Nuclear genes encoding plastid ribosomal proteins are more highly expressed in leaves than in roots. This leaf-specific induction seems to be light-independent. We have previously characterized a spinach nuclear factor S1F binding to a cis-element within the rps1 promoter, which negatively regulates both the rps1 and the cauliflower mosaic virus 35S promoters in transient expression assays. Here, we show that the S1F binding site is related to but different from the light-responsive Box II of the pea rbcS-3A promoter, which is recognized by the nuclear factor GT-1. Transgenic plant analyses showed that the S1F site tissue-specifically represses the rps1 promoter in roots as well as in etiolated seedlings. We suggest that the GT-1-related S1F binding site is responsible, at least in part, for the transcriptional repression of rps1 in nonphotosynthetic tissues such as roots.

The plastid ribosomal components are encoded by both the plastid and the nuclear genomes. The regulation of plastid ribonucleic acid synthesis is associated with the differentiation states of plastids in higher plants, which can be reflected by the differential regulation of both plastidic and nuclear gene expression in plant cells containing distinct plastid types (Deng and Gruissem, 1988; Bisanz-Seyer and Mache, 1992; Lagrange et al., 1993). We have previously shown that the transcript levels of nuclear gene rps1 encoding plastid ribosomal protein cS1 are much higher in chloroplast-containing leaf tissues than in proplastid amyloplast-containing root tissues (Franzetti et al., 1992). In contrast to many photosynthetic protein coding genes, the leaf-specific induction of rps1 transcription appears to be light-independent (Franzetti et al., 1992). This tissue-specific induction of rps1 expression would be achieved through a light-independent regulatory mechanism.

The light-dependent transcriptional regulation of chloroplastic protein coding genes, including those encoding ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) and chlorophyll a/b-binding proteins (cbb), seems to be mediated by specific interactions of nuclear factors with cis-acting DNA sequences in the promoter regions (Gilmartin et al., 1990). Nuclear factors interacting with conserved promoter elements have been characterized. One of those factors is GT-1 which binds to several degenerated DNA motifs, designated Box II, II', III, III', and III'', present within the pea rbcS-3A promoter (Green et al., 1987, 1988). Similar motifs have been identified within a number of other light-responsive genes (Stockhaus et al., 1987).

**S1F Binding Site Is Related to but Different from the Light-responsive GT-1 Binding Site and Differentially Represses the Spinach rps1 Promoter in Transgenic Tobacco**

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(Received for publication, January 12, 1994, and in revised form, March 14, 1994)

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Manzara and Gruissem, 1988; Dean et al., 1989; Elliott et al., 1989; Kay et al., 1990; Dehesh et al., 1990; Schindler and Cashmore, 1990; Lawton et al., 1991). Several studies have demonstrated that the GT-1 binding sites are important in the light-regulated expression (Gilmartin and Chua, 1990a, 1990b; Kuhlmeier et al., 1988; Lam and Chua 1990). One of the GT-1 factors which has high binding specificity to Box II has been recently cloned and named 2BF (Perisic and Lam, 1992) or GT-1a (Gilmartin et al., 1992).

Three binding sites (Site 1, 2, and 3) in the spinach rps1 promoter region and the cognate binding factor of Site 1 (S1F) have been previously characterized (Zhou et al., 1992). Here, we investigated further the relationship between these binding sites and the GT-1 binding site (Box II of the pea rbcS-3A promoter) and the function of Site 1 in transgenic plants. Our data demonstrate that the S1F binding site is related to the GT-1 binding site and preferentially represses the spinach rps1 promoter in nonphotosynthetic tissues such as roots.

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**MATERIALS AND METHODS**

**Probes Used in Gel Shift Assays—Synthesized oligonucleotides:** Site 1, 5'-CTGAGACTGTACATTGTGAACAG, and TAAACAAATGACATCTTGGATC-5'; Site 2, 5'-CTGAATTTCTCCGCAAATCTA, and AAAAGTCGATATCTTGATTTA; Site 3, 5'-CTGTAATTTCTCCGCAAATCTA, and AAAAGTCGATATCTTGATTTA.

