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Nucleotide Sequence of Leghemoglobin cDNA from *Canavalia lineata*

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Poly(A)$^+$ RNA was selected from *Canavalia lineata* root nodule RNA through oligo(dT) cellulose column and used for construction of a cDNA library using λgt10-*Eco*RI arms. The size of the library was 7.2×10^5 pfu/mL. A full length leghemoglobin (Lb) cDNA clone, pCILb1 (687 bp) isolated with soybean Lb probe, contained one open reading frame (ORF) of 447 bp with 54 bp plus 186 bp at 5′ and 3′ untranslated region, respectively. A consensus sequence of plant translation start region (AAAATGGG) was found at 5′ untranslated region, and two polyadenylation-related sequence (AATAAA, AATAAG) and a conserved motif between them (gACTTGTT) were found upstream of poly(A)$^+$ tail consisted of 13 (A)s at 3′ untranslated region. The ORF encoded a polypeptide consisted of 149 amino acids with a molecular weight of 16.2 kD. Deduced amino acid sequences showed high degree of homology values with those of other Lbs ranging from 66% (*Casuarina glauca*) to 85% (*Glycine max*).

Key words: root nodule cDNA library, Lb nucleotide sequence, *Canavalia lineata*

The establishment of effective nitrogen-fixing nodules on leguminous plants requires well-regulated interactions between *Rhizobium* and its host. A number of plant genes known as nodulin genes are specifically induced during the nodule development (Verma *et al.*, 1986). Leghemoglobins (Lb) constitute a major nodulins, whose expression is induced at the late stage of the nodule development just prior to the nitrogen fixation. The function of Lb is to facilitate the diffusion of oxygen at a low tension to the symbiont for metabolic process without inactivating highly oxygen-sensitive nitrogenase (Appleby, 1984).

Four major Lbs encoded by a multigene famlily have been identified in soybean (Verma *et al.*, 1986), and regulation of expression of Lbs have been studied by sequence comparison of 5′ region of the genes and by using transgenic plants carrying chimeric Lb promoter (Jensen *et al.*, 1988; Forde *et al.*, 1990).

However regulatory mechanism of Lb genes during nodule development is still uncertain. Moreover detecting Lb sequences in a variety of plant species is limiting due to only few Lb sequences are known to date (Arredondo and Escamilla, 1991).

Canavalia lineata has canavanine, a nonprotein amino acid, as a nitrogen source during germination (Rosenthal and Rhods, 1984) and grows at the southern part of Korea near Cheju Island. Papers related to the root nodules of *C. lineata* such as isolation of symbiotic *Rhizobium* sp. SNU003 (Kim and An, 1989), changes in nodule specific proteins (Choi and An, 1991) and cloning of *nifH, D* from the symbiont (Kang and An, 1993) have been published. However molecular biological studies on the host genes did not exist.

Accordingly, as a first step to investigate the structure of nodulin genes and regulation of their expression in *C. lineata*, we prepared a root nodule cDNA library and characterized a Lb cDNA clone, pCILb1.

MATERIALS AND METHODS

Enzymes and chemicals

Chemicals and enzymes were purchased from

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BRL (agarose), NEB (restriction enzymes, T4 DNA ligase, primer), Promega (nick-translation kit, Erase-a-base kit), Amersham (α-^{32}P-dCTP, nylon membrane, cDNA synthesis and cloning kit), Dupont (^{35}S-dATP), Pharmacia [Sephadex G-50 (medium)], and USB (DNA sequencing kit).

Plant materials

Canavalia lineata seeds were germinated in the dark for three days, and seedlings were grown in a controlled environment chamber with a photoperiod of 16h at 23℃ for three weeks (Choi and An, 1991). They were inoculated by watering nitrogen-free Hoagland solutions with *Rhizobium* sp. SNU003 (Kim and An, 1990). The nodules were harvested six weeks after inoculation and stored under liquid nitrogen until used.

