식물학회지 제32권 제1호
Korean J. Botany 32(1) : 33—40(1989)

Cloning of cDNA Encoding the Precursor to the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase in Pea (*Pisum sativum*)

Chang, Moo-Woong, Yong-Bum Koo and *Han-Jip Kim

(Department of Biology, Yeungnam University, Gyongsan)

완두콩(*Pisum sativum*)에서 Ribulose-1,5-Bisphosphate Carboxylase Small Subunit 유전자의 cDNA 클로닝과 광유도성 발현에 관한 연구

張 茂 雄 · 具 龍 範 · 金 翰 集*

(嶺南大學校 理科大學 生物學科)

ABSTRACT

Polysomal polyadenylated mRNAs which were purified from pea leaves were fractionated by sucrose grandient sedimentation. Fractions corresponding to the peak at 11.5S were found to contain mostly mRNA encoding the precursor polypeptide to the small subunit of ribulose bisphosphate carboxylase (rbcS) by *in vitro* translation in wheat germ extract.

Double-stranded cDNA which was synthesized from the 11.5S mRNA was cloned into Hind Ⅲ site of plasmid pBR 325. A cDNA clone, H24, was identified to code for rbcS. *In vitro* translation product of the hybridization-selected mRNA was molecular weight 20,000, presumably the precursor of rbcS. The nucleotide sequences of the H24 showed almost complete homology with the sequences encoding the transit peptide of the rbcS-3A gene which was reported by Fluhr *et al.* (1986).

INTRODUCTION

The chloroplast enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.4.4.39, also known as Fraction I Protein of chloroplast, here abbreviated to Rubisco) is a bifunctional enzyme that catalyzes the primary competing reactions in both photosynthetic and photorespiratory carbon metabolism. The Rubiscos from higher plants, algae and some bacteria are

This research was supported by a grant from the Ministry of Education, Korea, 1987.

*Present address: Department of Biology, Ajou University, Suwon.

composed of eight large (L, 55,000 daltons) and eight small (S, 15,000 daltons) subunits (Blair and Ellis, 1973; Miziorko and Lorimer, 1983).

Both the small subunit of the Rubisco and the light harvesting chlorophyll a/b-binding protein are the major components of the leaf proteins (Ellis, 1979) and specified by nuclear genes (rbcS and cab, respectively) whose transcript levels are light-regulated (Highfield and Ellis, 1978; Cashmore, 1979; Bedbrook *et al.*, 1980; Coruzzi *et al.*, 1984; Green *et al.*, 1978; Nagy *et al.*, 1986, 1987).

In the pea, the isolation of cDNA and genomic clones corresponding to rbcS and cab has facilitated investigations into their structure and expression (Bedbrook *et al.*, 1980; Coruzzi *et al.*, 1984; Fluhr *et al.*, 1986; Green *et al.*, 1987). Both rbcS and cab mRNAs are translated in cytoplasm as larger precursors containing amino-terminal transit peptides which are cleaved during or shortly after their post-translational transport into chloroplast (Apel and Kloppstech, 1978; Chua and Schmidt, 1978; Highfield and Ellis, 1978). The sizes of the precursor polypeptides are 20,000 daltons for the rbcS gene (Cashmore *et al.*, 1978; Highfield and Ellis, 1978) and 33,000 daltons for the cab gene (Cashmore *et al.*, 1978; Broglie *et al.*, 1981) when polyA-RNAs from pea cytoplasmic ribosomes are translated by a cell-free protein-synthesizing system.

In this study, we report the cloning of cDNA encoding the precursor to the small subunit of the Rubisco as a probe for screening nuclear genes from a genome library of the pea. We studied transcription of the light-regulated rbcS gene using an *in vitro* translation system, and characterized the cDNA for the rbcS gene by translating hybridization-selected mRNAs *in vitro* and sequencing the cDNA.

MATERIALS AND METHODS

Plant materials. The pea (*Pisum sativum* L. cv. Sparkle) seeds were purchased from Heung-Nong Seed Company and local markets and grown for 9 days in darkness followed by continuous illumination (2000 lux) for 48 hours for illuminated leaves or grown for 11 days in darkness for etiolated leaves.

