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Structural Analysis and Transcriptional Regulation of the Chloroplast *psbC* Gene from *Panax ginseng*

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The *psb*C gene, encoding the intrinsic chlorophyll-binding protein of CP43, one of the PS core complex polypeptides, was cloned from the *Panax ginseng* chloroplast, which is composed of 1,422 nucleotides and the overall nucleotide sequence shows more than 84% identity to those of eukaryotic photosynthetic organisms. The predicted topology of CP43, based on hydropathy analysis, includes six membrane-spanning α-helices resulting in three lumenal and four stromal loops. The putative translation start codon for the *psb*C gene is located at 48 nucleotides upstream from the stop codon of the *psb*D gene whose product is also a component of the PSII reaction center, implying that the promoter of the *psb*C gene is possibly located in the middle of the structural gene of the *psb*D gene. Northern blot analysis of the *in vivo* accumulation of the *psb*C transcript from the plants grown under the various growth light intensities (5%, 10%, 20%, and 100%) of daylight indicated that the steady-state level of the *psb*C transcript was not significantly affected by light intensity.

key words: photosystem II, *Panax ginseng*, *psb*C, transcription, light intensity

INTRODUCTION

Photosystem (PS) is a multisubunit complex consisting of more than 30 polypeptides in the thylakoid membranes of higher plants chloroplast. It uses light energy to catalyze a series of electron transfer reactions resulting in the water oxidation reaction of photosynthesis. The intrinsic polypeptides that are absolutely required for oxygen evolution are D1, D2, α- and β-subunits of cytochrome b_{559}, CP47, and CP43 [1]. The two chlorophyll-binding proteins of PSII, CP43 and CP47, are closely associated with the PSII reaction center [2] and form antennas within the PSII core complex [3]. One of the functions of CP43 and CP47 is thought to be the transfer of excitation energy to the PSII reaction center from chlorophyll a/b binding proteins in higher plants and green algae, or from phycobilisomes in cyanobacteria and red algae [4-7]. In addition, mutagenesis studies also suggested that CP43 may be partially involved in providing an environment for the Mn cluster [8, 9].

The evidence from light-induced cross-linking studies indicated that D1 and CP43 polypeptides are interacting dynamically at the lumenal side of PSII [10]. And under the condition of photoinhibition of PSII, not only were the PSII reaction center-forming D1 and D2 polypeptides degraded but also CP43 was also degraded [11]. However, the degradation products of CP47 were not detected under the same conditions. Both the degradation and the cross-linking of D1 and CP43 were prevented by the addition of electron donors to PSII, suggesting that these processes were caused by the donor-side mechanism of photoinhibition [11]. The D1 polypeptide is known to have a rapid turnover rate even in moderate light [12] and the turnover rate increases as light intensity increases [13].

Light plays a pivotal role in regulating the synthesis, accumulation, stoichiometry, and turnover of the protein components of PSII [14]. The chloroplast gene expression is strongly regulated by light in higher plants as well as other unicellular organisms such as *Chlamydomonas reinhardtii* and cyanobacteria. Light can modulate the overall plastid transcriptional activity and differentially stimulate the transcription of the PSII genes including the *psb*C gene. Therefore, the maintenance of CP43 in PSII under various intensities of light requires the transcriptional and translational regulation of the *psb*C gene [15].

Panax ginseng C. A. Meyer, a perennial herb in the family *Araliaceae*, is a shade plant so that high light intensity could be a limiting factor that affects the rate of growth. The most extensive studies were performed with regard to the effect of high light intensity on the light reaction of photosynthesis. The photosynthetic activity decreases [16] and the composition of chlorophyll-binding protein complexes is changed when light intensity is higher than 2,000 $\mu mol/m^2 \cdot sec$ [17]. Only a few studies have been, however, reported in the shade plants about the effects of high light intensity on the transcriptional regulations of the essential polypeptides of the PSII reaction center [16].

In the present study, the *psb*C gene of PSII was cloned from the chloroplast of *P. ginseng* and its primary structures and deduced amino acid sequences were analyzed. In addition, the effects of various intensities of daylight on transcriptional

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activity of the *psb*C gene were investigated. This would be helpful in part to understand the protection mechanism of the PSII from photoinhibition.

