The Mechanism of GT Element-mediated Cell Type-specific Transcriptional Control*

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Promoter studies have revealed that sequences related to the GT-1 binding site, known as GT elements, are conserved in plant nuclear genes of diverse functions. In this work, we addressed the issue of whether GT elements are involved in cell type-specific transcriptional regulation. We found that the inactivation of GT-1 site-mediated transcription in roots is correlated with the absence of the GT-1 binding activity in root extracts. In addition, the mutation of the related GT-1 (from the pea rbcS-3A) and the S1F (from the spinach rps1) sites resulted in an increase of their transcriptional activity in roots that contain a distinct GT element-binding factor, referred to as RGTF. Although specific to GT elements, RGTF has a different sequence requirement and a lower sequence specificity than GT-1. Interestingly, RGTF has a higher binding affinity to the mutant GT-1 and S1F sites than to the wild-type sequences. This correlation suggests that RGTF may have some role in transcriptional regulation in roots. Furthermore, root cellular protein extracts contain an inhibitory activity that prevents GT-1 from binding to DNA. This helps to explain the absence of the GT-1 binding activity in roots in which the gene of GT-1 is expressed. Together, these data suggest that the cell type-specific transcription modulation by GT elements is achieved by using two different strategies.

The expression of nuclear genes encoding both photosynthetic and plastid ribosomal proteins (r-proteins) is highly mesophyllous cell-specific. Plastid r-protein genes provide a good system to define cis-acting promoter elements and their binding factors involved in cell type-specific regulation. The transcription of these genes is highly activated in the leaf mesophyllous cells, compared with amyloplast-containing root cells and chromoplast-containing flower and fruit cells (1–3). In contrast to photosynthetic genes, the transcriptional activation of r-protein genes in the mesophyllous cells is light-independent (3). This specific pattern of transcriptional regulation of the r-protein genes suggests the existence of either specific transcriptional activators in chloroplast-containing cells and/or repressors in nonphotosynthetic cells. Studies on the rps1 gene coding for the r-protein CS1 have led to the identification of a promoter sequence that is specifically recognized by a leaf factor, known as S1F (4). It has been shown that the S1F binding site had a negative function in regulating the rps1 promoter in root cells and in protoplasts (4, 5). It looked likely that the S1F binding site was involved in the cell type-specific regulation of the rps1 gene.

Interestingly, the S1F binding site found also in other r-protein genes (2) is related to the GT-1 binding site of the pea rbcS-3A gene as shown by competition binding assays (5). The GT-1 binding site initially identified as Box II by Fluur et al. (6) and five other similar sequences found in the pea rbcS-3A gene (7) have been shown to play an important role in the light-regulated transcription of the gene (8). Sequences homologous to Box II have also been found within upstream regions of numerous other genes including ones not regulated by light (9). Specifically, cis-elements involved in tissue-specific (52/56 box in the pollen-specific lat52) (10), defense-related (SBF-1 binding sites in the soybean cha15) (11), light-repressed (the GT-2 binding sites in the rice phya) (12), and circadian clock-controlled (CGF site in cab2) (13) transcriptional regulation have been shown to be related to the GT-1 binding site as demonstrated by competition binding assays (Table I). These GT-1 binding site (Box II)-related elements are known as GT elements. The sequences of the defined GT elements show much variation when compared with the pea rbcS-3A gene Box II core sequence GTTAAA (Table I). This core sequence has been shown to be required for the GT-1 binding in vitro (14) and the light-responsive function in vivo (15). One common characteristic to all GT elements is a core sequence rich in nucleotides T and A rather than G and T, as indicated by the name. The flanking sequences are quite divergent. The diversity of GT elements suggests that a single binding factor such as GT-1 can bind to many related GT elements. Alternatively, there may be different GT element-binding proteins with different specificities and functions. A few GT element-binding factors have been characterized, among which GT-1 and GT-2 have been cloned (12, 16, 17). GT-1 and GT-2 are related proteins with one or two trihelical DNA-binding motifs, each of which recognizes different degenerated GT elements. For example, each of the two trihelical domains of GT-2 has a higher binding affinity to either GT2 or GT3 boxes of the rice phya gene, but both domains bind only poorly to the GT-1 site (18), whereas the trihelix of GT-1 has at least a 20-fold higher affinity to Box II than to any other tested GT elements (19). Interestingly, the genes for both GT-1 and GT-2 are expressed in all parts of the plants and are not regulated by light (17–19). These observations have led to the suggestion that either the transcriptional activity of the factors is regulated by post-translational modification or additional factors may be needed to regulate their activity.

