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Isolating *rbcL* gene and promoter of bell pepper (*Capsicum annuum* L.) and its sequence analysis using bioinformatic tools

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Abstract

Ru-BisCO (ribulose 1,5-bisphosphate carboxlyase) probobly is the most protein complex on the planet. Also it is the key enzyme in photosynthesis reactions in chloroplastic stroma to fix CO_2. It is usually consisted of eight small and eight large subunits encoded by nucleus and plastids respectively. In this research, we have cloned *rbcL* gene and promoter from a common sweet bell pepper by polymerase chain reaction using total cellular DNA. Our results showed that length of coding sequences of *rbcL* gene in pepper is 1434 bp with 478 deduced amino acid residues. The insilico analysis of promoter region showed that -10 and -35 regions contain TACAAT and TTGCGC boxes respectively. Further analysis of cloned *rbcL* promoter form this kind of non spicy pepper elaborate that this promoter comprised motifs such as CAAT-box, HSE and circadian.

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Introduction

One of the main features that distinguishes a plant cell from animal is the possession of plastids. Several types of plastids are existed in the various eukaryotic algae and plant tissues and organs, namely the chloroplast which contains the whole photosynthetic machinery system, the amyloplast which accumulates starch in storing organs, the chromoplast, which contains the attractive colors of fruits and flowers, and the etioplast, which is a dedifferentiated chloroplast found in dark-grown plants (Kirk and Tilney-Basset, 1978). Chloroplasts are the primary source of the world's food production which sustain life on this planet. Other important reactions that take place in plastids is oxygen evolution, carbon fixation, production of starch, synthesis of amino acids and fatty acids too (Verma and Daniel, 2007).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBis- Co) is the enzyme that facilitates the primary CO_2 fixation step in photosynthesis. The quaternary structure of the enzyme consists of eight large and eight small subunits (Palmer, 1985). Synthesis and assembly of functional Rubisco in plants and green algae require communication between organelles, because S subunits are encoded by the nuclear genome and synthesized in the cytosol, whereas large subunits are encoded by the chloroplast genome and synthesized on chloroplast ribosomes. Control of the expression of genes for Rubisco occurs both transcriptionally and post-transcriptionally, but apparently differs in the nucleus as compared to the chloroplast (Tabita, 1988). In algae, including Rhodophyta (Valentin and Zetsche, 1989; Kostrzewa *et al.,* 1990), both subunits are encoded by plastid genes.

Chloroplasts in higher plants possess their own genome composed of a circular, single, double-stranded DNA. In Contrast to cyanobacteria, although the plastid genome of higher plants is reduced in size (120 to 220 kb), but the existing genomic sequences still show some similarities. The chloroplast genomes in land plants typically contains 110 to 120 unique genes, whereas cyanobacteria contain more than 1500 genes. Emphasizing the point that many of missing genes were transferred into the nuclear genome of the host (Martin *et al*., 2002). In higher plants, rbcL gene is located in the plastid genome at long single copy (LSC) region. Since there is no report on rbcL gene sequence in bell pepper, this research was conducted and concentrated on isolation and in silico characterization of rbcL gene and promoter from a quite common sweet pepper.

Materials and methods

Materials

Bell papper (*Capsicum annuum* L. cv California Wonder) seeds were planted in plastic trays, and let to grow to 2-3 leaf stage before extracting their total cellular genomic DNA. In this study, the *E. coli* strain of DH5α was used for cloning purpose. The used restriction/cutting enzymes and *pfu* DNA Polymerase were purchased from SinaClon and Thermo corporation, respectively. Plasmid DNA extraction kits were provided from Bioneer Corporation (South Korea). The sequencing of the gene and promoter was carried out by Bioneer Co too.

DNA extraction

To prepare high quality DNA plants were incubated in a dark place for 24 hours for the full breakdown of cellular starch content. Total DNA was extracted from leaf sample at 2-3 leaves stage using CTAB (Saghaei *et al.*, 1984) method. Quality, quantity and concentration of the extracted DNA were evaluated by 0.8% agarose gel electrophoresis using DNA Weight Marker (SinaClon).

Designing the primers

The nucleotide sequence of the chloroplast genome of pepper with the accession number of NC_018552 was downloaded and used for designing the specific primers of F:5'-AAAAGCTT ACCACTGTCAAGGGGGAAGT-3´ and R:5'-AACTGCAGGGAACGGAACAAAGGGGA CA- 3' by the online software of Primer-Blast. The cleavage sites of *Hind*III and *Pst*I were embedded in the 5′ end of the forward and reverse primers to ensure the cloning procedure.