MATERIALS AND METHODS

Isolation of Chloroplast Genomic DNA

Plants of *P. ginseng* was grown under various intensities (5 %, 10%, 20% and 100%) of daylight (approximately 1,000 $\mu mol/m^2 \cdot sec$) with a 12hr-photoperiod at the field. The mature leaves from two-year old plants were collected, frozen in liquid nitrogen, and stored at -70°C for further preparations. The frozen leaves were ground in a mortar with liquid nitrogen and the powder was suspended in 100 mL of the extraction buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.35M sorbitol, 0.1% BSA, 0.1% β-mercaptoethanol, 10% PEG 4000) for every 10g of leaves. The chloroplast genomic DNA was isolated from this suspension based on the method described by Lee and Tae [18].

Cloning and Sequence Analysis of the psbC Gene

A polymerase chain reaction (PCR) was performed with the chloroplast genomic DNA as a template. The two different primers were used as follows: the forward primer (5'-CGAC TAGTTCCGGGTTCG-3') and the reverse primer (5'-GGC GAACGACGGGAATTG-3'). Thirty-five cycles of PCR (each of 1 min at 95°C for denaturation followed by 1.5 min at 55°C for annealing and 2 min at 72°C for the chain elongation) were performed in a reaction medium including 0.2 mM dNTP, 1mM the forward primer, 10mM the reverse primer, and 2 units of *Taq* DNA polymerase (Promega, Madison, USA). The PCR products were analyzed on 0.8% agarose gel. The DNA of approximately 1.8 kb in size was extracted with a Geneclean kit (BIO 101, CA, USA) and ligated to pGEM-T Easy vector (Promega, Madison, USA) with T4 DNA ligase (Promega, Madison, USA) in the presence of 10mM DTT, 30 mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, and 1mM ATP. The ligation mixture was then transformed into *Escherichia coli* strain JM109. A plasmid with an insert was extracted from a white colony grown in the presence of X-gal and the size of an insert was analyzed by the restriction digestion reactions. The DNA sequencing of the *psb*C gene in pGEM-T Easy vector was performed with the automated DNA sequence analyzer (LI-COR Biotechnology, Model long read IR 4200) and the DNA sequence data obtained was blasted to National Center for Biotechnology Information (NCBI) to estimate the degree of identity to the *psb*C gene from dicots, monocots, or a liverwort. The amino acid sequence alignment was performed with the shareware program, SeqPup.

Preparation and Labeling of the psbC Gene-Specific Probe

To prepare the *psb*C gene-specific DNA probe, a DNA fragment of the *psb*C gene was amplified by PCR with two different primers: the forward primer 5'-CCTGTTTCAGGG TCTCTAC-3', and the reverse primer 5'-GGATGTTGTGTC AGCCTGG-3'. The PCR product was subcloned into pGEM-T Easy vector. The insert was cleaved with *Eco*RI and separated on 0.8% agarose gel followed by labeling with a Digoxigenin (DIG) labeling and detection system (Boehringer Mannheim GmbH, Mannheim, Germany). One microgram of an insert was boiled for 10 min and placed on ice. Twenty microliters of the reaction mixture containing 50mM Tris-HCl, pH 7.2, 10mM $MgCl_2$, 0.1 mM dithioerythreitol, 0.2 mg/mL BSA, 0.1mM dATP, 0.1 mM dCTP, 0.065 mM dTTP, 0.035mM alkali-labile DIG-11-dUTP, pH 6.5, and 2 units of Klenow fragment was added and incubated for 20 hrs at 37°C. The DIG-labeled probe was then mixed with the hybridization liquid containing 50% (v/v) deionized formaldehyde, 5x SSC (150mM NaCl and 15mM sodium citrate), 0.1% (w/v) sodium lauryl sarcosine, 0.02% SDS and 2% (w/v) blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) and stored at -20°C.

Northern Blot Analysis

Total RNA was extracted from the leaves with an RNeasy mini kit (Qiagen, Valencia, USA). The integrity of mRNA was indirectly measured based on the intactness of rRNAs on 1.0% agarose/formaldehyde denaturing gel [19]. Non-radioactive Northern blot analysis was performed with DIG-labeled probes. Approximately 8 µg of total RNA was separated on 1.0% agarose/formaldehyde gel, transferred to Magnagraph nylon membrane (MSI, Westborogh, USA) in 10xSSC, pH 7.0, and then cross-linked with a UV crosslinker (Hoefer Scientific, San Francisco, USA). The membrane was hybridized with DIG-labeled probe for 12 hrs at 42°C in a solution containing 5xSSC, pH 7.0, 50% (v/v) deionized formamide, 0.1% (w/v) sodium lauryl sarcosine, 0.02% (w/v) SDS, and 2% (w/v) blocking reagent. Then, the blot was washed twice for 15 min at 42 in 2x SSC containing 0.1%(w/v) SDS and twice again for 15 min at 55°C in 0.5x SSC containing 0.1% (w/v) SDS. After blocking the membrane was incubated for 30 min with anti-DIG antibody conjugated with alkaline phosphatase (AP), washed twice with 100mM maleic acid, pH 7.5, 150 mM NaCl, 0.3% (v/v) Tween 20, incubated with the chemiluminescent AP substrate, CSPD (Boehringer Mannheim, Germany) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and exposed to the X-ray film.

