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Identification and Purification of *Trans*-Acting Factors Binding to the *rbcL* R2 Promoter Region in Maize (*Zea mays*)

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We reported previously that chloroplast DNA binding factors bind to the *rbcL* promoter R2 (–33 to 229 from ATG) region in *Zea mays*. In this study, we investigated transcriptional activity in the high salt extracts of chloroplast , and identified *rbcL* R2 region-specific binding proteins in high-salt extracts. In-vitro transcription and dot blot assays revealed that the extract was transcriptionally active. An electrophoretic mobility shift assay (EMSA) showed that R2 region Binding Factors 1 and 2 (named RBF-1, 2) bound specifically to the R2 region. RBF-1 and 2 were precipitated in 0 to 34% and 34 to 51% ammonium sulfate fraction, respectively; their binding activities were detected in 0.2 M and 0.4 M KCl fractions of DEAE cellulose chromatography, respectively. RBF-1 and RBF-2 were purified to about 140- and 66-fold from the chloroplast high-salt extract, respectively, and had specific binding activity to the R2 region. These factors may play an important role in regulating the transcription of *rbcL*.

Keywords: high-salt extract, *rbcL*, RBF-1, RBF-2, regulation of transcription

D-ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) is the key enzyme for CO_2 fixation in virtually all photosynthetic organisms. This major protein comprises up to 65% of the soluble protein in grasses or alfalfa leaves. As a bifunctional enzyme that catalyzes the carboxylation and oxygenolysis of RuBP, it is the primary enzyme of the competing reductive and oxidative pentose phosphate pathway (Tabita, 1988). In higher plants, algae, and most bacteria, Rubisco is composed of eight large subunits (55,000 daltons) and eight small subunits (13,000 daltons), with the active sites for carboxylase and oxygenase activities residing in the large subunits (Miziorko and Lorimer, 1983). Small subunits are necessary for maximum activity of the L8S8 form of Rubisco, but it is unknown how small a subunit may influence this activity (Andrews, 1988).

The large subunit gene (*rbcL*) is encoded by ctDNA; the small subunit gene (*rbcS*) by nuclear DNA in higher plants and green algae (Manzara and Gruissem, 1988). The small subunit is synthesized as a precursor, with a transit sequence of about 50 amino acids found at the amino terminus. The precursor form of the small subunit is synthesized in the cytoplasm, then transported into the chloroplast. After the transit sequence is removed, the mature large and small subunits are assembled into the Rubisco holoenzyme in the chloroplasts.

The *rbcL* promoter has a conserved sequence that shares homology with prokaryotic promoter-like chloroplast gene promoters. However, the *rbcL* 5'-promoter region sequence is >80% homologous to dicotyledonous plants. The three kinds of *rbcL* mRNA can be explained by mRNA 5'-end processing, or by one or more transcription initiation sites (Hanley-Bowdoin et al., 1985). Although the significance of this is not known, the 5'-end processing may act as regulatory mechanism of *rbcL* expression during chloroplast development (Sugiura, 1989). The ratio of 1.8 Kb to 1.6 Kb transcripts is reversed during light-induced development (Crossland et al., 1984).

Nuclear proteins interact with the conserved promoter sequence of a nuclear-photoregulated gene (Datta and Cashmore, 1989; Kuhlemeier et al., 1989; Schindler and Cashmore, 1990). Chloroplast proteins also interact with specific promoter sequences of plastid photogenes (Lam et al., 1988; Eisermann et al., 1990; Klein and Mullet, 1990). In the transcriptional regulation of *psbA*, *psbC*, *psbD*, *cab*, and *rbcS*, several *trans*-acting factors, such as GBF, GA-1, GC-1, AT-1, GT-1, ASF-2, TFIID, 3AF-1, 3AF-3, 3AF-5, and AGF, affect the photoregulated-gene activity (Datta and Cashmore, 1989; Donald and Cashmore, 1990; Gash et al., 1990; Lam and Chua, 1990; Shindler and Cashmore, 1990; Manzara et al., 1991; Sarokin and Chua, 1992). For example, DNA-protein interaction with the *psbA* promoter in mustard depends on

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the phosphorylation/dephosphorylation of *trans*-acting factors (Tiller and Link, 1993). Likewise, the pea chloroplast gene (*zfpA*) encodes protein with a zinc finger motif. This *zfpA* product may regulate gene expression by binding with another gene promoter. In addition, the *rbcL* promoter binding factor is found in maize (Lam et al., 1988; Lee and Sim, 1996).

