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Light Regulation of *rbcL* Transcript and Protein-binding Region on *rbcL* Promoter in Maize

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To know the changes of *rbcL* mRNA level by illumination, Northern hybridization analysis was performed with maize (*Zea mays* L. cv Golden X Bantam). The average level of *rbcL* mRNA in the light-grown shoots was 3.1 times higher than that of the dark-grown shoots after 6 to 10 growth days. The maximum difference of *rbcL* mRNA level between the dark-grown and the light-grown shoots was 5.1 folds. These results indicate that accumulation of *rbcL* mRNA in maize shoots is induced by light. Since the transcriptional control of gene expression commonly depends on an interaction between sequence-specific DNA binding proteins and their cognate promoter elements, we carried out gel-retardation assays to elucidate the specific binding proteins on the *rbcL* promoter. It was found that plastid proteins of light-grown shoots bound to the R2 DNA fragment (-33 to -229) and R3 DNA fragment (-230 to -418 from ATG) of the *rbcL* promoter. From the results of competitive binding assays and heat or protease treatments, it was demonstrated that the bindings were sequence-specific DNA-protein interactions. Therefore, it could be concluded that the *rbcL* promoter region has at least two specific recognition sites for plastid proteins.

Keywords : regulation of transcript, DNA-binding protein, light, *rbcL*, maize

Ribulose-1,5-bisphosphate carboxylase (RuBisCo) [RuBisCase; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] catalyzes the first step of the Calvin cycle, and also plays a role in photorespiration in C_3 plants (Kawashima and Wildman, 1970). In higher plants, this enzyme is a hexadecameric protein aggregate composed of eight identical 50-60 kDa large subunits (LSU) and eight identical 12-20 kDa small subunits (SSU) (Akazawa *et al.*, 1984). The small subunit gene (*rbcS*) is in the nucleus as a multigene family and the catalytic large subunit gene (*rbcL*) is present as an uninterupted single copy on the chloroplast genome (Coen *et al.*, 1977; Gutteridge and Gatenby, 1987; Link and Bogorad, 1980). The small subunit is synthesized as a large precursor polypeptide, which is processed to its matured form during its import into chloroplasts. And then, LSUs and SSUs are assembled into a holoenzyme (Kawashima and Wildman, 1972; Cashmore *et al.*, 1978).

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; Bedbrook *et al.*, 1979; Sasaki *et al.*, 1984; Inamine *et al.*, 1985; Berry *et al.*, 1986). The *rbcL* and *rbcS* genes show complex expression characteristics including developmentally regulated, photoregulated, and tissue-specific expression (Tobin and Silverthorne, 1985; Kuhlemeier *et al.*, 1987; Manzara and Gruissem, 1988). Although the regulation of *rbcL* and *rbcS* gene expression has been extensively studied in a number of plants, the regulatory mechanisms of these gene expressions remain controversial (Kawashima and Wildman, 1970; Berry *et al.*, 1985; Rodermel and Bogorad, 1985; Tobin and Silverthorne, 1985; Berry *et al.*, 1986). The expressions of *rbcL* and *rbcS* provide an important model system to study the developmental and light regulation of the 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).

Plants have evolved many ways of responding to their environment, and have frequently transduced

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environmental signals into their normal developmental pathways. One important environmental signal is light, which is used not only for photosynthesis, but also as a trigger and a modulator of complex developmental and regulatory mechanisms (Tobin and Silverthorne, 1985). Several studies concerning the differences in gene expression between light- and dark-grown plants have demonstrated that plant responses to light involve changes in the expression of specific genes at the mRNA level (Bedbrook *et al.*, 1978; Shinozaki *et al.*, 1982). According to the studies, the expressions of light regulated chloroplast genes are complicated. They are regulated by promoter strength, transcriptional activity, mRNA stability and translational activity. Moreover, they are regulated differentially in different species, tissues, genes and developmental stages (Sheen and Bogorad, 1986; Gruissem, 1989; Steinmetz and Weil, 1989; Gilmartin *et al.*, 1990).

