Journal of Biochemistry and Molecular Biology, Vol. 32, No. 2, March 1999, pp. 189–195

JOURNAL OF
Biochemistry
and
Molecular Biology

Cloning and Characterization of the *psb*EF Gene Encoding Cytochrome *b*-559 of the *Panax ginseng* Photosystem II Reaction Center

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Received 2 November 1998, Accepted 15 December 1998

From the *Panax ginseng* chloroplast, the *psb*E and *psb*F genes, encoding the α- and β-subunits of cytochrome *b*-559 of the photosystem II reaction center, respectively, were cloned and characterized. The *psb*E and *psb*F genes were composed of 252 and 117 nucleotides, respectively. The deduced amino acid sequence of the α-subunit showed 95%, 93%, and 91% homology to monocots, dicots, and liverwort, respectively, whereas the β-subunit showed approximately 98% to 95% homology to the same species. Southern blot analysis revealed that a single copy of the *psb*EF gene exists in the chloroplast plastid. Northern blot analysis indicated that the *psb*E and *psb*F genes are cotranscribed as a polycistron.

Keywords: Cytochrome *b*-559, Photosystem II, *psb*E, *psb*F.

Introduction

Cytochrome *b*-559 in the water-splitting photosystem II (PSII) reaction center of oxygenic photosynthesis makes the major structural and chemical differences (Tae *et al.*, 1988, Thompson and Brudvig, 1988) from the anoxygenic photosynthetic purple bacteria (Deisenhofer and Michel, 1989). Cytochrome *b*-559 is present in organisms ranging from the most primitive oxygenic photosynthetic reaction centers of cyanobacteria to those of higher plants. Removal of the heme-binding capacity of the cytochrome subunits is lethal to PSII function, implying that cytochrome *b*-559 plays a structural role in the functional integrity of the PSII reaction center (Pakrasi *et al.*, 1988). It is generally agreed that cytochrome *b*-559 does not participate in the main electron transport pathway of H_2O oxidation by the reaction center, but that its obligatory presence is related to the protection of PSII which is labile to environmental stresses such as heat and high light intensity (Jang and Tae, 1996).

Panax ginseng C. A. Meyer is a perennial herb in the family *Araliaceae*. The plant is cultivated for medicinal purposes under shaded conditions. Among the stresses, such as high light intensity, heat, and water deficiency, which affect the growth rate of *P. ginseng*, high light intensity is the most important factor and has been investigated most extensively with regard to its effect on photosynthesis. If the light intensity is higher than 2000 μEinstein/$m^2 \cdot sec^{-1}$, the photosynthetic activity decreases (Cheon, 1989) and the compositions of chlorophyll-protein complexes and integral proteins in thylakoids are changed (Degreef *et al.*, 1971; Bushmann *et al.*, 1978). It has also been observed that the chlorophyll content decreases while the chlorophyll a/b ratio increases when ginseng leaves are exposed to high light intensity (Park, 1980). However, very few studies have reported on the effect of high light intensity on chloroplast gene expression in shade plants, especially genes encoding the essential proteins of photosynthesis, and on the structural and functional integrity of the PSII reaction center in thylakoids.

In the present study, genes for the cytochrome *b*-559 α- and β-subunits, the *psb*E and *psb*F genes, were cloned from the chloroplast genome of *P. ginseng* and their primary structures and deduced amino acid sequences were analyzed. This information should be useful to understand the regulation of the *psb*EF gene under high light intensity and to elucidate the protective role of cytochrome *b*-559 in the PSII reaction center against photoinhibition in *P. ginseng*.

