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[CLONING AND SEQUENCING OF THE PROMOTER AND TERMINATOR REGIONS OF THE RBCL GENE FROM]

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Transformed bacteria were propagated and recombinant plasmids were extracted and sequenced. The resulted sequences were aligned and compared with the sequenced promoter and terminator of *rbcL* from cotton as the reference plant. The promoter region of *rbcL* gene from *G. perpensa* showed 81.5% identity with that of the cotton plant, while the terminator region of *rbcL* gene expressed 69.9% identity with that of cotton plant. The core sequences at -35 and -10 of the promoter from *G. perpensa* scored 54.44% of the default core sequences.



Cloning and sequencing of the promoter and terminator regions of the *rbcL* gene from *Gunnera perpensa* L.

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Abstract

Primers were designed based on the chloroplast genome sequence from the cotton *Gossypium barbadense* L. Specific restriction sites were included in the primers so as to facilitate the process of vector assembly for future chloroplast transformation. Polymerase chain reaction (PCR) was applied to amplify the promoter and terminator regions of the *rbcL* gene from the chloroplast DNA (cpDNA) of *G. perpensa*. These promoter and terminator regions of the *rbcL* gene were around 0.25 and 0.5 kilobase pair (kb) in length, respectively. The amplicons were purified and cloned into pGEM®-T Easy Vector System I, then transformed into DH5α strain of *E. coli* bacteria. Transformed bacteria were propagated and recombinant plasmids were extracted and sequenced. The resulted sequences were aligned and compared with the sequenced promoter and terminator of *rbcL* from cotton as the reference plant. The promoter region of *rbcL* gene from *G. perpensa* showed 81.5% identity with that of the cotton plant, while the terminator region of *rbcL* gene expressed 69.9% identity with that of cotton plant. The core sequences at -35 and -10 of the promoter from *G. perpensa* scored 54.44% of the default core sequences.

1. Introduction

Rubisco is ribulose-1,5-bisphosphate carboxylase/oxygenase and is the most abundant protein in the World. This protein catalyses and serves as the primary CO₂ assimilation enzyme during photosynthesis in all plants. This enzyme accumulates abundantly in the chloroplasts. Rubisco also initiate the process of photorespiration (Patel et al., 2008). Rubisco enzyme is a hexadecameric protein composed of eight large subunits arranged as four dimers chloroplast-encoded and eight small nuclear encoded subunits (Andersson, 2008). The *rbcL* and *rbcS* genes encode the large subunit (LSU) and small subunit (SSU), respectively (Spreitzer and Salvucci, 2002). The *rbcL* gene is located in the chloroplast genome, transcribed and translated on prokaryotic-like plastid ribosomes, while the *rbcS* gene occurs in the nuclear genome, transcribed and translated on cytoplasmic ribosomes as a precursor protein (Sasanuma, 2001). The precursor is imported into the chloroplasts due to a transit peptide and combined with LSU to form the functional enzyme (Rodermel et al., 1999).

The amount of Rubisco protein determines the potential activity of Rubisco enzyme. In turn, the amount of Rubisco protein is determined by the relative rate of biosynthesis and degradation. Biosynthesis and degradation of Rubisco are regulated by gene expression (Krapp et al., 1993) mRNA stability, polypeptide synthesis, post-translational modification, assembly of subunits into an active holoenzyme, and other various factors that affect protein degradation (Mehta et al., 1992; Eckardt and Pell, 1995; Desimone et al., 1996).

CA1P is the most known inhibitor of Rubisco and only found in the chloroplast formed by phosphorylation of 2-carboxy-D-arabinitol during low irradiance or darkness (Berry et al., 1987; Moore and Seemann, 1992).

Expression of Rubisco genes is mostly confined to leaves, occurs primarily in light, and is highly responsive to signals that affect growth and development of the plant. Expression of the *rbcL* and *rbcS* genes is highly regulated and influenced by a variety of intrinsic and external factors such as light (Shiina et al., 1998). Other factors include cell and tissue development (Wanner and Grussem, 1991; Furbank and Taylor, 1995; Patel et al., 2004; Majerana et al., 2005), photosynthetic metabolism (Acevedo-Hernandez et al., 2005), nutrients and hormones (Furbank and Taylor, 1995; Imai et al., 2005), as well as age (Hensel et al., 1993), senescence (Jiang et al., 1993), and diseases (Berger et al., 2004). Transcriptional and post-transcriptional levels mediate many aspects of *rbcL* and *rbcS* gene expression (Wanner and Grussem, 1991; Furbank and Taylor, 1995; Acevedo-Hernandez et al., 2005; Shiina et al., 1998; Patel et al., 2004, 2006).