The translation products were analyzed by SDS-PAGE and the gel was excised from the corresponding plasmids by restriction enzymes and ligated to either the pBluescript (Stratagene). Inserts containing dimer of each site were annealed and ligated to either the T7 RNA polymerase (Promega) in the presence of [3H]methylamine according to the manufacturer's procedure. The translation products were analyzed by SDS-PAGE and the gel was stained with Coomassie Blue. The translation products were then analyzed by gel electrophoresis.
Expression Analyses of transgenic Plants — The 0.4-kb spinach rps1 promoter fragment and the version bearing mutations in Site 1 were cut out, respectively, from the plasmids pHECAT and pM4CAT described in Zhou et al. (1992), and inserted into BamHI-SmaI sites of the binary plasmid pBI101 (Clontech) to direct the expression of the gene of P-glucuronidase. These constructs were introduced into Agrobacterium tumefaciens strain LBA4404 through triparental matings according to Fraley et al. (1985). Leaf discs of Nicotiana tabacum (Xanthi) were transformed by the constructs through Agrobacterium infections according to Jefferson et al. (1987).

For β-glucuronidase activity assays, crude protein extracts were taken from fully developed leaves and roots of mature transgenic plants. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad) according to the manufacturer's procedure. β-Glucuronidase enzyme activity was quantified according to Jefferson et al. (1987) by measuring the fluorescence of methylumbelliferone produced by β-glucuronidase cleavage of methylumbelliferyl-β-D-glucuronide.

RESULTS

Sites 1 and 3 of the rps1 Promoter Are Two Related Binding Sites — Examination of the nucleotide sequences of the three sites of the rps1 promoter (Zhou et al., 1992) showed that they are all rich in nucleotide T, A, and G in either the upper or the lower strand (Fig. 1). This similarity prompted us to examine whether they are related DNA binding sites. In the first experiment, dimers of Site 1 were 32P-labeled and used as probe in competition binding assays (Fig. 2A). The spinach leaf nuclear factor binding to Site 1 has been previously characterized and designated S1F (Zhou et al., 1992). Incubation of the probe with spinach leaf nuclear extracts gave rise to a shifted band which was supposed to be the S1F-Site 1 complex (lane 2). When increasing amounts (from 10–100-fold molar excess) of unlabeled DNA fragments corresponding to Site 1, Site 1 mutant (1m), Site 2, Site 2 mutant (2m), Site 3 and Site 3 mutant (3m) were used in A. 32P-labeled DNA fragment of Site 1 dimer was incubated with spinach leaf nuclear extracts in the absence of specific competitor (−) or in the presence of the indicated molar excess of unlabeled DNA fragments corresponding to dimers of Site 1, Site 1 mutant (1m), Site 2, Site 2 mutant (2m), Site 3 and Site 3 mutant (3m). B. 32P-labeled DNA fragment of Site 3 dimer was incubated with spinach leaf nuclear extract in the absence of specific competitor or in the presence of the same specific competitors as used in A.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Site 4</th>
<th>Site 5</th>
<th>Site 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar Excess</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

When dimers of Site 3 were used as probe in the competition binding assays, similar competition patterns were obtained with the same competitors as used in the previous experiment (Fig. 2B). In the case of competition with mutant Site 2 dimers, we observed some weak competition (lane 10). We reasoned

![Fig. 2. Relationship between the three binding sites from rps1 promoter for binding to spinach nuclear extracts. A, 32P-labeled DNA fragment of Site 1 dimer was incubated with spinach leaf nuclear extracts in the absence of specific competitor (−) or in the presence of the indicated molar excess of unlabeled DNA fragments corresponding to dimers of Site 1, Site 1 mutant (1m), Site 2, Site 2 mutant (2m), Site 3 and Site 3 mutant (3m). B, 32P-labeled DNA fragment of Site 3 dimer was incubated with spinach leaf nuclear extract in the absence of specific competitor or in the presence of the same specific competitors as used in A.](image)

