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SHORT COMMUNICATION

Subcloning and Sequencing of Maize *rbcL* Promoter Region

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pRLYS1 containing intact *rbcL* gene of maize (*Zea mays* L. cv Golden X Bantam T-51; Zm-A) was digested with several restriction enzymes to construct subclones carrying promoter region of *rbcL*. The DNA fragments of 0.20, 0.19, 0.92 and 1.55 kb among the *Eco*RI digests, the *Eco*RI-*Dde*I digests, the *Ava*I digests and the *Eco*RI-*Bam*HI digests of pRLYS1 were subcloned into pBluscriptSK+ and named pRLPS2, pRLPS3, pRLPS14 and pRLPS35, respectively. Four subclones contain the 1.92 kb portion from 136 nucleotide downstream to 1780 nucleotide upstream from the ATG initiation codon of *rbcL* gene. pRLPS2 (−29 to −229) and pRLPS3 (−239 to −420 from the ATG) were sequenced. When nucleotide sequence of Zm-A was compared with sequence of *rbcL* promoter region of a different cultivar of maize (*Zea mays* L. cv WFG TMS X BS7; Zm-B), the difference rate between two cultivars was 4.3%. The mean of sequence divergence between Zm-A and three grass species in the same tribe, Andropogoneae, in the upstream region from 29 to 420 of ATG was 1.8%, whereas between Zm-B and above-mentioned three species was 5.4%. Therefore, Zm-A seems to be evolutionarily closer to three other species in Andropogoneae tribe than Zm-B is.

Keywords: sequence analysis, promoter region, *rbcL*, *Zea mays* L.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is the major soluble protein in plants. The enzyme is composed of eight identical large subunits (LSU) of 53 kD and eight small subunits (SSU) of 12-14 kD. LSU is encoded in the chloroplast DNA and SSU is encoded in the nuclear DNA (Sugiura, 1989). The SSU is synthesized as a large precursor polypeptide on cytoplasmic ribosomes, which is processed to the mature form during the import into chloroplasts. Finally, LSUs and SSUs are assembled to the holoenzyme in chloroplasts (Kawashima and Wildman, 1972; Cashmore *et al.*, 1978). Rubisco fixes CO_2 in the Calvin cycle of photosynthesis and also plays a role in photorespiration in C_3 plants (Kawashima and Wildman, 1970). Active sites of Rubisco are found on the large subunits whereas the funtions of the small subunits remain unknown (Miziorko and Lorimer, 1983).

Because Rubisco is a highly abundant enzyme, *rbcL* and *rbcS* have been characterized early on and their expressions have been extensively studied in several higher plants (Bedbrook *et al.*, 1978; Bedbrood *et al.*, 1979; Sasaki *et al.*, 1984; Inamine *et al.*, 1985; Berry *et al.*, 1986). The expression of *rbcL* and *rbcS* provides an important model system to study the developmental and light regulation of genes for photosynthetic proteins. Especially, the study of *rbcL* expression will be applicable to the broader question of how chloroplast gene expression is regulated as a whole (Manzara and Gruissem, 1988).

Since the *rbcL* gene was first isolated from maize chloroplast DNA by Coen *et al.* (1977), it has been cloned from various plants and sequenced (McIntosh *et al.*, 1980: Zurawski *et al.*, 1981; Shnozaki and Sugiura, 1982; Zurawski *et al.*, 1984; Zurawski *et al.*, 1986). The nucleotide sequences are highly conserved and amino acid sequences show higher homology among *rbcL* genes of different species (Zurawski *et al.*, 1981). The rbcL and *rbcS* genes show complex expression characteristics including developmentally regulated, photoregulated, and tissue-specific expres-

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sion (Tobin and Silverthorne, 1985; Kuhlemeier *et al.*, 1987). Although the regulation of *rbcL* and *rbcS* gene expression has been extensively studied in a number of plants (Berry *et al.*, 1985; Inamine *et al.*, 1985; Rodermel and Bogorad, 1985; Berry *et al.*, 1986), the regulatory mechanisms of these gene expressions remain controversial.