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 Silverthorne, 1985; Maier *et al.*, 1987). Nuclear proteins interacting with conserved DNA motifs present in promoters of nuclear photoregulated genes have been described (Datta and Cashmore, 1989; Kuhlemeier *et al.*, 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 (Lam *et al.*, 1988; Eisermann *et al.*, 1990; Klein and Mullet, 1990). Although studies on the transcriptional control of nuclear genome expression have been greatly advanced,there is only limited information about the light-regulatory mechanisms of plastid gene transcription (Wada *et al.*, 1994).

Therefore, we investigated the light effect on the amount of *rbcL* mRNA in maize for further our understanding of the light-related regulation mechanism of *rbcL*. Also, we observed the sequence-specific DNA-protein bindings between the *rbcL* promoter and the plastid proteins from the light-grown seedlings.

MATERIALS AND METHODS

Plant material

Zea mays L. cv Golden X Bantam was purchased from Sakada Seeds Foundation (Japan). They were soaked with water in the dark for 24 h at 28°C. Plants were grown in a 16 : 8 h light-dark cycle in a 28°C growth chamber. For analysis of *rbcL* mRNA, plants were grown from 6 to 10 days in continuous light (greening seedlings) or complete darkness (etiolated seedlings). Light intensity in the growth chamber was 300 μmol m^{-2} s^{-1}. The seedlings grown under the light/dark cycle and continuous light condition were harvested in the middle of the day. All manipulations of the dark-treated seedlings were performed in complete darkness, and when required, light was provided by a dim safe-green light. Seedlings were immediately frozen in liquid nitrogen after harvesting and kept at -70°C prior to use.

Analysis of *rbcL* mRNA

Total RNAs were extracted from 6 to 10 day old maize shoots under continuous light and in darkness as described by Bogorad *et al.* (1983) with some modifications. Maize shoots were ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was transferred to a beaker and hot SDS lysis buffer (0.2 M sodium borate, pH 9.0, 1% SDS, 30 mM ethylene glycol bis (β-amino ethyl ether)-N,N'-tetraacetic acid (EGTA) was added to 1 mg/g of tissue and dithiothreitol was added 6 mg/ml of solution. The lysate was filtered through 4 layers of Miracloth (Calbiochem., USA) and extracted with an equal volume of phenol once, phenol-chloroform twice and chloroform once, and then RNA was precipitated with ethanol. The RNA pellet was suspended RNase-free water to 1.5 ml/g of tissue and an equal volume of 4 M LiCl was added. It was incubated at 4°C for 12-16 h and centrifuged. The RNA pellet was resuspended RNase-free water and reprecipitated with ethanol. The final RNA pellet was dissolve in RNase-free water and the RNA concentration was determined spectrophotometrically.

The RNAs were fractionated on 1% agarose-formaldehyde gel and transferred to nitrocellulose paper for Northern hybridization analysis. Hybridization was performed with ^{32}P-labeled 0.58 kb *Pst*I fragment of maize *rbcL* gene form pRLYS1 (Lee *et al.*, 1992) and in hybridization solution (50% formamide, 5×SSPE, 2×Denhardt's solution, 0.1% SDS). The amount of radioactivity was measured with probe-hybridized nitrocellulose filters in scintillation cocktail (diphenylozaxol 4 g, 1,4-bis [5-phenyl-2-oxazolyl] benzene 0.1 g, toluen to 1 L) using a Beckman LS 5000TA β-counter to determine the *rbcL* mRNA

levels.

Preparation of probe DNA

DNA probes for Northern hybridization were prepared using a nick translation kit (Bethesda Research Lab., USA). DNA probes used for gel retardation assays were prepared by 3'-recessed end labeling. The fragment R1 (0.17 kb) and R4 (0.36 kb) was made by cutting the pRLPS14 (Lee and Sim, 1995) with *Eco*RI and *Hind*III, and *Eco*RI and DdeI, respectively. The fragment R2 (0.20 kb) was made by cutting the pRLPS2 (Lee and Sim, 1995) with *Eco*RI, and the fragment R3 (0.19 kb) by cuttin the pRLPS3 (Lee and Sim, 1995) with *Eco*RI and *Hind*III. Locations of probes used in the upstream region of the *rbcL* gene are shown in Fig 2. 3'-Labeling reaction was performed with DNA polymerase I large fragment using [^{32}P]dNTP as the radioactive nucleotides.