RNA isolation

Total RNA from the root nodules was prepared according to the guanidinium thiocyanate method (Maniatis *et al.*, 1982), and Poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose (Ausubel *et al.*, 1987).

cDNA library construction

cDNA was synthesized from 5 μg of poly(A)$^+$ RNA from root nodules as template with a cDNA synthesis kit from Amersham by following recommendation from the manufacturer. After addition of *Eco*RI adaptor, cDNA was fractionated on a sephadex G50 column. The fraction containing cDNA longer than 500 bp was used to generate cDNA library in λgt10 as vector and strain NM514 of *E. coli* as host.

Screening and subcloning

Screening for Lb cDNA clones was done with an 0.51 kb *Pst*I fragment in pLb23, a soybean Lb cDNA clone (Sullivan *et al.*, 1981). Approximately 10,000 pfu (plaque forming unit) blotted onto 20 nylon membranes with a diameter 10 cm were screened with the probe by the plaque screening method (Benton and Davis, 1977). *Eco*RI fragments from the selected phage clones were subcloned into pUC19 by following the method of Maniatis *et al.* (1982).

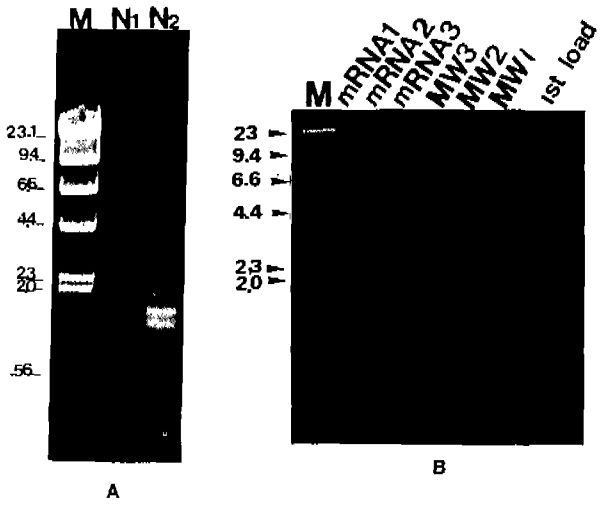

Fig. 1. Photographs of total RNAs extracted from *C. lineata* root nodule (A) and those passed through oligo(dT) cellulose column (B) on 0.8% agarose gel. M, λ/*Hin*dIII size marker; N_1 and N_2, total RNA extracted; mRNA 1, 2 and 3, 1st, 2nd and 3rd final wash eluant, respectively; MW 1, 2 and 3, 1st , 2nd and 3rd middle wash eluant; 1st load, 1st load buffer eluant.

Nucleotide sequencing

Recombinant DNA digested with *Xba*I and *Sph*I was used for constructing deletion series using Erase-a-base kit by following the method of Heinikoff (1984). Selected deletion series were sequenced by dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using M13 reverse primer. The sequencing mixture was resolved on 6% acrylamide/7 M urea gel. Sequences from autoradiogram were analyzed by using PC-GENE, DNASIS and BLAST (Altschul *et al.*, 1990) programs.

RESULTS AND DISCUSSION

Preparation of poly(A)$^+$ RNA

Total RNA from 2 g of *C. lineata* root nodules was separated on 0.8% non-denaturing agarose gel and stained with ethidium bromide (Fig. 1A). It showed several rRNA bands ranging from 0.5 kb to 2.3 kb, tightness of which indicated no noticeable degradation of RNAs during RNA extraction. Dige-

stion of the RNA with RNase-free DNase I did not cause any noticeable change in electrophotogram, showing no DNA contamination (data not shown).

Poly(A)$^+$ RNA was bound to oligo(dT) cellulose, while the remainder of the RNA washed out thoroughly (Fig. 1B, 1st load), resulting in similar band patterns with those of lanes N_1 and N_2 in Fig. 1. Then poly(A)$^+$ RNA was eluted by washing the column with a buffer containing 0.1% SDS to destabilize the dT : rA hybrid (Fig. 1B, mRNA 1, 2, 3). Total 1.2 μg of poly (A)$^+$ RNA was obtained from 2 g of root nodules.

