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Isolation of a Rice Genomic Clone Encoding Ribulose-1,5-bisphosphate Carboxylase

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Abstract : To study the light-induced expression mechanism and protein transport into the chloroplast, a rice genomic clone (*GrbcS*) for the small subunit of ribulose 1,5-bisphosphate carboxylase (*rbcS*) was isolated and its nucleotide sequence was determined. Nucleotide sequence analysis of *GrbcS* revealed that the gene consists of two exons interrupted by an intron, encoding a protein of 175 amino acids including a transit peptide of 47 amino acids. These structural features of *GrbcS* are consistent with those of other *rbcS* genes from monocot species. Genomic Southern blot analysis suggested that the *rbcS* genes are present as a relatively small multigene family in the rice genome. Comparison of the nucleotide and deduced amino acid sequences to other rice *rbcSs* shows close sequence similaritiy. Conserved DNA sequences present in other light-responsive genes are also found in the 5′ upstream region of *GrbcS* such as G-box, 3AF1-binding site and GATA site. The possible function of these putative regulatory elements are discussed (Received September 26, 1994; accepted October 16, 1994).

Introduction

Ribulose-1,5-bisphosphate carboxylase (Rbc), which is the most abundant protein on the earth, catalyzes the first step of Calvin cycle. In higer plants, Rbc is composed of eight large subunits, encoded by chloroplast DNA,[1)] and eight small subunits, encoded by nuclear DNA. The small subunit is translated in the cytoplasm and transported into chloroplast. Transit peptide mediates the transport of the nascent polypeptide into the chloroplast and is cleaved off during or shortly after transport.[2)] The mature small subunit assembles in the chloroplast stroma with the large subunit to form the holoenzyme which catalyzes the fixation of CO_2. The catalytic sites for both the carboxylase and oxygenase activities are present in the large subunit. *RbcS* is produced in very large amounts in leaves. The response of the gene to light is quite dramatic event in which its regualatory sequences exert powerful control on mRNA trapnscription. Plant grown in the dark contains less than one twentieth of the *rbcS* mRNA produced by plant grown in the light.[3)] Many *rbcS* genes from various species has been isolated and their nucleotide sequences were determined. In the case of dicot plants, 8 members of the genes in petunia,[4-7)] 5 genes in pea,[3,8)] 4 genes in tomato,[9)] 5 genes in potato[10)] have been characterized. In the case of monocot plants 5 genomic and 6 cDNA clones in lemna,[11)] one genomic and 4 cDNA clones in wheat[12,13)] and one genomic and 3 cDNA clones in maize[14,15)] have been identified and sequenced. To characterize the *rbcS* gene from rice, we have cloned a genomic clone. Nucleo-

Key words : Ribulose 1,5-bisphosphate carboxylase (*rbcS*), Nucleotide sequencing, Genomic Southern blot analysis, Multigene family, Light-responsive gene
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tide and deduced amino acid sequences of the clone were determined and analyzed together with those of other plants.

Materials and Methods

Bacterial strains

Bacterial strain used in these experiments is *E. coli* MC1061 [F^-, *ara*D139, (*ara*, Leu) 7696, *lac*Y74, *gal*U^-, *gal*K^-, *hsd*R^-, *str*A] for transformation and plasmid preparation.[16]

Culture media

E. coli MC1061 were grown in LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Transformed MC1061 was cultured in LB media containing ampicillin 50 ug/ml. Solid media were made with 1.5% (w/v) bacto-agar for plate.

Enzymes & chemicals

T4 DNA ligase, *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Sma*I, *Sph*I, *Xho*I and other restriction enzymes were purchased from New England Biolab. RNase A, proteinase K and agarose were from Sigma Chemical Co. [α-^{32}P] ATP were from Amersham.

Rice samples

Rice samples used in this experiment were the leaves of *Oryzae sativa* cultivar Nakdong which were grown for 15 days in the dark or under illumination at gorwth chamber.

Genomic DNA and mRNA preparation

Genomic DNA of rice was prepared by the procedure of Shure *et al.*[17] with minor modification. Total rice RNA was isolated by phenol extraction and poly(A)$^+$ RNA was by oligo (dT) chromatography as described previously.[18].

DNA hybridization

Southern blot analysis and colony hybridization were carried out according to the standard procedures described by Sambrook *et al.*[19]

Nucleotide sequencing

Nucleotides sequence of the *GrbcS* gene was determined by the dideoxynucleotide chain termination method[20] using the Sequenase kit (US Biochemical, U.S.A.). The double stranded plasmid DNA templates were sequenced using M13 forward and reverse primer. The nucleotides and amino acids sequence data were assembled and analyzed by DNASIS software package (Pharmacia-LKB).