In the present work, we have compared first the transcriptional function of the GT-1 binding site in leaf and root cells. Second, we have examined whether root cells contain any dis-
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**TABLE I**

| Motif      | Gene      | Sequence            | Factor | Reference |
|------------|-----------|---------------------|--------|-----------|
| Box II     | rbs-3A    | GGTGTTAATATG         | GT-1   | 14        |
| Box III    | rbs-3A    | TAGTGAATATG          | GT-1   | 14        |
| GT-2 box   | phyA      | GTAAAT              | GT-2   | 12        |
| Box 1      | chs-15    | TAAAGTTAAAAAC       | SBP-1  | 11        |
| Box 2      | chs-15    | CTGTTAATATATAT      | SBP-1  | 11        |
| Box 3      | chs-15    | TATGTGTTACTAA       | SBP-1  | 11        |
| Site 1     | rps1      | TACTGTTACAAAT       | SIF    | 5         |
| Site 2     | XbaI      | AGTATTGTTAAAAAG     | SIF    | 5         |
| CGF site   | ccb2      | TAGTTACTCGATAAAC    | CGF-1  | 13        |
| 52/56 box  | lat52     | TATTGTTATATA        | GT-1   | 10        |

**EXPERIMENTAL PROCEDURES**

**Transgenic Tobacco Plants and β-Glucuronidase (GUS) Activity Analysis**—Seeds of three individual tobacco lines transformed with 4II-90 or 4IIIm-90 kindly provided by Dr. N.-H. Chua (Rockefeller University, New York, NY) were germinated on Murashige-Skoog medium at 22 °C with a 16 h light cycle. Three-week-old seedlings were used to collect leaves and roots. Crude protein extracts were prepared according to Jefferson et al. (20) for measuring GUS activity. Protein concentrations were determined using the Bio-Rad protein assay reagents. GUS enzyme activities were quantified by measuring the fluorescence of 4-methylumbelliferyl-β-n-glucuronide produced by GUS cleavage of 4-methylumbelliferone.

**Preparation of Nuclear and Root Whole Protein Extracts**—Tobacco leaf nuclear extracts were prepared as described in Zhou et al. (4) using greenhouse-grown 3–4-week-old young tobacco leaves. Whole protein extracts from roots or leaves were prepared according to Green et al. (21) using greenhouse-grown tobacco plants. Protein concentrations were determined using the Bio-Rad protein assay reagents.

**DNA Probes**—DNA fragments containing tetramers of Box II or its mutant versions were cut out from their vector by the restriction enzymes HindIII and XhoI. Tetramers of the S1F site or its mutant version were cut out from the vector by XhoI and BamHI. The restriction fragments were separated by and purified from 6% acrylamide gels. The concentrations of the purified DNA fragments were determined by measuring their A260. For 32P labeling, aliquots of the above prepared DNA fragments were end-filled with the Klenow enzyme in the presence of [α-32P]dATP or [α-32P]dCTP.

**Gel Shift Assays**—Leaf or root protein extracts were incubated with 32P-labeled probes in a volume of 20 μl containing 12.5 mM Hepes-KOH, pH 7.9, 2.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCl, 10% glycerol, and 2 μg of poly(dI-dC) at room temperature for 30 min. The binding reactions were analyzed by electrophoreses on 6% native acrylamide gels in 0.5 TBE buffer. In some experiments, cold DNA fragments in 30-, 50-, or 100-fold molar excess were included as competitors. In some other experiments, root and leaf extracts were combined and/or treated by 50 mM potassium fluoride (KF) or calf alkaline phosphatase at room temperature for 10–15 min before adding the probes.