RNA extraction and fractionation. Illuminated pea leaves were pulverized and a polysomal pellet was obtained by centrifugation (Sorvall T875 fixed angle rotor; 40,000 rpm, 4 hours) with a 1.5 M sucrose cushion. Polysomal RNA was purifed from the polysome by sedimenting RNA through a 5.7 M CsCl cushion (Sorvall AH627 swing bucket; 27,000 rpm, 16 hours) (Ullrich *et al.*, 1977; Chirwin *et al.*, 1979). Poly(A)$^+$ mRNA was prepared by poly(U)-Sepharose chromatography of polysomal RNA (Cashmore *et al.*, 1978) and then fractionated by centrifugation (Sorvall AH627 swing bucket; 27,000 rpm, 22 hours) through sucrose gradients (5–20% w/v). The gradient was fractionated by an upward replacement method with continuous monitoring of absorbance at 254 nm by the LKB UV cord and recorder. Among 30 fractions obtained, fractions containing 11.5S and 14S mRNAs were

presumed to be mRNAs of the rbcS and cab genes, respectively, based on the translation products of the mRNAs in a cell-free translation system (Cashmore *et al.*, 1978; Cashmore, 1979).

Cloning of rbcS cDNA. The 11.5S mRNA was transcribed with AMV reverse transcriptase for synthesis of the first strand. The second stand was synthesized by Klenow fragment (Bedbrook *et al.*, 1980; Broglie *et al.*, 1981). The double strand cDNA was inserted into pBR325 using Hind Ⅲ linkers. Plasmids were transformed into *E. coli* HB101.

Identification of clones containing cDNA. Chimeric plasmids were detected by their sensitivity to tetracycline. Such clones were arrayed on a solid agar medium and grown. From tetracycline-sensitive colonies, recombinant plasmids were isolated by an alkaline lysis miniprep method (Maniatis *et al.*, 1982), and dot-blotted into a nitrocellulose filter for hybridization with a DNA probe (Belts *et al.*, 1983). The labelled DNA probe was prepared from the first strand cDNA which was synthesized from 11.5S peak mRNA. Two positive clones were selected by the dot-blot hybridization, and named H18 and H24.

Hybridization and elution of specific RNA. The plasmid DNA from the transformed H24 clone was denatured and adsorbed onto a nylon filter (NYX, Hoefer Scientific Instrument) and hybridized with total poly(A)$^+$ mRNAs from leaves (Broglie *et al.*, 1981; Maniatis *et al.*, 1982). The specific mRNA which was hybridized to the filter-bound DNA was eluted from the nylon filter and ethanol precipitated with 6 μg of wheat germ tRNA as a carrier (Grossman *et al.*, 1980).

Wheat germ cell-free translation. The specific mRNA (1 μg) was used to program a wheat germ protein-synthesizing system (Highfield and Ellis, 1978; Anderson *et al.*, 1983). The translation product which was labelled with ^{35}S-methionine was electrophoresed on slab SDS-polyacrylamide gradient (7–18% w/v) and analyzed by fluorography. In addition, total poly(A)$^+$ mRNAs from both illuminated and dark-grown leaves were translated *in vitro*. Light-induced transcription of mRNA was identified by comparing protein bands between the illuminated and the dark grown leaves by fluorography.

cDNA sequence analysis. The cDNA insert from clone H24 was used to determine the nucleotide sequence of a rbcS mRNA. The cDNA was subcloned into phage M13mp18 to prepare templates for DNA sequencing (Messing, 1983) by dideoxy chain termination and [α-^{35}S] dATP, and a forward sequencing primer (17–mer, New England Biolabs, 1212) was employed. About 100 nucleotides, one fourth of the cDNA, were sequenced.