Results and Discussion

Isolation of a *rbcS* genomic clone from rice

Previously, we reported cloning of *rbcS* cDNAs

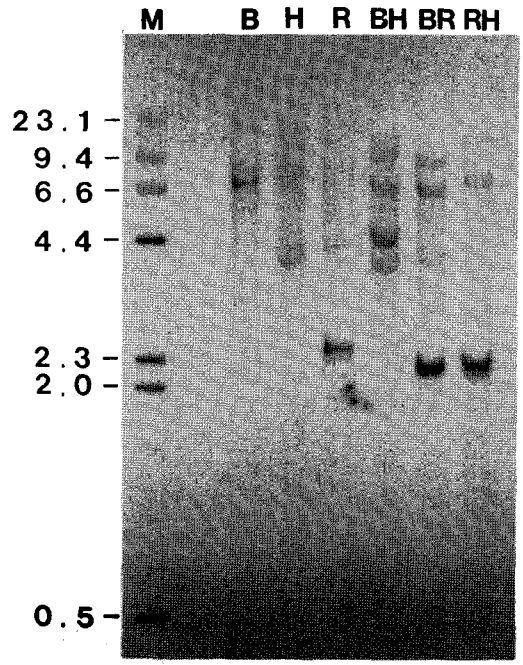

Fig. 1. Genomic Southern blot analysis of rice DNA. Genomic DNA was digested with *Bam*HI, *Hin*dIII, *Eco*RI or their combinations. It was electrophoresed in 1% agarose gel, transferred onto nylon membrane and hybridized with ^{32}P-labelled pB9 clone, a cDNA clone of *rbcS* from rice, as a probe. The restriction enzymes are abbreviated as follows: B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; BH, *Bam*HI and *Hin*dIII; BR, *Bam*HI and *Eco*RI; RH, *Hin*dIII and *Eco*RI. Size markers are shown in kb.

from rice by differential screening.[21] Genomic Southern blot analysis was carried out using the cDNA clone as a probe to determine the copy number of the gene in the rice genome (Fig. 1) revealing that the *rbcS* genes are present as a multigene family in rice genome like in other plant species.[22] To isolate *rbcS* genomic clone, relatively strongly hybridized 2.5 kbp *EcoR*I fragment was isolated from the agarose gel, ligated into pUC18 vector and transformed into *E. coli* MC1061. These transformants were screened for a genomic clone of *rbcS* with pB9, a partial cDNA clone for *rbcS*, as a probe. Restriction map of the cDNAs and a genomic clone were constructed by digesting with *Pst*I, *Sma*I, *Sph*I and *Xho*I as shown in Fig. 2. The genomic clone, named *GrbcS*, covers the entire coding regions of the gene including 3′ and 5′ untranslating regions.

Nucleotides sequence of the *GrbcS* clone

To charactscterize the *GrbcS* clone, nucleotide sequence was determined. A sequencing stratage is shown in Fig. 2 and nucleotide sequence of the *GrbcS* is shown in Fig. 3. The *GrbcS* clone contains about 1.0 kbp of 5′ upstream region, which presumably contains regulatory elements involved in its light responsive expression, and 387 bp noncoding region at 3′ downstream. There are TATA box and CAAT box at the 5′ upstream region and a poly adenylation signal, AATAAA, at the 3′ downstream region that are commonly found in most of eucaryotic genes.

Coding sequence of the *GrbcS* perfectly matches with those of partial cDNA clone, pB9 and pC10[21] and the full-length cDNA clone, rice 1.[23] Nucleotide sequence comparison of the *GrbcS* with cDNA clones pB9, pC10 and rice 1, reveals an intervening sequence of 103 nucleotide. The junction sequence between the exon and the intron is AG/GT, a consensus sequence of eucaryotic genes. All *rbcS* genes from dicots sequenced to date have at least two introns and those are always found in the same positions.[24,25] In the *rbcS* genes of the monocot species *Lemna gibba*, however, the first of these two introns is missing;[26] in two other monocot species, wheat and corn, the second intron is missing in at least one of *rbcS* genes.[2,14] *RbcS* genes in petunia, tomato, potato and tobacco contains an additional intron located at downstream from the second intron. As is shown in Fig. 2 and 3, *GrbcS* from rice contains only one intron.

