J. Biochem. Mol. Biol. Vol. 30, No. 3, pp. 173~176 May 31, 1997

Isolation and Characterization of a cDNA for a Ribulose-1,5-Bisphosphate Carboxylase Small Subunit in Spinach

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(Received February 17, 1997)

Abstract : We isolated a cDNA clone that encodes a ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) from spinach using a soybean rbcS cDNA as a probe. The small subunit consists of 180 amino acids including a transit peptide of 57 residues. Comparison of the amino acid sequence with those of other plant species shows a maximum of 70-80% identical residues. Southern blot analysis suggests the existence of multiple rbcS genes in the spinach genome. Northern blot analysis indicates that the rbcS gene is expressed predominantly in leaves and that the expression of the gene is induced by light.

Key words : gene expression, photoregulation, rbcS, spinach

Ribulose-1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39), one of the most abundant proteins in leaves of light-grown plants, plays an essential role in the photosynthetic pathway by incorporating CO_2 into ribulose-1,5-bisphosphate and yielding two molecules of 3-phosphoglycerate. In eukaryotes, this enzyme consists of eight large subunits and eight small subunits. The small subunits are encoded by nuclear genes (rbcS) and must be transported into the chloroplast to become the holoenzyme by assembling with the large subunits which are encoded by chloroplast genes (rbcL) (Miziorko and Lorimer, 1983). The rbcS genes have been cloned from a wide range of plant species (Dean *et al.*, 1989). Their sequence comparison reveals extensive similarity, indicating a strong evolutionary pressure for the structural/functional conservation of the polypeptide.

The rbcS genes, along with chlorophyll a/b-binding protein (cab) genes, have been used as a model system to investigate the molecular mechanisms underlying the light-regulated expression of plant genes. It is believed that stimulation of photoreceptors by light leads to the increased transcription of rbcS genes. However, the detailed signal transduction pathway from light capture to gene activation remains to be established.

In this report we present the nucleotide sequence of a spinach rbcS cDNA. The predicted amino acid sequence shows a high degree of homology with those of other plant species. The expression of the spinach rbcS gene was induced by light and the gene was expressed predominantly in leaves.

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Materials and Methods

Plant growth conditions

Seeds of *Spinacia oleracea* (cultivar Sakada) were purchased at a local market in the city of Cheongju. For cDNA library construction and tissue-specific gene expression experiments, seeds were germinated for 3 days and grown hydroponically for 7 days under continuous white light. For light induction, germinated seeds were grown for 4 days under continuous white light, transferred to the dark for 3 days, and then treated with white light for 1 day.

cDNA library construction and screening

Total RNA was prepared from light-grown spinach leaves using the phenol/SDS method (De Vries *et al.*, 1988). Polyadenylated RNA was isolated from the total RNA using oligo(dT) cellulose (Sambrook *et al.*, 1989). The cDNA was synthesized from 5 μg of the polyadenylated RNA using the Pharmacia cDNA synthesis kit. EcoRI/NotI-adapted cDNAs were cloned into a λgt10 vector and packaged using the Stratagene λgt10/EcoRI cloning kit. Approximately 2,000 recombinant phages were transferred to nitrocellulose filters and screened with

the insert of a soybean rbcS cDNA clone, pGSS8 (Cho *et al.*, 1992). The probe was labelled with the ECL direct nucleic acid labelling system (Amersham). The filters were hybridized at 37°C in the ECL hybridization solution containing 1.5 M NaCl and 5%(w/v) blocking agent overnight. The filters were then washed twice (10 min each) at 37°C in 0.4% SDS/0.5X SSC and twice (5 min each) at 37°C in 2X SSC. The filters were treated with detection reagents and exposed to X-ray film (Agfa). The insert of one positive clone was subcloned into the NotI site of pBluescript SK$^+$, resulting in plasmid pJA 300.

DNA sequence analysis

Restriction fragments of the pJA300 insert were subcloned into the pBluescript vector and the sequences of the subclones were determined by an automatic DNA sequencer at the Korea Basic Science Institute (Taejon, Korea). For manual DNA sequencing, the SILVER SEQUENCE™ DNA sequencing system (Promega) was used.