Rubisco activase is an important enzyme for complete activation of Rubisco (Somerville et al., 1982; Salvucci et al. 1985). Rubisco protein turnover is considerable under stress conditions that induce oxidizing environment (Feller et al., 2008). Oxidative modifications of specific residues on Rubisco mark the enzyme for degradation under stress conditions (Mehta et al., 1992; Desimone et al., 1996; Ishida et al., 1999; Moreno et al., 2008). C172S substitution (Cysteine 172 Serine) of the large subunit in *Chlamydomonas reinhardtii* demonstrated more resistance to proteinase K

at low redox potential *in vitro* and delayed stress-induced degradation by hydrogen peroxide or mannitol *in vivo* as well as faster inactivation at elevated temperatures; 40 °C and 50 °C (Moreno and Spreitzer, 1999). Substitution of Cysteine 449 and Cysteine 459 by Serine resulted in increased Rubisco degradation and polymerization during salt stress (Marin-Navarro and Moreno, 2006). Oxidative stress causes reduced transcript levels for the small subunit of Rubisco which in turn affects Rubisco abundance (Glick et al., 1995). Rubisco expression is regulated by elements that also influence the regulation of other chloroplast and cytosolic enzymes. High-light stress produces active oxygen species and/or reduction in stromal signals that cause a transient translation arrest of Rubisco large subunit transcripts in *Chlamydomonas* (Irihimovitch and Shapira, 2000). Drought induces decreased transcript level of *rbcS* accompanied by increased expression of cytosolic glutamine synthetase (Bauer et al., 1997). There is a very close correlation between CO₂ assimilation and Rubisco activity over a range of temperatures from 28 °C to 45 °C (Law and Crafts-Brandner, 1999). Sensitivity of Rubisco activase against elevated temperatures inhibits Rubisco and photosynthesis. Photosynthesis acclimation to heat stress in cotton and maize might be due to post-transcriptional modulation of Rubisco activase gene expression (Vargas-Suarez et al., 2004; DeRidder et al., 2007).

Hence, high abundance of *rbcL* gene transcripts and Rubisco protein are attributed to the strong promoter and terminator of this gene. Therefore, the far sighted aim of this study was to exploit the promoter and terminator regions of *rbcL* gene to over express target gene(s) in the chloroplast. This work was done to isolate, clone and sequence the promoter and terminator regions of *rbcL* gene from *G. perpensa*. These regions will subsequently be assembled into species-specific chloroplast transformation vector for the *G. perpensa*.

2. Material and Methods

2.1. Plant Material

Fresh young flower petals were peeled from an inflorescence of *G. perpensa* that was collected from the Botanical Garden at the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

2.2. Reagents and Stock buffers

Buffers and reagents for genomic DNA isolation from young flower petals of *G. perpensa* are listed in Table 1.

Table 1 Buffers and solutions for genomic DNA isolation from young flower petals of *G. perpensa*

Buffer	Contents
CTAB Extraction	2% (w/v) CTAB; 20 mM EDTA, pH 8.0; 100 mM Tris-HCl, pH 8.0; 1.4 M NaCl.
Polyvinylpyrrolidone (PVP)	4% (w/v) was added to CTAB extraction buffer just before extraction.
β-mercaptoethanol (βME)	3% (v/v) was added to CTAB extraction buffer just before extraction.
CTAB/NaCl	10% (w/v) CTAB; 0.7 M NaCl.
CTAB precipitation	1% (w/v) CTAB; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA.
High salt TE	10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1.0 M NaCl.
TE	10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA, pH 8.0.
Chloroform: isoamyl alcohol	24:1, (v/v).
Iso-propanol	Absolute.
Ethanol	80% and 99.99%.
Liquid nitrogen	To quick freeze plant material.

2.3. DNA extraction

The following protocol was applied step by step for genomic DNA extraction from young flower petals of *G. perpensa* (Table 2).