### Table I

**Alignment of GT elements with the three sites of the spinach rps1 promoter**

<table>
<thead>
<tr>
<th>Motif</th>
<th>Gene</th>
<th>Factor</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Box II</td>
<td>rbcS-3A</td>
<td>GT-1</td>
<td>GTGTTGTTTAAATAG</td>
<td>Green et al. (1988)</td>
</tr>
<tr>
<td>Box II* (rev)</td>
<td>rbcS-3A</td>
<td>GT-1</td>
<td>GTGAGGTTAAATCCC</td>
<td>Green et al. (1988)</td>
</tr>
<tr>
<td>Box III (rev)</td>
<td>rbcS-3A</td>
<td>GT-1</td>
<td>TAGTGAAAGATGTA</td>
<td>Green et al. (1988)</td>
</tr>
<tr>
<td>Box III* (rev)</td>
<td>rbcS-3A</td>
<td>GT-1</td>
<td>GGGTTGAATGTTGGT</td>
<td>Green et al. (1988)</td>
</tr>
<tr>
<td>Box III**</td>
<td>rbcS-3A</td>
<td>GT-1</td>
<td>TTTGGAATGAAACAG</td>
<td>Green et al. (1988)</td>
</tr>
<tr>
<td>GT-2 box</td>
<td>phyA</td>
<td>GT-2</td>
<td>GGTATT</td>
<td>Dehesh et al. (1990)</td>
</tr>
<tr>
<td>Box 1</td>
<td>chs15</td>
<td>SBF-1</td>
<td>TAAAGGTTAAAC</td>
<td>Lawton et al. (1991)</td>
</tr>
<tr>
<td>Box 2</td>
<td>chs15</td>
<td>SBF-1</td>
<td>CGTGTAAAATAT</td>
<td>Lawton et al. (1991)</td>
</tr>
<tr>
<td>Box 3</td>
<td>chs15</td>
<td>SBF-1</td>
<td>TATTGTTAATCAA</td>
<td>Lawton et al. (1991)</td>
</tr>
<tr>
<td>Site 1 (rev)</td>
<td>rps1</td>
<td>S1F</td>
<td>TTCTAGGTAAACACTTC</td>
<td>Zhou et al. (1992)</td>
</tr>
<tr>
<td>Site 2</td>
<td>rps1</td>
<td>S1F</td>
<td>GTGTTAATGTTAGAAA</td>
<td>Zhou et al. (1992)</td>
</tr>
<tr>
<td>Site 3 (rev)</td>
<td>rps1</td>
<td>S1F</td>
<td>AAGTTAGTTAAAGAT</td>
<td>Zhou et al. (1992)</td>
</tr>
</tbody>
</table>
Fig. 3. Relationship between the rps1 binding sites and the GT-1 binding site corresponding to Box II of the pea rbcS-3A promoter for binding to spinach leaf nuclear extracts. A, 32P-labeled dimers of Box II were incubated with spinach leaf nuclear extracts in the absence of specific competitor (−) or in the presence of the indicated molar excess of unlabeled DNA fragments corresponding to dimers of Box II (GT-1), Site 2, Site 3, Site 3 mutant (3m), Site 1 and Site 1 mutant (1m). B and C, 32P-labeled dimers of Site 3 (B) or Site 1 (C) of the rps1 promoter were incubated with spinach leaf nuclear extracts in the absence of specific competitor (−) or in the presence of the indicated molar excess of unlabeled DNA fragments corresponding to dimers of Box II (GT-1) and Box II mutant (gtm).

that the substitutions in Site 2 would have accidentally created a Site 3-like element.

Taken together, these data indicate that Sites 3 and 1 within the rps1 promoter are related binding sites.

Sites 1 and 3 of the rps1 Promoter Are Related to the Light-responsive Box II of the Pea rbcS-3A Promoter-The three binding sites within the rps1 promoter are T-, A-, and G-rich elements. This is reminiscent of the binding sites of GT-1 (Green et al., 1987, 1988), GT-2 (Dekesh et al., 1990), and SBF-1 (Lawton et al., 1991), which are involved, respectively, in the light-regulated expression of the ribulose-1,5-bisphosphate carboxylase small subunit (rbcS), the phytochrome A (phyA), and the chalcone synthase (chs15) genes. It has been suggested that GT-1, GT-2, and SBF-1 are related nuclear factors and probably belong to the same DNA-binding protein family: the GT-binding proteins (Lawton et al., 1991; Gilmartin et al., 1992). The core sequence of the GT-binding sites which are quite degenerated has been assessed as GGTTAA (Green et al., 1988). Sequence alignment shown in Table I suggests that the three sites of the rps1 promoter may be also GT-binding site-related elements.