Despite great progress in the regulation studies of higher plant genes, only limited information is available about the plastid gene transcription (Wada et al., 1994). Lee and Sim (1996) reported that a chloroplast DNA binding factor binds to the *rbcL* promoter R2 region (–33 to –229 from ATG) in *Zea mays*. This binding factor was extracted with a high-salt buffer from chloroplast membranes. In the current study, we used in-vitro transcription and dot blot assays to investigate whether the *rbcL* promoter R2 region-binding protein had transcriptional activity in the thylakoid membrane.

MATERIALS AND METHODS

Plant Material and Growth

Seeds of *Z. mays* L. cv Golden X Bantam were purchased from the Sakada Seeds Foundation (Japan). They were soaked in water at 28°C for 24 h in the dark. Afterward, they were planted in vermiculite. Seedlings were grown in a 28°C growth chamber under a 16-h light/8-h dark cycle, with light intensity of 10,000 lux. For the DNA-protein binding study, plants were grown for 8 d. Those produced under the light/dark cycle were harvested at mid-day.

Preparation of Probe DNA

The dot blot probe was labeled with [^{32}P]dCTP, using a Random Primer Labeling Kit ver. 2 (TaKaRa). The reaction mixture contained 25 ng template (0.58 Kb pRLYS1/pstI fragment), random primer, buffer, dNTP mixture, 50 μCi [α-^{32}P]dCTP, and a Klenow fragment. Our probe for EMSA was end-labeled with [α-^{32}P]dATP, using a Klenow fragment. For end-labeling, a reaction mixture containing 0.2 Kb of pRLPS2/EcoRI fragment, 20 μCi [α-^{32}P]dATP, and 1 unit of a Klenow fragment was incubated for 20 min at 37°C. The mixture was then extracted with phenol/chloroform/isoamylalcohol (PCI), and the probe was precipitated with ethanol.

pRLYS1 is a plasmid in which the maize chloroplast BamHI 9 DNA fragment (4.35 Kb) has been cloned into the BamHI site of a pUC19; pRLPS2 is a plasmid in which a 197-bp fragment (–33 to –229 from *rbcL* ATG) has been inserted into the EcoRI site of a pBluescriptIISK (Lee, 1992).

Preparation of Transcriptionally Active Extract from Plastids

The transcriptionally active high-salt extracts from plastids were prepared essentially according to Orozco et al. (1985). Intact chloroplasts were isolated on Percoll gradients, using the modified method of Bartlett et al. (1982). Specifically, each maize leaf was briefly homogenized and filtered through miracloth. The crude chloroplast fraction was collected by brief centrifugation at 4,500*g*. The pellet was then resuspended in GR buffer [330 mM sorbitol, 50 mM Hepes-KOH (pH 8.0), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 5 mM sodium ascorbate] and layered onto Percoll step gradients.

Broken and intact chloroplasts in each 3-mL tube were separated by centrifugation in an SW28 rotor at 2,500 rpm for 15 min. After centrifugation, the lower band (40 to 94% interface) containing intact chloroplasts was collected and diluted with six volumes of GR buffer. The diluted chloroplasts were concentrated by centrifugation at 4,000*g* for 5 min, and resuspended in 1mL of GR buffer per 2×109 plastids. Five volumes of a lysis buffer [20 mM Hepes-KOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 5 mM ε-amino-η-caproic acid] were added to the chloroplast suspension, which was then stored for 30 min at 4°C. The lysed chloroplasts were centrifuged for 5 min at 4000*g*. Afterward, the pellet was resuspended in 1 mL of a high-salt extraction buffer (lysis buffer component, 15% glycerol, 1 M NaCl). This mixture was gently stirred for 1 h, then centrifuged for 30 min at 80,000*g* (SW41 rotor; 25,500 rpm). The supernatant was collected and used for protein precipitation and in-vitro transcription.