Deduced amino acid sequence of *GrbcS*

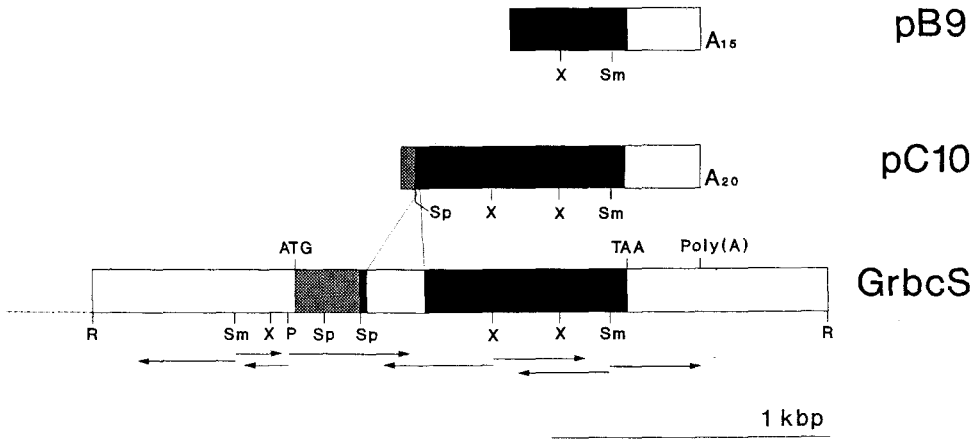

Fig. 2. Restriction enzyme maps of rice *rbcS* clones. The maps of partial cDNA clone pB9 and pC10 are shown relative to that of *GrbcS*. The 5′-upstream region, 3′-downstream region and intron are shown in open box. Hatched box is for transit peptide and closed box for mature protein of *rbcS*. Restriction enzyme sites are indicated as follows: R, *Eco*RI; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I. Sequencing strategy of *GrbcS* is shown in arrows.

```
-318                                                                    ga
-316  tgtgcagaggagcaaaggtgatctggcagtggatatctccccatccatcctcacccggctgcccatcactcgccgccgc
-237  atactacatcatgtggagagaggaagacgaggaccacagccagagcccgggtcgagatgccaccacggccacaatccac
-158  gagcccggcgcgacaccaccgcgcggcgtgagcagccacaaacgcccgcggataggcgcgcacggcCAATcctaccaca
 -79  tccccggcctcgctccgagccgctgccatccgatccgctgagttttggcTATTTATAcgtaccgcgggagcctgtgtgc

   1  agagcagtgcatctcaagaagtactcgagcaaagaaggagagagcttggtgagctgcagag  ATG GCC CCC TCC
 -47                                                                 Met Ala Pro Ser

  74  GTG ATG GCG TCG TCG GCC ACC ACC GTC GCT CCC TTC CAG GGG CTC AAG TCC ACC GCC GGC
 -43  Val Met Ala Ser Ser Ala Thr Thr Val Ala Pro Phe Gln Gly Leu Lys Ser Thr Ala Gly

 134  ATG CCC GTC GCC CGC CGC TCC GGC AAC TCC AGC TTC GGC AAC GTC AGC AAT GGC GGC AGG
 -23  Met Pro Val Ala Arg Arg Ser Gly Asn Ser Ser Phe Gly Asn Val Ser Asn Gly Gly Arg

 194  ATC AGG TGC ATG CAG  gtaataacctactgacccggcacacattattcttcttcttcttcttcttcttctt
  -3  Ile Arg Cys Met Gln

 268  cttcttcttcaacattaaccaataattcaattatcgtttatttag  GTG TGG CCG ATT GAG GGC ATC AAG
   3                                                Val Trp Pro Ile Glu Gly Ile Lys

 337  AAG TTC GAG ACC CTC TCC TAC CTG CCA CCG CTC ACC GTG GAG GAC CTC CTG AAG CAG ATC
  11  Lys Phe Glu Thr Leu Ser Tyr Leu Pro Pro Leu Thr Val Glu Asp Leu Leu Lys Gln Ile

 397  GAG TAC CTG CTC CGT TCC AAG TGG GTG CCC TGC CTC GAG TTC AGC AAG GTC GGA TTC GTC
  31  Glu Tyr Leu Leu Arg Ser Lys Trp Val Pro Cys Leu Glu Phe Ser Lys Val Gly Phe Val

 457  TAC CGT GAG AAC CAC AGA TCC CCC GGA TAC TAC GAT GGC AGG TAC TGG ACC ATG TGG AAG
  51  Tyr Arg Glu Asn His Arg Ser Pro Gly Tyr Tyr Asp Gly Arg Tyr Trp Thr Met Trp Lys

 517  CTG CCC ATG TTC GGG TGC ACT GAC GCC ACC CAG GTG CTC AAG GAG CTC GAG GAG GCC AAG
  71  Leu Pro Met Phe Gly Cys Thr Asp Ala Thr Gln Val Leu Lys Glu Leu Glu Glu Ala Lys