2.4. DNA quantification

UV-Visible spectrophotometer (Varian, Australia) was used to measure the absorbance of isolated genomic DNA at A₂₆₀ and A₂₈₀ nm. While the purity of extracted

DNA was determined based on the ratio of A₂₆₀/A₂₈₀, the yield was measured according to the formula (DNA (μg) = A₂₆₀ x 50 x Dilution factor). A sample run on 0.8% agarose gel was utilized to have a visible test of quantity and quality of extracted genomic DNA. The gel was run in 1 x TAE (40mM Tris-acetate and 1mM EDTA) buffer for ~50 min and stained in 0.5 μg/ml Ethidium bromide solution. The isolated genomic DNA was compared with

1 kb GeneRulerTM DNA Ladder Mix (Fermentas, USA) as a DNA marker. The gel was visualized and photographed under a UV light source, UVitec Ltd, BTS-20 M model (Cambridge, UK).

2.5. PCR amplification

Primers for the well conserved promoter (PLf and PLr) and terminator (TLf1 and TLR1) regions of the chloroplast/chromoplast *rbcL* gene were designed based on the chloroplast genome sequence from the cotton *G. barbadense* (Ibrahim et al. 2006). During primers design, G+C contents, melting temperature, primer length, and primer-dimer formation were carefully considered (Table 3). These primers were employed in combination with templates from isolated genomic DNA for PCR amplification of the promoter and terminator regions of *rbcL* gene. Each PCR reaction mixture was 25 µl in 200 µl thin-walled tubes and contained ~50 ng of template genomic DNA, 0.625 U *Taq* DNA polymerase (Fermentas, USA), 4 mM MgCl₂, 0.4 mM of each dNTP,

Table 2 Protocol steps for genomic DNA isolation from young flower petals of *G. perpensa*

Step No.	Details
1	0.02 g of PVP were weighed in sterile 1.5 ml Eppendorf tube, 500 µl of CTAB extraction buffer were added and incubated in a water bath at 65 °C. The solution was occasionally mixed by inverting the tube to dissolve PVP.
2	Young flower petal tissues were ground to a fine powder under liquid nitrogen in pre-chilled mortar and pestle.
3	Powder of young flower petal tissues was weighed in sterile pre-chilled 1.5 ml tubes (100, 200, 300 and 400 mg) and kept in liquid nitrogen until next step.
4	15 µl of βME were added to the hot mix of CTAB extraction buffer and PVP, mixed well and the tube was returned to the water bath at 65 °C.
5	The hot mix of CTAB extraction buffer, PVP and βME was added to the frozen powder, mixed well and incubated at 65 °C for 30 min with occasional mixing to avoid aggregation of homogenate.
6	One volume of chloroform:iso-amyle alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min.
7	The upper phase was carefully transferred to a new sterile 1.5 ml tube. The volume of supernatant was determined.
8	One tenth volume of CTAB/NaCl at 65 °C was added and mixed gently.
9	One volume of chloroform:iso-amyle alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min.
10	The upper phase was carefully transferred to a new sterile 1.5 ml tube and the volume was determined.
11	One volume of CTAB precipitation solution was added, mixed gently and stored at -20 °C for 20 min.
12	The extracted DNA was fished out using micropipette into a new tube or precipitated by centrifugation at 14,000 rpm for 5 min.
13	The pellet was carefully recovered and dissolved in 300 µl of high salt TE buffer.
14	200 µl of ice-cold Iso-propanol were added, mixed and followed by centrifugation at 14,000 rpm for 10 min
15	The solution was carefully decanted and pellet was recovered.
16	The pellet was washed in 80% ice-cold ethanol, spun down and carefully recovered.
17	A second wash of pellet was carried out with 99.99% ice-cold ethanol, spun down and carefully recovered.
18	To dry up the DNA pellets, tubes with their lids open were inverted on sterile tissue paper for 10 min, then DNA pellets were dissolved in 50 µl TE buffer for each.

and 0.2 µM of each forward and reverse primer. PCR reactions were run in a Corbett Research PC 960 Thermal Cycler (Corbett Research, Mortlake, Sydney, Australia) and subjected to the following PCR protocol:

one initial denaturizing cycle at 94 °C for 2 min, initial annealing step at 50 °C for 30 s, and elongation step at 72 °C for 30 s, which was followed by 35 cycles at 94 °C for 1 min, 50 °C for 30 s, 72 °C for 30 s, then followed by a final extension step at 72 °C for 5 min. Amplified PCR products were analyzed using electrophoresis in agarose gel (1.5%), stained with Ethidium bromide, visualized and photographed under UV