**RESULTS**

**A Mutant Sequence of the GT-1 Binding Site (Box II) Has a Positive Effect on Transcription in Transgenic Roots**—Transgenic tobacco lines produced with either 4II-90 or 4IIIm-90 plasmid construct (15) were used in this study. In the 4II-90 construct, a tetramer of the wild-type pea rbs-3A Box II (GTGTTAATATG) ligated upstream to the cauliflower mosaic virus 35S promoter region (from −90 to +8) was used to drive transcription of the reporter gene uidA coding for GUS, whereas in 4IIIm-90, a mutant version of Box II (GTGTTAAATATG) was used. This mutant with G to C changes has been previously shown to be inactive in binding to GT-1 in vitro (14) and in conferring light response in leaves in vivo (8, 15). Seeds from three independent transgenic lines transformed with each construct were germinated on synthetic media. Three-week-old seedlings were used for preparing leaf and root protein extracts. GUS enzymatic activities of both types of extracts from each line were measured. As shown in Fig. 1, in the 4II-90 transgenic lines, GUS activities in leaf extracts are over 10–15 times higher than in root extracts. In leaves, GUS activities from lines transformed with 4II-90 are 3–4-fold higher than from the 4IIIm-90-transformed lines, confirming the previous results (15), although the difference is not as great as previously published. These data indicate that the GT-1 binding site is a leaf positive cis-element. However, in roots, the average GUS activity from the 4IIIm-90-transformed lines is about 3 times higher than in the 4II-90-transformed lines. In order to minimize the effects of copy number and insertion position of the transgenes, we compared the root:leaf ratio of GUS activity from individual lines. We found even greater differences (5–6-fold) between both types of transgenic lines. However, this increased promoter activity in roots is still lower than both the wild-type and the mutant promoters in leaves. These observations are consistent with our previous results obtained with the S1F binding site (5). Therefore, GT elements in general may be involved in the root-specific inactivation of nuclear genes encoding chloroplast proteins.

**Root Extracts Contain No GT-1 Binding Activity but Contain a Distinct GT Element-binding Protein**—Since GT-1 has been shown to be ubiquitously expressed in all parts of plants (16, 17, 19), it is unlikely that GT-1 acts as a root-specific transcriptional repressor. A distinct negative transcription factor may bind to GT elements, resulting in transcription repression in root cells. In order to identify such a factor, we have prepared whole protein extracts of tobacco roots from greenhouse-grown plants. The tobacco root protein extracts were incubated with 32P-labeled Box II tetramers and analyzed by gel shift assays in comparison with leaf nuclear extracts. As shown in Fig. 2, GT-1 binding activity detected in leaf extracts (lane 2) was absent in root extracts (lane 4). However, in root extracts a shifted band with a higher mobility than the leaf GT-1-DNA complex was observed (Fig. 2, lane 4). This shifted band seems to be GT element-specific, since the presence of a 30-fold molar excess of the S1F site (lane 5) or a 50-fold molar excess of Box II (lane 6) could reduce or eliminate the shifted complex. A 100-fold excess of an unrelated DNA was unable to compete with the probe for binding (lane 7). This root GT element-binding factor is called RGTF (for root GT element factor).

**RGTF Has a Distinct Sequence Requirement for Binding**—The GT-1 factor binding to the pea rbs-3A gene Box II (GTGTTAATATG) was first identified in pea leaf nuclear extracts (7). The pea GT-1 requires the core sequence GGTAA of Box II for an efficient fixation (14). However, the GT-1 binding activity characterized in tobacco leaf nuclear extracts requires the two additional nucleotides T and A downstream of the GGTAA core (17). In contrast, the cloned tobacco GT-1a requires only the core sequence GGTAA for interaction (17). The
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The root RGTF (fragment (100-fold molar excess). Specific shifted leaf GT-1 (of the RGTF binding strength to the wild-type and the mutant GG to CC changes in the core sequence (see above). The order II but rather the mutant sequence 5, which bears the crucial strongest RGTF binding activity was not the wild-type Box II (top) and the root RGTF (bottom) complexes are indicated by arrows.