Gel retardation assay

The chloroplast extract was prepared from plants which were grown for 8 days under a 16 h-light/8 h-dark cycle. Chloroplasts were isolated according to the method of Orozco *et al.* (1986) with some modifications. Isolated chloroplasts were lysed and precipitated with 0 -70% solid $(NH_4)_2SO_4$ and dialysed against 50 mM Tris-HCl pH7.6, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.1% Triton X-100, 50% glycerol and 10 mM $(NH_4)_2SO_4$. Amount of protein in the extract was measured by the method of Bradford (1976).

The binding reaction was performed with the method of Eisermann *et al.* (1990). The reaction was carried out in a final volume of 50 μl binding buffer (30 mM Tris · Cl, pH 7.0, 0.5 mM β-mercaptoethanol, 0.5 mM EDTA, 80 mM $(NH_4)_2SO_4$) containing a 2.5 ng 3'-labeled probe, 10 μl (20 μg protein) chloroplast extract and 2 μg poly(dI · dC) for 30 minutes at 25°C. Poly(dI · dC) was used as a competitor to reduce the buildup of nonspecific aggregates. Equal amount of protein in the chloroplast extract was always added to each binding reaction mixture. For nonspecific competition analysis, a 100-fold excess of unlabled mechanically sheared salmon sperm DNA or *Eco*RI-*Dde*I digested pBluscriptSK+ was added to the binding reaction mixture. Protease-treated and heat-inactivated chloroplast extract were also used for binding reactions. The DNA-protein complexes were electrophoresed on a 5% (30 : 0.8 acrylamide : bisacrylamide) polyacrylamide gel by the method of Hennighausen and Lubon (1987). After soaking the gel in 5% glycerol solution, the gel was dried and autoradiographed.

RESULTS

The amount of *rbcL* mRNA can be influenced by light

To determine the effect of illumination on the *rbcL* mRNA level of maize, we examined *rbcL* mRNA level under various conditions. Total RNA was isolated from maize shoots grown for 6 to 10 days in complete darkness or continuous light. Equal amounts of total RNA was hybridized with labeled *rbcL* DNA fragments of maize. The effect of light on the levels of *rbcL* mRNA in maize shoots is shown in Fig. 1A. From the quantitation results of each blots using a liquid scintillation counter, the average level of *rbcL* mRNA in the dark-grown shoots is 32% that of the light-grown shoots on 6 to 10 growth days (Fig. 1B). The minimum and maximum difference of *rbcL* mRNA level between the dark-grown and the light-grown shoots was observed on 6 and 7 growth days as 2.4 and 5.1 folds, respectively. An equal amount of loading on each lane was shown in Fig. 1A. We also checked the level *rbcL* mRNA in shoots which were transferred from complete darkness to light for only 16 h before harvesting. The level of *rbcL* mRNA in transferred shoots was less than or similar with that in light-grown shoots depending on their growth days. Transferred shoots were presented similar *rbcL* expression with light-grown shoots at 8 and 9 growth days (Fig. 1B).

Identification of protein binding regions in the *rbcL* promoter

In order to find out whether the *rbcL* promoter has sites for sequence-specific DNA binding proteins, gel retardation assays were performed. The R1, R2, R3 and R4 DNA fragments of the *rbcL* promoter (Table 1) were incubated with chloroplast extracts from light-grown seedlings in a binding buffer. The *Hind*III-*Bam*HI fragment of pBR322 was used as a negative control DNA. As shown in Fig. 2, the R1 fragment exhibited two retarded bands, the R2 and R3 fragments had one each, while the R4 fragment had no retarded bands. None of the *rbcL* promoter fragments had retarded bands in a binding

