Sequence-specific Interaction between S1F, a Spinach Nuclear Factor, and a Negative cis-Element Conserved in Plastid-related Genes*

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The nuclear gene rps1 coding for the spinach plastid ribosomal protein CS1 exhibits both a constitutive and leaf-specific expression pattern. In contrast to other chloroplast-related genes like rbcS and cab, the leaf induction of rps1 expression is light-independent. These unique features of rps1 expression provide good models to study the mechanisms regulating plastid development and differentiation in higher plants. We report on the identification of a spinach leaf nuclear factor, designated S1F, interacting with the rps1 promoter. The S1F binding site is conserved in the promoter region of many plastid-related genes, including rbcS, cab, and rpl21. A binding activity similar to S1F was detected in nuclear extract from dark-grown de-differentiated soybean suspension cells. Through site-specific mutagenesis and transient expression in soybean cell protoplasts, we show that the S1F binding site is a negative element down-regulating the promoter activity of rps1. A ligated tetramer of S1F site was able to repress activity of the cauliflower mosaic virus 35 S promoter extending the negative function of the S1F binding site on promoter activity.

Sequence-specific DNA binding transcription factors play an important role in regulating developmental and differential processes in eukaryotes. Although the mechanism is not fully understood, it seems that these factors mediate their effects by directly or indirectly interacting with the general transcription apparatus (for review, see Refs. 1 and 2). In higher plants, differentiation of plastids depends essentially on plant cell types in which they reside and environmental conditions such as light and nutrition (for review, see Ref. 3). Sequence-specific transcription factors regulating the expression of nuclear genes coding for plastid proteins would play an important role in responding to these internal and external conditions. Recently, a number of cis-acting elements and trans-acting factors have been identified through studies on photosynthesis-related genes like rbcS and cab whose expression appears limited to chloroplast-containing cells (for review, see Ref. 4). There have been a number of reports demonstrating the existence of both positive and negative cis-elements in these promoters (5–9). Most of the studies, however, have been focused on the light responsiveness and/or organ specificity of these elements. Little is known about the regulatory elements mediating expression of plastid-related genes in a constitutive and/or developmental manner.

We have previously shown that the nuclear rps1 gene coding for the spinach plastid ribosomal protein CS1 is constitutively expressed in most tissues and at most development stages (34). The constitutively expressed rps1 is, however, differentially and light-independently up-regulated in tissues such as leaves that contain photosynthetic competent cells (34). Unlike multilocus families such as rbcS, in which each member has been shown to be expressed differentially with respect to the level of expression and developmental and/or organ specificity (10, 11), the differential expression of rps1 in leaves is achieved through employing an additional transcription start site, from which transcription is much more active than from the constitutive one. This kind of differential regulation through alternative usage of two initiation sites has been observed in another plastid ribosomal protein coding gene rpl21. The mechanisms regulating this kind of differential expression are unknown.

With the aim of elucidating the cis-acting elements and trans-acting factors that would be involved in the overlapped differential and constitutive regulation of rps1 gene expression, we have searched for the presence of cis-elements and their binding proteins in the upstream region between nucleotides +23 and −400 of spinach rps1 promoter through in vitro DNA-protein interaction and in vivo transient expression assays. In this work we have identified a spinach nuclear factor, designated S1F, binding to the rps1 promoter. The S1F binding site is conserved in the promoter region of many other plastid-related genes, including rpl21. Through transient transfection assays in soybean protoplasts in which a binding activity similar to S1F was detected, we showed that the S1F site acted as a strong negative element in the native rps1 promoter context. Furthermore, a tetramer of the S1F site was able to repress the CaMV235 S promoter by about 3 times. The possible function of the S1F site on the transcription of rbcS and cab genes is discussed.