Southern blot analysis

Genomic DNA was isolated from spinach leaves as described by Razin (1988). Genomic DNA (10 μg) was digested with EcoRI or HindIII, run on a 0.8% agarose gel, and transferred to a nitrocellulose filter using 20X SSC as described by Sambrook *et al.* (1989). The filter was hybridized to the ^{32}P-labelled spinach rbcS cDNA at 37°C in a hybridization solution (6X SSC, 30% formamide, 0.5% SDS, 5X Denhardt's solution, 0.1 mg/ml salmon sperm DNA) and washed twice (10 min each) at 37°C in 0.5% SDS/2X SSC and twice (20 min each) at 50°C in 0.1% SDS/0.2X SSC. The rbcS probe was prepared by cutting pJA300 with NotI. This probe DNA (pJA300 insert) was labelled with [α-^{32}P] dCTP (3,000 Ci/mmol, Amersham) using a random primed DNA labelling kit (Boehringer Mannheim).

Northern blot analysis

Total RNA (20 μg) was electrophoresed in a 1.2% agarose/2.2 M formaldehyde gel, transferred to a nitrocellulose filter, and hybridized with the insert of pJA300. Probe labelling, hybridization, and washing were performed using the ECL direct nucleic acid labelling system (Amersham). Hybridization was performed at 37°C in the ECL hybridization solution containing 0.5 M NaCl and 5% (w/v) blocking agent overnight. Washing was carried out twice (10 min each) at 55°C in 0.4% SDS/0.5X SSC and then twice (5 min each) at 25°C in 2X SSC. The RNA content loaded was checked by ethidium bromide staining of the gel or by methylene blue staining of the blot.

Results and Discussion

To isolate rbcS cDNA clones in spinach, a cDNA library was constructed with mRNA isolated from light-grown spinach leaves. The cDNA library was screened for rbcS cDNA clones using a soybean rbcS cDNA as a probe. Four positive clones were identified from approximately 2,000 recombinant cDNA clones screened. When DNAs isolated from these positive clones were analyzed by restriction enzyme digestion followed by Southern hybridization, all of the clones were found to contain a rbcS cDNA insert of about 0.8 kb. The insert of one of these clones was subcloned into the vector pBluescript SK$^+$ and this resulting plasmid was named pJA300.

The nucleotide and predicted amino acid sequences of the cDNA insert of pJA300 are shown in Fig. 1. The insert, which contains 729 bp, has an open reading frame of 180 amino acids. It encodes a transit peptide of 57 amino acids and a mature rbcS polypeptide of 123 amino acids. The transit peptide of the Rubisco small subunit plays a key role in chloroplast import and processing (Van den Broeck *et al.*, 1985). A putative polyadenylation signal (AATAAT) is found within 30 nucleotides from the 3' end of the cDNA (Fig. 1). This sequence differs from the consensus AATAAA motif of animal cells on position 6.

Recently, a group at the University of Geneva report-

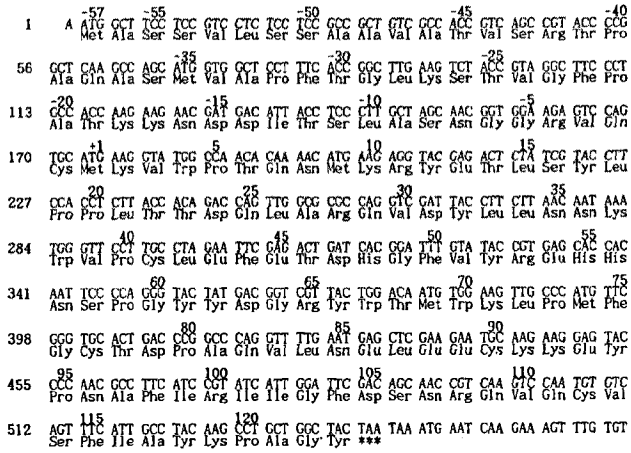

Fig. 1. Nucleotide sequence and the deduced amino acid sequence of a spinach rbcS cDNA. The Rubisco small subunit consists of a transit peptide of 57 amino acids and a mature polypeptide of 123 amino acids. The first amino acid of the mature polypeptide is numbered +1. A plausible polyadenylation signal (AATAAT) is underlined. Arrowhead (▶) indicates the start site of poly(A) tail according to the report by a group at the University of Geneva (see text). ***, Stop codon.

