 Isolation of *Hevea* Genomic DNA

For this application, the GF-1 Plant DNA Extraction Kit from Vivantis was used. 20 mg of *H. brasiliensis* leaves were ground in liquid nitrogen into fine powder. 250 µl Buffer PL and 20 µl of Proteinase K were added into 2.0 ml collecting tube containing the ground leaf sample and was incubated at 65°C for 1-2 hours. After incubation, the mixture was centrifuged at 16000 *g* for 5 minutes and the resulting supernatant was transferred into a fresh collecting tube. A double volume of Buffer PB was added into the supernatant and was mixed through vortexing which afterwards was incubated at 65°C for 10 minutes. 200 µl of analytical grade absolute ethanol was then added into the mixture and then immediately mixed, the mixture was then loaded into the GF-1 column to be centrifuged at 10000 *g* for 1 minute of which the resulting flowthrough was discarded. 650 µl wash buffer that had been diluted with absolute ethanol as per manufacturer's recommendation was added into the column and then centrifuged at 10000 *g* for 2 minutes and the flowthrough again was discarded. The centrifugation step was repeated to remove any wash buffer residue. 40 µl of Elution buffer was added directly onto the column and left to incubate in room temperature for 2 minutes. The column was centrifuged at 10000 *g* for 1 minute and the resulting supernatant containing DNA was stored in -20°C until further use. It is advisable to repeat the elution step twice to ensure maximum yield.

2.2 Detection of Genomic DNA

Extracted DNA samples were detected using agarose gel electrophoresis. A gel concentration of 1% were used and was prepared by dissolving 1 g of agarose powder into 100 ml of 1X TAE buffer. To facilitate dissolving, the mixture was heated in a microwave under medium-high heat for approximately 2 minutes. Ethidium bromide (EtBr) was added into the gel solution and act as DNA staining dye for visualization. The gel solution was poured into a gel casting tray with a well forming comb attached, once solidified, the comb was removed and the gel was placed fully submerged inside electrophoresis tank containing 1X TAE buffer. The top part of the gel where the well are aligned, was positioned at the negative electrode while the bottom part at the positive electrode. 4 µL of 1kb DNA ladder was loaded onto the first well. Then, 3 µL of the extracted DNA with 1 µL of 6x DNA loading dye were placed onto adjacent wells. The electrophoresis was performed at 150 V for 50 minutes. After electrophoresis, the gel was visualized under UV light using Alpha Imager™ 2200.

2.3 Determination of Concentration and Purity of Nucleic Acid

The concentration and purity of the DNA extract were determined using Nanodrop™ 2000 UV-Vis Spectophotometer (Thermo Scientific). The elution buffer solution provided with the DNA extraction kit was used as the blanking solution. The absorbance ratio of A_{260}/A_{280} at values of 1.8 to 2.0 is considered highly pure. Determination of concentration of nucleic acid is essential to ascertain the amount of DNA template to be used in polymerase chain reaction (PCR).

2.4 Amplification of Promoter Region by Polymerase Chain Reaction (PCR)

Three putative promoter regions of three genes, *rbcL*, *psbA*, and *rrn16* were amplified using PCR. Primers were designed via the NCBI Primer BLAST application based on published *Hevea* chloroplast genome

sequence by Tangphatsornruang *et al*. (2011) [7] (refer Table 1). MyTaq™ PCR Master Mix from Bioline was used for the PCR (Table 2) with slight modification of the PCR cycle profile (Table 3) to accommodate our sample. Gradient PCR was first done using Eppendorf Mastercycler® gradient thermocycler to ascertain the optimum annealing temperature for each primer pair. Fragment length verification was done via UV light visualization of electrophoresed agarose gel of the PCR product. The confirmed PCR products were then sent for sequencing.