Materials and Methods

Chloroplast genomic DNA isolation The frozen leaves were ground with a mortar and pestle in liquid nitrogen and the powder

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was suspended in 100 ml of the extraction buffer (50 mM Tris·HCl, pH 8.0, 5 mM EDTA, 0.35 M sorbitol, 0.1% BSA, 0.1% β-mercaptoethanol, 10% PEG 4000) for every 10 g of leaves. The homogenate was filtered through several layers of cheesecloth and one layer of miracloth. Chloroplasts were pelleted by centrifugation for 15 min at 8000 × *g* and resuspended in 5 ml of washing buffer (10 mM Tris, pH 8.0, 20 mM EDTA, 0.35 M sorbitol, 0.1% β-mercaptoethanol). One ml of 5% sarkosyl was added and incubated for 15 min at room temperature. Then, 860 μl of 5 M NaCl and 686 μl of 8.6% CTAB/0.7 M NaCl were added. The samples were incubated at 60°C for 15 min and extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). After centrifugation for 10 min at 5000 × *g*, the upper aqueous phase was collected and the nucleic acids were precipitated by the addition of 2/3 volume of isopropanol. After incubation for 10 min at room temperature, nucleic acids were pelleted by centrifugation for 20 min at 14,300 × *g*. The pellet was washed with 70% ice-cold ethanol, air-dried, and resuspended in an appropriate volume of water or TE buffer.

Labeling of the *psb*EF gene probe To prepare the *psb*EF-specific DNA probe, the primers, designed to bind to highly-conserved regions of the *psb*E and *psb*F gene, were used to amplify the DNA fragment including the two genes. The PCR fragment was subcloned into a pGEM T-easy vector (Promega, Madison, USA) using the *Eco*RV site to generate the pGEM T-easy/*psb*EF plasmid, which was then linearized with *Nco*I and *Pst*I. The insert was separated from the agarose gel and labeled with DIG-labeling and detection system (Boehringer Mannheim GmbH, Mannheim, Germany). One μg of the insert was boiled for 10 min and placed on ice. Twenty μl of the reaction mixture containing 50 mM Tris·HCl, pH 7.2, 10 mM $MgCl_2$, 0.1 mM dithioerythreitol, 0.2 mg/ml BSA, 0.1 mM dATP, 0.1 mM dCTP, 0.065 mM dTTP, 0.035 mM alkali-labile DIG-dUTP, pH 6.5, and 2 units of Klenow fragment were added and incubated for 20 h at 37°C. The DIG-labeled probe was then mixed with the hybridization fluid containing 50% (v/v) deionized formaldehyde, 5× SSC (150 mM NaCl and 15 mM sodium citrate), 0.1% (w/v) sodium lauryl sarcosine, 0.02% SDS, and 2% (w/v) blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany) and then stored at −20°C.

Southern blotting analysis Approximately 10 μg of the purified chloroplast genomic DNA was digested with *Bam*HI/*Hin*dIII or *Eco*RI/*Pst*I, electrophoresed on a 0.8% agarose gel, transferred to a Magnacharge nylon membrane (MSI, Westborogh, USA) in 10× SSC, pH 7.0 and then cross-linked with UV light (UV Cross-Linker, Stratagene, La Jolla, USA). Protocols and reagents for the chemiluminescent identification of DNA were supplied as components of the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim GmbH, Mannheim, Germany). The prehybridization was performed for 3 h at 42°C in 5× SSC, 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. The hybridization at 42°C for at least 16 h was carried out with the DIG-labeled probe of the *psb*EF gene. The blot was washed twice for 15 min at 42°C in 2× SSC containing 0.1% (w/v) SDS and twice for 15 min at 55°C in 0.5× SSC containing 0.1% (w/v) SDS. After exposing the blot to a blocking solution for 1 h, the anti-digoxigenin antibody was added to the solution and incubated for 30 min. The blot was washed five times for 5 min at room temperature in 0.1 M maleic acid and 0.15 M NaCl (pH 7.5), rinsed for 30 s in 0.1 M Tris·HCl, pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$, and incubated with 1/100 diluted CSPD (Boehringer Mannheim GmbH, Mannheim, Germany) in 0.1 M Tris·HCl, pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$. After incubation for 15 min at 37°C, the labeled blots were exposed to x-ray film for 1 h.