RESULTS AND DISCUSSION

Most of eukaryotic mRNAs contain poly(A) sequence which facilitates isolation of this RNA fraction by affinity chromatography. Light increases transcription of some poly(A)-containing RNA during the first 6 hours of illumination in barley (Apel and Kloppstech, 1978). The authors also reported that poly(A)-containing RNA levels remained constant during 18 hours

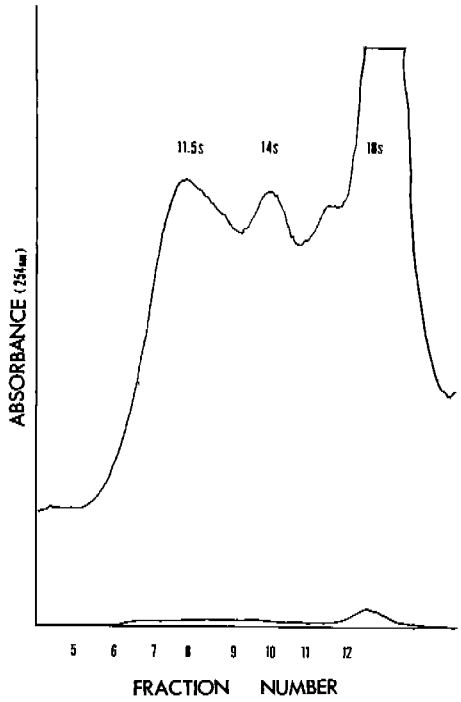


Fig. 1. Fractionation of poly(A)$^+$ mRNA by 5–20% sucrose gradients and continuous monitoring of absorbance at 254 nm. Two prominent mRNA peaks. 11.5S and 14S, which corresponded to the fractions 7, 8, 9 and 10 were detected in addition to 18S rRNA.

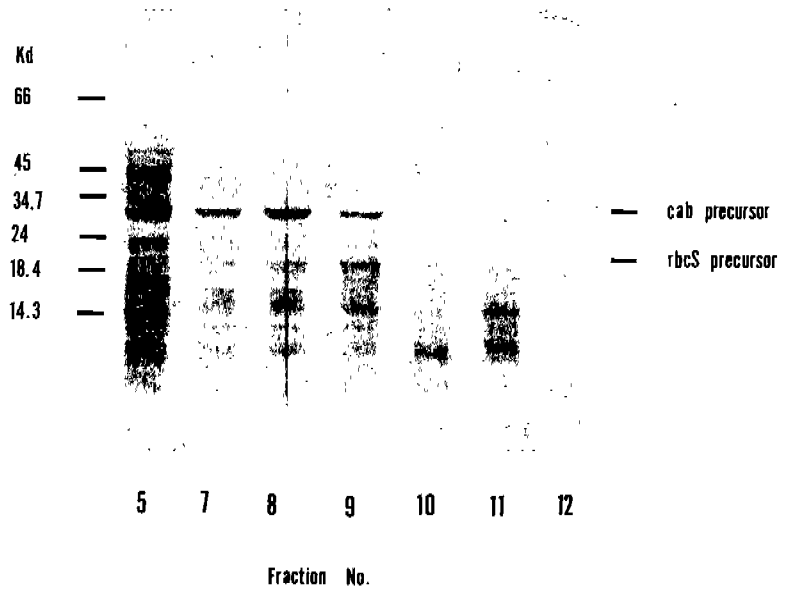
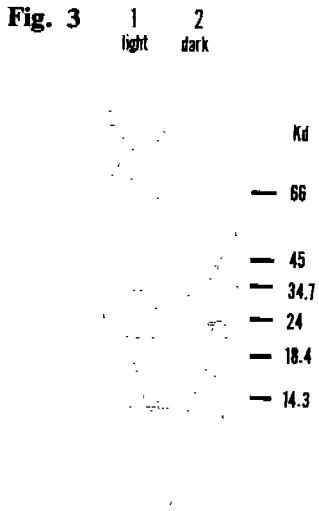


Fig. 2. Fluorography of cell-free translation products from fractionated poly(A)$^+$ mRNAs which were described in Fig. 1. Translation products were analyzed by SDS-polyacrylamide gradient (7–18%) gel electrophoresis and fluorography. Translation products of fractions 7 and 8 contained the rbcS precursor polypeptide as the most porminent species. Kd: kilo dalton.

of illumination after the initial increase. Since the major cytoplasmically synthesized leaf proteins are the small subunit of Rubisco and the cab protein, the mRNAs coding for these polypeptides form the major components of leaf cytoplasmic poly(A)$^+$ mRNA. In our experiment, the different poly(A)-containing RNA fractions were isolated from the pea leaves which were illuminated for 48 hours (Fig. 1), and mRNA of each fraction was translated in a heterrologous cell-free wheat germ system (Fig. 2).