Table 3 Primers used in this study, their sequences, lengths and embedded restriction enzymes (underlined)

Name	Sequence	Length (bp)	Restriction Enzyme
PLf	5'- GATCAGGTTGCCATACATAT <u>G</u> -3'	23	(+NdeI)
PLr	5'- CTTAACACA <u>AAG</u> CTTGAATCCAACAC -3'	27	(+HindIII)
TLf1	5'- GAG <u>G</u> TAGCAAATGGAGTCCTGAAC -3'	25	(+NheI)
TLr1	5'- CAC <u>GTC</u> GACAAAAGGACAACATACAGG -3'	27	(+SalI/HincII)

2.6. DNA cloning

The Wizard® SV Gel and PCR Clean-Up System (Promega, USA) was used to purify PCR products. Then, these purified PCR products were cloned into pGEM®-T Easy Vector System I (Promega, USA). DH5 α strain of *E.coli* competent cells were the hosts for recombinant plasmids. Boiling lyses mini-preparations of white colonies in STET buffer (8% Sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris pH 8.0) and Lysozyme (20mg/ml stock) followed by restriction digestions with *Eco*RI (NEB, England) were the method that was followed to confirm successful transformation of bacteria. PureYield™ Plasmid Miniprep System (Promega, USA) was employed to extract recombinant plasmids from colonies that were successfully transformed. Extracted plasmids were the templates for the modified dideoxy chain termination method of DNA sequencing. ABI 373 DNA sequencer (Applied Biosystems, USA) was the instrument used to call the sequence of bases.

2.7. Data analysis

The obtained sequences were analyzed using Genetyx software (Genetyx, Tokyo, Japan) and the (Blast) Basic Local Alignment Search Tool (Altschul et al. 1990) feature on the website of the National Center for Biotechnology Information.

3. Results

3.1. DNA extraction

Gel electrophoresis test showed a high molecular weight of well intact genomic DNA (Fig. 1). This could be attributed to the naturally soft flower petal tissues compared to photosynthetic leaves or succulent tissues. It was found much easier to crush, grind and lyses young petals than leaves or succulent tissues. It was also found that the quantity of extracted genomic DNA is proportional to the amount of petal tissues from 100 to

400 mg (Ibrahim, 2011; this study). It was reported that genomic DNA extracted from photosynthetic leaves decreases with the increase of leaf tissue amount per extraction buffer unit (Moyo et al., 2008).

3.2. PCR amplification

Successful PCR amplification of the promoter and terminator segments of chloroplast/chromoplast *rbcL* gene from *G. perpensa* indicated that organelles' genomic DNA was co-extracted with nuclear genomic DNA and of high quantity and quality as well as amplifiable (Fig. 2). Despite a wide difference, as being from far related families, between *G. barbadense* as the reference for primers design and *G. perpensa* as the source of template DNA, the promoters and terminators of plastid *rbcL* gene expressed similar lengths in both

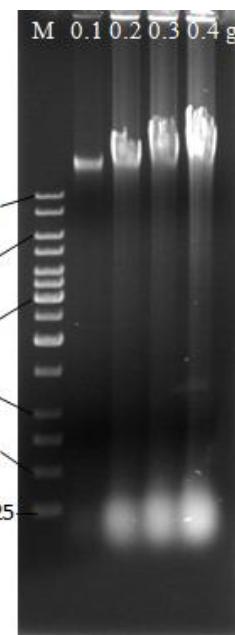


Fig. 1. Gel photo shows DNA extracted from 100, 200, 300 and 400 mg of flower petal tissues from *G. perpensa*. (M) refers to 1 kb GeneRuler™ DNA Ladder Mix (Fermentas, USA).

plants. This could be due to the well conserved nature of plastid genomes and genes (Shimada and Sugiura, 1991).

3.3. DNA cloning

The promoter and terminator regions of the chloroplast/chromoplast *rbcL* gene were successfully cloned into pGEM®T Easy plasmids and successfully transformed into the host DH5α competent bacteria. Boiling lyses mini-preparations of the plasmids from transformed bacteria and digestion with *Eco*RI showed that the correct promoter and terminator segments were recombined into the plasmids (Fig. 3). These plasmids were extracted, purified (Fig. 4) and were successfully used as templates for the modified dideoxy chain reactions and sequences of the inserted segments were obtained (Fig. 5). The promoter region was of 285 bp, while the terminator region was of 439 bp, compared with 261 bp and 454 bp for the promoter and terminator of cotton *G. barbadense*, respectively.