RESULTS AND DISCUSSION

Nucleotide and Its Deduced Amino Acid Sequence Analysis of the psbC Gene

Analysis of approximately 1.8kb DNA fragment from the *P. ginseng* chloroplast chromosome has disclosed at least one uninterrupted open reading frame (ORF). Comparisons of nucleotide and its deduced amino acid sequences with those from monocots and dicots in database have revealed that the

ORF shows significant degree of identity to the *psb*C gene of PSII, which is composed of 1,422 nucleotides (Fig. 1). The deduced amino acid sequence of the *psb*C gene showed 99%, 98%, 96%, 95%, and 95% identities to tobacco, Arabidopsis, rice, maize, and liverwort, respectively, whereas it showed only 82% identity to *Synechocytis* sp. PCC 6803. This result implies that the structure of CP43 from *P. ginseng* is similar to those from higher sun-plants. The predicted topology of CP43, based on hydropathy analysis, includes six membrane-spanning α-helices as well as five hydrophilic loops (Fig. 2). In particular a large hydrophilic loop E connecting helices V and VI, which is exposed to the lumen, has been suggested to be crucial for function and stability of CP43 based on the site-directed mutagenesis analysis of *Synechocytis* sp. PCC 6803 [20].

An intriguing aspect of CP43 is the distribution of histidyl residues, a primary candidate of axial ligands for chlorophyll molecules. In CP43, thirteen histidyl residues are conserved. Ten residues are located in the predicted membrane spanning helices, especially near the membrane surface, and three residues are found in the hydrophilic loops. Seven His residues are close to the stromal surface, whereas six residues reside near the luminal surface in a proposed topolographical model (Fig. 2). The locations of histidyl resides in CP43 show a striking similarity to those located in the membrane spanning regions of CP47, another chlorophyll-binding protein of the PSII core complex, even though these two polypeptides exhibit only limited overall primary sequence homology. The fact that the histidyl residues are well-conserved in the predicted membrane spanning helices, of which the primary sequence homology is only about 30%, implies that they are crucial to the function of CP43. One of possible functions could be a ligand for a chlorophyll molecule, since CP43 has been estimated to bind 9-25 molecules of chlorophyll *a* [21, 22].

Putative translation start codon for the *psb*C gene is located at 48 nucleotides upstream from the stop codon of the *psb*D gene which encodes the D2 polypeptide of the PSII reaction center, indicating that the promoter region of the *psb*C gene is possibly located in the structural gene of the *psb*D gene. Indeed, it is predicted that -35 (TTGAGA) and -10 (TATTC T) elements with 16bp spacing, the putative prokaryotic promoter of the *psb*C gene was found in the structural gene of the *psb*D gene (Fig. 4).

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1   M K T L Y S L R R F Y P V E T L F N G T L A L A G R D Q E T   30
    ATGAAAACCTTATATTCCCTGAGGAGGTTCTACCCCGTGGAAACGCTCTTTAATGGAACTTTAGCTTTAGCTGGTCGTGACCAAGAAACC

31  T G F A W W A G N A R L I N L S G K L L G A H V A H A G L I   60
    ACTGGTTTCGCTTGGTGGGCCGGGAATGCCCGGCTTATCAATTTATCCGGTAAACTACTCGGGGCTCATGTAGCCCATGCCGGATTAATT

61  V F W A G G M N L F E V A H F V P E K P M Y E Q G L I L L P   90
    GTATTCTGGGCCGGAGGAATGAACCTATTTGAAGTGGCTCATTTCGTACCAGAGAAGCCTATGTATGAACAAGGATTAATTTTACTTCCC

91  H L A T L G W G V G P G G E V I D T F P Y F V S G V L H L I  120
    CACCTAGCTACTCTAGGTTGGGGGGTAGGTCCTGGTGGGGAAGTTATAGACACTTTTCCATACTTTGTATCTGGAGTACTTCATTTAATT

121 S S A V L G F G G I Y H A L L G P E T L E E S F P F F G Y V  150
    TCCTCTGCAGTATTGGGCTTTGGTGGTATTTATCATGCACTTCTAGGGCCTGAGACGCTTGAAGAATCTTTTCCATTCTTCGGTTATGTA