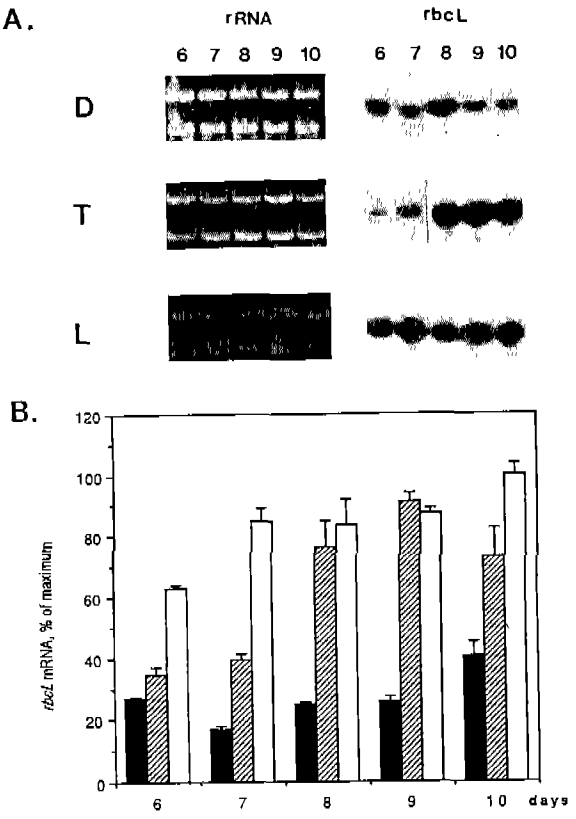

Fig. 1. Northern blot analysis of *rbc*L mRNA levels from the dark-grown, light-grown and transferred seedlings. A. Total RNA isolated from 6- to 10-day-old dark-grown (D), light-grown (L) and transferred (from dark to light) seedlings (T). Equal amounts of RNA were separated on 1% formaldehyde gels, transferred to nylon membranes and hybridized with a nick-translated 0.58 kb *Pst*I fragment of maize *rbc*L. Ethidium bromide stained gels show equal amount of loading to each gel. Numbers at the top are growth days of plants. B. Blots were quantitated by scintillation counting of excised blots. Each data point represents the mean of three experiments±S.E. Empty bar, filled bar and lined bar designate mRNA levels in light-grown, dark-grown and transferred seedlings, respectively.

reaction without chloroplast extract. The *Hind*III-*Bam*HI fragment of pBR322, as negative control, did not show any retarded band regardless of the presence of chloroplast extracts. Therefore, we found that chloroplast proteins could be bound to the R1 (+136 to -32), R2 (-33 to -229) and R3 (-230 to -418 from ATG) fragments of the *rbcL* promoter.

Competitive binding assays with unlabeled DNA fragments

To determine whether the bindings of chloroplast proteins to R1, R2, and R3 DNA fragments were

Table 1. Location of DNA fragments, R1, R2, R3 and R4

Fragment	location on upstream of *rbc*L			size (bp)
R1	+136	to	-32	168
R2	-33	to	-229	197
R3	-230	to	-418	189
R4	-419	to	-780	362

- and + mean upstream and downstream from the translational initiation site (ATG), respectively.

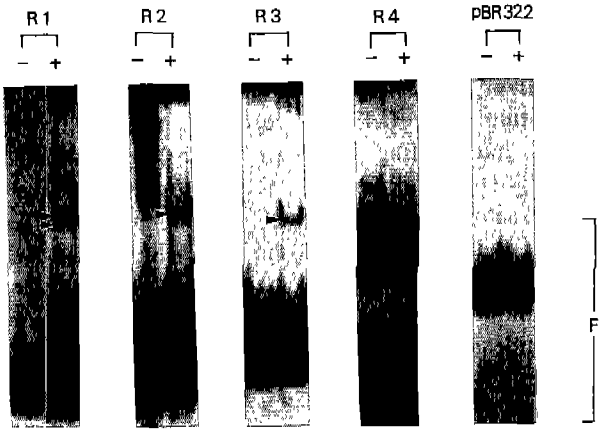

Fig. 2. Gel retardation assay of *rbc*L promoter with chloroplast extract. The labeled DNA fragment and poly(dI.dC) were subjected to 5% polyacrylamide gel electrophoresis with chloroplast extract (+) or without chloroplast extract (-). The *Hind*III-*Bam*HI DNA fragment of pBR322 was used as a negative control DNA. The arrows point to the retarded band from the DNA-protein complex formation. F designates free probe.

sequence-specific or not, unlabeled nonspecific DNA was added to a binding reaction mixture. Since a 100-fold excess of nonspecific competitors, such as mechanically sheared salmon sperm DNA and *Eco*RI-*Dde*I digested pBluescriptSK+ had no effect on the band migration seen with labeled R2 and R3 fragments (Fig. 3), it was known that the retarded bands of the R2 and R3 fragments were formed by the sequence-specific interactions. Two retarded bands of the R1 fragment were eliminated by the addition of nonspecific competitors. Therefore, the binding between the R1 fragment and the chloroplast extract was not regarded as being sequence-specific.