The *psb*EF DNA sequence analysis The DNA fragments of the chloroplast genome digested with *Bam*HI and *Hin*dIII were ligated into pBluescript II SK^+ (Stratagene, La Jolla, USA) which had been previously digested with *Bam*HI and *Hin*dIII and recovered from a 0.8% agarose gel, and transformed to the *E. coli* MV1190. The PCRs were performed directly with white colonies as templates and with primers for the highly-conserved regions of the *psb*E and *psb*F genes in the reaction mixture containing 0.8 M dNTP, 1 μM forward primer, 1 μM reverse primer, and 2 units of *Taq* DNA polymerase. The plasmid was extracted with a Midiprep kit (Qiagen, Valencia, USA) from the colony which showed a positive PCR band for *psb*EF and the DNA sequence analysis of the insert was performed with an automated DNA sequence analyzer (Applied Biosystem, Model 373A). The DNA sequence data obtained were blasted to the NCBI (National Center for Biotechnology Information) through the internet to estimate the degree of homology to the previously sequenced *psb*EF genes from dicots, monocots, and liverwort. The amino acid sequence alignment was performed with the shareware program, SeqPup.

RNA extraction and Northern blot analysis Total nucleic acid samples were isolated from the liquid-nitrogen-frozen leaves of *P. ginseng* by extraction in water-saturated phenol:chloroform:isoamyl alcohol (50:49:1) and the extraction buffer (100 mM Tris·HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, and 1.0% SDS). Total RNA was obtained by extraction of total nucleic acid samples with acid phenol, pH 4.5, followed by extraction with chloroform:isoamyl alcohol (49:1, v/v) and precipitation with 2 M LiCl. The size and the integrity of total RNA were analyzed based on their intactness of rRNAs on a 1.0% agarose/formaldehyde denaturing agarose gel. For Northern blot analysis, approximately 20 μg of the purified RNAs were separated on a 1.0% agarose/formaldehyde gel (Sambrook *et al.*, 1989), transferred to a Hybond N nylon membrane (Amersham, Buckinghamshire, UK) in 10× SSC, pH 7.0, and then cross-linked with UV light. The same methods as in Southern blot analysis were applied to detect the message of the *psb*EF gene.

Results

Identification of *psb*EF by PCR Part of the *psb*EF gene for the cytochrome *b*-559 α- and β-subunits was amplified by PCR from a chloroplast genomic DNA isolated from the leaves of *P. ginseng* by using the forward primer EF-1 [5′-AGCATGTCTGGAAGCACGGG-3′] and the reverse primer EF-2 [5′-GTTCTATA(A/G)TT(C/G)G (G/A)ATT(A/T)GG-3′], in which the nucleotide sequences in parenthesis indicate the degenerate bases. These primers

were designed on the basis of highly-conserved regions of the cytochrome *b*-559 gene from higher plants, were supposed to bind to the upstream of *psb*E and to the downstream of *psb*F, respectively, and resulted in the production of an approximately 0.5 kb DNA fragment after PCR (data not shown).

The PCR-amplified DNA fragment was subcloned into pGEM T-easy vector with additional dT residues in its *Eco*RV cleavage site, which could increase the blunt end ligation efficiency at least 10 times (Mezei and Storts, 1994). The plasmid was extracted from each of the white colonies grown in the presence of X-gal in a plate, and digested with *Eco*RI. Since the pGEM T-easy vector has two *Eco*RI cleavage sites flanking the PCR-amplified *psb*EF gene, the *Eco*RI digestion made it possible to examine the presence and the size of the *psb*EF gene insert (Nam and Tae, 1998). The nucleotide sequence of the insert was partially determined to confirm the *psb*EF gene encoding the cytochrome *b*-559 α- and β-subunits.