 577  AAG GCG TAC CCT GAT GCA TTC GTC CGT ATC ATC GGC TTC GAC AAC GTC AGG CAG GTG CAG
  91  Lys Ala Tyr Pro Asp Ala Phe Val Arg Ile Ile Gly Phe Asp Asn Val Arg Gln Val Gln

 637  CTC ATC AGC TTC ATC GCC TAC AAG CCC CCG GGC TGC GAG GAG TCT GGT GGC AAC TAA  gc
 111  Leu Ile Ser Phe Ile Ala Tyr Lys Pro Pro Gly Cys Glu Glu Ser Gly Gly Asn ***

 696  cgtcatcgtcatatatagccttgtttaattgttcatctctgattcgatgatgtctcccaccttgtttcgtgtgttccca
 775  gtttgttcatcgtcttttgattttaccggccgtgctctgcttttgtttttgtttcacctgatctctctctgacttgatg
 854  taagagtgtggtatctgctacgactatatgttgttgggtgaggcatatgtgAATGAAatatatggaagtccggctatat
 933  atatttatacaaagggtacgagatggatgtgaatcctagagcatatgtgtccaacaatcaattcgtcgtcaatgaaaat
1012  ttgatcatggaattaaaaaatcatgcttctgttgttcatcggaaatgcctttctatactgattagtggattt
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Fig. 3. Nucleotides sequence of the *GrbcS* clone. The *GrbcS* clone contains 5′-upstream region, a coding region and 3′ downstream region. The coding region is interupted by one intron into two exons which encods 175 amino acids. The transit peptide (47 amino acids long) is shown in shade. Nucleotide sequence is numbered from a putative transcriptional initiation site as +1. Nucleotide sequence of exon region is shown in uppercase letter whereas 5′-upstream region, 3′-downstream region and intron are shown in lowercase letter. Amino acid numbering starts from mature protein of *rbcS* as +1. Canonical promoter elements and a polyadenylational signal are shown in open letter.

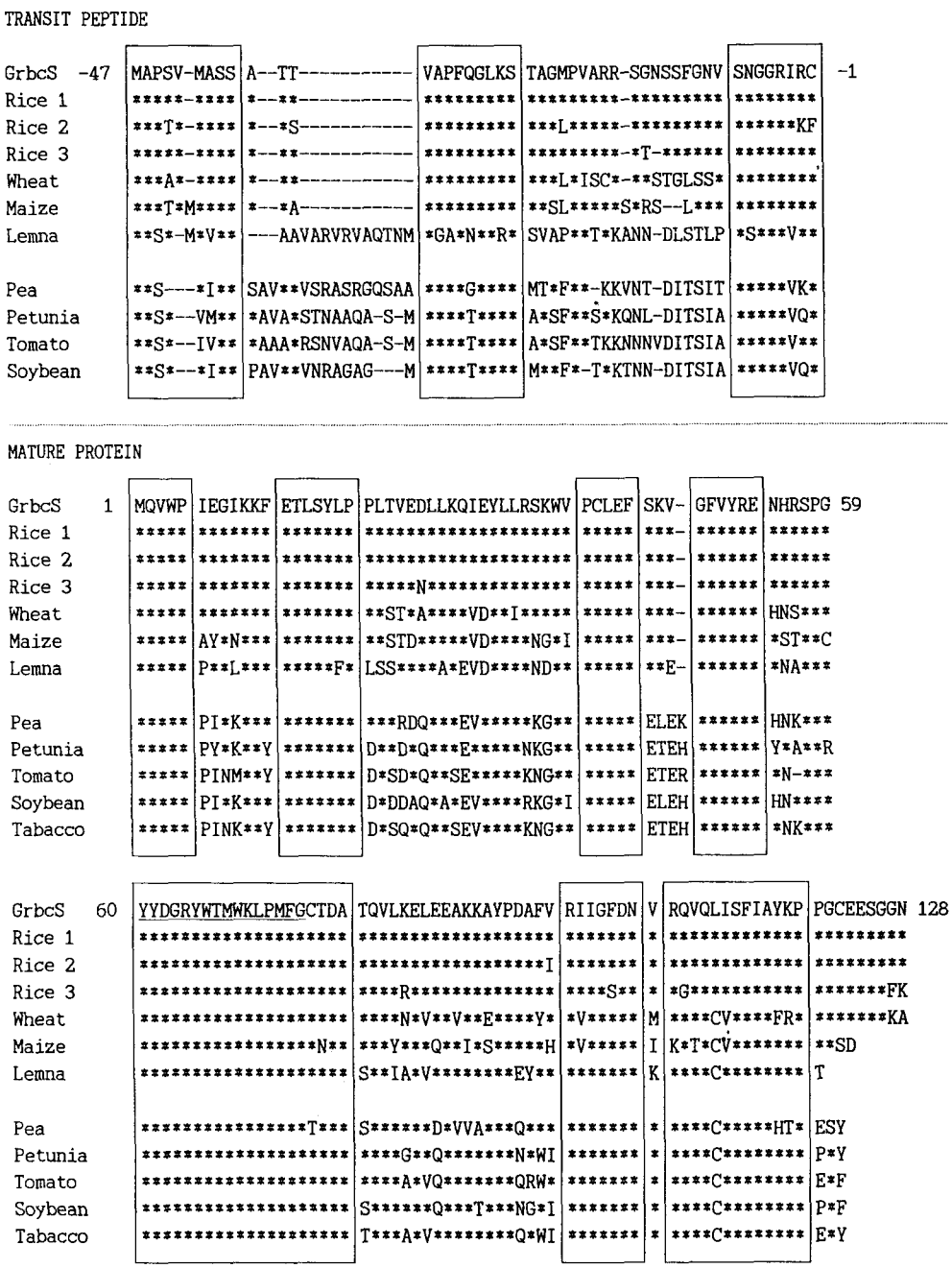

Fig. 4. Comparison of the amino acid sequences of the transit peptide and mature protein of *GrbcS* with those from various species. Sequences were taken from Matsuoka *et al.*[23] for rice 1 (pOSSS1139) and rice 2 (pOSSS2106), Moon *et al.*[27] for rice 3 (pS10), Broglie *et al.*[2] for wheat, Matsuoka *et al.*[28] for Maize, Stiekema *et al.*[11] for *Lemna*, Cashmore[25] for pea, Dean *et al.*[5] for petunia, Mcknight *et al.*[33] for tomato, Berry-Lowe *et al.*[24] for soybean, and Mazur and Chui[34] for tobacco. The highly conserved regions are shown in boxes and absolutely conserved sequence in all of the *RbcS*[22] are underlined. Positional identity was shown by asterisk (*) and spaces (−) were introduced for maximum similarity.

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-318                                                                GA -317
