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CLONING, SUBCELLULAR LOCALIZATION AND EXPRESSION OF *CHLI*, A SUBUNIT OF MAGNESIUM-CHELATASE IN SOYBEAN

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Mg-insertion is the first committed step in chlorophyll synthesis and is catalyzed by Mg-chelatase. In photosynthetic bacteria, *bchI* gene product was suggested to be a subunit of Mg-chelatase. We isolated a *bchI* homolog from a soybean cDNA library and designated it as *chlI*. CHLI consisted of 421 amino acid residues and the sequence exhibited a high similarity to other BchI homologs. CHLI contained an ATP-binding motif found in other BchI homologs. CHLI was localized in the soluble fraction in soybean chloroplasts, suggesting that it was a stromal subunit of Mg-chelatase. *chlI* mRNA in cell culture (SB-P) of soybean was reversibly induced by light.

In chloroplasts of higher plants, biosyntheses of chlorophyll and heme share a common pathway up to the level of protoporphyrin IX. The insertion of magnesium (Mg) into protoporphyrin IX is the first committed step in the chlorophyll branch of tetrapyrrole biosynthetic pathway, and is catalyzed by Mg-chelatase. Despite the central importance of the Mg chelation, there is little information on the biochemical properties of Mg-chelatase due to the instability of the activity.

By genetic analyses of pigment-deficient mutants of photosynthetic bacteria, it has been suggested that three genetic loci (*bchI*, *bchH* and *bchD*) are involved in the reaction of Mg-chelatase (1). In higher plants, *bchI* and *bchH* homologs have been isolated by gene tagging methods (2,3). It is, therefore, likely that Mg-chelatase in higher plants is also composed of three subunits similar to those of photosynthetic bacteria.

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cs is a *bchI* homolog and was cloned from *Arabidopsis thaliana* by T-DNA tagging (2). Analysis of the deduced amino acid sequence suggested that the gene product (CS) is a water-soluble protein, but the function is not yet clear (2). In higher plants, Mg-chelatase activity has only been successfully measured in intact chloroplasts (4). The activity of Mg-chelatase was strictly dependent on ATP (5,6). Recently, Walker and Weinstein reported that both stromal and membrane fractions were required to reconstitute Mg-chelatase activity, indicating that Mg-chelatase consisted of soluble and membranous subunits (6). It is possible that *cs* gene product functions as a water-soluble subunit of Mg-chelatase.

Here we describe the cloning, localization and expression of CHLI (*chlI*), which is a homolog of BchI, isolated from cDNA library of soybean (*Glycine max* var. Resnik).

MATERIALS AND METHODS

Plant materials

Seeds of soybean (*Glycine max* L. var. Resnik) were soaked in water and germinated on wet vermiculite at 27°C in the dark for 5 days. The seedlings were then illuminated at 27°C with continuous white fluorescent light (5 $W \cdot m^{-2}$) for 5 days.

Soybean (*Glycine max*) photoautotroph cell culture (SB-P) was first described by Horn et al.(7). It was grown photomixotrophically in 300 ml of KN1 medium containing basic Murashige-Skoog salts (Hazleton Biologics), 0.03 mg of thiamine, 0.3 mg of α-naphthaleneacetic acid, 0.06 mg of kinetin, and 3 g of sucrose (pH 5.7 to 5.9) in 1-liter flasks. The cells were shaken at 105 rpm under continuous white fluorescent light (9.2 $W \cdot m^{-2}$) at 25°C and subcultured every 14 days. In time-course experiment, the cell cultures were incubated for 7 days under continuous light after inoculation. The flasks of cell cultures were then wrapped with two layers of aluminum foil and were shaken for additional 2 days to adapt in the dark (8). After dark adaptation, cells were re-illuminated and incubated up to 24 h.