The wild-type (G) and its dinucleotide substitution mutants (3–9) of Box II (A, right) were 32P-labeled and incubated with either 10 μg of leaf nuclear extracts (A, left) or 18 μg of root extracts (B). C, a 50-fold molar excess of unlabeled wild-type (G) or mutant (3–9) sequences of Box II were included as competitors as indicated in binding reactions between the 32P-labeled wild-type Box II and roots extracts. The GT-1 complexes in A and the RGTF complexes in B and C are indicated by arrows. The asterisks in B and C indicate nonspecific bands. The shifted bands in B and C were quantified using an Imager machine (Appligene). The reproduction of photographs does not faithfully reflect the autoradiographs of the gels with regard to the relative intensities of the shifted bands. The quantitations should be taken into consideration.

The wild-type Box II (Fig. 3C). These data revealed three binding characteristics of RGTF. First, RGTF has a different sequence requirement from GT-1 for binding to Box II. The core sequence GGTATAA required by GT-1 is not completely required by RGTF. Second, RGTF has a lower sequence specificity than GT-1, due to its ability to bind to most mutant Box II sequences. This indicates that RGTF can recognize, with different affinities, a large spectrum of degenerated GT elements. Third, RGTF has an even greater affinity to the mutant sequence 5 (GTGTGGTTAATAG) than to the wild-type sequence of Box II. This point is interesting, since the higher binding activity of RGTF to mutant 5 correlates with its higher transcriptional activity in roots of transgenic tobacco plants (Fig. 1).

RGTF Has a Higher Binding Affinity to the Mutant than to the Wild-type S1F Binding Site—We have previously shown that the S1F binding site (ATGTTACAAAT) within the rps1 promoter is related to the rbcS-3A Box II (5). In addition, the rps1 promoter with the mutated S1F site (ATGTCGGAGAAT) had a higher transcriptional activity in transgenic tobacco roots than the wild-type promoter (5). This led us to consider the S1F binding site as a root-specific negative element. Since RGTF binds also to the S1F binding site (Fig. 2, lane 5), the following assays were performed to determine whether the mutant S1F site is recognized also by RGTF. We performed gel shift assays with tetramers of the S1F site as probe using root protein extracts. We observed a similar shifted band as ob-

![Figure 1](image1.png)  
**Fig. 1.** GUS activities from leaves (**black bars**) or roots (**open bars**) of individual tobacco transgenic lines transformed with either the wild-type (TGTTGTTAATAG, 4II-90) or the mutant (TGTTGGTTAATAG, 4Ilm-90) Box II promoter construct. The root:leaf ratios of GUS activities from each line are indicated above the bars.

![Figure 2](image2.png)  
**Fig. 2.** Detection of a distinct GT-1 site (Box II)-binding factor in root extracts. 32P-Labeled tetramers of Box II DNA were incubated with 10 μg of leaf nuclear protein extracts (L) (lane 2) or with 18 μg of root whole protein extracts (R) (lanes 4–7). Lanes 1 and 3 contain the probe only. Where indicated, competitor DNAs were included in the binding reactions. Lane 5 (S), S1F binding site (30-fold molar excess); lane 6 (O), Box II (50-fold molar excess); lane 7 (T), an unrelated DNA fragment (100-fold molar excess). Specific shifted leaf GT-1 (top) and the root RGTF (bottom) complexes are indicated by arrows.