Ammonium Sulfate Fractionation of the Crude Extract

Solid ammonium sulfate (0.32 g/mL) was added to the supernatant. This solution was gently shaken at 4°C for 60 min. Afterward, the precipitated protein was collected by centrifugation for 15 min at 60,000*g* (SW41 rotor; 22,000 rpm). The pellet was suspended in a minimum volume of DE-52 buffer [50 mM Tricine-KOH (pH 8.00, 50 mM KCl, 0.5 mM EDTA, 0.5

mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-η-caproic acid, and 5% glycerol], and dialyzed twice against 2 L of DE-52 buffer with buffer change.

To prepare for loading on the DEAE column, the proteins in the high-salt extract were first precipitated with 0.2 g/mL $(NH_4)_2SO_4$ (0 to 34%). The protein pellets were collected by centrifugation at 60,000*g* in an SW41Ti rotor. The resultant supernatant was further fractionated by the addition of 0.12 g/mL $(NH_4)_2SO_4$ (34 to 51%), and the pellet was collected. To each supernatant, 0.13 g/mL or 0.15 g/mL $(NH_4)_2SO_4$ was sequentially added to achieve 51 to 68% or 68 to 85% precipitation (Lam et al., 1988).

DEAE-Cellulose Chromatography

DEAE-cellulose chromatography was performed using the automated fast protein liquid chromatography system (Pharmacia). A DEAE column (DE-52 resin from Whatman) was packed and washed, at a flow rate of 1.2 mL/min, with 10 column-volumes of DE-52 buffer. Proteins that had been precipitated with $(NH_4)_2SO_4$ were loaded onto the column at a flow of 0.5 mL/min. The column was washed with 25mL of DE-52 buffer, and protein was then stepwise eluted from the resin at the same flow rate, using DE-52 buffers containing 50 mM, 200 mM, 400 mM, or 1,000 mM KCl. Each fraction was dialyzed and concentrated by Centricon-10 devices (Amicon), then frozen in liquid nitrogen and stored at –70°C until further use.

In-Vitro Transcription

In-vitro transcription was performed using the methods of Orozco et al. (1985) and Kim and Mullet (1995). Activity in high-salt extracts of 1.2 x 108 plastids was assayed using 200 ng of exogenous superhelical DNA (pRLYS1) template in 40 µL of a reaction buffer that contained 12 mM Hepes-KOH (pH 8.0), 60 mM KCl, 10 mM $MgCl_2$, 10.25 mM DTT, 25 mM Tricine-KOH (pH 8.0), 0.25 mM EDTA, 2.5% glycerol, 0.25 mM ε-amino-η-caproic acid, 0.5 mM GTP, 0.5 mM CTP, 0.05 mM ATP, 0.05 mM UTP, and 12.5 unit of RNase inhibitor (Sigma). Our probe, pRLYS1, contained a 0.58-Kb *rbcL* gene fragment. After a 60-min incubation at 30°C, the reaction mixture was extracted with PCI, and the nucleic acids in the supernatant were precipitated with ethanol. As a control, several in-vitro transcription reactions were further treated with 10 µg/mL of RNase A for another 30 min at 30°C, before the PCI extraction. The resulting nucleic acids were treated with DNase, phenol-extracted, and ethanol-precipitated.

Dot Blotting

Dot blotting was performed according to the methods of Thomas (1983) and Sambrook et al. (1989). The in-vitro transcription mixture was heated to 65°C, cooled on ice, and spotted onto a nitrocellulose membrane. The nitrocellulose blots were dried and cross-linked for 5 min of UV irradiation at 254 nm. RNA dot blots were prehybridized in a buffer (6X SSPE, 5X Denhardt's Solution, 0.5% SDS, 100 g/mL salmon sperm DNA, and 50% formamide) for 4 h at 42°C. Hybridization was performed for 36 h at 42°C, in a prehybridization buffer containing denatured probe. The hybridized membrane was washed twice in 2X SSPE for 10 min at 42°C, and four times in 0.1X SSPE. Washed membranes were autoradiographed on X-ray film (Agfa) at –70°C.