Studies of gene expression have been advanced recently by identification of DNA-protein interactions between trans-acting factors and specific cis-acting DNA sequences (Maier *et al.*, 1987). The binding of trans-acting factors can turn transcription on or off and can influence the level of transcription (Tobin and Silverthrone, 1985). Nuclear proteins interacting with conserved DNA motifs present in promoters of nuclear photoregulated genes have been described (Kuhlemeier *et al.*, 1989; Datta and Cashmore, 1989; Schindler and Cashmore, 1990). However, only a few reports indicate that chloroplast proteins interact with specific DNA sequences of the promoter of plastid photogenes.

We had previously reported the new genomic clone pRLYS1 which contains the intact *rbcL* gene of maize (*Zea mays* L. cv Golden X Bantam T-51) (Lee *et al.*, 1992). In this study, we constructed four different subclones carrying promoter region of *rbcL* from pRLYS1 and sequenced a part of *rbcL* promoter region.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction enzymes, DNA polymerase I large fragment and T4 DNA ligase were obtained from New England Biolabs (USA). Calf intestinal phosphatase and [α-^{35}S] dATP (1,000 Ci/mmol) were purchased from Amersham (UK) and DNA sequencing was performed with DNA sequenase kit of United States Biochemical Corporation (USA). Most of other chemicals were obtained from Sigma Chemical Co. (USA).

Bacterial strains and plasmids

Escherichia coli strain DH5α and *E. coli* strain JM 109 (Sambrook *et al.*, 1989) were used for host cells. They were cultured at 37℃ in LB media (Trypton 10 g, yeast extract 5 g, NaCl 10 g, D.W. to 1 L, pH 7.0) with vigorous shaking. pBluescriptSK+ (Sambrook *et al.*, 1989) was a plasmid vector for subcloning and sequencing. pRLYS1 containing intact *rbcL* gene of maize (Lee *et al.*, 1992) was used for subcloning of *rbcL* promoter region to make pRLPS2, pRLPS3, pRLPS14 and pRLPS35.

Plasmid manipulation

Large scale isolation of plasmids was performed by the method of Marco *et al.* (1982), and small scale isolation of plasmids followed a modified procedure from Birnboim and Doly (1979). Plasmid isolation for sequencing was performed by the method of Mierendorf and Pfeffer (1987).

Subcloning of *rbcL* promoter region

pRLYS1 was digested with several restriction enzymes to construct subclones which carry promoter region of *rbcL*. The restriction sites in *rbcL* promoter region and the subcloning scheme are shown in Fig. 1. The DNA fragments of 0.20, 0.19, 0.92 and 1.55 kb among the *Eco*RI digests, the *Eco*RI-*Dde*I digests, the AvaI digests and the *Eco*RI-*Bam*HI digests of pRLYS1 were recovered from the 1.7% agarose gel, respectively. The 0.20 kb *Eco*RI fragment and the 1.55 kb *Eco*RI-*Bam*HI fragment were subcloned into pBluescriptSK+ to make pRLPS2 and pRLPS35, respectively.

Recessed 3′-termini of the 0.19 kb *Eco*RI-*Dde*I fragment and of the 0.92 kb AvaI fragment were filled by large fragment of the *E. coli* DNA polymerase I in the presence of the appropriate dNTPs (Sambrook *et al.*, 1989). End-filled fragment was ligated into the *Eco*RV site of the pBluescriptSK+. The recombinant plasmids containing end-filled 0.19 kb *Eco*RI-*Dde*I fragment and 0.92 kb *Ava*I fragment were called pRLPS3 and pRLPS14, respectively. Four different recombinant plasmids were transformed into *E. coli* DH5 or *E. coli* JM109. Transformants were selected from the LB media containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal), isopropyl-thio-β-D-galactoside (IPTG) and ampicillin (100 μg/mL).