![Figure 3](image3.png)  
**Fig. 3.** RGTF has a distinct sequence requirement for binding. The wild type (G) and its dinucleotide substitution mutants (3–9) of Box II (A, right) were 32P-labeled and incubated with either 10 μg of leaf nuclear extracts (A, left) or 18 μg of root extracts (B). C, a 50-fold molar excess of unlabeled wild-type (G) or mutant (3–9) sequences of Box II were included as competitors as indicated in binding reactions between the 32P-labeled wild-type Box II and roots extracts. The GT-1 complexes in A and the RGTF complexes in B and C are indicated by arrows. The asterisks in B and C indicate nonspecific bands. The shifted bands in B and C were quantified using an Imager machine (Appligene). The reproduction of photographs does not faithfully reflect the autoradiographs of the gels with regard to the relative intensities of the shifted bands. The quantitations should be taken into consideration.
FIG. 4. The mutant S1F site has a higher binding activity to RGTF than the wild type. A, equal amounts of root extracts were incubated with 32P-labeled wild-type S1F binding site. Where indicated, specific competitor DNAs in 50-fold molar excess were included in the binding reactions; S, the wild-type S1F site (ATGTAACAAT); Sm, the mutant S1F site (ATGTTAACAAAT); G, the wild-type Box II; G5, mutant 5 of Box II (see Fig. 3); G7, mutant 7 of Box II (see Fig. 3). B, 6, 12, or 18 μg of root extracts were incubated with labeled wild-type (S) or mutant (Sm) S1F site. The shifted bands were quantified as in Fig. 3.

FIG. 5. Root extracts contain an inhibitory activity that prevents GT-1 from binding to Box II. Gel shift assays of root extracts (18 μg lanes 2 and 3), leaf extracts (10 μg; lanes 4, 5, and 9), or mixtures of both (lanes 6–8 and 10–12) with the wild-type Box II as probe are shown. Lane 1 contains the probe only. Where indicated, a 50-fold molar excess of unlabeled probe (G) was included as competitor DNA (lanes 3 and 5). In lanes 6–8, 18 μg of root extracts were incubated with 10, 20, and 30 μg of leaf extracts at room temperature during 10 min before adding the probe. In lanes 9–12, 40 μg of leaf extracts were incubated with 0, 9, 18, and 36 μg of root extracts at room temperature for 10 min before adding the probe. The arrows indicate the GT-1-DNA or the RGTF-DNA complexes. The asterisk indicates nonspecific bands.

The activity of many DNA-binding proteins is regulated by phosphorylation/dephosphorylation. It is possible that a root-specific activity may phosphorylate or dephosphorylate GT-1 and inhibit its binding activity. It has been reported that the binding activity of the
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soybean GT element-binding protein SBF-1 is inhibited by dephosphorylation (22). In order to test this hypothesis, we treated the root extracts with either 50 mM potassium fluoride (Fig. 7, lane 4), an inhibitor of phosphatases, or with up to 2 units of calf alkaline phosphatase (Fig. 7, lanes 5 and 6) before incubating with the leaf extracts. Neither treatment was effective in preventing the inhibition of the GT-1 binding by the root extracts.

**DISCUSSION**

The highly cell type-specific nature of the expression of genes encoding both the photosynthetic and the plastid ribosomal proteins indicates that internal developmental signals are involved in the regulation. These internal signals include both cell identity and a possible “chloroplast factor,” which seems to be important to couple gene expression of photosynthetic proteins with the presence of chloroplasts in the cell (23). However, the expression of nuclear genes encoding plastid r-proteins is not dependent on the presence of chloroplasts (24). Therefore, cell type-specific transcription factors are likely to be involved in the regulation of both types of nuclear genes. Promoter studies have shown that the GT-1 binding site is a cis-element with several copies in the promoters of both photosynthetic and ribosomal protein genes (5, 9). Previous studies have shown that the GT-1 site confers light responsiveness to a chimeric promoter construct (15). Data presented in this article show that the mutation of the GT-1 site created a positive function in transcription in leaves. On the other hand, this mutation provoked an increase of transcription activity in roots (Fig. 1). Similar results were previously obtained with the S1F binding site in activating transcription in roots, although the mutation of the S1F binding site within a short rps1 promoter fragment did not affect the promoter activity in leaves (5). The divergence between the GT-1 and the S1F sites concerning their activity in leaves is not understood at present. However, these data together support the notion that the GT-1 binding site is involved in leaf cell activation and that in roots wild-type GT elements have lower transcription activity than their mutant versions. Based on these data only, one can make the conclusion that GT elements are root negative transcription elements.