-316 TGTGCAGAGGAGCAAAGGTGATCTGGCAGTGGATATCTCCCCATCCATCCTCACCCGGCTGCCCATCACTCGCCGCCGC -238

-237 ATACTACATCATGTGGAGAGAGGAAGACGAGGACCACAGCCAGAGCCCGGGTCGAGATGCCACCACGGCCACAATCCAC -159

-158 GAGCCCGGCGCGACACCACCGCGCGGCGTGAGCAGCCACAAACGCCCGCGGATAGGCGCGCACGGCCAATCCTACCACA -80

 -79 TCCCCGGCCTCGCTCCGAGCCGCTGCCATCCGATCCGCTGAGTTTTGGCTATTTATACGTACCGCGGGAGCCTGTGTGC -1

   1 AGAGCAGTGCATCTCAAGAAGTACTCGAGCAAAGAAGGAGAGAGCTTGGTGAGCTGCAGAG  ATG GCC CCC TCC  73
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Fig. 5. Putative light responsive elements in the 5′ upstream region such as box II, G-box, 3AF1-box, GATA-box and canonical CAAT, TATAAA are noted. Box II is presented with light shadow, G-box and GATA box with underline, 3AF1 box with inversion, CAAT and TATAAA in open letter. Numbering starts from putative translational initiation site as +1.

Deduced amino acid sequence from the *GrbcS* was compared with other *RbcS* sequences from various species (Fig. 4). A transit peptide of the *GrbcS* contains 47 amino acid residues as in wheat and maize. In contrast, the transit peptides of *Lemna* and dicot plants are made up of more than 55 amino acid residues. The amino acid sequence of the transit peptide from *GrbcS* shows 90% and 96% similarities to those of rice 2[23] and rice 3,[27] respectively, whereas 74% to that of wheat,[2] 76% to maize,[28] 33% to Lemna[11] and 33% to average of dicot plants as shown in Fig. 4. There are three highly conserved regions in the transit peptides which have been described by Karlin-Neumann and Tobin.[29] They proposed that they might be involved in the recognition and transport across the chloroplast envelope.

In all of the plant species analyzed, amino acid sequences of the mature *RbcS* are more conserved compared to the transit peptides. The similarities of the mature protein sequence of *GrbcS* to those of rice 2 and rice 3 were 99% and 95%, respectively, and 74% to that of maize, 73% to Lemna and 74% to average of dicot plants as shown in Fig. 4. An invariant hexadecapeptide motif, YYDGRYWTMWKLPMFG,[22] was located between amino acid residues 58 and 73.

5′ upstream region of *GrbcS* contains light-responsive regulatory elements

Regulatory element (consensus sequence)	Location	Motif/trans-acting factor	Reference
GATCTGGCAGT (CACGTGGCAYY)	−287~−297	G-box CG-1, GBF	Giuliano *et al.*[31]
GGATAT (GGTTAA)	−281~−286	Box II GT-1	Green *et al.*[35]