Nucleotide sequencing

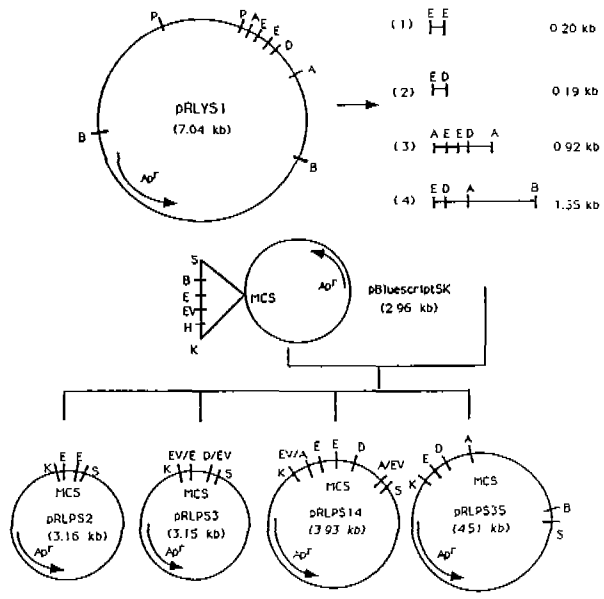

Fig. 1. The restriction sites of *rbcL* promoter region in pRLYS1 and the subcloning scheme. The recombinant plasmid, pRLYS1, containing the intact genomic *rbcL* gene from maize was constructed by the ligation of a *Bam*HI 9 fragment of chloroplast DNA to pUC19 (Lee *et al.*, 1992). For making subclones of the *rbcL* promoter region, the four above-denoted types of restriction enzyme fragments from the pRLYS1 were recovered from the agarose gel. The recessed 3′-termini of the 0.19 kb *Eco*RI-*Dde*I fragment and the 0.92 kb *Ava*I fragment among the recovered fragments were filled with Klenow fragment of *E. coli* RNA polymerase I. And then, all types of recovered DNAs were ligated to pBluscriptSK which were treated with proper restriction enzymes. The names and sizes of four subclones were shown in Fig. 1. The restriction sites are denoted by A, *Ava*I; B, *Bam*HI; D, *Dde*I; E, *Eco*RI; EV, *Eco*RV; K, *Kpn*I; P, *Pst*I; S, *Sac*I. The MCS means multicloning site.

The promoter region of *rbcL* in pRLPS2 and pRLPS3 was sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977). [α-^{35}S]dATP was used, and the DNA was electrophoresed on a 6% polyacrylamide gel at 40 mA. The gel was exposed to X-ray film (AGFA) for 16 hr at −70℃.

RESULTS AND DISCUSSION

Subcloning of *rbcL* promoter region

pRLYS1 containing intact *rbcL* gene of maize (*Z. mays* L. cv Golden X Bantam T-51) had been constructed (Lee *et al.*, 1992). To analyze nucleotide sequence of promoter region of *rbcL* and study DNA-protein interaction between promoter region of *rbcL* and chloroplast extract, we decided to construct subclones which carry the promoter region of *rbcL* from pRLYS1.

The restriction sites of *rbcL* promoter region in pRLYS1 were determined as shown in Fig. 1 from the result of restriction enzyme digestion and report of Link and Bogorad (1980). The pRLYS1 was digested with several restriction enzymes to construct subclones carrying *rbcL* promoter region. The construction of recombinant plasmids and the selection of transformants were performed as described in Materials and Methods. The selected transformants were certified with the presence of inserted fragments after the digestion of restriction enzymes. As pRLPS2 and pRLPS35 were constructed with cohesive-end ligation, each of them was digested with the same enzymes which were used for making a recombinant plasmid. pRLPS3 and pRLPS14 were constructed with blunt-end ligation, because pBluscriptSK+ does not have restriction sites for *Dde*I and *Ava*I. The end-filled 0.19 kb *Eco*RI-*Dde*I fragment and the 0.90 kb *Ava*I fragment of pRLYS1 were ligated to *Eco*RV site of pBluscriptSK+ to make pRLPS3 and pRLPS 14. Therefore, the presence of inserted fragments in these two recombinant plasmids were confirmed by cutting of either *Eco*RI site or *Hin*dIII site which located at the nearest site to the *Eco*RV site in the multicloning site of pBluscriptSK+.