3.4. Data analysis

The resultant sequences of the promoter and terminator of the *G. perpensa* were compared with the reference sequence of the chloroplast *rbcL* gene from cotton *G. barbadense* (Fig. 6). The promoter region of *rbcL* gene from *G. perpensa* showed 81.5% identity compared with that of the cotton plant, though it was slightly longer by 24 bp, while the terminator region of *rbcL* gene expressed 69.9% identity compared with that of cotton plant and showed a shorter length by 15 bp. The core sequences at -35 and -10 of the promoter from *G. perpensa* scored 54.44% identity of the default core sequences.

4. Discussion

Flower petal tissues were used in this study due to the results of a previous report that proved extraction of genomic DNA from flower petals is much easier and

genomic DNA is more pure than that extracted from photosynthetic tissues such as leaves (Ibrahim 2011). Pašakinskienė and Paplauskienė (1999) reported that the proportion of DNA to other contents in a cell is very low in highly differentiated plant leaves, while rapid cell division and extensive DNA synthesis in floral meristems increased the proportion of extracted genomic DNA. It was also reported that medicinal plants, crop plants, fruit trees, ornamental plants and desert shrubs contain high amount of secondary metabolites, polysaccharides and polyphenolics (Khanuja et al. 1999; Aljanabi et al. 1999; Pirttilä et al. 2001; Cheng et al. 2003; Horne et al. 2004; Hameed et al. 2004; Jabbarzadeh et al. 2009). High contents of polyphenolics and polysaccharides in plant leaves are problematic and complicate the process of isolation of high-quality/-quantity intact genomic nucleic acids (Michiels et al. 2003; Puchooa et al. 2004; Karaca et al. 2005; Angeles et al. 2005). Secondary metabolites, polyphenolics and polysaccharides co-extracted with genomic nucleic acids cause contamination and interfere in downstream enzymatic applications such as PCR and restriction digestion (Porebski et al. 1997).

Chloroplast genomes as well as other organelle genomes evolve slower than nuclear genomes (Palmer 1987). This conservation phenomenon of organelle genomes was exploited to design universal primers that were capable of amplifying polymorphic non-coding regions of cpDNA of some algae, bryophytes, pteridophytes, gymnosperms and angiosperms (Taberlet et al. 1991), non-coding regions of mitochondrial and cpDNA (Demessure et al. 1995), coding regions of cpDNA (Badenes and Parfitt 1995; Tsumura et al. 1996) as well as whole chloroplast genomes (Dhingra and Folta 2005; Ibrahim et al. 2007). A database for some of these primers was created by Heinze (2007).

Based on comparative studies, the chloroplast genomes of higher plants are highly conserved in size, general structure, gene content, gene order and DNA sequence (Palmer 1985; 1991; Shimada and Sugiura, 1991).

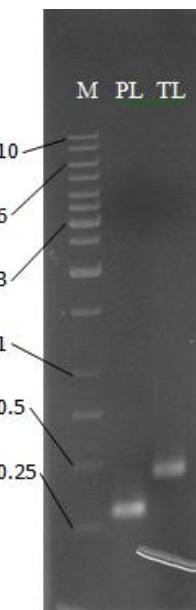


Fig. 2 Gel photo shows PCR amplified cpDNA for the promoter and terminator regions of *rbcL* gene from *G. perpensa*. (M) refers to 1 kb GeneRuler™ DNA Ladder Mix (Fermentas, USA).