Chloroplast genomic DNA analysis The DNA fragment from the vector pGEM T-easy/*psb*EF was DIG-labeled and used as a probe for the detection of the *psb*EF gene in the chloroplast genomic DNA. As a result of Southern blot analysis using approximately 10 μg of the purified chloroplast DNA digested with *Bam*HI/*Hin*dIII or *Eco*RI/*Pst*I, a single band positioned approximately 3.5 kb in size was observed in lane B, in which the *Bam*HI/*Hin*dIII-digested chloroplast genomic DNA was loaded (Fig. 1). On the other hand, the *Eco*RI/*Pst*I-digested DNA generated two bands, as seen in lane C (Fig. 1), approximately 2.2 kb and 1.4 kb in size, implying that there may be *Eco*RI or *Pst*I sites within the *psb*EF gene. These results also indicate that the 3.5 kb fragment of the *Bam*HI/*Hin*dIII-digested chloroplast genomic DNA contains the *psb*EF gene. Therefore, the chloroplast genomic DNA was digested with *Bam*HI and *Hin*dIII and the DNA fragments 3.0 kb to 4.0 kb in size were subcloned into a pBluescript II SK$^+$ vector and its nucleotide sequence was determined.

Northern blot analysis The message of the *psb*EF gene was analyzed with Northern blot analysis. The integrity and size distribution of the total RNA purified from the leaves of *P. ginseng* with an RNeasy mini kit (Qiagen, Valencia, USA) was checked by using a 1.0% agarose/formaldehyde gel and ethidium bromide staining. The relevant rRNAs species of 16S, 18S, 23S, and 25S appeared as sharp bands on the stained gel, indicating the intactness of mRNAs in the total RNA preparations (data not shown). Since the *psb*EF gene is shown to be cotranscribed with the *psb*LJ gene as a polycistron, the PCR-amplified and DIG-labeled DNA fragment of the *psb*EF gene was used as a probe and a single band was detected at a position a little smaller than 16S rRNA, of which the size is approximately 1.5 kb in size (Fig. 2).

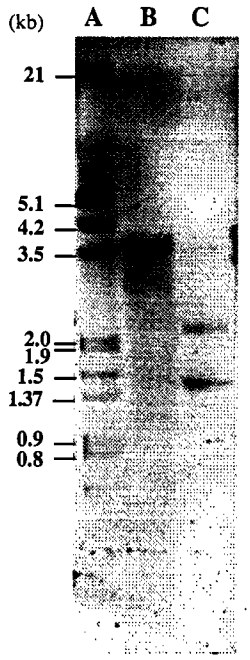

Fig. 1. Genomic Southern blot analysis of the chloroplast *psb*EF gene. The chloroplast genomic DNA from *P. ginseng* was digested with *Bam*HI and *Hin*dIII (lane B) or *Eco*RI and *Pst*I (lane C), separated on a 0.8% agarose gel, and transferred onto a Megnacharge nylon membrane. The probe was prepared with the 0.5 kb DNA fragment of the *Eco*RI-digested pGEM T-easy/*psb*EF vector by the random hexamer priming method. The DNA size marker in lane A includes fragments 21 kb, 5.1 kb, 4.2 kb, 3.5 kb, 2.0 kb, 1.9 kb, 1.5 kb, 1.37 kb, 0.9 kb, and 0.8 kb in size ordered from the top to the bottom.

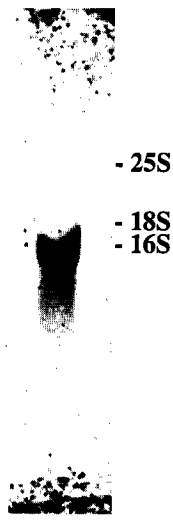

Fig. 2. Northern blot analysis of the chloroplast *psb*EF gene. Total RNA from *P. ginseng* leaves was extracted and approximately 20 μg of the purified RNA was separated on a 1.0% agarose/formaldehyde denaturing gel and transferred to a Hybond N nylon membrane. The PCR-amplified and DIG-labeled DNA fragment of the *psb*EF gene was prepared as a probe by the random hexamer priming method.

