The Plant Cell, Vol. 3, 1305–1316, December 1991

Developmental and Organ-Specific Changes in Promoter DNA-Protein Interactions in the Tomato *rbcS* Gene Family

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The five genes encoding ribulose-1,5-bisphosphate carboxylase (*rbcS*) from tomato are differentially expressed. Transcription of the genes is organ specific and developmentally regulated in fruit and light regulated in cotyledons and leaves. DNase I footprinting assays were used to map multiple sites of DNA-protein interaction in the promoter regions of all five genes and to determine whether the differential transcriptional activity of each gene correlated with developmental or organ-specific changes in DNA-protein interactions. We show organ-specific differences in DNase I protection patterns, suggesting that differential transcription of *rbcS* genes is controlled at least in part at the level of DNA-protein interactions. In contrast, no changes were detected in the DNase I footprint pattern generated with nuclear extracts from dark-grown cotyledons versus cotyledons exposed to light, implying that light-dependent regulation of *rbcS* transcription is controlled by protein-protein interactions or modification of DNA binding proteins. During development of tomato fruit, most DNA-protein interactions in the *rbcS* promoter regions disappear, coincident with the transcriptional inactivation of the *rbcS* genes. In nuclear extracts from nonphotosynthetic roots and red fruit, the only detectable DNase I protection corresponds to a G-box binding activity. Detection of other DNA binding proteins in extracts from these organs and expression of nonphotosynthetic genes exclude the possibility that roots and red fruit are transcriptionally inactive. The absence of complex promoter protection patterns in these organs suggests either that cooperative interactions between different DNA binding proteins are necessary to form functional transcription complexes or that there is developmental and organ-specific regulation of several *rbcS*-specific transcription factors in these organs. The DNase I-protected DNA sequences defined in this study are discussed in the context of conserved DNA sequence motifs and previously characterized binding sites.

INTRODUCTION

The molecular basis for transcriptional regulation of gene expression is now emerging in a number of biological systems and involves a complex array of DNA-protein and protein-protein interactions that vary in response to environmental conditions and developmental cues. The multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*) of tomato offers the opportunity to study differential and organ-specific transcription, as well as light and developmental regulation in a single, well-characterized system. The tomato *rbcS* gene family consists of five members (*rbcS1*, *2*, *3A*, *3B*, and *3C*), which are encoded at three genetic loci (Vallejos et al., 1986). *rbcS3A*, *3B*, and *3C* are tandemly arranged within 10 kb at a single locus (Sugita et al., 1987). Although the coding regions of the five genes are highly conserved, each locus encodes a unique protein. It is unknown, however, whether these small subunit isoforms are functionally distinct.

We have demonstrated previously that the tomato *rbcS* genes are differentially expressed and that the mRNAs of individual genes accumulate to different levels in characteristic developmental and organ-specific patterns (Sugita and Gruissem, 1987; Wanner and Gruissem, 1991). For example, *rbcS1*, *2*, and *3A* mRNAs accumulate in cotyledons of dark-grown seedlings, but expression of *rbcS3B* and *3C* is strictly light dependent. In leaves, all five genes are active but their mRNAs accumulate to different levels. In young tomato fruit, which are photosynthetically active (Piechulla et al., 1987), only *rbcS1* and *2* mRNAs accumulate, and their levels decrease during fruit maturation and ripening. No *rbcS* mRNAs are detectable in roots. In an accompanying report (Wanner and Gruissem, 1991), we showed that the qualitative differences in *rbcS* mRNA accumulation are controlled at the transcriptional level but that quantitative differences are regulated by

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post-transcriptional mechanisms. Thus, the *rbcS* genes in tomato constitute a good model system to determine how light-dependent, developmental, and organ-specific processes control differential transcription.

Our transcriptional studies of tomato *rbcS* genes, together with data published for *rbcS* genes from other plants, suggest that *rbcS* promoter regions are critical in controlling the different patterns of expression. In general, DNA sequences 5′ proximal to the transcription start site of different *rbcS* genes are sufficient to direct organ-specific expression of reporter genes in transgenic plants (for review, see Dean et al., 1989). This is also the case for the five tomato *rbcS* promoter regions, which direct light-regulated and organ-specific expression of the β-glucuronidase reporter enzyme in transgenic tomato plants (T. Manzara, K. Callan, and W. Gruissem, unpublished data). Differences in the transcriptional regulation among the tomato *rbcS* genes are likely to be a consequence of the unique spatial arrangements of conserved DNA sequence motifs in the promoter regions.