Although poly(A)$^+$ RNA was purified by two rounds of poly(U)-Sepharose column choromatography, a large amount of 18S ribosomal RNA was shown in Fig. 1. The two prominent mRNA peaks corresponding to 11.5S and 14S mRNAs produced proteins of 20,000 daltons and 33,000 daltons, respectively, in the wheat germ cell-free translation system as shown in Fig. 2. Cashmore *et al.* (1978) and others previously found that polypetides of molecular weights 20,000 daltons and 33,000 daltons were the precursors to the small subunit of Rubisco and cab proteins, respectively.

Cell-free translation products of the total leaf mRNAs were compared with the difference sought between the illuminated and the dark-grown etiolated leaves in Fig. 3. Although the dark room failed to provide complete darkness, the etiolated leaves showed absence of the band



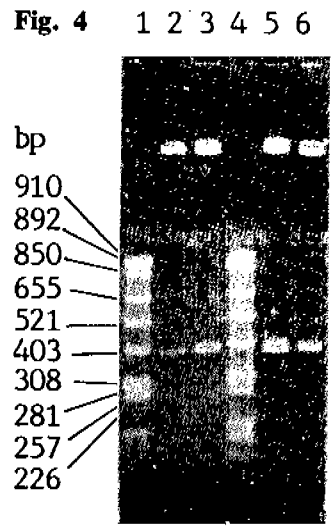


Fig. 3. Fluorography showing light-inducible expression of rbcS gene. Cell-free translation products of mRNAs between light-grown and dark-grown pea leaves were compared. The light-grown leaves had the rbcS precursor of 20,000 daltons whereas the dark-grown leaves showed no band in the position for the rbcS precursor.

Fig. 4. Hind Ⅲ cleavage of recombinant plasmids of two positive clones, H24 and H18. Plasmids (pH24 and pH18) were purified by EtBr-CsCl equlibrium density gradient centrifugation, and digested with Hind Ⅲ. The excised DNA inserts were separated from the vector by agarose gel electrophoresis. Both plasmids carry about 380 base paris of cDNA inserts.
bp, base pair; lanes 1 and 4, Alu I digests of pBR 325; lanes 2 and 5, pH24; lanes 3 and 6, pH18.

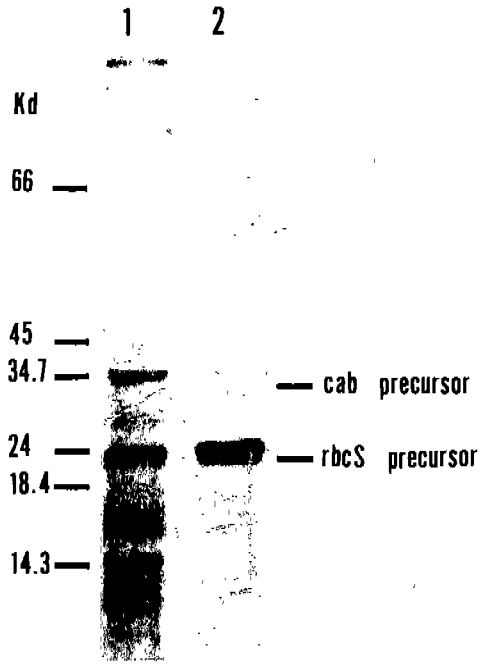


Fig. 5. Comparison of translation products between the total mRNA and the mRNA which was hybridized to filter-bound cDNA of H24 clone.
Lane 1 : Cell-free translation products of light-grown pea leaf mRNA.
Lane 2 : Cell-free translation products of mRNA which was isolated from DNA-RNA hybridization.

in the position presumed for the precursor to the small subunit of the Rubisco, indicating light-inducible expression of the rbcS gene. However, these etiolated leaves had a polypeptide of 33,000 daltons (Fig. 3, lane 2) which was presumed to be the precursor to the light-harvesting chlorophyll a/b-binding (cab) protein whose transcript levels were also regulated by light as well. It appeared that induction of cab genes requires much lower amount of light than that for rbcS genes.