Nucleotide and deduced amino acid sequences of the *psb*EF gene Analysis of the 3.5 kb DNA insert from the *P. ginseng* plastid chromosome has disclosed at least two uninterrupted open reading frames (ORFs). Comparisons of nucleotide and deduced amino acid sequences with those from monocots, dicots, and liverwort in the database (Fig. 3) revealed that the two ORFs have a high degree of homology to the photosystem II genes, *psb*E and *psb*F, which encode the apocytochrome *b*-559 α- and β-subunits, respectively (Fig. 4). The deduced amino acid sequence of the α-subunit showed 95%, 93%, and 91% homology to monocots, dicots, and liverwort, respectively, whereas the β-subunit showed approximately 95% to 98% homologies. The *psb*E and *psb*E gene are composed of 252 and 117 nucleotides which can be translated into proteins composed of 83 and 39 amino acid residues, respectively. The ATG initiation codons positioned at +1 and +262 in Fig. 4 are preceded by the potential ribosome-binding sites, GGAG (−10 to −7) and GAGG (253 to 256), located 10 and 9 nucleotides prior to the start codons of *psb*E and *psb*F, respectively. The open reading frames of *psb*E and *psb*F end with the amber (TAG) and the ochre (TAA) stop codons, respectively, at positions +250 and +379. The *psb*E and *psb*F genes are connected by only 9 nucleotides (+283 to +291) in which the putative ribosome binding site is included.

Secondary structure of cytochrome *b*-559 The hydropathy analysis revealed that each of the cytochrome *b*-559 α- and β-subunits contains a long stretch of hydrophobic domain, spanning Tyr-19 to Pro-50 in the α-subunit and Trp-14 to Gln-38 in the β-subunit (Fig. 5). These hydrophobic domains are expected to span the thylakoid membranes as α-helix, based on the rise distance per each residue, and the positively-charged Arg residue (Arg-18 and Arg-51 in the α-subunit and Arg-13 and Arg-39 in the β-subunit), flanking these hydrophobic

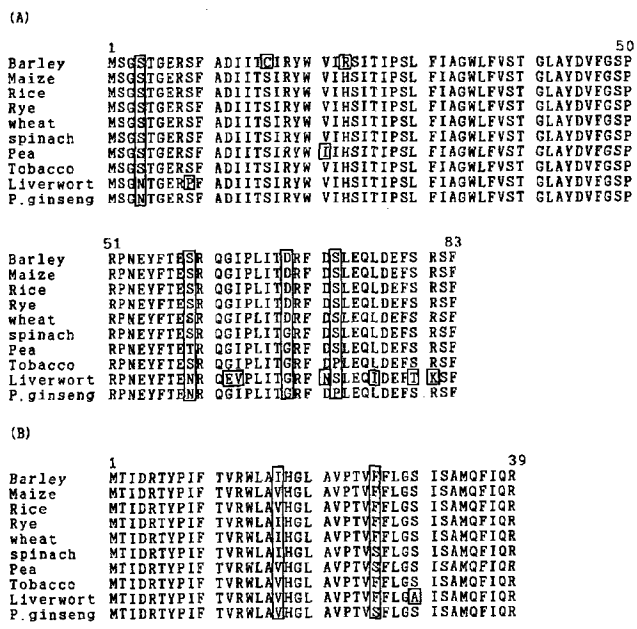

Fig. 3. Multiple amino acid sequence alignments of the cytochrome *b*-559 α- and β-subunits. The cytochrome *b*-559 α- and β-subunits from *P. ginseng* is aligned with those from monocots (barley, maize, rice, rye, and wheat), dicots (spinach, pea, and tobacco), and liverwort. The amino acid sequences that are different from that of *P. ginseng* are boxed.