In order to determine whether the rps1 binding sites are related to the GT motifs, we used dimers of the authentic GT-1 binding site corresponding to Box II of the pea rbcS-3A promoter as probe for competition binding assays (Fig. 3A). Box II of the pea rbcS-3A has the perfect GGTTAA core sequence. Dimers of wild-type or mutant Box II were excised from plasmids. Incubation of 32P-labeled dimers of Box II with spinach nuclear extracts gave rise to a shifted complex (lane 1). This shifted band could be efficiently competed away by unlabeled probe in excess (lanes 2–4), but not by dimers of the mutant Box II at 100-fold molar excess (lane 5). The mutant Box II
bearing two nucleotide substitutions has been shown to be functionally inactive in vivo and inactive in binding GT-1 in vitro (Green et al., 1988; Kuhlemeier et al., 1988). These data suggest the existence of a spinach version of GT-1. Excess amounts of Site 2 dimers from the rps1 promoter did not compete efficiently with dimers of Box II for binding to GT-1 (lanes 6-8), suggesting that Site 2 within the rps1 promoter is not related to the GT-1 binding site. However, dimers of either Site 1 or 3 could compete with the probe for GT-1 binding (lanes 9-11 and lanes 13-15). Dimers of either Site 1 or 3 did not compete with the probe at 100-molar excess (lanes 12 and 16).

Similarly, dimers of the GT-1 site in excess amount could efficiently compete with either Site 3 (Fig. 3B) or Site 1 (Fig. 3C) for binding to their cognate factors. Dimers of mutant GT-1 site did not compete at 100-fold molar excess (lane 6).

These data together indicate that Sites 1 and 3 within the spinach rps1 promoter are related to the GT-1 binding site for binding to spinach nuclear factors.

The Box II Binding Factor B2F (or GT-1a) Does Not Bind to Site 1 or 3 of the rps1 Promoter—One of the GT-1 factors has been recently cloned from tobacco. This cloned factor named B2F (Perisic and Lam, 1992) or GT-1a (Gilmartin et al., 1992) has high binding specificity to Box II of the pea rbcS-3A promoter. However, it is unable to bind to other GT-1-related binding sites (Gilmartin et al., 1992). Here, we examined whether or not B2F/GT-1a binds to Site 1 or 3 of the spinach rps1 promoter. B2F/GT-1a cDNA was isolated using reverse transcriptase-polymerase chain reaction and cloned downstream of the T7 promoter (see "Materials and Methods"). The B2F/GT-1a protein was then produced by in vitro transcription/translation. SDS-PAGE analyses of the translation products revealed a major 35S-labeled band at about revealed a major 35S-labeled band at about 43-kDa position (Fig. 4A), corresponding to the calculated molecular mass from the cDNA. The translation mixtures were then used to incubate with 32P-labeled DNA probes and analyzed by gel shift assays. The B2F/GT-1a cDNA-directed translation products bound only to dimers of Box II of the pea rbcS-3A, but did not recognize Site 1 or 3 of the spinach rps1 promoter (Fig. 4B), suggesting that Sites 1 and 3 of the rps1 are different from Box II of the rbcS-3A in regarding to the binding specificity to tobacco B2F/GT-1a.

Site 1 Preferentially Represses the rps1 Promoter Activity in Roots and in Etiolated Seedlings of Transgenic Tobacco—To test the function of Site 1 in vivo, we inserted the 0.4-kb wild-type or the Site 1-mutated rps1 promoter fragment to control the expression of uidA gene (encoding β-glucuronidase, GUS) (Fig. 5A). The constructs were then introduced into tobacco leaf discs. 15-18 transformed lines were generated with each construct. Protein extracts of both leaf and root tissues were from the CaMV 35S promoter, data not shown, suggesting that more upstream elements are required to ensure high level expression of rps1 in leaves. The mutant promoter had a comparable activity as the wild-type promoter, with a slight increase in plants transformed with the mutant promoter construct (Fig. 5B). These data suggest that Site 1 is not required for activating the rps1 promoter in leaves. However, in root extracts, the β-glucuronidase activity was about 6-fold higher from plants transformed by the mutant promoter construct than from those transformed by the wild-type promoter constructs.
Structure (Fig. 5B), showing that Site 1 has negative effect on the 0.4-kb rps1 promoter in roots. We examined further whether Site 1 modulate the rps1 promoter activity in etiolated plants. Seeds from 10 independent plants transformed by each construct were germinated in semi-solid Murashige-Skoog medium under either light or dark conditions. Aerial parts of the seedlings were collected and extracted for β-glucuronidase activity assays. In light-grown seedlings, the β-glucuronidase activity produced by the mutant promoter-β-glucuronidase construct was about twice as much as produced by the wild-type promoter construct. In etiolated seedlings, the mutant promoter activity was about 5-fold higher than the wild-type promoter (Fig. 5C), suggesting that Site 1 also has a relatively strong negative function on the transcription from the 0.4-kb rps1 promoter in etiolated plants.