Construction of cDNA library

The constructed cDNA library was titered on wild type L87 and NM514 (high frequency lysogeny mutant) *E. coli* host, respectively. The ratio of titers on L87 : NM514 was 442, while titers of the background and the ligation control reactions on both host were in the order of 10^3 and 10^6 (pfu/ug arms), respectively. These results showed that each step of library construction was performed successfully, and cDNA library size was calculated as 7.2×10^5 pfu/mL.

DNAs from ten individual plaques randomly selected from 5×10^4 plaques/plate were digested with *Eco*RI and analyzed on 1.0% agarose gel (data not shown). It showed 8 out of 10 plaques had inserts ranging from 0.5 kb to 2 kb, meaning 80% of recombination rate.

The library was also screened with several heterologous probes, such as soybean Lb, alfalfa aspartate aminotransferase (1.7 kb *Not*I/*Bam*HI fragment in pAAT37 (Gantt *et al.*, 1992)) and soybean uricase II (0.85 kb *Pst*I fragment in pNOD35 (Nguyen *et al.*, 1985)). Each of these was represented by 5×10^3 (10%), 33 (0.07%) and 50 (0.1%) clones, respectively, out of 5×10^4 plaques (data not shown).

These results showed that the construction of cDNA library was successful enough to select low-frequency mRNAs in the root nodules of *C. lineata*.

Isolation of Lb cDNA clones

About 5×10^4 plaques were screened with the soybean Lb cDNA as a probe at a plate density of 500-1,000 phages/90 mm plate. About 10% (5×10^3 clones) of the plaques gave strong signals, which

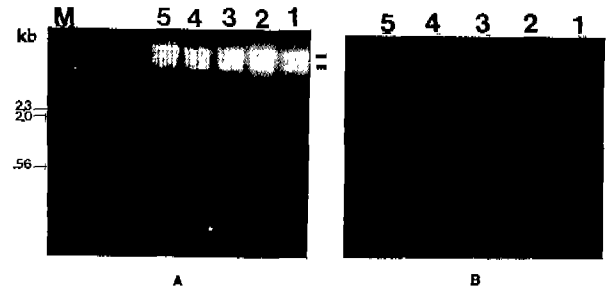

Fig. 2. Electrophoresis of *Eco*RI digested phage DNAs from clones λCILb 1-5 (A) and corresponding blot hybridization pattern with soybean Lb probe (B). M, λ/*Hin*dIII size marker. Bars indicate 32.7 and 10.6 kb λgt10 arms.

was a little lower value than 12-15% of the soybean nodule cDNA library (Fuller *et al.*, 1983). Following secondary screening of these 20 clones, five clones were finally selected for further characterization by Southern hybridization (Fig. 2). The insert size of the recombinant phage DNAs digested with *Eco*RI was ranging from 450 bp to 780 bp (Fig. 2A). Corresponding blot hybridized with the probe showed that all inserts had similar sequences with the Lb (Fig. 2B). Among them, inserts of 700 bp, 780 bp and 700 bp from λCILb 1, 2, 5 were subcloned into pUC 19, and resulting recombinant plasmids were named as pCILb 1, 2, 5, respectively.

Nucleotide sequence of pCILb1

Deletion series of pCILb1 with about 150 bp differences (data not shown) were obtained and used for sequencing 687 bp (Fig. 3). It is consisted of one open reading frame (ORF) of 447 bp encoding 149 amino acids with 54 bp at 5′ plus 186 bp at 3′ untranslated region.

A putative translation start region AAAATGGG was found at 52 nucleotide (nt), which is very similar to that (ANNATGGC) of higher plant genes (Elliston and Messing, 1988).

Two putative poly(A) addition signals, AATAAA and AATAAG, and poly(A)$^+$ tail of 13As were found 21 nt, 55 nt and 151 nt downstream of the termination codon, respectively. Although functional analysis on them remains to be carried out, existance of more than two signals for polyadenylation and importance of interaction between them were reported in plants (Joshi, 1987).