PCR amplification and Bacterial Transformation

Total Genomic DNA was used as a template for amplification of target fragment in the concentration of 5 ng/µl. The PCR program consisted of an initial denaturing at 94 °C for 5 min, continued by 35 cycles of 94°C for 60s, 60.4°C for 30s and 72°C for 60s, with a final extension step at 72°C for 2 min. The quality and quantity of PCR amplificant were evaluated by electrophoresis on 0.8% agarose gel using weight marker DNA. Target amplificant was eluted using extraction kit and used at concentration of 38 ng/µl in ligation reaction with pTG19-T vector at 4°C for 24 hrs.

E.coli Competent bacterial cells were prepared using TSS protocol and transformation was done by 5 µl of ligation reaction using heat shock procedure. pTG19-T plasmid contains a ampicillin resistance gene, so the plasmid-free bacteria do not live on medium containing ampicillin antibiotic in contrast to transformants. The resultant white colonies on media containing x-gal and IPTG were farther confirmed by direct colony PCR technique before inoculation of liquid bacterial culture. The plasmid DNA was extracted from the liquid culture of PCR positive colonies using plasmid extraction kit.

Finally, the recombinant plasmids DNA were reconfirmed with *Eco*RI digestion and was sent for sequencing to Bioneer Co, South Korea.

Results and discussion

Cloning of rbcL gene

To amplify the *rbcL* gene region with designed specific primers, the lengths and nucleotide composition as well as Tm of the primers were considered to ensure the efficiency of the PCR. The length of the predestinated fragment was 2139 nts, which resulted as such and was shown in Figure 1. The fragments were ligated to the cloning vector of pTG19-T illustrated in Figure 2. Inserted fragment in the vector pTG19-T was further corroborated by PCR and digestion by restriction enzyme of *Eco*RI (Fig 3).

Table 1. The results of pair Blast of Bell papper (*Capsicum annuum)* with some other species.

	PrbcL	SrbcL	TrbcL	NrbcL	GrbcL	BrbcL
CrbcL	89	98	99	98	91	90
PrbcL		89	89	90	92	90
SrbcL			99	98	91	90
TrbcL				99	91	90
NrbcL					91	90
GrbcL						91

CrbcL (*Capsicum annuum*), NC_018552; PrbcL (*Pisum sativum*), NC_014057.1; SrbcL (*Solanum tuberosum);* NC_008096.2; TrbcL (Tomato: *Solanum lycopersicum*), NC_007898.3 NC_007898.3; NrbcL (*Nicotiana tobaccum);* NC_ 001879.2; GrbcL (*Glycine max*), NC_007942.1; BrbcL (*Brasica napus*), NC_016734.1.

Sequence analysis of rbcL promoter

The size of the pepper's plastid genome is 156,781 bps which is the largest among known Solanaceous plastomes. The quadripartite structure includes 87,366 bps of LSC and 25,783 bps of SSC that are separated by a pair of 17,849 bps of IR copies. According to chloroplast gene mapping, rbcL gene is placed at the large single copy (Jo *et al.*, 2011). Using the BLAST online software, the rbcL coding sequence of pepper was compared with some other plants that is shown in Table 1 with highest similarity with Tomato's rbcL (99%), and followed by 98% with potato and Nicotiana.

The promoters of chloroplast genes are typically composed of two hexamer sequences, ctpl and ctp2, separated on average by 16-18 nucleotides and resembling the -35, -10 prokaryotic core promoter (Handley-Bowdoin and Chua, 1987). The ctp1-ctp2 sequence is TTGCGC-18nts-TACAAT (Fig 4).

The promoter region of the cloned sequence were analyzed using plantcare bioinformatic software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and regulatory elements as well as conserved motifs in promoter region were identified (Table 2).

Table 2. Regulatory elements in rbcL promoter sequence of pepper.