These results suggest that in contrast to the GT-1 binding sites, the S1F binding site has no positive effect in light-grown leaf tissues, but acts as a negative element differentially repressing the spinach rps1 promoter in transgenic plants.

**DISCUSSION**

Two binding sites (Sites 1 and 3) within the rps1 promoter are related to the light-responsive GT-1 binding site (Box II of the pea rbcS-3A) in respect to their competitive binding activity to spinach leaf nuclear factors. However, the cloned tobacco B2F/GT-1a which has high specificity to Box II of the pea rbcS-3A could not bind to Site 1 or 3 of the rps1 promoter. Thus, the rps1 sites are different from the GT-1 site. It has been reported that B2F/GT-1a binds only to Box II, but do not bind to Box III of the pea rbcS-3A promoter (Gilmartin et al., 1992), which had been, however, shown to be relevant GT-1 binding (Dehesh et al., 1992). Therefore, there may exist a group of GTA-rich sequence binding factors which have distinct protein structure and accurate DNA sequence specificity. The rps1 Site 1 binding factor S1F and the factor binding to Site 3 are either members of the GT-1 factors, or different factors having an overlapped recognition sequence with the GT-1 factors.

The GT-1 binding sites had been initially identified in the pea rbcS-3A promoter and had been shown to be implicated in the light-dependent activation of rbcS-3A transcription in green tissues (Gilmartin and Chua, 1990a, 1990b; Kuhlemeier et al., 1988; Lam and Chua, 1990). In contrast, GT-related binding sites identified in rice phytochrome A gene (phyA) promoter function as positive elements in transcriptional activation in dark (Dehesh et al., 1990). Furthermore, Lawton et al. (1991) have shown that three GT-1 related SBF-1 binding sites are located within a silencer region of the chalcone synthase gene (chs15). However, our results have demonstrated that the GT-1 related S1F site has distinct functions. It does not activate the rps1 promoter in light-grown leaves, but has negative function on the 0.4-kb promoter in roots and in etiolated seedlings. During the differentiation of nonphotosynthetic plastids (amyloplasts in roots, chromoplasts in fruits, and etioplasts in dark-grown leaves), many genes, including those encoding plastid ribosomal proteins are repressed. The fact that the S1F binding site specifically represses the rps1 promoter in roots as well as in nonphotosynthetic cell cultures (Zhou et al., 1992) suggests that the S1F binding site of the rps1 promoter is a negative element functioning specifically in nonphotosynthetic tissues. This would explain at least partially how the non-light regulated rps1 gene is up-regulated in leaves but down-regulated in roots as well as in other nonphotosynthetic tissues (Franzetti et al., 1992). This root-specific repression by Site 1 would be achieved through interactions with distinct S1F binding activities, since Site 1 seems to have no function in light-grown leaves from which the S1F is characterized. Indeed, we have found a different S1F binding activity in root extracts. Whether this root-specific S1F binding activity is a different factor or a post-transcriptionally modified version of the light-grown leaf S1F is actually under investigation.

In addition, Site 1 also has relatively strong negative function in etiolated plants (Fig. 5). This is intriguing, since the spinach rps1 mRNA seems to be accumulated at the same levels in both light- and dark-grown plants (Franzetti et al., 1992). We suggest the existence of other upstream elements which activate the transcription of rps1 in dark. This kind of dark repression by GT elements has been also reported by Kuhlemeier et al. (1987).

Therefore, GT elements may have distinct functions in modulating transcriptional activity of a subset of genes in response to diverse physiological conditions. Specific functions of the GT elements and their cognate binding factors would be determined by specific promoter context and particular combination of binding factors present in different cell types.

**Acknowledgment**—We thank Dr. N.-H. Chua for providing clones containing tetramers of wild-type and mutant GT-1 binding sites.

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