The presence of 0.20 kb *Eco*RI fragment from pRLYSI in pRLPS2 is shown in Fig. 2A. pRLPS2 has noncoding region spanning from −29 to −229 from the ATG translation initiation codon. pRLPS3 containing the end-filled 0.19 kb *Eco*RI-*Dde*I fragment of pRLYS1 was identified by the presence of the same-sized fragment after the digestion of *Eco*RI and *Hin*dIII (Fig. 2B). However, the 0.20 kb *Eco*RI fragment could not be excluded from the *Eco*RI-*Dde*I digests of pRLYS1 during the recovery of 0.19 kb *Eco*RI-*Dde*I fragment from the agarose gel because of their similar size. Therefore, the transformants were certified again by nucleotide sequencing. pRLPS3 carries promoter region of *rbcL* from −230 to −420 from the ATG codon. The pRLPS14 carrying the end-filled 0.92 kb *Ava*I fragment of pRLYS1 was identified with the same manner as pRLPS3. The presence and orientation of inserted fragment

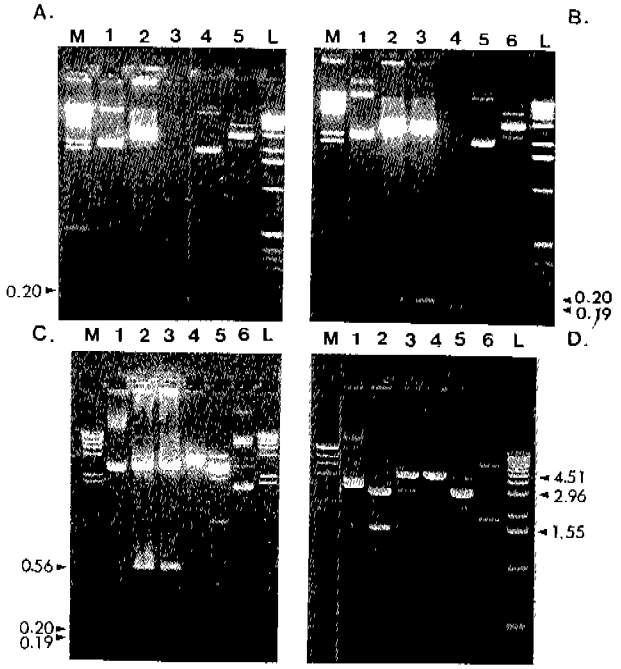

Fig. 2. Electrophoresis patterns of the digests of recombinant plasmids. Electrophoresis was carried out at 8 V/cm in 1.7% agarose gel. A. pRLPS2, lane 1, pRLPS2 (uncut); 2, pRLPS2 digests with *Eco*RI; 3, 0.20 kb *Eco*RI fragment eluted from pRLYS1; 4, pBluescriptSK+ (ccc form); 5, pBluescriptSK+ (L form). B. pRLPS3, lane 1, pRLPS3 (uncut); 2 and 3, pRLPS3 digests with *Eco*RI and *Hin*dIII; 4, 0.19 kb *Eco*RI-*Dde*I digests and 0.2 kb *Eco*RI digests eluted from pRLYS1; 5, pBluescriptSK+ (ccc form); 6, pBluescriptSK+ (L form). C. pRLPS14, lane 1, pRLPS14 (uncut); 2, pRLPS14 digests with *Eco*RI and *Hin*dIII; 3, pRLPS14 digests with *Eco*RI; 4, pRLPS14 digests with *Hin*dIII; 5, pBluescriptSK+ (L form); 6, pBluescriptSK+ (ccc form). D. pRLPS35, lane 1, pRLPS35 (uncut); 2, pRLPS35 digests with *Eco*RI and *Bam*HI; 3, pRLPS35 digest with *Eco*RI; 4, pRLPS35 digest with *Bam*HI; 5, pBluescriptSK+ (L form); 6, pBluescriptSK+ (ccc form). L and ccc mean covalently closed circle and linear. M and L mean λ digests with *Hin*dIII and 1 kb ladder, respectively, as a size marker.