GGAGAGAGGAA (TAGAGATCTA)	−213~−223	3AF1 box 3AF1	Lam and Chua[30]
GATA (GATA)	−105~−108	GATA motif ASF-2	Gidoni *et al.*[32]
CAAT (CAAT)	−89~−92	CAAT box C/EBP	Bakker and Parker[36]
TATTTATA (TATAAA)	−23~−30	TATA box TFIID	Gasch *et al.*[37]

To understand the light-responsive expression mechanism of the *rbcS* gene, the 5′ upstream region of the *GrbcS* gene was searched for the presence of possible regulatory elements (Table 1). In addition to canonical promoter elements, such as 'TATA' and 'CAAT' boxes, the sequence homologous to the box II, G-box, GATA box and 3AF-1 box were found in the 5′ upstream region. The GT-1 binding site in box II (−281~−286) is present more than once in most of *rbcS* genes. They interact with the same protein factor and are functionally redundant in the *rbcS*-3A promoter.[30] The tetramer of box II can confer light responsiveness and tissue specificity on the expression of a truncated cauliflower mosaic virus 35S promoter (−90 version) in transgenic tobacco. The G-box (−287~−297) interacting with CG-1 or GBF, is another

conserved sequence in *rbcS* genes that is required for high efficiency of the Arabidopsis *rbcS*-1A promoter in transgenic tobacco.[31] The GATA box (−105~−108), which is observed in most of *rbcS* and *cab* genes, was also found.[32] The 3AF1 binding site (−213~−223) seems to confer transcriptional activity of light independently but in quantitative fashion.[30] To understand the light responsive gene expression of *GrbcS*, futher studies on the function of those putative regulatory elements and the mechanisms of post-translational transit of precursor protein into chloroplast is required.

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리불로스 1,5- 이인산 탄산화효소 유전자의 분리 및 특성규명

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초록 : Ribulose 1,5-bisphosphate carboxylase small subunit(*rbcS*)의 광유도 발현과 엽록체로의 단백질 이동 메카니즘을 연구하기 위해 벼의 게놈으로부터 *rbcS* 유전자를 분리하여(*GrbcS*) 그의 염기서열을 결정하였다. *GrbcS*의 유전자 염기서열 결정 결과, 단백질 암호부위는 한개의 intron과 두개의 exon으로 이루어져 있고 이들은 47개의 transit peptide를 포함하는 175개의 아미노산을 암호화하는 것으로 밝혀졌다. *GrbcS*의 이러한 구조적인 성질은 다른 단자엽 식물의 그것과 비교적 일치하고 genomic Southern blot analysis 결과 *rbcS* 유전자는 벼의 게놈상에 상대적으로 적은 규모의 multigene family로 존재한다는 것이 밝혀졌다. *GrbcS*의 유전자 염기서열과 그로부터 유추된 아미노산의 염기서열은 벼로부터 분리된 다른 *rbcS*와 매우 유사함을 보였고 다른 식물체로부터 분리된 그것과도 높은 유사성을 보였다. *GrbcS*의 5′ 앞쪽 부분에는 G-box, 3AF1-binding site, GATA site와 같은 광유도 발현 유전자에 공통적으로 존재하는 염기서열을 지니고 있었다.