Gel shift assay analyses of root protein extracts have led to the identification of an additional GT element-binding protein, RGTF, which has 1.5–2 times higher binding activity to the mutant sequences than to the wild types of both the GT-1 and the S1F sites. This higher RGTF binding activity of the mutant sequences is well correlated to their higher transcriptional activity in roots. The higher root transcriptional activity of the mutant GT elements was presumably due to the fact that the mutations have created higher affinity binding sites of RGTF than the wild-type sequences. This suggests that the GT-1 site is not an optimal or a major binding site of RGTF. Based on the relatively low root activity of the wild-type Box II, or even of mutant 5 (Fig. 1), we suggest that the transcriptional activation activity of RGTF is relatively weak.

RGTF seems not to be a highly sequence-specific DNA-binding factor, based on the observations that it recognizes most of the dinucleotide substitution mutants of the GT-1 binding site (Fig. 3, B and C). However, the RGTF binding activities of those degenerative Box II sequences differ over 10-fold when comparing the strongest (mutant 5) to the weakest (mutant 7) (Fig. 3B). The two nucleotides AA in the core sequence (GGTTAA) of Box II are crucial for the binding of RGTF. Substitutions of AA by GG decreased by about 5-fold the binding activity compared with the wild-type sequence (Fig. 3B). In contrast, the GG to CC changes in the core sequence increased the RGTF binding activity about 1.7-fold (Fig. 3B). These data indicate that, although with a relatively low binding specificity, RGTF has specific binding sequence requirements. These binding features of RGTF might allow it to bind to a large spectrum of promoter sequences. RGTF seems not to be a root-specific factor. It was detected also in leaf extracts (Fig. 6, lane 6), especially when the GT-1 binding activity was inhibited (Figs. 5–7), suggesting that RGTF may be a kind of general factor existing in different cell types. It is unlikely that the GT-1-Box II complex seen in gel shift assays with leaf extracts contains also RGTF, since the mobility of the leaf complex was not affected by the competition of the Box II mutant 5 sequence, which is specific to RGTF but not to GT-1 (Fig. 6, lanes 8 and 9). Therefore, the binding of RGTF seems to be GT-1-independent. It is also unlikely that RGTF is a different form of GT-1, since RGTF binds to different sequences. RGTF might play some role in ensuring a lower but adequate level of expression in nonmesophyllous cells of GT element-containing genes including plastid r-protein and photosynthetic genes. Lower transcriptional activity of Box II in roots correlates with the absence of the GT-1 binding activity in root protein extracts. This strongly suggests that GT-1 is a leaf-specific activator. Based on the results obtained from the cloned tobacco or Arabidopsis GT-1 factors, GT-1 is transcribed in roots (17, 22). The absence of GT-1 binding activity in root extracts can be explained by modifications at either post-transcriptional or post-translational levels. The data shown in Figs. 5–7 demonstrated that a
root cellular activity could inhibit the binding activity of GT-1. This root inhibition seems to be specific to GT-1, since the root extracts gave rise to similar shifted bands as the leaf extracts when incubated with $^{32}$P-labeled *Arabidopsis* GBF binding sites (G-box) (Ref. 25; data not shown). The studies with calf alkaline phosphatase or a phosphatase inhibitor (KF) showed that phosphorylation/dephosphorylation may not be involved in the inhibition, although our data do not rule out the possibility that specific phosphatases insensitive to KF are involved.

It is not clear at this stage whether the inhibitory activity is localized in the cytoplasm or in the nucleus of root cells, since the root extracts we used in this study are whole cellular protein extracts. Nevertheless, this inhibitory activity seems to be root cell-specific. Gel shift assays performed with leaf whole protein extracts gave rise to the same shifted band as leaf nuclear extracts (data not shown).

Based on the above discussion, we would reason as follows (Fig. 8). In leaf (mesophyllous) cells, GT-1 binds to Box II or related GT elements and strongly activates transcription. In root cells, the binding activity of GT-1 is prevented by an inhibitor, and the transcriptional activation mediated by GT-1 is abolished. However, RGTF can bind to GT elements and very moderately activate transcription to ensure an adequate expression of different genes in roots. The activation strength would depend on the binding affinity of RGTF to the element. This mode of regulation might help to determine the fine tuning of expression of individual genes in root cells.

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