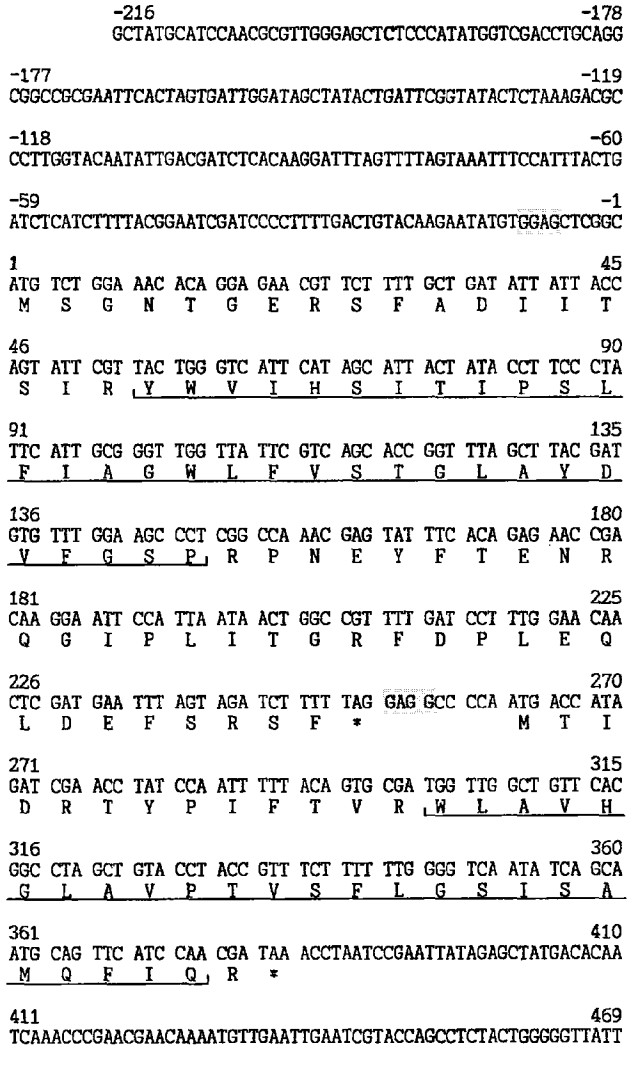

Fig. 4. Nucleotide and deduced amino acid sequence of the *psb*EF gene of *P. ginseng*. The deduced amino acid sequences contain the full length of the cytochrome *b*-559 α- and β-subunits. The sequence of the nontranscribed strand of genes are arranged in codons and the corresponding amino acids are indicated. The number of nucleotides presented is approximately 680 and numbered in the 5′ to 3′ direction with the first nucleotide coding the initiative Met-1 in the cytochrome *b*-559 α-subunit designated +1. Possible ribosome binding sites are shaded. Hydrophobic domains of α- and β-subunits, which are expected to span the thylakoids as α-helices, are underlined.