151 W K D R N K M T T I L G I H L I L L G I G A F L L V F K A L  180
    TGGAAAGATAGAAATAAAATGACTACAATTTTAGGTATTCACTTAATCTTATTAGGTATAGGTGCTTTTCTTCTAGTATTCAAGGCTCTT

181 Y F G G V Y D T W A P G G G D V R K I T N L T L S P S I I F  210
    TATTTTGGTGGCGTATATGATACCTGGGCTCCGGGGGGGGGAGATGTAAGAAAAATTACCAACTTGACCCTTAGCCCAAGTATTATATTT

211 G Y L L K S P F G G E G W I V S V D D L E D I I G G H V W L  240
    GGTTATTTACTAAAATCGCCCTTTGGAGGGGAAGGATGGATTGTTAGTGTAGATGATTTGGAAGATATAATCGGAGGACATGTATGGTTA

241 G S I C I L G G I W H I L T K P F A W A R R A L V W S G E A  270
    GGTTCCATTTGTATACTTGGTGGAATCTGGCATATCTTAACCAAACCCTTCGCATGGGCTCGACGCGCACTTGTGTGGTCTGGGGAGGCT

271 Y L S Y S L G A L S V F G F I A C C F V W F N N T A Y P S E  300
    TACTTATCTTATAGTTTAGGGGCTTTATCCGTCTTTGGTTTCATTGCTTGTTGTTTTGTCTGGTTCAATAATACCGCCTATCCTAGCGAG

301 F Y G P T G P E A S Q A Q A F T F L V R D Q R L G A N V G S  330
    TTTTACGGACCCACTGGACCAGAAGCTTCCCAAGCTCAAGCATTTACTTTTCTAGTTAGAGACCAACGCCTTGGGGCTAACGTGGGATCC

331 A Q G P T G L G K Y L M R S P T G E V I F G G E T M R F W D  360
    GCTCAAGGACCTACTGGTTTAGGTAAATATTTAATGCGTTCCCCGACCGGAGAAGTCATTTTTGGAGGAGAAACTATGCGTTTTTAGGAT

361 L R A P W L E P L R G P S G L D L S R L K K D I Q P W Q E R  390
    CTGCGTGCCCCTTGGTTAGAACCTCTAAGGGGTCCAAGTGGGTTGGACTTGAGTAGGCTGAAAAAAGACATACAACCTTGGCAAGAACGG

391 R S A E Y M T H A P L G S L N S V G G V A T E I N A V N Y V  420
    CGTTCTGCAGAATATATGACCCATGCTCCTTTAGGTTCTTTAAATTCTGTGGGTGGCGTAGCTACCGAGATCAATGCAGTCAATTATGTC

421 S P R S W L A T S H F V L G F F L F V G H L W H A G R A R A  450
    TCTCCTAGAAGTTGGTTAGCTACTTCTCATTTTGTTCTAGGATTCTTCTTGTTCGTAGGTCATTTGTGGCACGCGGGAAGGGCTCGTGCA

451 A A A G F E K G I D R D F E P V L S M T P L N *              473
    GCTGCAGCAGGGTTTGAAAAAGGAATTGATCGTGATTTTGAACCTGTTCTTTCCATGACCCCTCTTAATTGA
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Figure 1. Nucleotide and its deduced amino acid sequences of the *psb*C gene. The total 1,422 nucleotide sequence of the *psb*C gene from *P. ginseng* is presented and numbered 5' to 3' direction. Its deduced amino acid sequence is shown in one-letter code. The predicted membrane spanning regions are underlined.

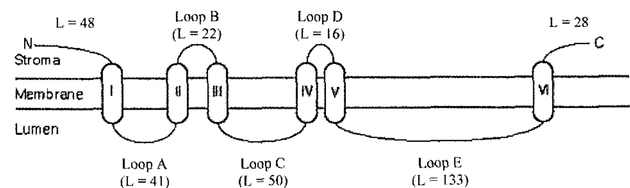

Figure 2. Predicted folding pattern of CP43. Based on hydropathy analysis, CP43 has six membrane spanning domains which span the thylakoid membrane as a-helices (indicated by Roman numerals I-VI). The proposed membrane spanning regions of helix I to IV are as follows: helix I, L-49 to F-70; helix II, F-112 to P-137; helix III, I-160 to F-182; helix IV, I-233 to T-254; helix V, Y-271 to W-291, and helix VI, W-425 to A-445.