were determined from the result of restriction enzyme digestions (Fig. 2C). The pRLPS14 has a region of *rbcL* from +136 to −780 from the ATG initiation codon. To construct a subclone having a further upstream promoter region of *rbcL*, 1.55 kb *Eco*RI-*Bam*HI fragment of pRLYS1 was ligated into pBluscriptSK and this recombinant plasmid was named pRLPS35. Inserted DNA fragment (from −230 to −1780 from the ATG) was identified by the restriction enzyme digestions (Fig. 2D). Four subclones were constructed from pRLYS1 and contain an 1.92 kb portion from 136 nucleotide downstream to 1780 nucleotide upstream from the ATG initiation codon of the *rbcL* gene.

Nucleotide sequence analysis of *rbcL* promoter region

Because all reports about the cloning and the sequencing of the maize *rbcL* gene were performed with *Z. mays* L. cv WFG TMS X BS7 (Illinois Foudation Seeds, Inc. USA) (Coen *et al.*, 1977; Link and Bogorad, 1980; McIntosh *et al.*, 1980), we constructed a new recombinant plasmid from the *rbcL* gene of *Z. mays* L. cv Golden X Bantam T-51 (Sakada Seeds Foundation, Japan) and sequenced a part of the promoter region of *rbcL* to compare nucleotide sequence between two different cultivars.

The inserts of pRLPS2 (−29 to −229) and pRLPS 3 (−230 to −420 from the ATG) were sequenced. *Z. mays* L. cv Golden X Bantam T-51 (mentioned as a Zm-A below) had 17 different nucleotides among 392 nucleotides in the *rbcL* noncoding region, when it was compared with *Z. mays* L. cv WFG TMS X BS7 (mentioned as a Zm-B below) (McIntosh *et al.*, 1980) (Fig. 3). Difference rate between two was 4.3%. In order to compare the *rbcL* noncoding region of several species in the same tribe, Andropogoneae, DNA sequences of *Sorghum macrospermum* (Sm), *Sorghum bicolar* (Sb) and *Tripsacum dactyloides* (Td) (Golenberg *et al.*, 1993) were alinged together in Fig. 3. The positions which have different nucleotides between two maize cultivars (Zm-A and Zm-B) in the *rbcL* noncoding region are shown in Table 1 with previously mentioned three grass species. In the case of Zm-A, 15 nucleotides among the 17 nucleotides differing from Zm-B show 100 percent homology with three grass species, Td, Sm and Sb. However, the position 383 was homologous with two species among three grass species and the position 196 was different from all other species (Table 1). When sequence difference percentage was calculated from the aligned nucleotide sequences of Fig. 3, the long insertion in Sm and Td (207 to 236 and 322 to 342) and the deletion of several nucleotieds in Zm-A and Zm-B (39 to 44 and 79 to 83) were not included in the counting. The mean of sequence difference between Zm-A and