Isolation of cDNA

A soybean cDNA library (Clontech, Palo, Alto, CA) was screened with radiolabeled probe of *ch*42 gene (*bchI* homolog) of *Arabidopsis*. Two positive lambda clones were isolated and cloned into plasmid vectors (pUC118, pUC119 or pBluescript II SK). These two clones were partially overlapped. One cDNA fragment of 1.8 kb contained most of an open reading frame but lacked the 3' end, and the other fragment of 1.0 kb contained the 3' end region including the termination codon. These sequences were consolidated and analyzed.

Isolation of intact chloroplasts and fractionation

Intact chloroplasts were isolated according to the method of Douce and Joyard (9), except that the homogenation of soybean cotyledons was performed with a food processor (MX-X10; National Electric Co., Tokyo) which had been modified by incorporation of razor blades as described by Kannangara et al. (10). Purification of the stromal fraction and membranes of intact chloroplasts was performed according to the method of Douce and Joyard with slight modifications (9). Ten ml of a 0.6 M sucrose solution were prepared in the centrifuge tubes (6 tubes for RPS40T swinging bucket rotor, HITACHI). One ml of the suspension of intact chloroplasts which were osmotically bursted in a swelling medium was then layered onto the sucrose solution and the tubes were centrifuged at 72,000 X g for 1 h at 3°C. The 0.6 M sucrose solution contained 0.6 M sucrose, 10 mM tricine (pH 7.8 at 2°C) and 4 mM $MgCl_2$. The swelling medium contained 10 mM tricine (pH 7.8 at 2°C) and 4 mM $MgCl_2$. After centrifugation two fractions were clearly separated: a tightly-packed, dark green pellets at the bottom of the tube (thylakoid- and envelope-membrane fractions) and a clear slightly brown supernatant (soluble fraction). The dark green pellet was resuspended in 1 ml of the swelling medium, layered onto the sucrose solution in the same manner, and centrifuged at 72,000 X g for 1 h at 3°C again.

Nucleic acid sequence

Dideoxy sequencing (11) was carried out on both strands of double-strand DNA templates using a *Bca*BEST Dideoxy Sequencing Kit (TAKARA SHUZO Co., Japan). On the basis of the partial sequence, oligonucleotides (20 mer) were synthesized and used for sequencing the internal regions.

Western blot analysis

SDS-PAGE was done according to the method by Laemmli (12). Total polypeptides in the intact chloroplasts and the two fractions (soluble and membrane fractions) which were separated by SDS-PAGE were trasferred onto a nitrocellulose membrane. CHLI band was detected using antisera raised against recombinant CHLI protein of *Anabaena variabilis* (accession number: D49426). Localization markers used were the large subunit of ribulose bisphosphate carboxylase (LSU) and the light-harvesting chlorophyll binding protein (LHCP).

Northern blot analysis

Total RNA was isolated according to the procedure of guanidine thiocyanate and phenol-chloroform extraction (13). Total RNA (20 μg/lane) was denatured, subjected to electrophoresis on 1.2% agarose gel and transferred onto a nylon membrane. One of the blots was hybridized with ^{32}P-labeled probe (1 kb) of 5'-noncoding region in the soybean *chlI* cDNA for 16 h at 60°C, and another blot was hybridized with actin cDNA under the same condition as a loading control. After hybridization, both blots were washed twice with 2 X SSC/0.1% SDS for 2 min at 60°C, and twice for 30 min at 60°C.

RESULTS AND DISCUSSION

Structure of a chlI clone and its predicted amino acid sequence

The cloned *chlI* cDNA (Accession number of DDBJ: D45857) was composed of 1953 bp and contained 569 bp of 5'-untranslated region, 1263 bp of an open reading frame and 121 bp of 3'-untranslated region. The deduced amino acid sequence showed that the open reading frame encoded 421 amino acid residues with a predicted molecular mass of 45,871 Da. (Fig. 1)