ed the nucleotide sequence of a spinach rbcS cDNA (EMBL accession number X97600). Their nucleotide sequence of the coding region is identical to ours. However, a major difference was found in the 3' untranslated region (UTR). In their sequence the poly(A) tail starts from the 162nd nucleotide downstream of the stop codon (Fig. 1, arrowhead), whereas no poly(A) tail was observed in our sequence. Probably there are two alternative polyadenylation sites in the spinach rbcS transcript. Plant polyadenylation signals are complex and it is not unusual that plant cells have multiple polyadenylation sites (Hunt, 1994). The functional significance of this differential polyadenylation site choice is not currently known. It is conceivable, however, that the prevalence levels of the two gene products might be different as the difference in the 3' UTR sequence is thought to affect the transport, stability, and/or translation efficiency of mRNA (Jackson and Standart, 1990).

In Fig. 2, we compared the amino acid sequence of the spinach Rubisco small subunit to those of other plant species. The spinach rbcS protein is most similar to ice plant rbcS5 protein. The mature polypeptides encoded by the spinach rbcS and the ice plant rbcS5 genes show 75.6% sequence identity, while the transit peptides show 80.7% sequence identity. Comparison of the amino acid sequence of the mature polypeptide with those of *Arabidopsis thaliana, Brassica napus*, and lettuce exhibits about 75% identity.

The amino acid sequence of a purified Rubisco small subunit (mature polypeptide only) from spinach was reported by Martin (1979). This sequence differs from our sequence by 35 amino acids out of 123 residues (Fig. 2), demonstrating that at least 2 rbcS genes exist in spinach. The large sequence difference between the two spinach rbcS polypeptides is noticeable. Analysis of amino acid sequences of previously reported rbcS proteins reveals little intraspecific divergence although interspecific divergence is extensive. Five rbcS genes of tomato and 5 rbcS genes of potato, for example, encode mature polypeptides which differ from each other by at most 4 amino acids (Sugita *et al.*, 1987) and 7 amino acids (Wolter *et al.*, 1988), respectively.

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1. MASSVLSSAAVATVSRTPA*QASMVAPFTGLKSTVGFPATKK*NDDITSLASNGGRVQCMKVWPTQNMKR  -68
2. ----MV--------N----*--------N----VAA--V---*-N----V-T--------Q---PLGK-K  -68
3. ----M----TM**-A*S--*--T-----N----SAA----R-A-N----1T------N--Q---PIGK-K  -66
4. ----M------**-T*S--*--T----------SAA--V-R-A-N----I-------S------PVGK-K  -66
5. ---*I*--S-I---N--TST---IA--------N-A--V---A-N-FS--P----------PIGL-K  -68
6. ----MI--F--T--N-**-*G-G----------MA---TR-T*-N----I----------Q---PVGK-K  -66
7.                                                              -Q---PLGL-K  -11

1. YETLSYLPPLTTDQLARQVDYLLNNKWVPCLEFETDHGFVYREHHNSPGYYDGRYWTMWKLPMFGCTDPA -138
2. F---------SEES-MKE-Q-----G--------PT--------G-T----------------------S -138
3. F-------D--DSE--KE----IR---I--V---LE--------G----------------L-----S- -136
4. F-------D--EVE-GKE-----R---I--V---LE--------GST----------------L-----S- -136
5. ----------SDEA-SKEI---IR---I------LE---------H----------------------S- -138
6. F-------D-DDA---KE-E---RKG-I------LE---------NR---------------------AS -136
7. F-----------E--LAE-N---VKG-I-P----VKD-------DK-------------------G----  -81