We previously aligned the DNA sequences of the five tomato *rbcS* promoter regions with the corresponding regions from other known dicot *rbcS* genes and identified several conserved DNA sequence motifs (Manzara and Gruissem, 1988). The spatial arrangement of the conserved DNA sequence motifs in the five tomato *rbcS* promoter regions is shown in Figure 1, and the corresponding DNA sequences are listed in Table 1. The comparison of the *rbcS* promoter regions in Figure 1 shows that each promoter region contains a unique pattern of conserved DNA sequence motifs and that the spatial arrangement between different DNA sequence motifs varies among the promoters. Certain DNA sequence motifs are conserved only among a subset of the tomato *rbcS* promoters and in some cases correlate with a specific transcription pattern. For example, sequences 9 and 10 are found only in *rbcS3B* and *3C*, which represent the subset of *rbcS* genes whose expression is light dependent in cotyledons. Other sequence motifs are present in the five promoter regions, including the CAAT and TATA boxes, the "box II" sequence that binds to GT-1 (Green et al., 1988), and a putative light-regulatory element (LRE; Grob and Stüber, 1987). A small number of the DNA sequence motifs have been functionally defined (for review, see Gilmartin et al., 1990), but the function of several other conserved DNA sequence motifs and the significance of their distinct spatial arrangements or interaction with DNA binding proteins are unknown.

We demonstrated in this report that most, but not all, of the conserved DNA sequence motifs in the five tomato *rbcS* promoters interact with DNA binding proteins and that these DNA-protein interactions are specific. We found that the strict light activation of *rbcS3B* and *3C* in cotyledons is apparently controlled by protein-protein interactions or modification of DNA binding proteins because no differences were detected in the DNase I footprint patterns after illumination. By contrast, distinct organ-specific DNase I footprint patterns were detected, indicating that organ-specific control of *rbcS* transcription may be controlled at least in part at the level of DNA-protein interactions. We determined that the developmental inactivation of *rbcS1* and *2* transcription in ripening tomato fruit is correlated with the loss of all but one DNA binding protein. Our analysis of all promoters from the *rbcS* gene family demonstrated that the light-dependent, developmental, and organ-specific transcriptional regulation of *rbcS* genes is complex and is reflected by differences in spatial and temporal interaction of DNA binding proteins with several different DNA sequence motifs.

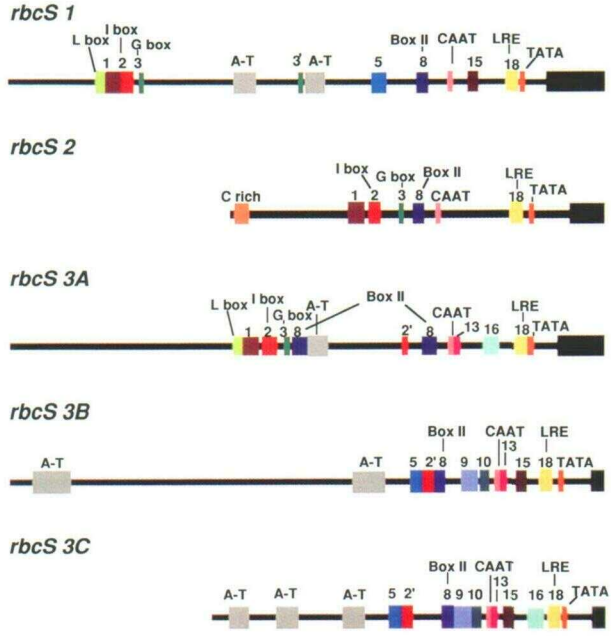

Figure 1. Conserved DNA Sequence Motifs in the 5′ Upstream Regions of the Tomato *rbcS* Genes.

Heavy black lines represent the 5′ upstream region of each gene. Conserved sequences are designated by colored boxes (for key, see Table 1). Solid black boxes at the 3′ end (right) of each upstream region represent the 5′ untranslated region of each gene. The numerical designations for conserved sequences are from Manzara and Gruissem (1988). L-box, I-box, and G-box are the terminology of Ueda et al. (1989), box II is the terminology of Fluhr et al. (1986), and LRE is the terminology of Grob and Stüber (1987).