The deduced amino acid sequence of CHLI of *Glycine max* exhibited high similarity to BchI of *Arabidopsis thaliana* (80.4%)(2), *Euglena gracilis* (14), *Cryptomonas Φ* (15) and *Rhodobacter capsulatus* (16) (Fig. 1). Douglas and Reith indicated that there were four highly conserved regions (I-IV) in BchI family (15), which were also conserved in soybean CHLI. They also reported that in the region I, nine consecutive amino acids that were completely conserved among all of BchI homologs showed a significant similarity to a consensus sequence, AGXXXXGKST, which is present in the ATP/GTP-binding site A (P loop) of ATP-binding proteins (15). Moreover, it was indicated that the consensus sequence matched exactly to ATP-binding motif (GDRGTGKST) of the nitrogenase iron protein family (*nifH/chlL*) (15) and that of CobS, which is a subunit of cobaltochelatase from *Pseudomonas denitrificans* (17). In the alignment of the *chlI* gene products which we described here, the nine amino acids which are located between positions 116 and 124 were also completely conserved, suggesting that this CHLI requires ATP to express the activity. Indeed, Pardo et al. and Walker et al. reported that in vitro activity of Mg-chelatase required ATP (5,6).

As shown in Fig. 1, CHLI of soybean as well as of *Arabidopsis* has a long N-terminal sequence which was not present in the algal and bacterial counterparts. The sequence could function as a transit sequence for targetting to chloroplasts (see the next section). The *chlI* genes of *Euglena gracilis* and *Cryptomonas Φ* are encoded in chloroplast genome (14,15). Accordingly, it can be speculated that the *chlI* genes in plastid genome was transferred to the nuclear DNA in higher plants but remained in the algal plastid genome. Interestingly, phylogenetic analysis (data not shown) indicated that the *chlI* gene family was separeted into four groups, i.e., *Rhodobacter capsulatus* (prokaryote), *Euglena gracilis* (chloroplast-encoded), other algae such as *Cryptomonas Φ* (chloroplast-encoded) and *Anabaena*, and land