1. QVLNELEECKKEYPNAFIRIIGFDSNRQVQCVSFIAYKPAGY    -180
2. --VA----A--A--E---------NV-----I--------S-DA  -182
3. ---K-V-----------------NT-----I--V----PSFTG   -180
4. ---K-VQ---T-------------N------I-------PSFTGA -181
5. --MK-VG------------V----NI-----I---VA--P-VL   -181
6. ---K--Q-A-TA---G--------NV-----I-------P-F    -178
7. --V--V--V--AP-D--V-F----NK-E---I---------      -123
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Fig. 2. Comparison of Rubisco small subunit amino acid sequences. 1, Spinach (this study); 2, Ice plant (SWISS-PROT accession number P16032); 3, *Arabidopsis thaliana* (SWISS-PROT accession number P10795); 4, *Brassica napus* (SWISS-PROT accession number P27985); 5, Lettuce (DDBJ accession number D14001); 6, Soybean (Cho *et al.*, 1992); 7, Mature polypeptide portion of a spinach Rubisco small subunit (SWISS-PROT accession number P00870; Martin, 1979). Identical residues are indicated by dashed lines and gaps (*) are introduced to maximize homology. Arrowhead (▶) indicates the first amino acid of the mature polypeptide.

In most higher plants, rbcS genes are known to exist as a multigene family of more than 4 members (Dean *et al.*, 1989). To examine whether the spinach genome contains multiple rbcS genes, we carried out a genomic Southern hybridization experiment using the spinach rbcS cDNA as a probe. Genomic DNA was isolated from spinach leaves, digested with EcoRI or HindIII, and hybridized with the pJA300 insert as described in Materials and Methods. More than 5 hybridizing bands were detected (Fig. 3), implying that multiple rbcS genes are present in the spinach genome.

Expression of rbcS genes has been shown to be regulated by tissue-specific programs and by light. To examine the tissue-specific expression pattern of rbcS genes in spinach, a Northern blot hybridization experiment was performed using the insert of pJA300 as a probe. As shown in Fig. 4, the rbcS transcripts were detected at a high level in leaves (lane LF), at a low level in stems (lane SM), and undetectable in roots (lane RT). Light-regulated expression of the rbcS genes was also analyzed by Northern hybridization. When light-grown

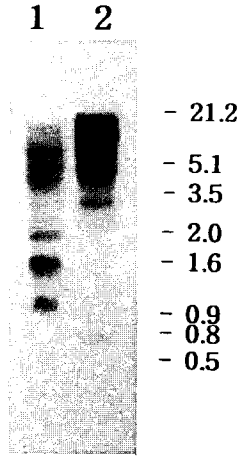

Fig. 3. Genomic Southern analysis of spinach rbcS genes. Spinach genomic DNA (about 10 μg) digested with EcoRI (lane 1) or HindIII (lane 2) was analyzed by Southern blot hybridization using a spinach rbcS cDNA as a probe. The positions of molecular weight markers (in kilobases) are shown.

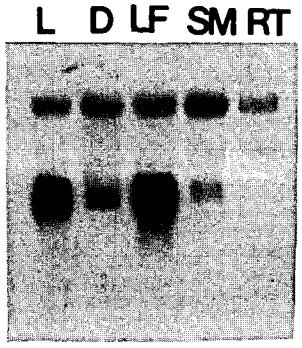

Fig. 4. Light-induced and tissue-specific expression of spinach rbcS genes. Total RNA (about 20 μg) from leaves of light-treated (L) or dark-treated (D) spinach seedlings was analyzed by Northern blot hybridization using a spinach rbcS cDNA as a probe. Total RNA (about 20 μg) from leaves (LF), stems (SM), or roots (RT) of light-grown plants was analyzed by Northern blot hybridization. The upper hybridizing bands, that appear to result from cross-hybridization of the probe to large ribosomal RNA, were observed in some experiments.

spinach seedlings were placed in the dark for 3 days, the amount of rbcS transcripts was reduced to the basal level (Fig. 4, lane D). Exposure of the dark-treated seedlings to light for 1 day resulted in a substantial increase of the rbcS transcripts (Fig. 4, lane L), indicating that light activates rbcS gene expression in spinach. The activation of rbcS genes in spinach is believed to be mediated by phytochrome based on our previous observation that a red light pulse induced an increase in the amount of rbcS transcripts and a subsequent far-red light pulse treatment reversed this effect (Park *et al.*, 1995).

Acknowledgement

We thank Suck Jong You for manual DNA sequencing.

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