MATERIALS AND METHODS

Plasmid Constructions, Site-directed Mutagenesis, and Polymerase Chain Reactions (PCR)—The plasmid pH6 was constructed as follows. The rps1 promoter region from +23 to −400 was generated by EcoIII and EcoRI digestion from the original genomic clone (34) and

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1 The abbreviations used are: CaMV, cauliflower mosaic virus; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction.
inserted into the EcoRI and Smal sites of pTZ191U (Bio-Rad). For plasmids pHmCAT and pHm4CAT, the HindIII-BamHI fragment from the plasmid pCaMV (Pharmacia LKB Biotechnology Inc.), containing the structural gene of chloramphenicol acetyltransferase and the rpsl transcription terminator, was ligated into BamHI- and HindIII-digested pHE or pHm4 (see below). For plasmids p2C35CAT, a ligated tetramer of the synthesized double-stranded oligonucleotide (named S1) was inserted into the XbaI site upstream to the 35 S promoter of plasmid pCaMV.

SEQUENCE 1

For clustered point mutagenesis in the promoter region of rpsl (M1, M4, and M7, see Fig. 2B), the plasmid pHmE was converted into single strands, annealed respectively with three oligonucleotides bearing nucleotide substitutions (see Fig. 2B) and synthesized with Kleenow enzyme as described (12). The mutants were identified in sequencing (13). Amplification of the region between nucleotides -176 and -257 of wild type and mutant rpsl promoters was done by PCR with the following oligonucleotides as primers.

5'-CTAGATTTGACATGAAAAC-3' 3'-TTAACAATGGTACTTTGTGATC-5'.

SEQUENCE 2

5'-AGCTTTGACAAACAGATG-3'.

SEQUENCE 3

Preparation of Nuclear Extracts—Nuclear extracts from mature spinach leaves were prepared essentially as described by Green et al. (14). Spinach leaves bought from a local market were washed with cold water and homogenized at 4 °C in buffer I (1 M hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl2, 0.5% Triton X-100, 5 mM 2-mercaptoethanol, and 0.8 mM PMSF) at 4 °C. Homogenates were filtered through 100 μm nylon meshes, precipitated by centrifugation at 10,000 g for 15 min. Pellets were resuspended with buffer II (0.5 M hexylene glycol, 10 mM PIPES/KOH, pH 7.0, 10 mM MgCl2, 5 mM 2-mercaptoethanol, and 0.8 mM PMSF) plus 0.5% Triton X-100. Nuclei were centrifuged again at 3000 × g for 10 min and washed with buffer II without Triton. After centrifugation at 3000 × g for 10 min, nuclei were then lysed with buffer III (110 mM KCl, 15 mM HEPES/KOH, pH 7.5, 5 mM MgCl2, and 1 mM DTT), and chromatin was precipitated by addition of 0.1 volume of 4 M ammonium sulfate at 4 °C for 30 min and eliminated by centrifugation at 40,000 × g for 90 min. The nuclear proteins in the supernatants were precipitated by addition of ammonium sulfate to a final concentration of 0.9 g/mL. After incubation at 4 °C for 30 min, precipitated proteins were collected by centrifugation at 10,000 × g for 15 min. Pellets were resuspended with a minimal volume of buffer IV (40 mM KCl, 25 mM HEPES/KOH, pH 7.5, 0.1 mM EDTA, 10% glycerol, and 1 mM DTT) and dialyzed against buffer IV for 3 h with two changes of buffer.

For nuclear extracts of soybean cells, dark-grown soybean suspension cells were harvested at exponential growth phase and subjected to digestion by cellulase (0.2%) and pectolyase (0.02%) in buffer CPW13 for 3 h. After filtering through 80-μm nylon mesh, and washing with buffer CPW13, about 1,000,000 protoplasts were transfected with 2 μg of plasmid DNA by the calcium-polycytochrome gel method (20). The transfected protoplasts were incubated at 28 °C in the dark for 24 h and then extracted for CAT assays as described (21).

RESULTS

Identification of Three cis-Elements in the S1 Promoter—We have previously observed that nuclear factors interact with the upstream region from nucleotide +23 to -400 of the spinach rpsl promoter (34). To define protein factors and their DNA binding sequences, we analyzed this upstream DNA fragment by DNase I protection assay. As shown in Fig. 1, three sites of protection by nuclear extracts from mature spinach leaves were observed on both strands (Fig. 1A). Resolution of these sites revealed three sequences as indicated in Fig. 1B.