RESULTS

We determined the interaction of DNA binding proteins with their cognate DNA sequences in the promoter regions

Table 1. Conserved Sequence Motifs in *rbcS* Promoters

Number/ Symbol	Motif	Sequence	Binding Factor	Putative Function	References
	L box	AATTAACCAA		unknown	Ueda et al. (1989)
1		pyrimidine rich		unknown	
2	I box	GGATGAGATAAGATTA (C C)	GA-1	unknown	Ueda et al. (1989)
2	I box	GATAAG	GA-1	"enhancer"	Donald and Cashmore (1990)
2	GATA	GATGAGATA (T)	ASF-2 [a]	leaf-specific	Lam and Chua (1989)
3	G box	CACGTGGC (T)	GBF	"enhancer"	Donald and Cashmore (1990)
5		TTAAATAGAGGGCGTAA		unknown	
8	Box II	TTGTGPuTAATPuT (GA A A A)	GT-1	light regulation	Lam and Chua (1990)
9		TTTGTAATGTCAA (A C)		unknown	
10		GAGCCACA (A)		unknown	
12	CAAT box	ATCCAAC (T)		unknown	
13		GGTTAC		unknown	
15		AGATGAGG	LRF-1 (?)	light regulation	Buzby et al. (1990)
16		TTTGTGTCCGTTAGATG (T A A)		unknown	
18	"LRE"	CCTTATCAT		unknown	Grob and Stuber (1987)
19	TATA box	TTATATAAA (C T)	TFIID [a]	transcription initiation	Gasch et al. (1990)
	A-T rich	(varies)		unknown	
	A-T rich	AATATTTTTATT	AT-1	unknown	Datta and Cashmore (1989)
	A-T rich	AAATAGATAAATAAAAACATT	3AF-1 [a]	"enhancer"	Lam et al. (1990)

[a] These genes have been cloned.

of all five *rbcS* genes using mobility-shift and DNase I footprinting assays. Gel-shift assays were used primarily to test for the presence of DNA binding proteins in the nuclear extracts and to determine the specificity of binding. DNA-protein interactions were investigated during light induction of *rbcS* transcription in cotyledons by comparing DNase I footprint patterns generated by nuclear extracts from cotyledons of 7-day-old dark-grown seedlings, seedlings exposed to light for 6 or 12 hr, and light-grown seedlings. Organ-specific differences in *rbcS* transcription were correlated with changes in DNA-protein interactions among extracts derived from cotyledons and roots of 7-day-old dark-grown seedlings and from leaves and fruit of mature tomato plants. In addition, transcriptional inactivation of *rbcS* during tomato fruit development was examined at the level of DNA-protein interactions by comparing footprints generated by extracts derived from young fruit (5 to 8 mm in diameter, approximately 10 days post-pollination) and mature fruit (orange/firm red). Finally, specific conserved DNA sequences were studied with respect to their associated binding activities and possible involvement in differential transcription among the five genes.

Specific and Unique Binding Complexes Are Formed between *rbcS* 5′ Upstream Regions and Nuclear Extracts Derived from Different Organs of Tomato

As a first step in correlating the differential transcription patterns of the tomato *rbcS* genes with specific DNA-protein interactions of their promoter regions, we tested nuclear extracts for DNA binding activities capable of interacting with the 5′ upstream sequences of the *rbcS* genes and determined the specificity of the DNA-protein complexes. DNA fragments derived from the 5′ upstream regions of the five tomato *rbcS* genes, ranging in size from 200 to 500 bp, were single-end labeled and used as probes in mobility-shift assays. A representative mobility-shift experiment is shown in Figure 2 in which increasing concentrations of extracts derived from nuclei of dark-grown cotyledons, leaves, young fruit, mature fruit, and roots were incubated with the 3A-4 DNA fragment. The 3A-4 DNA fragment shown in Figure 2A contains the L-, I-, and G-box sequences (Giuliano et al., 1988) and the box II sequence (Fluhr et al., 1986). Based on mutational analysis of the Arabidopsis *rbcS1A* promoter, it appears that the G- and I-boxes are required for full promoter activity (Donald and Cashmore, 1990). The G-box sequence interacts with the G-box binding factor (GBF; Giuliano et al., 1988). The box II DNA sequence motif interacts with the GT-1 binding activity (Green et al., 1987) and is required for full