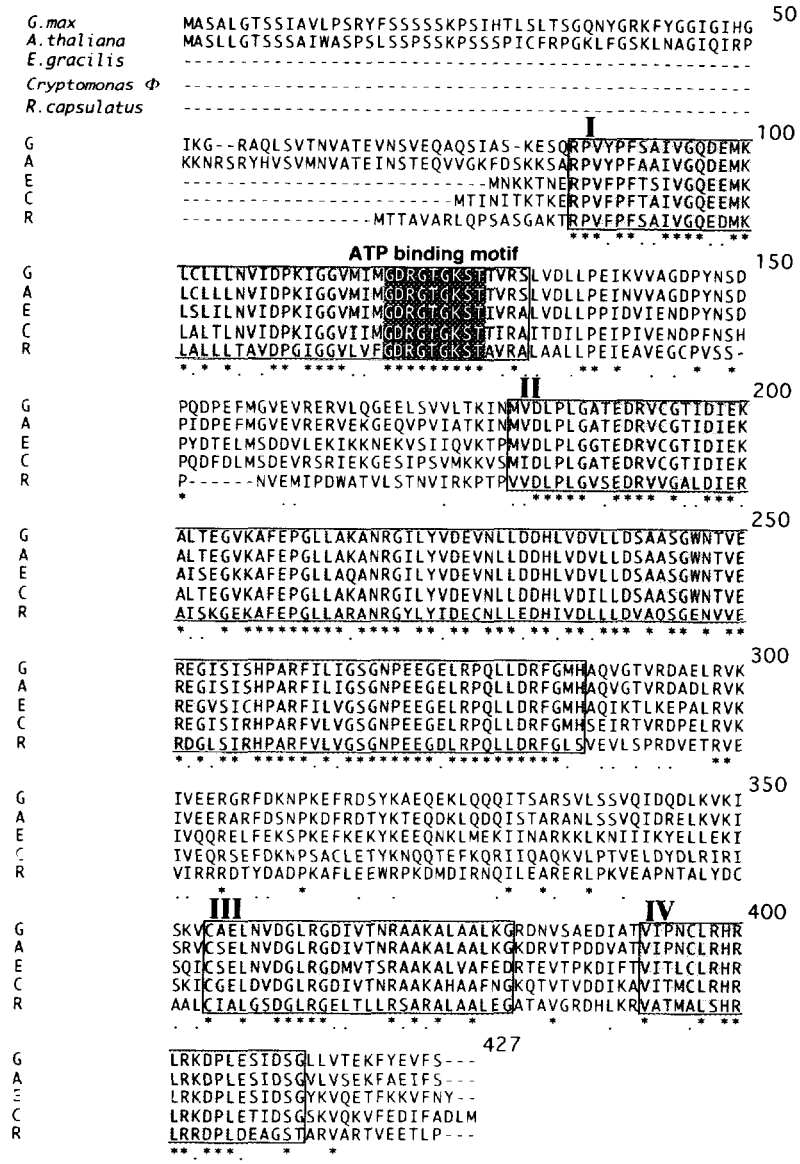

Fig. 1. Alignment of the deduced amino acid sequence of CHLI from *Glycine max* (G), with those of *Arabidopsis thaliana* (A), *Euglena gracilis* (E), *Cryptomonas Φ* (C), and *Rhodobacter capsulatus* (R). Deletion of amino acid is represented by a dash. Four highly conserved regions are designated as I, II, III and IV. The consensus sequence of the ATP-binding protein is indicated in the black box. The bottom line of the alignment shows identical residues present in all sequence (asterisks) and conservative replacements (dots) according to Schwartz and Dayhoff (20).

plants such as *G. max* and *A. thaliana* (nuclear-encoded). This emphasized a close evolutionary relationship.

All three *chlI* gene products displayed similar hydropathy profiles (Fig. 2), suggesting that the tertiary structure of the polypeptides is conserved. Hydropathy plots of *chlI* gene products

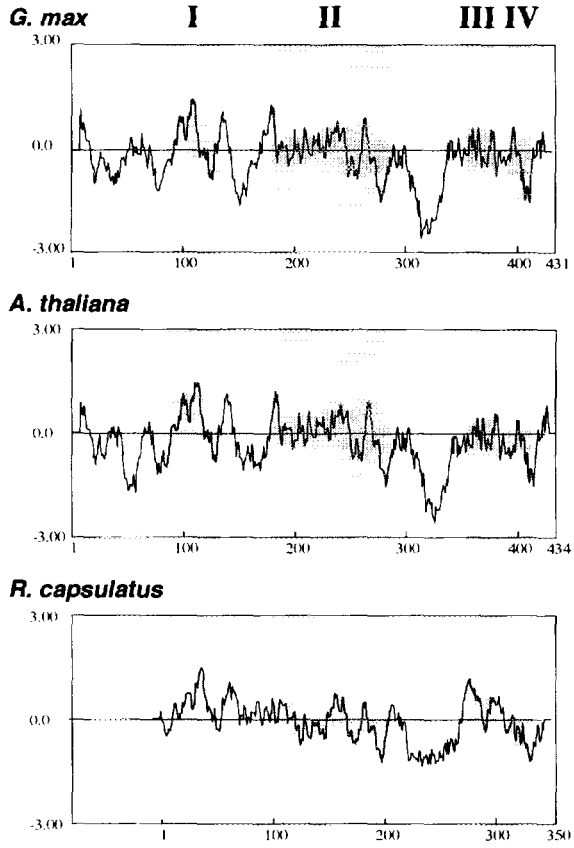

Fig. 2. Comparison of hydropathy plots of *chlI* gene product from *Glycine max* with those of *Arabidopsis thaliana* and *Rhodobacter capsulatus*. Plots were obtained using the Kyte-Doolittle option of DNA strider (21). The positions of the four conserved domains are indicated by Roman numerals I-IV. The values on the Y axis represent the Kyte-Doolittle estimate of hydrophobicity, and the values on the X axis represent the number of amino acid in the gene products.