EMSA (Electrophoretic Mobility Shift Assay)

EMSA was done according to the procedures of Eiserman et al. (1990) and Kim and Mullet (1995). A typical reaction mixture contained 1 ng (about 50,000 cpm) radiolabeled DNA probe, 1 g poly(dI-dC) (Sigma), 1X DEAE buffer [50 mM Tricine-KOH (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-η-caproic acid, 5% glycerol, and 10 mM $MgCl_2$], and 10 µg protein in 20 µL of the total reaction volume. The mixture was incubated for 40 min at 37°C, then loaded onto a 5% polyacrylamide gel. Electrophoresis at 4°C was performed at 25 mA in a 0.5X TBE running buffer (Sambrook et al., 1989). The gel was then dried and exposed to X-ray film.

RESULTS

In-Vitro Transcription and Dot Blotting

(We would not like to delete this sentence.) No transcript accumulated in the absence of template DNA (Lane 1) or high-salt extract (Lane 2), or after the transcription reaction with RNase (Lane 3). Moreover, pUC19 template did not produce any detectable transcripts under the same reaction condition. Only the pRLYS1 (probe) template generated a considerable amount of *rbcL* transcript. These results sug-

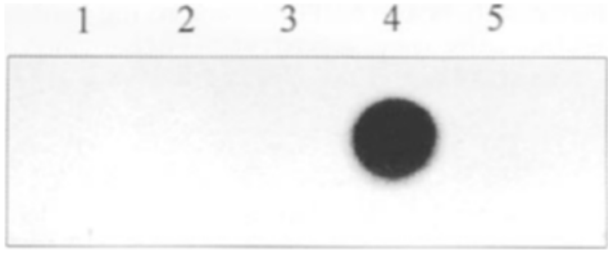

Figure 1. Dot blotting after in-vitro transcription with pRLYS1 and high-salt extract. Lane 1, (–) template; Lane 2, (–) extract; Lane 3, Treated with RNase after in-vitro transcription; Lane 4, in-vitro transcription mixture, including all components; Lane 5, in-vitro transcription mixture, including pUC19 as a template.

gest that the high-salt extract contained transcriptionally active components that specifically drove *rbcL* gene expression.

Specific DNA-Protein Complex Formation

We used EMSA to confirm that proteins prepared from chloroplast membranes bind specifically to the *rbcL* promoter R2 region. No retarded band was detected in the absence of our protein extract (Fig. 2A). However, a 1,000- or 5,000-fold excess amount of poly(dI-dC) did not abolish the retarded bands. This specificity of DNA-protein complex was further substantiated by the fact that increasing amounts of the unlabeled, specific competitor R2 fragment gradually reduced the amount of retarded bands (Fig. 2B). Interestingly, two kinds of retarded bands were observed in approximately two-fold greater amounts of the fast-migrating band than with the slow-migrating band. Although the significance of these two bands is unknown, this perhaps could be explained by cooperative binding of protein factors to a specific R2 sequence or to two different R2-specific sequences.