Table 2 PCR Master Mix components

Component	Total Volume (50 µl reaction)	Final concentration
Ultrapure water	Add to 50 µl	Add to 50 µl
2X MyTaq PCR Master Mix	25 µl	1X
Forward primer (25 µM)	1 µl	0.5 µM
Reverse primer (25 µM)	1 µl	0.5 µM
Template DNA	Based on DNA extract	≥200 ng

Table 3 PCR thermocycler profile

Cycle step	Temperature (°C)	Time
Initial denaturation	95	3 mins
Denaturation	95	30 sec
Annealing	X	1 min
Extension	72	1 min
*Repeat for 35 cycles		
Final extension	72	10 mins
Hold	4	∞

*X : Based on primer pair optimum annealing temperature

2.5 Analyzing Putative Promoter Region

Once the sequence of each PCR products were obtained, they were verified using NCBI BLAST application to determine whether the sequence matches the desired region of amplification. Promoter consensus sequence were analyzed by finding conserved regions upstream of each gene. To that end, a short fragment of the amplified putative promoter region was compared with the same chloroplast genome region of 6 different species of plants, which were obtained from NCBI database, using T-Coffee multiple alignment application (European Bioinformatics Institute). Among the 6 species, *Jatropha* sp. was included as this plant as it is under the same family with *Hevea* which is Euphorbiacieae.

Table 1 List of designed primers with sequence and annealing temperature

Primer name	Primer Sequence	Annealing Temperature	Product length
rbcL F	5'-CTGCAGATTTTCACATCTCGGATT-3'	53.6°C	550bp
rbcL R	5'-ATCGGTCCACACAGTTGTCC-3'		
psbA F	5'-AATATCTACCGGAGGGGCAG-3'	53.0°C	524bp
psbA R	5'-TGAAAAATGCAAGCACGGC-3'		
rrn16 F	5'-ATTTTGACTTGTTCCCCCGC-3'	54.9°C	555bp
rrn16 R	5'-AAAATTCCCCACTGCTGCCT-3'		

3.0 RESULTS AND DISCUSSION

The genes of the chloroplast genome are mainly divided into photosynthesis related genes and non-photosynthesis related genes [8], [9].

Photosynthesis related genes are mainly transcribed by plastid-encoded RNA polymerase (PEP) which requires prokaryotic σ-like factors for promoter recognition [8]. Thus, in photosynthesis related genes, the regions responsible for correct promoter recognition are the -10 and -35 promoter elements.

The *rbcL* gene is a photosynthesis related gene and the multiple alignment result shows a highly conserved -10 region with a consensus sequence of GGAGGG. This is similar to the discovery made by Cheong and colleagues regarding a putative ribosome binding site of *Hevea rbcL* [10]. The -35 region of *Hevea* is AGAATT which is similar to *Arabidopsis* sp., *Jatropha* sp, *Nicotiana tabacum*, and *Solanum tuberosum* (Figure 1). The two remaining species which are *S. lycopersicum* and *Zea mays* differs only on the first two bases of the -35 region; 5'-ATAATT-3' and 5'-TGAATT-3' respectively.