Site 2, which is slightly protected from DNase I digestion by nuclear extract, contains a GT-rich sequence (GAGTTATGAGA)5. It is not clear at this stage whether this element is related to the previously characterized GT-1 sites, which are probably involved in the light responsiveness of many light-regulated genes (24–26). Interestingly, site 3 (AAGTTATGAGAAGAT) is highly similar to one of the binding sites (AAGTTAAAAA) of chalcone synthase silencer binding protein SBF-1, which has been shown to be related to GT-1 (27).

Our attention was turned to site 1, which appears to be a new plant cis-element, as judged by its nucleotide sequence: GAAGTTATGACATGAAAG. To define the nucleotides essential for binding activity, we introduced three sets of site-specific mutations in this element by using oligonucleotide-directed mutagenesis (Fig. 2B). DNA fragments spanning from -176 to -257 were prepared by PCR amplification from wild type and mutated clones and used as probes in gel shift assays. As shown in Fig. 2A, when incubated with spinach leaf nuclear extract, the 32P-labeled wild type fragment was nearly completely shifted. The DNA fragments bearing nucleotide substitutions on both sides of the element (M1 and M7) maintained their binding activity as the wild type.
type, demonstrating that these nucleotides are not important for binding. In contrast, when nucleotides -233, -234, -235, and -237 were changed (M4), binding activity of the DNA fragment was almost completely lost. At least one of the mutated base pairs must be required for binding activity.

Presence of S1F Site in Other Plastid-related Genes—
Searches in other well documented plant genes allowed us to discover in the promoter region of pea rbcS-3A a sequence homologous to site 1 over a stretch of 18 base pairs with only two nucleotides changed (Fig. 3B). Pea rbcS-3A promoter has been extensively studied before (4). This element had not been, however, identified. To ascertain that site 1 binding factor (S1F) binds indeed to pea rbcS-3A promoter, we performed competition experiments with gel shift assays. In these experiments, we used as probes two 22-mer double-stranded oligonucleotides, one (SI) corresponding to site 1 of rps1 and the other (3A) to the homologous region of pea rbcS-3A. When extracts from leaves were used, a similar shifted band was observed with either probe (Fig. 3A). This shifted band can be competed away by a 50-fold excess of unlabeled probe.

Sequence comparison further revealed that this element is conserved also in the promoter region of other rbcS and cab genes of many dicot plants (Fig. 3B and Refs. 30-33). A highly conserved S1F binding site was found in the upstream region of another plastid ribosomal protein gene, rpl21. The consensus sequence deduced from the comparison corresponds well to the core sequence of the S1F site determined in Fig. 2.

Conservation of a S1F-related Binding Activity in Dark-grown Dedifferentiated Soybean Suspension Cells—
Dark-grown soybean suspension cells were available in our laboratory. Those cultured cells contain mainly amyloplasts. It was interesting to know whether these single type plastid-contain-

FIG. 1. DNase I protection analysis of the promoter region between nucleotide -400 and +23 of the spinach rps1 gene. A, an EcoR I and BamHI I DNA fragment containing the promoter region between -400 and +23 of rps1 was labeled by Klenow filling in the presence of [32P]dATP for the upper strand or [32P]dCTP for the lower strand. After incubation with (+) or without (−) spinach nuclear extracts, the DNA fragment was partially digested with transcription start sites at +1 and bound

FIG. 2. Determination of nucleotides essential for the binding activity of site 1. A, DNA fragments corresponding to the rps1 promoter region from -176 to -257 were generated from wild type (W) or mutated DNA clones M1, M4, and M7 (see B) by PCR amplifications. The PCR fragments were then cut by XbaI, labeled by Klenow filling with [32P]dCTP, and incubated with equal amount of spinach leaf nuclear extracts. The binding reactions were electrophoretically separated on a 4% native polyacrylamide gel. Free (F) and bound (B) DNA are indicated by arrows. B, alignment of wild type and point mutations of site 1. Substituted nucleotides in the mutants are indicated by boldface letters.