The tranformed clone H24 contained cDNA chimeric plasmids. H24 cDNA was about 380 base pairs long, a half-length of a rbcS mRNA (Fig. 4). The cDNA insert was excised and separated from the plasmids of clone H24 by 1.5% agarose gel electrophoresis and specific mRNA which was isolated from RNA-DNA hybridization on the nylon filter produced only the precursor protein of the rbcS gene when the mRNA was translated *in vitro* (Fig. 5, lane 2).

The partial sequence (100 nucleotides) of H24 cDNA and the deduced amino acid sequence from the sequence were compared with those of the five known genomic DNAs for rbcS genes which were reported by Coruzzi *et al.* (1984), Timko *et al.* (1985) and Fluhr *et al.* (1986). H24 cDNA contained a coding sequence for the additional amino acids (a transit peptide) located at the N-terminus of the matured small subunit peptide (data not shown). Among the five genomic rbcS genes, the rbcS-3A which was reported by Fluhr *et al.* (1986) showed 100% homolgy with the H24 cDNA for the nucleotide encoding the transit peptide. The result indicated that the poly(A)$^+$ mRNA which was utilized as a templated for cDNA synthesis was the mRNA of the rbcS-3A gene. Further study is in progress by utilizing this cDNA as a

probe to screen the nuclear genes from a genome library of the pea.

적 요

엽록체 효소인 ribulose-1,5-bisphosphate carboxylase의 small subunit 유전자(rbcS)의 광유도성 발현을 조사하고, 핵유전자를 분리하기 위한 probe로 사용하고자 rbcS cDNA를 클로닝하였다.

빛을 조사한 완두콩 잎에서 polysomal poly(A)$^+$ mRNA를 분획하고 wheat-germ extract를 사용하여 번역시킨 결과, 11.5S peak에 해당하는 분획에 rbcS 유전자의 mRNA가 주종을 이루고 있는 것이 밝혀졌다. 11.5S peak mRNA로부터 cDNA를 pBR325의 Hind Ⅲ 부위에 클로닝하고 이 cDNA와 혼성화반응시켜 얻어진 mRNA의 *in vitro* 번역 단백질이 rbcS 전구체 단백질 (20000 dalton)임을 확인하였다.

이 rbcS cDNA의 염기서열은 rbcS 전구체 단백질의 transit peptide에 해당하는 부분으로서 Fluhr등 (1986)이 발표한 rbcS-3A 유전자의 염기서열과 100% homology를 보였다.

REFERENCES

Anderson, C.W., J.W. Straus and B.S. Dudock. 1983. Preparation of a cell-free protein-synthcizing system from wheat germ. *In*, Methods in Ezymology. R. Wu, L. Grossman and K. Moldave (eds.). Vol 101, Academic Press, New York. pp. 635–644.

Apel, K. and K. Kloppstech. 1978. The plastid membranes of barley (*Hordeum vulgare*) light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll a/b protein. *Eur. J. Biochem.* **85**: 581–588.

Bedbrook, J.R., S.M. Smith and R.J. Ellis. 1980. Molecular cloning and sequencing of cDNA encoding the precursor to the small subunit of chloroplast ribulose-1,5-bisphosphate carboxylase. *Nature* **287**: 692–697.

Belts, G.A., K.A. Jacob, T.H. Eichbush, P.T. Cherbas and F.C. Kafatos. 1983. Isolation of multigene families and determination of homologies by filter hybridization methods. *In*, Methods in Enzymology. R. Wu, L. Grossman and K Moldave (eds.). Vol. 100, Academic Press, New York. pp. 266–285.