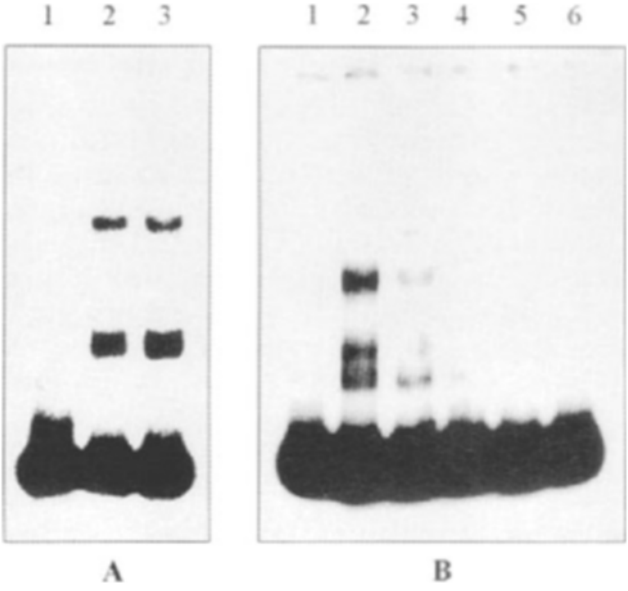

Figure 2. EMSA in the presence of various amounts of non-specific competitor (poly(dI-dC)) and specific competitor. **A.** Lane 1, (–) protein; Lane 2, (+) protein, 1 μg poly(dI-dC); Lane 3, (+) protein, 5 μg poly(dI-dC). **B.** Lane 1, (–) protein; Lane 2, (+) protein; Lane 3, (+) protein, 50X unlabeled R2; Lane 4, (+) protein, 100X unlabeled R2; Lane 5, (+) protein, 150X unlabeled R2; Lane 6, (+) protein, 200X unlabeled R2. In all lanes, the probe was197 bp long.

Purification of DNA Binding Proteins Using $(NH_4)_2SO_4$ Precipitation

We isolated two retarded bands that had been generated by either *R*2 Binding Factor-1 or Factor 2 (subsequently named RBF-1 and -2). The first was in the 0 to 34% ammonium sulfate fraction, the second in the 34 to 51% fraction (Fig. 3: Lanes 2, 3). This suggests that RBF-1 and RBF-2 specifically bind to two independent sequences of the R2 promoter.

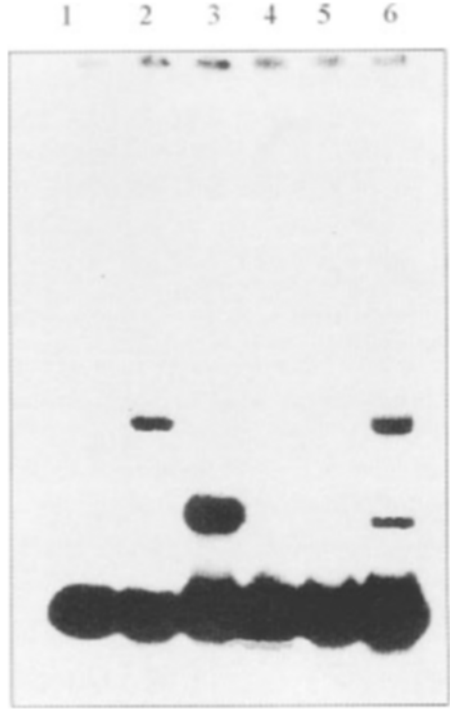

Figure 3. A. EMSA with differently precipitated ammonium sulfate fractions. Lane 1, (–) protein; Lane 2, (+) protein precipitated with 0 to 34% $(NH_4)_2SO_4$; Lane 3, (+) protein precipitated with 34 to 51% $(NH_4)_2SO_4$; Lane 4, (+) protein precipitated with 51 to 68% $(NH_4)_2SO_4$; Lane 5, (+) protein precipitated with 68 to 85% $(NH_4)_2SO_4$; Lane 6, (+) protein precipitated with 0 to 85% $(NH_4)_2SO_4$.

Purification of DNA Binding Proteins Using DEAE-Cellulose Chromatography

To further purify the RBF-1 and RBF-2 factors, the proteins precipitated with 0 to 34% or 34 to 51% $(NH_4)_2SO_4$ were fractionated by step-wise KCl gradient elution of the DEAE-cellulose column. Most of the RBF-1 binding activity was detected in the 200-mM KCl fraction, although residual activity was found in the 400-mM KCl fraction (Fig. 4A). In contrast, RBF-2 binding activity was exclusively detected in the 400-mM KCl fraction (Fig. 5A).

Comparison of Mobilities among the Complex RBF-1, RBF-2, or *Escherichia coli* RNA Polymerase

The plastid promoters, *psbA*, *rps16*, and *trnQ*, appear to be recognized by *E. coli* RNA polymerase (Lam et al., 1988; Baeza et al., 1991; Tiller and Link, 1993). *E. coli* and chloroplast RNA polymerases are similar in size, and *E. coli* RNA polymerase binding activity to the plastid promoter of *rps16* is greatly diminished in a buffer containing 1.85 mM $MgCl_2$. We reasoned that if RBF-1 or 2 were a chloroplast RNA polymerase, the R2 promoter DNA complexes with RBF-1 or 2 should migrate at a distance similar to that with the *E. coli* RNA polymerase.