Site	Positin	Strad	Sequence	Function
Box 4	159	+	ATTAAT	Part of a conserved DNA module involved in light responsiveness
Box I	44	+	TTTCAAA	Light responsive element
CAAT-box	70	-	CCCAATTT	Common cis-acting element in promoter and enhancer regions
	127	+	CAAAT	
	116	-	CAAAT	
	163	-	CAAT	
	72	-	CCAAT	
	162	-	CAATT	
	122	+	CAAAT	
	71	-	CAATT	
	104	+	CAAT	
ERE	43	+	ATTTCAAA	Ethylene-responsive element
HSE	186	-	AAAAAATTTC	Cis-acting element involved in heat stress responsiveness
Sp1	283	-	CC(G/A)CCC	Light responsive element
TATA-box	16	-	TAATA	Core promoter element around -30 of transcription start
	100	+	TATA	
	85	+	ATATAT	
	177	-	TAATA	
	67	-	TTTTA	
	168	+	TAATA	
	87	+	ATATAT	
	14	+	TAATA	
	60	-	TTTTA	
	171	-	TAATA	
	86	+	TATA	
	84	+	TATATATA	
	88	+	TATA	
Unnamed__4	284	-	CTCC	
as-2-box	105	+	GATAatGATG	Involved in shoot-specific expression and light responsiveness
circadian	111	-	CAANNNNATC	Cis-acting regulatory element involved in circadian control

CrbcL (*Capsicum annuum*), NC_018552; PrbcL (*Pisum sativum*), NC_014057.1; SrbcL (*Solanum tuberosum);* NC_008096.2; TrbcL (Tomato: *Solanum lycopersicum*), NC_007898.3 NC_007898.3; NrbcL (*Nicotiana tobaccum);* NC_ 001879.2; GrbcL (*Glycine max*), NC_007942.1; BrbcL (*Brasica napus*), NC_016734.1.

CAAT box plays an important role in determining the efficiency of promoter (Lewin, 2009) and was found in *Brassica rapa*, *A. thaliana*, *Glycine max*, *Petunia hybrid* and *Hordeum vulgare* too (Shirsat *et al.*, 1989).

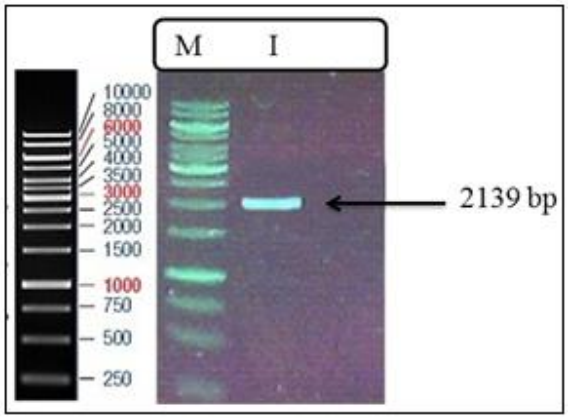

Fig. 1. After PCR, products run on an agarose gel. M) DNA marker, I) Amplified fragments.

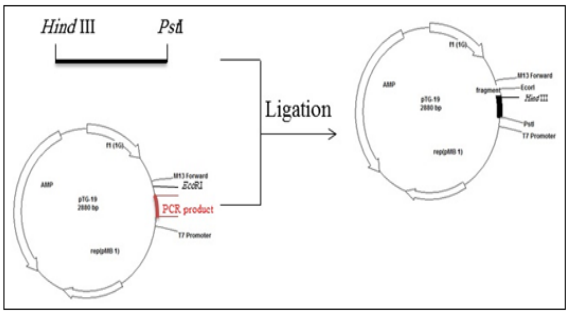

Fig. 2. Ligation reaction. using heat shock method. The pTG19-T is a linear-type vector which takes a circular form after ligation.

According to data for in vitro interactions of a tomato (*Lycopersicone sculentum*) HSF with the apx1 promoter and mutational analysis, the HSE is responsible for the heat-shock induction of the gene and partially contributes to the induction by oxidative stress (Storozhenko *et al.,* 1998). The first-identified and best known core promoter element is the TATA box, which was discovered in the course of sequencing the histone genes in *Drosophila* (Goldberg, 1979). TATA box alone can confer core promoter activity. A TATA box sequence has been found in almost all plant genes (Mesing *et al.,* 1983). In eukaryotic promoters, between 10 and 20% of all genes (Gershenzon and Ioshikhes, 2005) contain a TATA box (sequence TATAAA), which provides for a TATA binding protein and assists the formation of the RNA polymerase transcriptional complex (Smale and Kadonaga, 2003). The TATA box typically lies very close to the transcription initiation site (often within 50 bases), and tends to be surrounded by GC rich sequences.