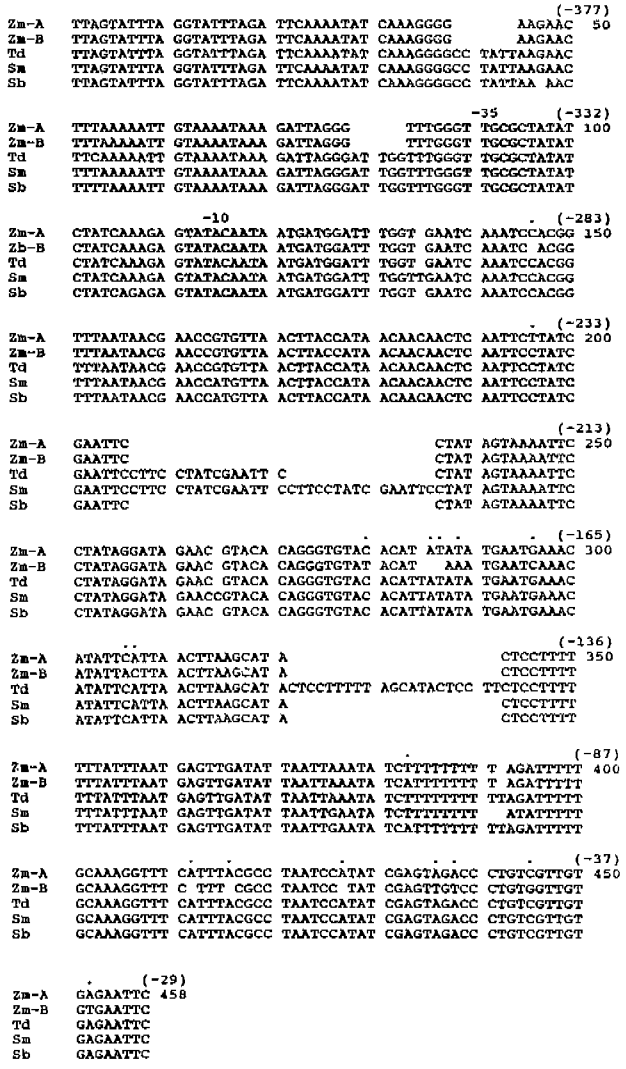

Fig. 3. Nucleotide sequence of the noncoding region of the *rbc*L gene for four grass species containing two varieties of maize. Each abbreviated species name and sequence source are as follow: Zm-A, *Z. mays* L. cv Golden X Bantam T-51 (in this study); Zm-B, *Z. mays* L. cv WFG TMS X BS7 (McIntosh *et al.*, 1980); Sm, *Sorghum macrospermum* L. (Golenberg *et al.*, 1993); Sb, *Sorghum bicolar* L. (Golenberg *et al.*, 1993); Td, *Tripsacum dactyloides* L (Golenberg *et al.*, 1993). All are classified to the same tribe, Andropogoneae (Golenberg *et al.*, 1993). The 420 nucleotide site upstream from the Zm-A initiation ATG codon is numbered as one and the subsequent numbers of nucleotides are increased in the downstream orientation. The numbers in parentheses indicate the upstream position of Zm-A from the ATG codon. Gaps are necessary to align the sequences for increasing the percentage of similarity. A′-35′ element and a′-10′ element are shown in bold. Dots designate different nucleotides between Zm-A and Zm-B.

Table 1. Different sequence positions between Zm-A and Zm-B in noncoding region of *rbcL*

No.	Position in sequence	Nucleotide				
		Zm-A	Zm-B	Td[a]	Sm[a]	Sb[a]
1	146	C	–	C	C	C
2	196	T	C	C	C	C
3	280	C	T	C	C	C
4	286	A	–	A	A	A
5	287	T	–	T	T	T
6	289	T	A	T	T	T
7	296	G	C	G	G	G
8	306	C	A	C	C	C
9	307	A	C	A	A	A
10	383	T	A	T	T	A
11	412	A	–	A	A	A
12	416	A	–	A	A	A
13	427	A	–	A	A	A
14	436	A	T	A	A	A
15	438	A	T	A	A	A
16	445	C	G	C	C	C
17	452	A	T	A	A	A

[a]In order to compare the different nucleotides between Zm-A and Zm-B with the nucleotieds of three other grass species in the same tribe, *Tripsacum dactyloids* (Td), *Sorghum macrospermum* (Sm) and *Sorghum bicolar* (Sb) (Golenberg *et al.*, 1993) are shown together.
–, denotes base deletion.

above-mentioned three grass species was 1.8%, whereas between Zm-B and three grass species was 5.4%.