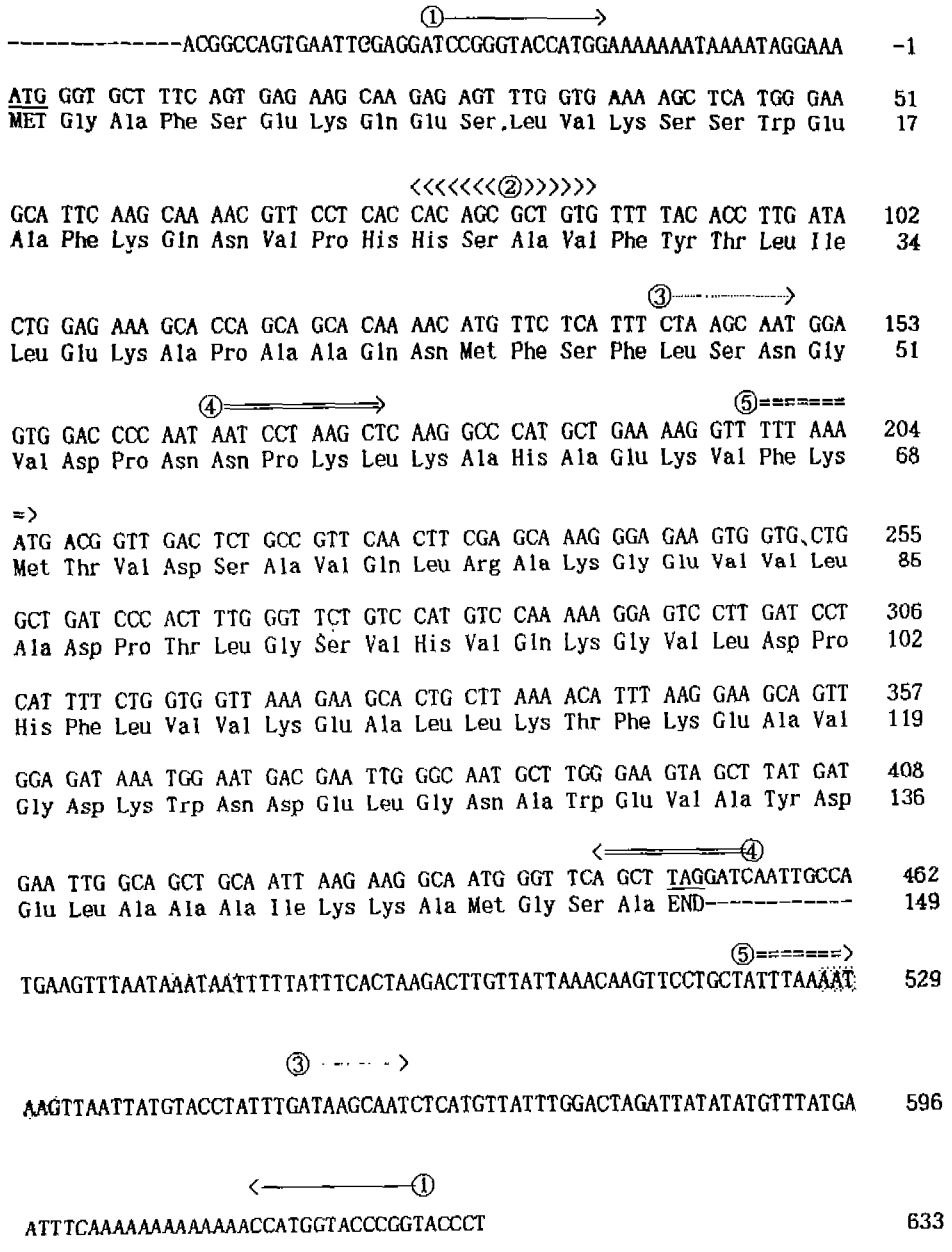

Fig. 3. The nucleotide sequence of pC1Lb1 encoding *C. lineata* leghemoglobin and deduced amino acid sequence. Translation start and termination codon are underlined and consensus element related to polyadenylation highlighted. Arrows above the sequence indicate ①, inverted repeat (CCGGGTACCATGG); ②, palindromic repeat (CACAGCGCTGTG); ③, direct repeat (TAAGCAAT); ④, inverted repeat (ATCCTAAGCT); ⑤, direct repeat (TTAAAAT). GenBank accession number of this sequence is U09671.