The mobilities of the DNA complexes with *E. coli* RNA polymerase, RBF-1, or RBF-2 differed considerably in the current study. No DNA binding of RBF-1 and *E. coli* RNA polymerase occurred in the presence of 1.85 mM $MgCl_2$ in a buffer. However, this low-magnesium condition did not eliminate the capacity of RBF-2 binding. This could have resulted partly because RBF-2 was more cationic than RBF-1; anion exchange chromatography caused the RBF-2 factor to be eluted at a two-fold higher KCl concentration than for the RBF-1 factor..

DISCUSSION

We investigated whether transcriptionally active high-salt extracts from chloroplast thylakoid membranes bind to the *rbcL* R2 promoter region. Here, activity was measured by in-vitro transcription and dot blot assays (Fig. 1). The results clearly show that the high-salt extract contained transcriptionally active factors to drive *rbcL* gene expression.

Many efforts have been made to characterize the *cis*-acting regulatory DNA sequence and the *trans*-acting protein factors that are involved in the expres-

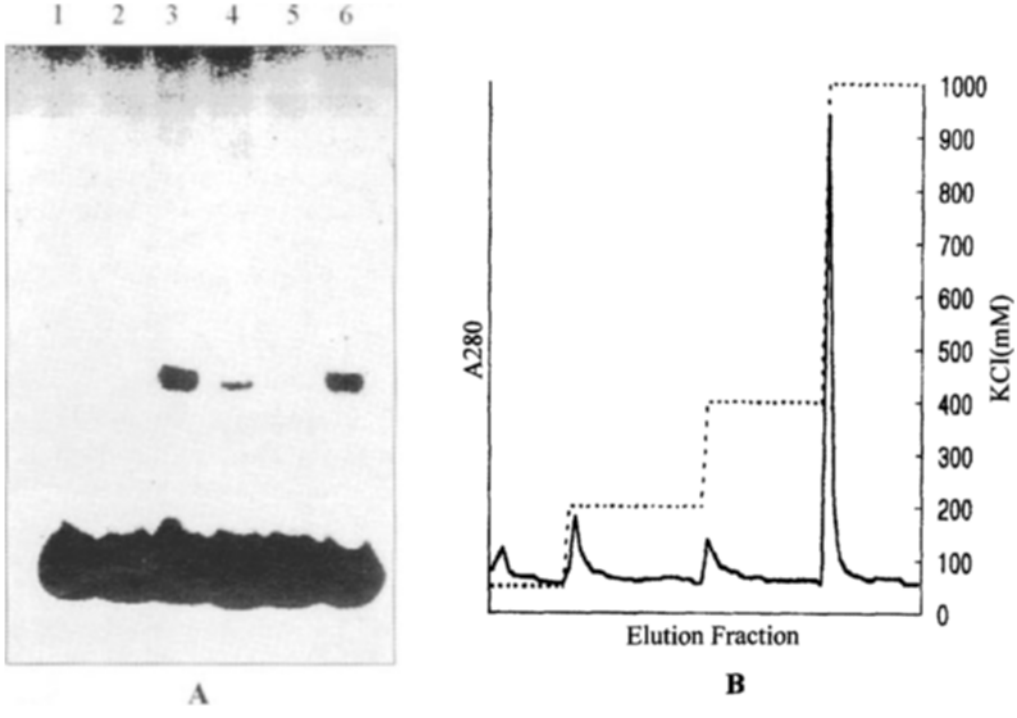

Figure 4. A. EMSA with ion-exchange chromatography elution fractions of 0 to 34% $(NH_4)_2SO_4$ precipitate. Lane 1, (–) protein; Lane 2, (+) protein eluted with buffer containing 50 mM KCl; Lane 3, (+) protein eluted with buffer containing 200 mM KCl; Lane 4, (+) protein eluted with buffer containing 400 mM KCl; Lane 5, (+) protein eluted with buffer containing 1000 mM KCl; Lane 6, (+) protein precipitated with 0 to 34% $(NH_4)_2SO_4$. **B.** Protein peak of chromatography elution fractions.