Fifteen nucleotides in Zm-A among 17 nucleotides differing from Zm-B were the same as three other grass speices, Td, Sm and Sb as in Table 1. These 15 positions were compared with 8 closely related species in grass family in Golenberg *et al.* (1993), 100% homology was found in all positions except two position. The position 306 was homologous with 7 species and the position 445 with 5 species among 8 related species in grass family. Zm-A had 6 less deletion positions and 7 less substitution positions than Zm-B, as compared to the 8 grass species. The mean of sequence divergence between Zm-A and Td, Sm and Sb in the upstream region from 29 to 420 of initiation ATG codon is 3.6% less than between Zm-B and above-mentioned three species. Therefore, Zm-A seems to be evolutionarily closer to three other species in the Andropogoneae

tribe than Zm-B is. It is interesting that different cultivars of maize have 4.3% nucleotide difference in 392 nucleotides of *rbcL* noncoding region. We could not surmise exactly the reason of difference like this. One possibility of such a difference may be that the two maize cultivars were grown in different regions, i.e., Japan and USA.

The transcriptional control of gene expression commonly depends on an interplay between multiple sequence-specific DNA binding proteins and their cognate promoter elements. Several regulatory elements have been identified in the photoregulated genes. Despite of recent advance of identification of DNA-protein interactions, there is no report that specific DNA sequence in the *rbcL* promoter is assumed as a binding site for transcriptional regulatory factor. We tried to find specific DNA sequences in *rbcL* promoter region, which were homologous with other known cis-acting elements in photogenes. It was found that a homologous sequence with GATA for the nuclear binding factors GA-1 among the reported specific DNA elements (Schindler and Cashmore, 1990) was located at position −206 to −203 from ATG within the *rbcL* promoter. GATA element could be found within the promoters of the *cabE*, all other LHCII Type I CAB, *rbcS-1A* and CaMV 35S (Schindler and Cashmore, 1990). Of course, GATA element in *rbcL* is need to determine whether this sequence is involved in DNA-protein binding reaction for transcriptional regulation or not. To elucidate transcriptional regulation mechanism of plastid genes at the molecular level, it is very useful to investigate DNA-protein interaction of *rbcL* as a model system. We are doing the DNA-protein binding assay with subclones which were obtained in this study. These subclones could be used for further studies to elucidate transcriptional regulation mechanism of *rbcL* gene.

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옥수수 *rbcL* 遺傳子 프로모터 地域의 Subcloning 및 塩基序列 決定

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적 요

옥수수(*Zea mays* L. cv Golden X Bantam T-51; Zm-A)의 *rbcL* 프로모터 지역을 subcloning하기 위해 완전한 옥수수 *rbcL* 유전자를 가지고 있는 pRLYS1을 몇몇 제한효소로 절단하였다. pRLYS1의 *Eco*RI 절편, *Eco*RI-*Dde*I 절편, *Ava*I 절편 그리고 *Eco*RI-*Bam*HI 절편들 중 0.20, 0.19, 0.92 그리고 1.55 kb 크기의 DNA 절편을 pBluescriptSK+에 결합시켜 각각 pRLPS2, pRLPS3, pRLPS14 그리고 pRLPS35라는 재조합 플라스미드를 합성하였다. 네 개의 subclone은 *rbcL* 유전자의 ATG 개시 코돈 하류 136 누클레오티드부터 상류 1780 누클레오티드까지의 1.92 kb 부위를 포함한다. pRLPS2(ATG로부터 −29부터 −229)와 pRLPS3(−230부터 −420)의 염기서열을 결정하였다. 이들의 염기서열을 *Zea mays* L. cv WFG TMS X BS7(Zm-B)의 *rbcL* 프로모터 지역 염기서열과 비교했을 때 두 재배종간의 염기서열이 4.3% 다르다는 것을 알 수 있었다. ATG 코돈으로부터 상류 29 누클레오티드로부터 420 누클레오티드 지역에서 Zm-A와 같은 Andropogoneae 족(族)으로 분류되는 다른 세 종과 Zm-A간의 평균 염기서열 차이는 1.8%였으나, Zm-B와 위에서 비교된 다른 세 종간의 평균 염기서열 차이는 5.4%였다. 따라서 Zm-A는 Zm-B보다 같은 족(族)내의 다른 세 종과 진화적으로 더 연관되어 있다는 것을 알 수 있었다.

주요어: 염기서열 분석, 프로모터 지역, *rbcL*, *Zea mays* L.

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