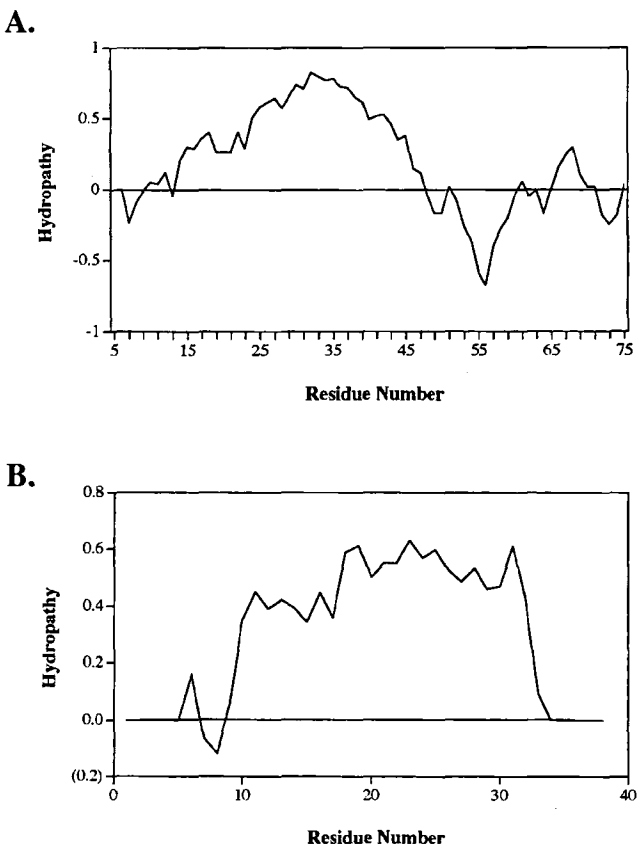

Fig. 5. Hydropathy plot of the cytochrome *b*-559 α- and β-subunits. The values of hydropathy were calculated based on the Eisenberg consensus index for each amino acid with a window size of 9, according to the algorithm discussed by Shiver *et al.* (1989). The total number of amino acid residues analyzed are (A) 83 for the α-subunit and (B) 39 for the β-subunit, respectively.

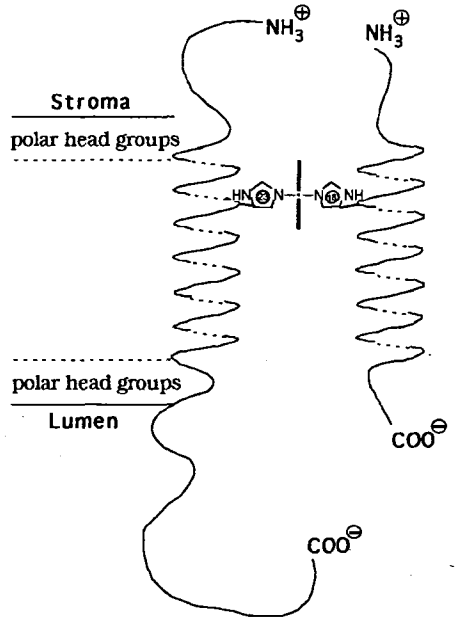

Fig. 6. The $\alpha\beta$-heterodimeric model of cytochrome *b*-559 in the PSII reaction center of *P. ginseng*. The heme iron, symbolized by a dot in the middle of the vertical bar, is coordinated by His-23 and His-18 of the α- and β-subunits, respectively. The NH_2- and COOH-termini of the α-subunit are positioned on the stromal and the lumenal sides of the thylakoid membranes, respectively (Tae *et al.*, 1988), resulting in the same orientation of the β-subunit in this model.

domains function as anchors to prevent these domains from popping out of the membranes. Moreover, each subunit has a single histidine residue positioned at the fifth residue from the amino termini of the hydrophobic domains (His-23 in the α-subunit and His-18 in the β-subunit). Since cytochrome *b*-559 is composed of two subunits (α- and β-) and a heme group, the two His residues are reported to function as ligands of the heme group through bis-histidine ligation (Babcock *et al.*, 1985), resulting in the $\alpha\beta$-heterodimeric cytochrome *b*-559 in the PSII reaction center in higher plants (Tae and Cramer, 1994; Cha and Tae, 1997). Since the amino terminal domain of the α-subunit is shown to be exposed to the stromal side (Tae *et al.*, 1988), the amino terminal domain of β-subunit is also directed to the stromal side (Fig. 6).