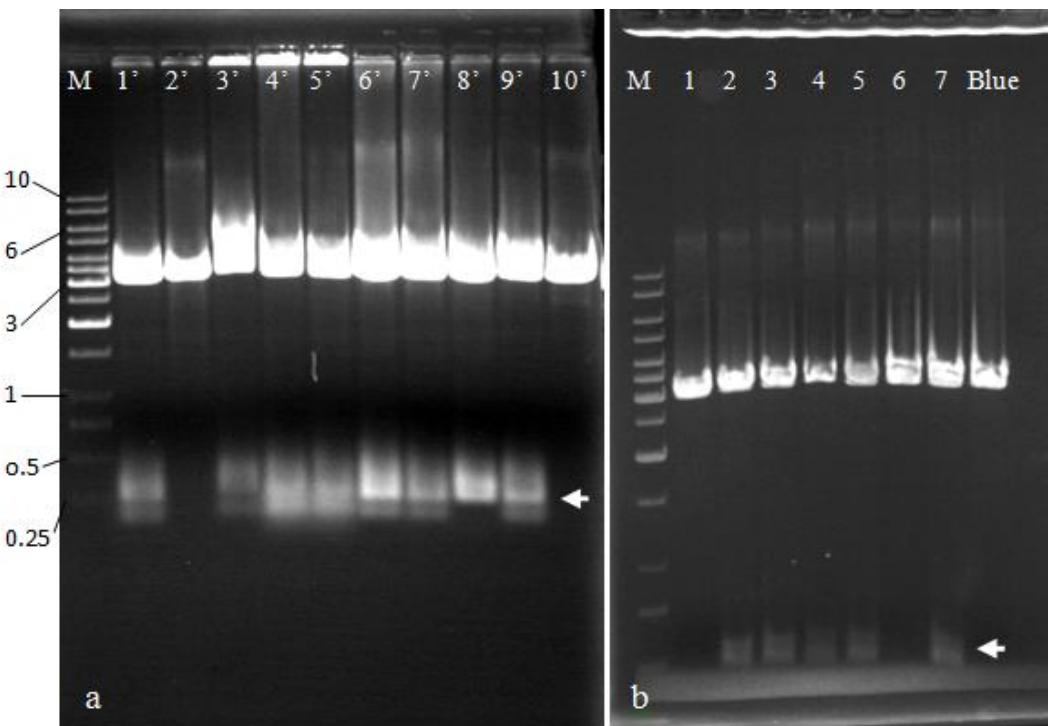


Fig. 3 Boiling lyses mini-preparations of transformed colonies that contained the recombinant plasmids and digested with *Eco*RI. The correct promoter (a) and terminator (b) regions of *rbcL* gene from *G. perpensa* that were excised from recombinant plasmids are indicated by arrows. (M) refers to 1 kb GeneRuler™ DNA Ladder Mix (Fermentas, USA).