FIG. 3. Conservation of S1F site in other plastid-related gene promoters. A, gel shift assays with synthesized double-stranded oligonucleotides. S1 represents the double-stranded oligonucleotide to the spinach rps1 S1F site. 3A represents the double-stranded oligonucleotide corresponding to the pea rbcS-3A promoter region. Labeled S1 or 3A were incubated with (+) or without (−) spinach leaf nuclear extracts and electrophoresed. Where indicated, a 50-fold molar excess of unlabeled DNA was added in the incubation mixture for competition. NS represents an unrelated 25-mer double-stranded oligonucleotide. B, comparison of putative S1F sites found in the rbcS, cab, and rpl21 genes with the rps1 S1F site. Conserved nucleotides are marked by boldface letters.
Binding of S1F to a Negative Element of rps1 Promoter

ing cells had maintained the S1F-like binding activity. For this purpose, we performed gel shift assays using nuclear extracts from these soybean cells. Incubation of wild type and mutated DNA fragments used in Fig. 2 with soybean nuclear extracts gave rise to a shifted pattern similar to that with spinach leaf extracts (Fig. 4B), implying that S1F activity was preserved in these dedifferentiated cells. The soybean shifted band migrated slightly faster than the spinach leaf band (Fig. 4A). It is not clear whether this difference of mobility was due to differences in size and/or physiological state of the factors from those two materials. These data suggest that S1F is an ubiquitous sequence-specific factor present in different cell types.

S1F Site Down-regulates S1 Promoter as Well as CaMV 35 S Promoter in Protoplasts of Soybean Suspension Cells—Since S1F site binding activity exists in soybean cells, which are convenient materials for protoplast preparation and transient expression assays, we decided to use these cells to determine the function of the S1F site in the context of the rps1 promoter in vivo. For this purpose, we transfected the soybean protoplasts with, respectively, plasmid constructs containing the bacterial chloramphenicol acetyltransferase gene driven by the upstream region from +23 to -400 of the rps1 promoter or by its mutated version (M4). Activities of these constructs were determined by CAT assays using the protoplast extracts 24 h after transfection. As shown in Fig. 5A, the mutated promoter was 7 times more active than the wild type, demonstrating negative function of the S1F site of rps1 promoter in the soybean protoplasts.

In order to confirm its negative function, we fused a tetramer of S1F site to the upstream region -343 to +8 of the cauliflower mosaic virus 35 S promoter, which acts as a strong constitutive promoter in most organs of plants (28) and in protoplasts of cultured cells. The chimeric and the original (35 S) promoters were used, respectively, to drive CAT gene expression in protoplasts of soybean suspension cell. The activities of both promoters were determined by CAT assays of extracts from transiently transfected protoplasts. As shown in Fig. 5B, the fused S1F sites-35 S promoter was about 3 times less active than the 35 S promoter, indicating that S1F sites were able to repress 35 S promoter in transient expression.

Taken together, these data strongly support the notion that the S1F site is a negative element in soybean suspension cells.

Determination of the Molecular Size of S1F—In the first attempt to characterize S1F, we used Southwestern blotting to determine the molecular mass of S1F. Spinach leaf nuclear proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then incubated with the 32P-labeled tetramer of rps1 S1F site and revealed by autoradiography. Lanes 1, 2, and 3 show the result for wild type, while lane 4 shows the result for a band of about 30 kDa was revealed by the radioactive DNA probe. To confirm this observation, UV cross-linking technique was used. After incubation of the leaf nuclear extract with 32P-end-labeled 22-base pair synthetic oligonucleotide of the S1F site, the binding

**FIG. 4.** Demonstration of S1F binding activity in dark-grown dedifferentiated soybean suspension cell extracts. A, **32**P-labeled PCR amplified wild type DNA fragment used as in Fig. 2 was incubated without (-) or with nuclear extracts from spinach leaves (SL) or soybean cells (SC) and electrophoresed. B, gel shift assays with nuclear extracts from soybean cells incubated with the wild type and the mutated probes used in Fig. 2.