Discussion

The *psb*EF gene is located in the chloroplast genome (Westhoff *et al.*, 1985) and encodes cytochrome *b*-559, which is found in all the known photosynthetic organisms, implying its functional importance in the conversion of light to electrochemical energy. *P. ginseng* is not exceptional, although it is a shade plant. The *psb*EF gene is located in the chloroplast genome and the copy number turns out to be one as determined by Southern blot analysis. The *Bam*HI/*Hin*dIII digestion of the purified chloroplast DNA gave a single band positioned at approximately 3.5 kb in size (Fig. 1, lane B), whereas the *Eco*RI/*Pst*I digestion DNA generated two bands whose sizes were approximately 2.2 kb and 1.4 kb (Fig. 1, lane C). The nucleotide sequence of the chloroplast DNA fragment including the *psb*EF gene (Fig. 4) revealed that one *Pst*I site (5′-CTGCAG-3′) at −184 to −179 and two *Eco*RI sites (5′-GAATTC-3′) at −170 to −165 and 185 to 190 were found, whereas neither *Bam*HI nor *Hin*dIII sites are present. The *Eco*RI/*Pst*I double digestions could also generate the *Pst*I-*Eco*RI (379 bp) or the *Eco*RI-*Eco*RI (355 bp) DNA fragment but these fragments seemed to be too small to be detected in the gel system used in this study. However, at least the *Eco*RI site at 185 to 190 located in the structural gene of *psb*E seemed to be cleaved and generated two bands of 2.2 kb and 1.4 kb in sizes, suggesting that the chloroplast genome contains a single copy of the *psb*EF gene.

The deduced amino acid sequence comparison of the α-subunit showed 91 to 98% homology to monocots, dicots, and liverwort, whereas the β-subunit showed approximately 95 to 98% homology to them. The β-subunit seemed to be in general more conserved than the α-subunit. Among monocots, dicots, and liverwort, dicots showed higher homology to *P. ginseng* in terms of the α-subunit amino acid sequence and, even though liverwort is known to grow in shaded areas, it showed the lowest homology to *P. ginseng*. The amino acid sequence variations in the α-subunit seemed to occur more frequently in the hydrophilic domains than in the hydrophobic membrane spanning domain (Tyr-19 to Pro-50), implying that changes in the interactions of these hydrophilic domains of the α-subunit with other polypeptides of the PSII reaction center in *P. ginseng* might be responsible for the photoinhibition by high intensity light. In particular, both Asn-4 and Asn-59 are conserved in the shaded plants, *P. ginseng* and the liverwort, whereas they are replaced by Ser-4 and Ser-59 or Thr-59 in monocots and dicots.

The hydropathy analysis revealed that each of α- and β-subunits has a single hydrophobic domain which is expected to span the thylakoid membrane as an α-helix (Figs. 5A and 5B) and that the His residues (His-23 and His-18 in the α- and β-subunits, respectively) are positioned at the fifth residue from the NH_2-termini of the membrane spanning domains of the α- and β-subunits, which are believed to ligate the heme group through hydrophobic interactions. The His residues are all conserved in the known photosynthetic organisms including *P. ginseng*, indicating that they are essential for the cytochrome *b*-559 function.

Northern blot analysis revealed that the *psb*EF gene was detected as a single band roughly 1.2 to 1.3 kb in size (Fig. 2), implying that it is cotranscribed. Since the *psb*EF gene is also known to be cotranscribed with the *psb*L gene and the *psb*J gene, the size of the transcript should be larger than the sum of the number of nucleotides from the *psb*EFLJ gene, which is approximately 600 bases. Therefore, the extra 600 to 700 nucleotides may come from the nontranslated upstream downstream sequences of the *psb*EFLJ operon. Based on the fact that in the biogenesis of PSII in C3 and C4 plants, the *psb*EF gene is known to be expressed in the dark, before the greening of leaves, to prepare for photosynthesis (Westhoff *et al.*, 1990) and the fact that the *psb*EF gene product, cytochrome *b*-559, plays a structural role in the assembly of the functional PSII complex in thylakoids (Pakrasi *et al.*, 1988), the hypothesis is nowadays widely accepted that cytochrome *b*-559 along with D1 and D2 is inserted into thylakoids and assembled to the PSII reaction center. Then, the chlorophyll binding protein such as CP-47 and CP-43 come together and form a functional PSII complex.

Acknowledgment This work was supported by a Genetic Engineering research grant (1996) from the ministry of Education, Korea.

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