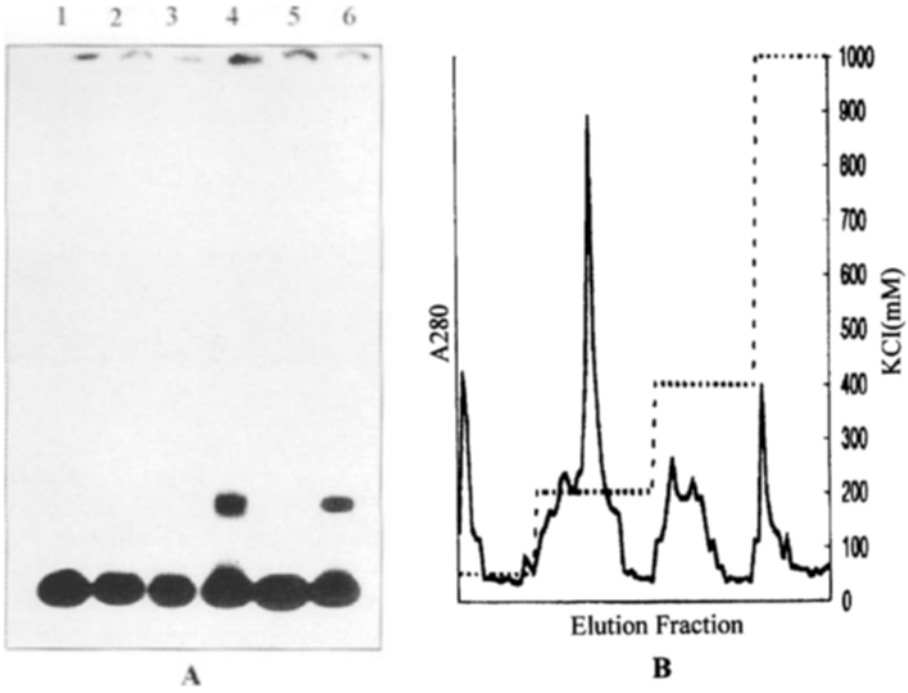

Figure 5. A. EMSA with ion-exchange chromatography elution fractions of 34 to 51% $(NH_4)_2SO_4$ precipitate. Lane 1, (–) protein; Lane 2, (+) protein eluted with buffer containing 50 mM KCl; Lane 3, (+) protein eluted with buffer containing 200 mM KCl; Lane 4, (+) protein eluted with buffer containing 400 mM KCl; Lane 5, (+) protein eluted with buffer containing 1000 mM KCl; Lane 6, (+) protein precipitated with 34 to 51% $(NH_4)_2SO_4$. **B.** Protein peak of chromatography elution fractions.

sion of chloroplast genes. Several nuclear binding proteins and cognate promoter elements have been identified. For example, chloroplast proteins (ACF, CDF-2) bind to the promoter of chloroplast *psbD*, *rps16*, *atpB*, and *rbcL* genes (Lam et al., 1988; Baeza et al., 1991; Lee, 1992; Kim and Mullet, 1995). In this study, two factors (RBF-1 and RBF-2) were identified that bind to the *rbcL* R2 region spanning from 33 to 229bp upstream of the ATG codon. Our competitive gel mobility shift assays demonstrated that RBF-1 and RBF-2 are R2 sequence-specific DNA binding proteins (Fig. 2).

Maize plants express three kinds of *rbcL* transcripts, with 5'-ends corresponding to positions –300, –105, and –63 (Crossland et al., 1984; Hanley-Bowdoin et al., 1985). The longest RNA can be specifically cleaved to a shorter RNA (Hanley-Bowdoin and Chua, 1989). Based on our results, we believe that the binding of RBF-1 and RBF-2 to the R2 region may affect *rbcL* RNA expression. However, we don't know which of the three transcripts is expressed by RBF-1 and RBF-2.