Between two putative poly(A) additional signals, additional motif (gACTTGTT) was found, which is very similar to AACTTGTT motif known to be present at homologous positions in Lb genes from soybean, kidney bean and broad bean (Kuhse and Pühler, 1987). However inverted form of this sequence was found 5 nt downstream of this motif in contrast to other Lb genes in which inverted repeats were found in the coding region.

There are two direct repeats, one palindrome sequence and two inverted repeat sequnces in the whole sequences (Fig. 3), the roles of which are not

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                1                                                  50

                   *  * * **   F       *  G    L*          *   *G ** *
Consensus       GAFTEKQEA LVNSSWEAFK QNIPQYSVVF YTSILEKAPA AKNLFSFLKD
                  +    +                                     +
Cl Lb1          MGAFSEKQES LVKSSWEAFK QNVPHHSAVF YTLILEKAPA AQNMFSFL--
Gm Lbc2         MGAFTEKQEA LVSSSFEAFK ANIPQYSVVF YTSILEKAPA AKDLFSFL--
Gm Lba          -GAFTEKQEA LVSSSFEAFK ANIPQYSVVF YTSILEKAPA AKDLFSFL--
Ms Lb3          MG-FTDKQEA LVNSSWESFK QN-PGNSVLF YTIILEKAPA AKGMFSFLKD
Ps Lb1          -G-FTDKQEA LVNSSSE-FK QNLPGYSILF YTIVLEKAPA AKGLFSFLKD
Cg Lb           --ALTEKQEA LLKQSWEVLK QNIPAHSLRL FALIIEAAPE SKYVFSFLKD

                51                                                 100

                   PQT *  *  **   *        *A *    *V   G    A    *  *A
Consensus       SNGVDPNNPK LQAHAEKVFG LVRDSAGQLR ATGTVVLADA TLKRLGSIHV

Cl Lb1          SNGVDPNNPK LKAHAEKVFK MTVDSAVQLR AKGEVVLADP TL---GSVHV
Gm Lbc2         SNGVDPSNPK LTGHAEKLFG LVRDSAGQLK ANGTVVVADA AL---GSIHA
Gm Lba          SNGVDPSNPK LTGHAEKLFG LVRDSAGQLK ANGTVV-ADA AL---GSIHA
Ms Lb3          SAGVQDS-PK LQSHAEKVFG MVRDSAAQLR ATGGVVLGDA TL---GAIHI
Ps Lb1          TAGVEDS-PK LQAHAFQVFG LVRDSAAQLR TKGEVVLGNA TL---GAIHV
Cg Lb           SNEIPENNPK LKAHAAVIFK TICESATELR QKGHAVWDNN TLKRLGSIHL

                101                                                150

                      *  *   * * *  *       **E * S**  ** * *   **     PSS
Consensus       QKGVTDPHFV VVKEALLKTI KEAVGDKWSD ELSTAWEVAY DELAAAIKKA MKDAAT (155)
                                                +
Cl Lb1          QKGVLDPHFL VVKEALLKTF KEAVGDKWND ELGNAWEVAY DELAAAIKKA MGSA   (149)
Gm Lbc2         QKAITDPQFV VVKEALLKTI KEAVGDKWSD ELSSAWEVAY DELAAAIKA        (144)
Gm Lba          QKAITDPQFV VVKEALLKTI KEAVGDKWSD ELSSAWEVAY DELAAAIKA        (143)
Ms Lb3          QKGVVDPHFA VVKEALLKTI KEVSGDKWSE ELNTAWEVAY DALATAIKKA M     (145)
Ps Lb1          QKGVTNPHFV VVKEAILQTI KKASGNNWSE ELNTAWEVAY DGLATAIKKA MKTA  (147)
Cg Lb           KNKITDPHFE VMKGALLGTI KEAIKENWSD EMCQAWTEAY NQLVATIKAE MKE   (151)
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Fig. 4. Comparison of amino acid sequences from different leghemoglobins (Lbs). All Lbs except the *Glycine max* (Gm) Lba and *Casuarina glauca* (Cg) are deduced from the nucleotide sequences (Consensus, Arredondo-Peter and Escamilla 1991; *Canavalia lineata* (Cl), this work; Gm Lbc2, Wiborg *et al.*, 1982; Gm Lba, Ollis *et al.*, 1983; *Medicago sativa* (Ms), Kiss *et al.*, 1987; *Pisum sativum* (Ps), Lehtovarra *et al.*, 1980; Cg, Kortt *et al.*, 1988). Thirty nine amino acids highly conserved are marked with asterisks and interchangeable residues are shown, respectively, above the consensus sequences. Four residues of Cl Lb1, which are differnt from the highly conserved residues but changed to similar one, are marked with plus sign above the Cl Lb1 sequences. Numbers at the end of the sequences indicate the total number of amino acids.