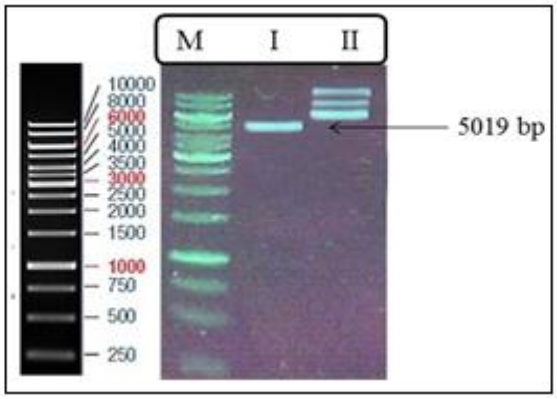

Fig. 3. Gel agarose description of digested fragments. The extracted plasmid were digested with enzyme *Eco*RI. Because one restriction sites are present, only a linear fragment is produced. M) DNA marker, I)cut, II) uncut.

Elements putatively involved in light mediated regulation are pictured as Box 4 (ATTAAT), (Sarvestani *et al.*, 2014).

Biological clocks have been demonstrated to regulate gene expression and to coordinate metabolic and physiological reactions in several eukaryotes as well as in some prokaryotes too (Harmer *et al.*, 2000; Schaffer *et al.*, 2001). Circadian expression of a gene encoding chlorophyll a/b-binding protein (CAB) was widely observed in dicotyledonous and monocotyledonous plants (Meyer *et al.*, 1989). Modulation of gene expression has been typically regarded as a key event in the establishment of circadian rhythmicity. After all, many clock genes (CG) and clock controlled genes (CCG) display robust oscillations in steady-state mRNA levels (Takahashi, 1995). These observations led naturally to the concept of a circadian cis acting regulatory element, originally coined “circadian clock-responsive element” [CCRE; (Takahashi, 1995), or “time-box” (Ishida, 1995)]. Among these motives, the as-2 box is involved in shoot-specific expression and light responsiveness (Diaz-De-Leon *et al.*, 1993).

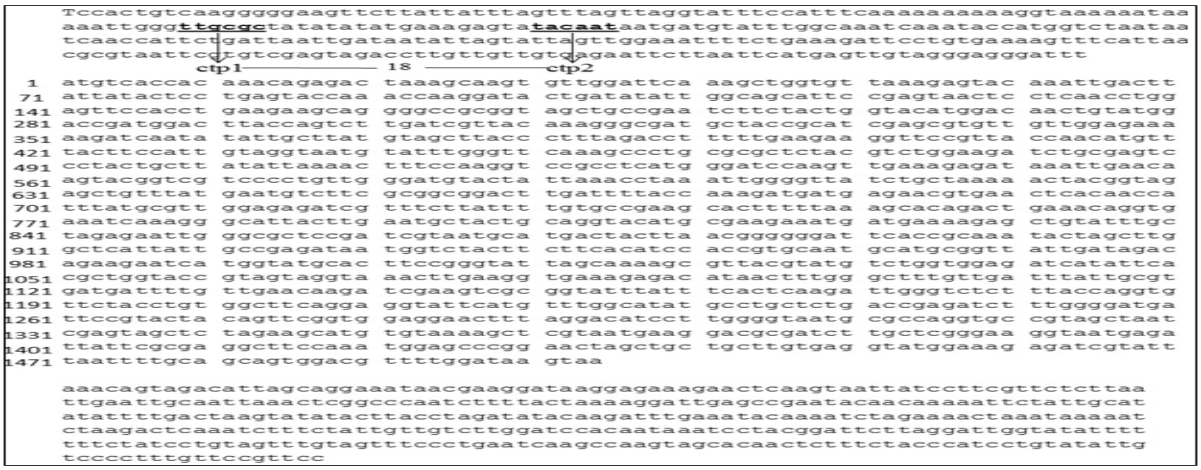

Fig. 4. Cloned rbcL sequences shows -10 and -35 sequences in agreement of prokaryotic motives of TACAAT and TTGCGC.

Conclusion

The aim of this research was to clone and characterize *rbcL* gene of bell pepper plastid by specific primers. Amplified fragment was ligated and cloned into pTG19-T vector, then was transformed to *E. coli* . Enzyme *Eco*RI was used to perform plasmid Digestion. Finally, admission of truth, the cloned fragments were sent for sequencing. The obtained results were compared with the sequences in NCBI. The promoter region was analyzed and found that the motifs like CAAT-box, TATA-box, HSE and Box 4 is present in bell pepper plastome.

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