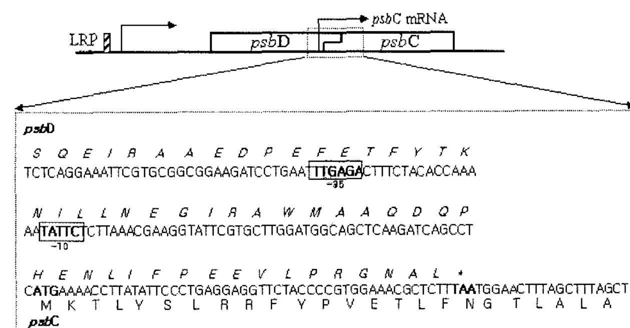

Figure 3. Partial Nucleotide and amino acid sequences of the overlapping region of the *psb*D and *psb*C genes. The possible promoter regions of the *psb*C gene, located in the structural gene of *psb*D, are written in bold letters and boxed.

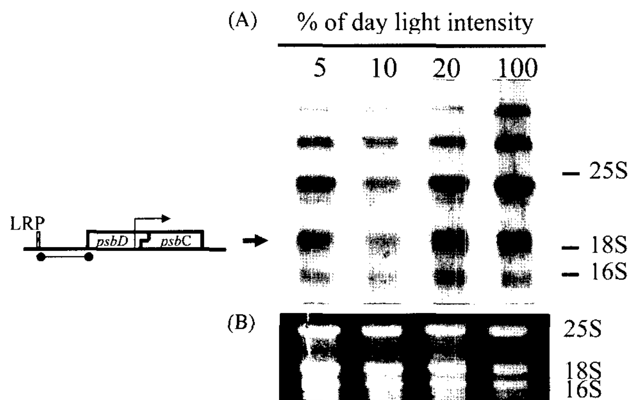

Figure 4. Effect of light intensity on the *in vivo* accumulation of the *psb*C mRNA in mature leaves from *P. ginseng*. Total RNA samples were extracted from mature leaves grown under various intensities (5, 10, 20, and 100%) of daylight and analyzed by Northern hybridization experiments using the DIG-labeled *psb*C gene-specific probe (A). 8 μg of total RNA was loaded per each lane and the degree of total RNA degradation was indirectly indicated by the intactness of rRNAs (B). The band intensities were measured with the program of Multigauge (version. 2.02).

Transcriptional Regulation of the psbC Genes by Light Intensity

The *in vivo* accumulation of the *psb*C mRNA was analyzed with total RNA extracted from the leaves of *P. ginseng* grown under various intensities (5%, 10%, 20%, and 100%) of daylight. The intactness and the size distribution of the relevant species of 16S, 18S and 25S rRNAs on 1.2% agarose/formaldehyde gel indicated that mRNA was relatively intact (Fig. 4B). The equal amounts of mRNAs were loaded onto the gel for Northern blot analysis. In order to detect the transcript of the *psb*C gene, the PCR-amplified and DIG-labeled DNA fragment of the *psb*C gene-specific probe was used. Several bands were detected in Northern blot analysis (Fig. 4). Since the size of 16S rRNA band is approximately corresponding to 1.5kb, mRNA from the 1,422 bp of the *psb*C gene could be the band indicated by the arrow in Fig. 4(A). The upper band of the possible *psb*C mRNA, indicated by the arrow head, is likely to be the *psb*D/*psb*C mRNA, of which the transcription is activated by a blue light and ultraviolet A [15].

Since *P. ginseng* is a shade plant, high intensity of light causes the photoinhibition of PSII. The *psb*A gene product is known to be involved in protecting the PSII complex from photoinhibition by replacing the damaged D1 polypeptide very rapidly. Since CP43 is a member of the PSII core complex together with D1 and D2 and its Loop E in the lumen is interacting with the D1 polypeptide, it is not surprising to observe that CP43 is also undergo light-mediated turnover similar to light-labile D1, although its decay is about half of D1 decay [23]. The *in vivo* accumulation of the *psb*C transcript, after being cultivated under various intensities of daylight (5%, 10%, 20%, and 100%), was measured and the results indicated that light intensity did not significantly affect the steady-state level of the *psb*C mRNA, although 20% and 100% intensities of daylight caused amounts of message to increase up to 12% (Fig. 4A). This phenomenon was also observed in the *psb*A transcript [18]. This observation can be explained by the fact that the transcription of *psb*C is not affected by light intensity. Therefore, it can be postulated that the transcriptional regulation of the *psb*C gene expression is not responsible for the loss of CP43 in PSII under high light intensity.

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