Similar to *rbcL* gene, the *psbA* gene is a photosynthesis related gene. A research done to improve the efficiency of foreign protein expression in *E.coli* using chloroplast *psbA* promoter have found out that by using the promoter sequence consisting of sequences 10bp and 35bp upstream of *psbA* transcription initiation site, expression could be increased 18.5 fold more than the expression using *E.coli* T7 promoters [11]. The sequence of the -10 region of *Hevea* is AGATTT and multiple alignment results shows that the first four bases is conserved with a single gap introduced between the first base and the second base (Figure 2). The sequence of the -35 region of *Hevea* is CCTGAT, however there is an addition of bases from -18bp to -23bp which is absent from the upstream sequence of the other plant species tested. This means that, discounting the gap, the -35bp element of *Hevea* is very much different from the -35bp element of other comparative species. The -35 region of the other species has a consensus sequence of AGTCCC with the only variation being *Zea mays* with a sequence of AGTCCT.

```
                                  -35                      -10
Hevea                 TTGTTGTTGTGAGAATTCTTAATTCATGAGTTGTAGGGAGGGACTT

Arabidopsis           GTTTTATTGCAAGAATTCTAAATTCATGACTTGTAGGGAGGGACTT
Jatropha              TTGTTGTTGTGAGAATTCTTAATTCATGAGTTGTAGGGAGGGACTT
Nicotiana             TTGTTGTTGTGAGAATTCTTAATTCATGAGTTGTAGGGAGGGATTT
S.tuberosum           TTGCTGTTGTGAGAATTCTTAATTCATGAGTTGTAGGGAGGGATTT
S.lycopersicum        TTGTTGTTGTGATAATTTTTAATTCATGAGTTCTAGGGAGGGATTT
Zea mays              CTGTGCTTGTGTGAATTCTTAATTCATGAGTTGTAGGGAGGGACTT
                       *    ***    **** * ********* ** ********** **
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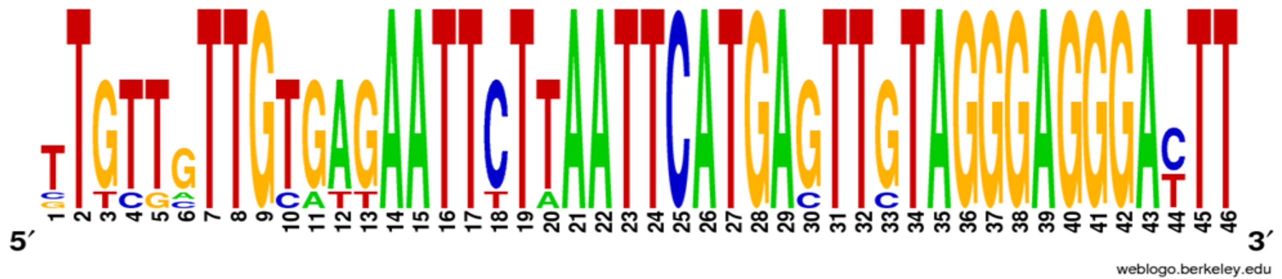

Figure 1: Multiple alignment of *rbcL* upstream sequence (-1bp to -46bp)

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                                 -35            -23            -10
Hevea            -----GAGTCCCTGATGATTAAATAAAATAAACCAA-GATTTTACC

                            -35
Arabidopsis      CTTGGGAGTCCCTGATTATTAAA-----TAAACCAAGGATTTTACC
Jatropha         CTTGGGAGTCCCTGATGATTAAA-----TAAACCAA-GATTTTACC
Nicotiana        CTTGGGAGTCCCTGATGATTAAA-----TAAACCAA-GATTTTACC
S.lycopersicum   CTTGGGAGTCCCTGATGATTAAA-----TAAACCAA-GATTTTACC
S.tuberosum      CTTGGGAGTCCCTGATGATTAAA-----TAAACCAA-GATTTTACC
Zea mays         CTTGGGAGTCCTTGCAATTTGAA-----TAAACCAA-GATCTTACC
                       ****** **     ** **      ******** *** *****
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5′ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 3′

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Figure 2 Multiple alignment of *psbA* upstream sequence (-1bp to -40bp)

```
                         -35                            -10
Hevea            TGTCTACGAACAAGGAAGCTATAAGTAATGCAACTAAGAA

Arabidopsis      TGTCTACGAAGAAGGAAGCTATAAGTAATGCAACTATGAA
Jatropha         TGTCTACGAACAAGCAAGCTAAAAGTAATGCAACTAAGAA
Nicotiana        TGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAA
S.lycopersicum   TGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAA
S.tuberosum      TGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAA
Zea mays         TGTCTACGAATAAGGAAGCTATAAGTAATGCAACTATGAA
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5′ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 3′

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Figure 3 Multiple alignment of *rrn16* upstream sequence (-1bp to -40bp)

The *rrn16* gene encodes for the production of ribosomal RNA for the 16S ribosome subunit and unlike rbcL and psbA, the rrn16 gene is not photosynthesis related. However, in most higher plants, the *rrn* operon is transcribed by plastid encoded polymerase (PEP) which recognizes σ-type promoters [12]. Based on the multiple alignment result of *rrn16* upstream region, the sequences are highly conserved. The -10 region of *rrn16* has a consensus sequence of CAACTA with 100% homology among the species tested (Figure 3). The -35 region has a consensus sequence of ACGACC with the only difference being *Zea mays* which has a sequence of ACGAAT.

4.0 CONCLUSION

From the experiment, the results show that there is a considerable degree of conservation in the putative promoter regions of each genes tested to, to verify whether the regions indeed corresponds to a functional transcription recognition site, in-vitro study must be done to corroborate the findings. Therefore, in-vitro transcription test is planned as next step of this research.

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