To confirm whether the retarded bands were formed by the binding of plastid proteins to DNA, we treated plastid extract with either proteinase K or heat. The electrophoretic band retardations of the R2 and R3 DNA fragments were not seen in a binding reaction with either proteinase K or heat treated plastid extracts (Fig. 4). These results provided evidence that these retarded bands were DNA-protein com-

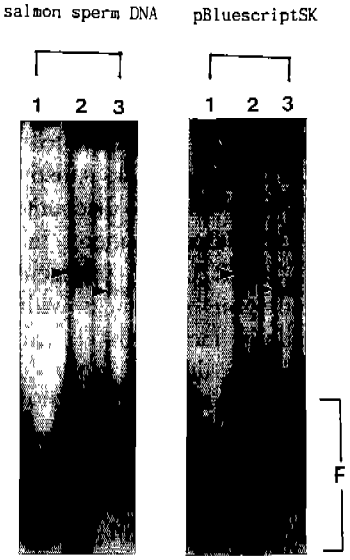

Fig. 3. Competitive binding assay of *rbc*L promoter with nonspecific competitors. Competition with 100-fold excess mechanically sheared salmon sperm DNA and *Eco*RI-*Dde*I digested pBluescriptSK+. Nonspecific competitors were added to the binding reactions containing probe R1 (lane 1), probe R2 (lane 2) and probe R3 (lane 3), respectively. The arrow indicates the retarded band by DNA-protein complex formation. F designates free probe.

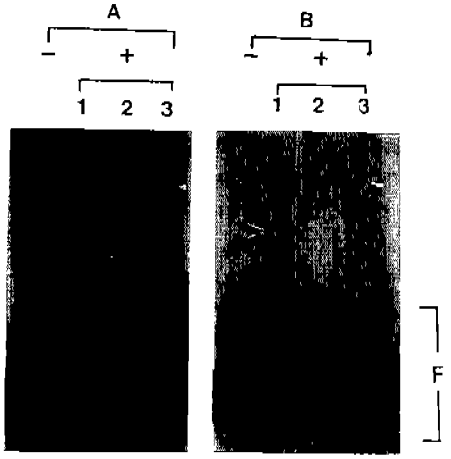

Fig. 4. Gel retardation assay of *rbc*L promoter with inactivated chloroplast extract. ^{32}P-labeled R2 and R3 were incubated with chloroplast extract (lane 1), denatured chloroplast extract (lane 2) and proteinase K-treated chloroplast extract (lane 3) in a binding buffer. - and + mean binding reactions which were performed without and with chloroplast extract, respectively. The arrow indicates the retarded band by DNA-protein complex formation. F designates free probe.

plexes.

Discussion

In order to study the effect of light on the levels of *rbcL* mRNA, *rbcL* mRNA levels in shoots grown under dark or light condition were measured by Northern hybridization (Fig. 1). *rbcL* mRNA levels from the light-grown shoots were 2.4 to 5.1 times higher than those from the dark-grown shoots depending on their developmental stages. In the case of dark- and light-grown shoots, there was no significant difference in the *rbcL* mRNA accumulation during growth. The *rbcL* mRNA accumulation in transferred shoots varied depending on their growth days. The *rbcL* mRNA level in transferred shoots was dramatically increased only on 8 days. It means that induction of *rbcL* by light can be influenced at different extent depending on its developmental stage. The average difference of *rbcL* mRNA levels between light- and dark-grown shoots was 3.1 times during growth days. These results evidently indicate that the *rbcL* mRNA level in maize shoots can be increased by illumination. It is known that the mRNA levels for many plastid genes can be regulated at both the transcriptional and post-transcriptional level (Manzara and Gruissem, 1988). Klein and Mullet (1990) also reported that a detectable increase in *rbcL* mRNA levels was observed by 4 h of illumination in barley and maize. They suggested that additional regulation points likely exist to control RNA accumulation, including transcript stability in maize. The differential stability of RNA is also an important determinant of transcript levels during plastid development in barley. In spinach, the differential stability of *psb*A mRNA has been reported to be the sole reason for differential accumulation of *psb*A mRNA in illuminated plants (Deng and Gruissem, 1987). So we are currently studying the change of *rbcL* mRNA stability by illumination to demonstrate the relation between the change of *rbcL* mRNA stability and transcription rate during light-induced development of chloroplasts in maize.