| PLASMID CONSTRUCT | RELATIVE CAT ACTIVITY |
|-------------------|-----------------------|
| pHECAT 32-23 | 1.0 |
| pHEM4CAT 32 | 7.2 ± 1.4 |

**FIG. 5.** Effect of the S1F site on transcription from the rps1 and CaMV 35 S promoters. A, CAT plasmids driven by the rps1 promoter (pHECAT) or the S1F site mutated version (pHEM4CAT), were transfected into protoplasts of soybean suspension cells, and CAT activity was measured after a 24-h incubation. The results represent mean values ± standard deviations of five independent series of transfection. B, CAT plasmids directed by the CaMV 35 S promoter (pCaMVCN) or the fused S1F sites-35 S promoter (p2/3-3SSCAT) were transfected and the CAT activities were measured as in A. The results represent mean values ± standard deviations of four independent series of transfection experiments.

**FIG. 6.** Determination of the molecular size of S1F by Southwestern blot or UV cross-linking analysis. Lane 1, blot of spinach leaf nuclear extracts probed with the 32P-labeled tetramer of rps1 S1F site and revealed by autoradiography. Lanes 2–4, UV cross-linking assays. End-labeled 22-mer oligonucleotide S1 (see Fig. 3) was incubated with (lanes 2 and 3) or without (lane 4) spinach leaf nuclear extract for binding in the presence of 2 μg of poly(dI-dC), and the binding mixtures were irradiated by UV light. The radiolabeled proteins were separated by electrophoresis and revealed by autoradiography. In lane 2, a 50-fold molar excess of cold probe DNA was added for competition. Arrows indicate the protein bands revealed by both methods. Numbers are the molecular size markers in kDa.
complexes were subsequently irradiated with UV light and then separated by SDS-polyacrylamide gel electrophoresis. In these experiments, four radioactive bands were observed in the absence of specific competitors (Fig. 6, lane 3). When 50 times the amount of cold probe was added during the binding reaction, the band migrating at about 37 kDa disappeared (Fig. 6, lane 2), indicating that this band was the specific SIF-SIF site complex, and that the others were produced by nonspecific DNA-protein interactions. Taking the DNA weight into account, the size of this band is consistent with the Southwestern result. These data indicated that SIF is a protein of about 30 kDa.

**DISCUSSION**

Studies on the expression of nuclear genes coding for plastid ribosomal proteins provide good opportunities in searching for nuclear transcription factors involved in biogenesis and development of plastids in higher plants. In this paper we have described the identification of a spinach leaf nuclear factor SIF that binds to the rps1 promoter. The SIF binding site is conserved in the promoter region of many other plastid-related genes including rbcS, cab, and rpl21. Presence of the SIF binding site in those functionally different promoters and the preservation of the SIF binding activity in the differentiated soybean cells imply that SIF would be a ubiquitous factor involved in regulation of plastid differentiation and development.

Site-specific mutagenesis and transient expression assays have shown that the SIF site down-regulates the promoter activity of rps1 in the protoplasts of dark-grown differentiated soybean suspension cells. This negative function is mediated through unique combinations of a common negative cis-element (e.g. SIF site) with other different cis-elements present in those promoters. In eukaryotes, many negative transcription factors can also function as activators, depending on the nature of the DNA binding site and interaction with other proteins (reviewed in Ref. 29). Specifically, it has been shown that GT-1 binding sites, which are involved in the up-regulation of many light-responsive genes, could repress constitutive transcription in the dark when fused to CaMV 35 S promoter (6). For SIF, we do not know presently whether it functions differently in regulating rps1 expression in different tissues in which different rps1 promoter binding activities have been detected (34).

As described previously, differential expression of rps1 and rpl21 is regulated through alternative usage of two start sites. Since the SIF site is present in both promoters, it will be interesting to know whether SIF plays a role in transcription start site selection. These problems can be solved only through experiments with transgenic plants which are now in progress.

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