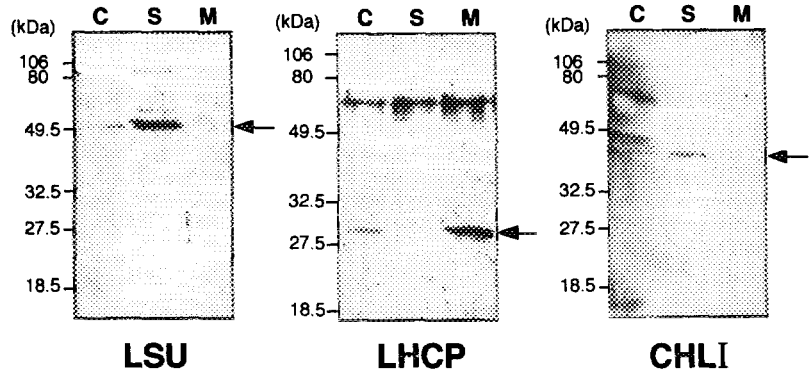

Fig. 3. Western blot analysis of CHLI in chloroplast fractions. Chloroplast (C), soluble fraction (S) and membrane fraction (M). Proteins were probed with antisera against LSU, LHCP and CHLI . Each lane (C, S, M) for LSU and LHCP contains 1 μg of protein, whereas the lane for CHLI contains 75 μg of protein. Arrows denote the migration positions of LSU, LHCP and CHLI.

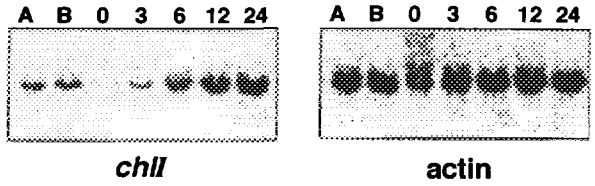

Fig. 4. Effect of light on the expression of *chlI* in SB-P cells. Cell cultures were incubated for 7 days after inoculation and further incubated under dark for 2 days. After the dark adaptation, the cell cultures were exposed to continuous white light for 0 h, 3 h, 6 h, 12 h, 24 h. A is the cell culture incubated in the light for 7 days after inoculation. B was illuminated 3 days longer than A. Actin was used as a control.

including soybean CHLI showed the absence of some strongly hydrophobic region that could constitute membrane-spanning domains, suggesting that soybean CHLI was a soluble protein or loosely membrane-bound.

Western blot analysis

As demonstrated in Fig. 3, the CHLI of soybean cotyledon was localized in the soluble fraction of the chloroplast as was LSU. This is the first direct demonstration that CHLI is localized in the soluble fraction of chloroplast. The CHLI band appeared at approximately 40 kDa which is lower than 46 kDa of the predicted molecular mass from the deduced amino acid sequence, suggesting that CHLI had a chloroplast-targeting signal in the N-terminal region. The difference in molecular masses (6 kDa) between the two polypeptides suggested that the cleavage site was located between 60 th and 70 th amino acid residue from the N-terminus.

Northern blot analysis

chlI mRNA levels increased when dark-adapted SB-P cells were exposed to light (Fig. 4). The levels of *chlI* mRNA before dark adaptation (Fig. 4, A) and after further incubation for 3 days in the light (Fig. 4, B) were comparable and much higher than that just after dark adaptation (Fig. 4, 0), indicating that the expression of *chlI* was reversible in the light-dark transition. The light-induced transcription of *chlI* of soybean was in agreement with the result of *cs* of *Arabidopsis* (2) but not with *Euglena ccs*A gene (14), and with algae (15). The differential effects of light on the expression of this gene may be correlated with the presence of phytochrome mediated signal transduction pathways (18,19).

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