Numerous laboratories have made efforts to characterize the DNA sequences that mediate gene expression and the protein factors that bind to regulatory sequences. Several nuclear binding proteins and their cognate promoter elements have been identified in recent works. The well characterized nuclear factors which interact with the promoters of several light-regulated genes are GT-1, GBF, AT-1, GA-1 and GC-1 (Datta and Cashmore, 1989; Kuhlemeier *et al.*, 1989; Schindler and Cashmore, 1990). Most reports about the binding proteins are restricted to nuclear

proteins and only a few reports have described the interaction of chloroplast proteins with defined sequence elements of chloroplast genes (Lam *et al.*, 1988; Zaitlin *et al.*, 1989; Eisermann *et al.*, 1990). In the present work, we found the binding of chloroplast proteins to the maize *rbcL* promoter region. Chloroplast proteins specifically bind to the R2 and R3 DNA fragments which span from 33 to 229 and from 230 to 418 upstream of the ATG codon, respectively.

It was reported that three *rbcL* transcripts, with 5'-ends corresponding to positions -300, -105, and -63, are present in maize. The -105 RNA is a minor species and the ratio of the -300 RNA and -63 RNA varies during the developmental stages (Crossland *et al.*, 1984; Hanley-Bowdoin *et al.*, 1985). There is a report that the longest RNA can be specifically cleaved to a shorter RNA (Hanley-Bowdoin *et al.*, 1985; Hanley-Bowdoin and Chua, 1989). However, transcription initiation at the -300 region of *rbcL* was a result of an *in vitro* system. According to our results showing the bindings of plastid proteins around the 5'-end region of -300 RNA (R3 fragment) and -63 RNA (R2 fragment), it cannot be discounted that the 5'-end heterogeneity of *rbcL* RNA could have resulted from multiple transcriptional start sites *in vivo*. In addition, even though the promoter for maize *rbcL* gene is located with the DNA region surrounding the -300 site, the possibility that regulatory factors bind downstream from the promoter region can not be excluded. Recently, Klein *et al.* (1994) reported that the basic *rbc*L promoter is located within the region of the gene extending from positions -18 to + 63, taking position +1 as the site of initiation of transcription. They also showed that deletion of the sequence between positions +170 and +126 within the protein-encoding region reduces the full rate of transcription. They concluded that part of the promoter of the *rbc*L gene is downstream of the transcription start site and the enhancing element lies within the protein coding region of the gene in *Chlamydomonas*. Therefore, although the R2 fragment does not contain a basic *rbcL* promoter, it can not be excluded that the R2 fragment may act as a downstream regulatory region and has binding sites for regulatory factors.

A typical plastid gene promoter region contains sequences homologous to the canonical '-35' and '-10' elements (Allison and Maliga, 1995; Hanley-Bowdoin and Chua, 1989). However, it was reported that the regulation of *psb*D by light acts mainly through repeated sequences upstream of the -10/-35 promoter elements (Akazawa *et al.*, 1984). Results of Allison and Maliga (1995) and Klein *et al.* (1994) indicated that chloroplast promoter is more complex than previously assumed, and may comprise gene enhancer and regulatory elements in addition to the core promoter motifs (Klein *et al.*, 1994; Allison and Maliga 1995). Therefore, we could not discount the importance of addtional regulatory elements in plastid gene expression. Also, the R2 DNA fragment in this study has two homologous sequences with 'GATA' for the nuclear binding factor GA-1 (Schindler and Cashmore, 1990) at position -235 to -238 and -121 to -118 from ATG (Lee and Sim, 1995). Based on sequence similarity to the upstream regions of several light- regulated nuclear genes, a conserved GATA sequence found near the transcription initiation sites may be involved in the light-responsive transcription from the 90 bp region encompassing transcription initiation sites of the plastid *psb*D gene (Allison and Maliga, 1995). Therefore, the binding factor to the R2 in our study could be related to GA-1 or another kind of binding factor involving light-regulation. To know more detail about the regulation of *rbc*L expression, we are currently working on characterization of *rbc*L promoter and its binding factors.

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