certain, though inverted repeats were suggested to be involved in secondary structure of mRNA and translational regulation of Lb genes (Kuhse and Pühler, 1987).

The nucleotide sequence of ORF showed identity values ranging from 66% with *Casuarina glauca* to 85% with *Glycine max* Lbs (data not shown).

Amino acids sequence of pClLb1

The ORF encodes a polypeptide of 149 amino acids with a molecular weight of 16.2 kD, little bigger than 15.7 kD estimated from SDA-PAGE of *C. lineata* root nodules (Choi and An, 1991). Deduced amino acid sequences showed high degree of homo-

logy values with those of other Lbs, ranging from 66% with *Casuarina glauca* to 85% with *G. max* (data not shown). According to the consensus plant Lb, proposed by Arredondo-Peter and Escamilla (1991) and composed of 465 nt or 155 amino acids, there are 39 amino acids common to all Lbs (Fig. 4). Among them, four amino acids of *C. lineata* Lb were different from the common amino acids. However all the changes were recorded positive by BLAST program; Thr-Ser (5th), Ala-Ser (10th), Lys-Asp (42 nd), Ser-Asn (129th).

Hydropathy analysis of this Lb (data not shown) also revealed that there was no signal peptide or membrane-associated signal, which is consistent with the fact that Lb can not be transported into peribacterial membrane, limiting its location to cytoplasm of plant cell (Robertson *et al.*, 1984).

These results suggested that the Lb in *C. lineata* is a typical plant Lb in its nucleotide and amino acid sequences. The studies on the identification of this Lb by *in vitro* translation and 2-D gel electrophoresis and isolation of a genomic sequence from the genomic library are being undertaken.

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해녀콩의 Leghemoglobin cDNA 염基序列

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적 요

Oligo(dT) column을 이용하여 해녀콩(*Canavalia lineata*)의 뿌리혹 RNA로부터 poly(A)$^+$ RNA를 선별한 후 λgt10-*Eco*RI arm을 이용하여 cDNA library를 작성하였으며, 작성된 library는 7.2×10^5 pfu/mL의 titer 값을 나타냈다. 콩의 leghemoglobin(Lb) 유전자를 탐침으로 사용하여 선별한 해녀콩의 Lb cDNA 클론인 pCILb1은 447 bp의 open reading frame (ORF), 54 bp 및 186 bp의 5'와 3'의 비해독 부위로 구성된 687 bp의 삽입 DNA를 갖고 있었다. 염기서열 분석 결과 식물 유전자의 개시 코돈 부위 보존서열인 AAAATGGG가 5' 비해독 부위에, poly(A) 첨가 관련 서열인 AATAAA와 AATAAG 및 이들 사이에 보존된 서열인 gACTTGTT가 13개의 A로 구성된 poly(A)$^+$ tail 상류 3' 비해독 부위에 존재하였다. ORF는 149개의 아미노산으로 구성된 16.2 kD의 단백질을 암호화하고 있었으며, 추론된 아미노산 서열은 다른 Lb 서열과 66%(*Casuarina glauca*)에서 85%(콩)까지의 높은 유사성을 보여주었다.

주요어 : 뿌리혹 cDNA library, Lb 염기서열, 해녀콩

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