Blair, G.E. and R.J. Ellis. 1973. Protein synthesis in chloroplast. I. Light-driven synthesis of large subunit of Fraction I Protein by isolated pea chloroplasts. *Biochem. Biophys. Acta* **319**: 223–234.

Broglie, R., G. Bellemare, S.G. Bartlett, N.-H. Chua and A.R. Cashmore. 1981. Cloned DNA sequences complementary to mRNAs encoding precursors to the small subunit of ribulose-1,5-bisphosphate carboxylase and a chlorophyll a/b binding polypeptide. *Proc. Natl. Acad. Sci.* USA **78**: 7304–7308.

Cashmore, A.R. 1979. Reiteration frequency of the gene coding for the small subunit of ribulsoe-1, 5-bisphosphate carboxylase. *Cell* **17**: 383–388.

Cashmore, A.R., M.K. Broadhurst and R.E. Gray. 1978. Cell-free synthesis of leaf protein: Identification of an apparent precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. USA* **75**: 655–659.

Chirwin, J.M., A.E. Przybyla, R.J. MacDonald and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299.

Chua, N.-H. and G.W. Schmidt. 1978. Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci.* USA **75**:

6110–6114.

Coruzzi, G., R. Broglie, C. Edwards and N.-H. Chua. 1984. Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose–1,5–bisphosphate caboxylase. *EMBO J.* **3**: 1671–1679.

Ellis, R.J. 1979. The most abundant protein in the world. *Trends in Biochem. Sci.* **4**: 241–244.

Fluhr, R., P. Moses, G. Morelli, G. Coruzzi and N.-H. Chua. 1986. Expression dynamics of the pea rbcS multigene family and organ distribution of the transcripts. *EMBO J.* **5**: 2063–2071.

Green, P.J., S.A. Kay and N.-H. Chua. 1987. Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbcS–3A gene. *EMBO. J.* **6**: 2543–2549.

Grossman, A., S.G. Bartlett and N.-H. Chua. 1980. Energy dependent uptake of cytoplasmically synthesized polypeptides by chloroplasts. *Nature* **285**: 625–628.

Highfield, P.E. and R.J. Ellis. 1978. Synthesis and transport of the small subunit of chloroplast ribulose-bisphosphate carboxylase. *Nature* **271**: 420–424.

Maniatis, T., D.F. Fritsch and J. Sambrook. 1982. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p. 545.

Messing, J. 1983. New M13 vectors for cloning. *In,* Methods in Enzymology. R. Wu, L. Grossman and K. Moldave (eds.). Vol. 101, Academic Press, New York. pp. 20–78.

Miziorko, H.M. and G.H. Lorimer. 1983. Ribulose–1,5–bisphosphate carboxylase-oxygenase. *Ann. Rev. Biochem.* **52**: 507–535.

Nagy, F., S.A. Kay, M. Boutry, M.-Y. Hsu and N.-H. Chua. 1986. Phytochrome-controlled expression of a wheat cab gene in transgenic tobacco seedlings. *EMBO. J.* **5**: 1119–1124.

Nagy, F., M. Boutry, M.-Y. Hsu, M. Wong and N.-H. Chua. 1987. The 5'-proximal reglon of the wheat cab–1 gene contains a 268–bp enhancer-like sequence for phytochrome for phytochrome response. *EMBO J.* **6**: 2537–2542.

Timko, M.P., A.P. Kausch, J.M. Hand, A.R. Cashmore, L. Herrera-Estrella, G. van den Broeck and M. van Montagu. 1985. *In.* Molecular Bilogy of Photosynthetic Apparatus. K.E. Steinbeck, S. Bonitz, C.J. Arntzen and L. Bogorad (eds.). Cold Spring Harbor Press, New Yrok. pp. 381–396.

Ullich, A., J. Shine, J.M. Chirwin, R. Pictet, E. Tischer, W.J. Rutter and H.M. Goodman. 1977. Rat insulin gene: Construction of plasmid containing the coding sequences. *Science* **196**: 1313–1319.

(Received December 7, 1988)