In addition, we cannot exclude the possibility that the two factors bind to a downstream sequence for –300 RNA expression. Klein et al. (1994) reported that the basic *rbcL* promoter is located within the region of the gene between positions –18 and +63. A part of the *rbcL* promoter in *Chlamydomonas* is extended downstream of the transcription initiation site, and an enhancer element is located within the coding region of the gene. Although such a basic *rbcL* promoter is missing in the R2 region, this region may

Table 1. *E. coli* RNA polymerase not only recognizes and binds to plastid promoters such as *psbA*, *rps16*, and *trnQ* (Baeza et al., 1991; Tiller and Link, 1993) but is also similar in size to chloroplast RNA polymerase (Baeza et al., 1991; Tiller and Link, 1993). Thus, we performed EMSA with *E. coli* RNA polymerase, RBF-1, and RBF-2. EMSA showed that *E. coli* RNA polymerase differs in size from RBF-1 and RBF-2 (Fig. 6). Taken together, RBF-1 and RBF-2 are not likely to be chloroplast RNA polymerases, but R2 region-specific *trans*-acting factors involved in transcriptional initiation of the *rbcL* gene.

	High-salt extract	$(NH_4)_2SO_4$ precipitation	Ion-exchange chromatography	Fold Purification
RBF1	40 mg	5.56 mg	0.288 mg	139.2
RBF2	40 mg	4.21 mg	0.607 mg	65.9

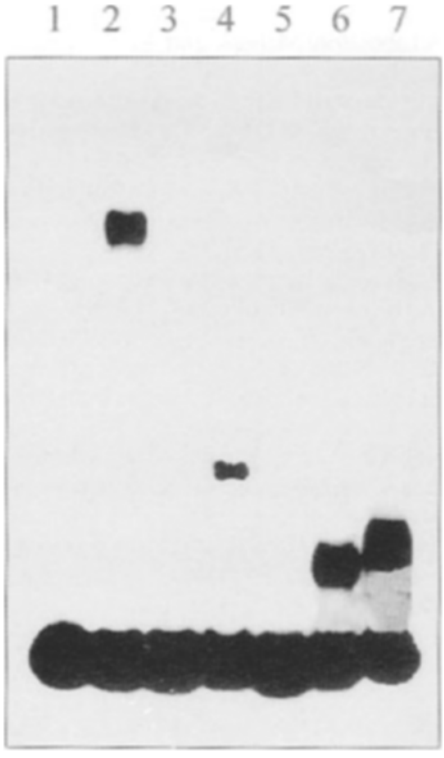

Figure 6. EMSA with RBF-1, RBF-2, and *E. coli* RNA polymerase. Lane 1, (–) protein; Lane 2, *E. coli* RNA polymerase, binding buffer containing 10 mM $MgCl_2$; Lane 3, *E. coli* RNA polymerase, binding buffer containing 1.85 mM $MgCl_2$; Lane 4, 0 to 34% $(NH_4)_2SO_4$ precipitation fraction, binding buffer containing 10 mM $MgCl_2$; Lane 5, 0 to 34% $(NH_4)_2SO_4$ precipitation fraction, binding buffer containing 1.85 mM $MgCl_2$; Lane 6, 34 to 51% $(NH_4)_2SO_4$ precipitation fraction, binding buffer containing 10 mM $MgCl_2$; Lane 7, 34 to 51% $(NH_4)_2SO_4$ precipitation fraction, binding buffer containing 1.85 mM $MgCl_2$. In all lanes, the probe was a 197-bp EcoRI fragment isolated from plasmid pRLPS2.

control transcription of the *rbcL* gene. The R2 region has two homologous sequences, at –235 to –238 and –118 to –121 from ATG, with 'GATA' as the nuclear binding factor GA-1 (Schindler and Cashmore, 1990).

In the current study, the binding activities of RBF-1 and RBF-2 were detected in 0.2 M and 0.4 M KCl fractions, respectively, of DEAE cellulose chromatography. Likewise, RBF-1 and RBF-2 were purified to about 140- and 66-fold from the chloroplast high-salt extract, respectively (Table 1), and had specific binding activity to the R2 region. These factors may play an important role in regulating the transcription of *rbcL*.

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