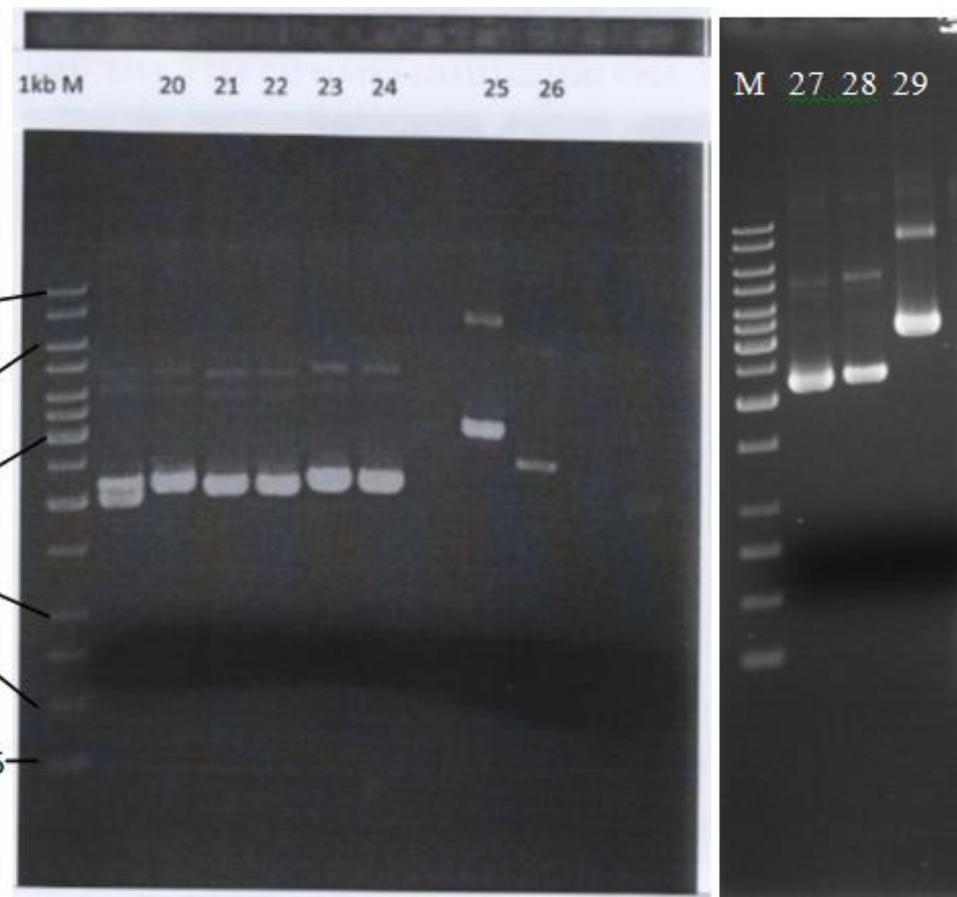


Fig. 4 Extracted and purified recombinant plasmids that contain inserts of promoter and terminator regions of *rbcL* gene from *G. perpensa* ready for sequencing.

(a)

TGATCAG GTT GCGCCATACA TATGAAAGAG TATACAATAA TAATAATGAT GTGTTGGTA 60
 AATCAAATAC CATGGTCTAA TAATCAATCT GATTAGTTGA TAATATTAGT ATTAGTTGGG 120
 AAGTTTTGA AAGATTCCTG TGAATAGTTT CATTAAACGCC TAATTCAATTG GTGTCGAGTA 180
 GACCTTGTG TTGTCAGAAT TCTTAATTCA TGAGTTGTAG GGAGGGATT ATGTCACCCAC 240
 AACACAGAGAC TAAAGCTTGT GTTGGATTCA AAGCTTGTGT TAAAG 285

(b)

TGAGGCTAGC AAATGGAGTC CTGAACTAGC TGCCGCTTGT GAAGTATGGA AAGAGATCAG 60
 ATTTGAGTTT AAAGCGGTGG ATACTTTGGA TAAATAATAA AGAGGATCAA CAGCCAGATA 120
 ACAAAATAATT ACTCTCCGTT TTCTTAATTG AATTGCACTC GGCTCAATCT TTTAGTAAA 180
 AGGATTGGGC CAGATTCTAT TGCATGTATT TCGCCAAAAT ACATATATCT CTAGATATAG 240
 AATAGAAGAT TTGAAGAATA GAAGATTGA AATAGAAAAT CGAATACTCA AATGTTCTA 300
 TTGTTGTCTT GGATCCACAA TTAAACCGAC GGATGCTCTG GATTGGTATA TTCTTTATAG 360
 ATCCTGTGTT TTCCCTGAAT CAAGCGAAGT ATCACAAATC TTTAACCCA TCCTGTATGT 420
 TGTCCCTTTG TCGACGTGA 439

Fig. 5 The sequences of the promoter (a) and terminator (b) regions of *rbcL* gene from the cpDNA of *G. perpensa*. Restriction enzymes *Nde*I, *Hind*III, *Nhe*I, and *Sal*I/*Hinc*II are sequentially in double-lined rectangles. Possible ribosome-binding site is underlined. Possible translation start codon of *rbcL* gene is in rectangle in the promoter region (ATG), while the termination codon (TAA) is in rectangle in the terminator region.

Ibrahim et al. (2007) used these conserved genes to design international primers that are suitable for the amplification of the whole circular cpDNA from different species of cultivated cotton. In this study a set of primers were designed based on the same phenomenon of conserved chloroplast/chromoplast genes among different species of higher plants and they were used to execute an experiment that included successful genomic DNA extraction from young flower petals, PCR amplification of the promoter and terminator regions of *rbcL* gene from the cpDNA of *G. perpensa*, and the PCR amplicons were purified. Then the purified amplicons were cloned into a suitable vector, transformed into a suitable bacterial host, extracted again and finally sequenced. The results proved further that the cpDNA of *G. perpensa* is not an exception of the well conserved cpDNA among divert species of plants.

The amplified, cloned and sequenced promoter and terminator regions of *rbcL* gene of the cpDNA from *G. perpensa* will be used to assembly a species-specific chloroplast transformation vector and subsequently genetic manipulation of the chloroplast genome (Kumar et al. 2004a; 2004b). The final goal of this series of experiments is to transform the chloroplast genome of *G. perpensa* for the production of pharmaceutical and industrial proteins as well as insertion of genes for abiotic and biotic stress.

Acknowledgments

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