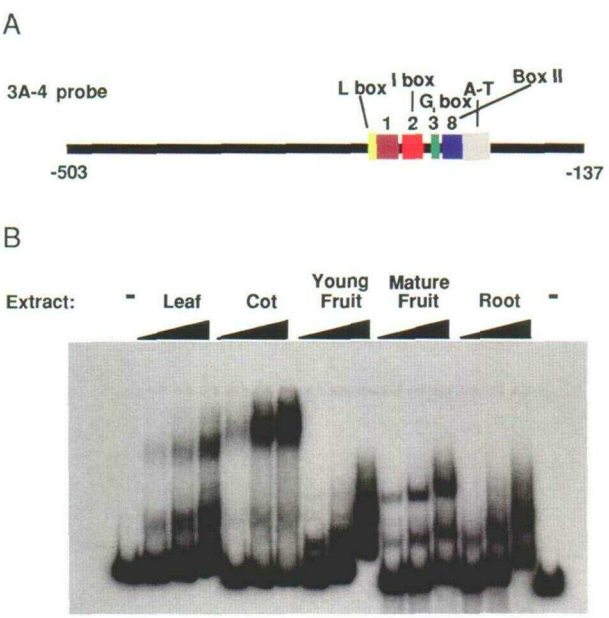

Figure 2. Organ-Specific Mobility Shifts for the −503 to −137 Region of the *rbcS3A* Gene.

(A) The 3A-4 probe is shown, including conserved sequence motifs.

(B) Shifts in mobility of the 3A-4 probe produced by increasing amounts of nuclear extracts from different organs. Within a group of three lanes, the protein concentration is doubled for each consecutive lane. The amount of protein per reaction in the first lane of each group is as follows: leaf, 0.2 μg; cotyledon (cot), 0.9 μg; young fruit, 0.4 μg; mature fruit, 1.7 μg; and root, 1.8 μg. Each lane contains 1 fmol of the 3A-4 probe.

activity of the pea *rbcS3A* promoter in transgenic tobacco plants (Kuhlemeier et al., 1988). The function of these DNA sequence motifs in the tomato *rbcS3A* promoter is unknown.

As shown in Figure 2B, changes in fragment mobility were observed with the tomato *rbcS* 3A-4 DNA fragment using all five nuclear extracts. In each case, the complexes formed appeared to be different, except for root and mature fruit extracts, which generate at least one complex of comparable mobility. We found that increasing the concentration of the extract did not significantly change the qualitative pattern of the mobility shifts generated by the different extracts, although sometimes the mobilities of all bands were affected (see young fruit extract, Figure 2B). DNA binding is sensitive to proteolytic digestion (data not shown), suggesting that the complexes formed result from DNA-protein interactions. Additional mobility-shift analyses using fragments from the five tomato *rbcS* promoter regions (see Figure 6) yielded similar results, except that in some cases no significant mobility-shift pattern was observed with root or mature fruit extracts (data not shown). From these data, we concluded that the nuclear extracts from different tomato organs contain DNA binding proteins that form distinct and organ-specific complexes, with the exception of red fruit and root extracts, which appear to form similar complexes.

To assess the specificity of binding for each extract, competition mobility-shift assays were performed using the 3A-4 fragment as the probe. Figure 3 shows an example of a competition mobility-shift assay in which DNA fragments unrelated to the *rbcS* 3A-4 DNA fragment were used as binding competitors in the presence of extract derived from dark-grown cotyledons. The results demonstrated that there is little or no competition for DNA binding proteins between the 3A-4 DNA fragment and DNA fragments derived from pUC19, the 5′ upstream region of the cauliflower mosaic virus (CaMV) 35S promoter, exon 1 and a part of intron 1 of the *rbcS2* gene, and a cDNA clone of the *rbcS2* gene. In contrast, the *rbcS1* promoter DNA fragment 1-4, which extends from −627 to −146 of the *rbcS1* gene and contains several DNA sequence motifs in common with 3A-4 (see Figures 1 and 6), competed for protein binding to the 3A-4 fragment as efficiently as the 3A-4 fragment itself. A fragment derived from the promoter region of *rbcS3C* also competed for protein binding to the 3A-4 fragment but less efficiently than 1-4 or 3A-4. Parallel experiments using extracts derived from leaves, roots, and fruit yielded results comparable with those shown in Figure 3, except that the *rbcS3C* DNA fragment did not compete efficiently in assays with fruit and root extracts (data not shown). Together, these experiments indicated that nuclear extracts derived from organs in which the tomato *rbcS* genes are differentially transcribed contain DNA binding proteins that yield organ-specific and distinct DNA-protein complexes with *rbcS* promoter regions.

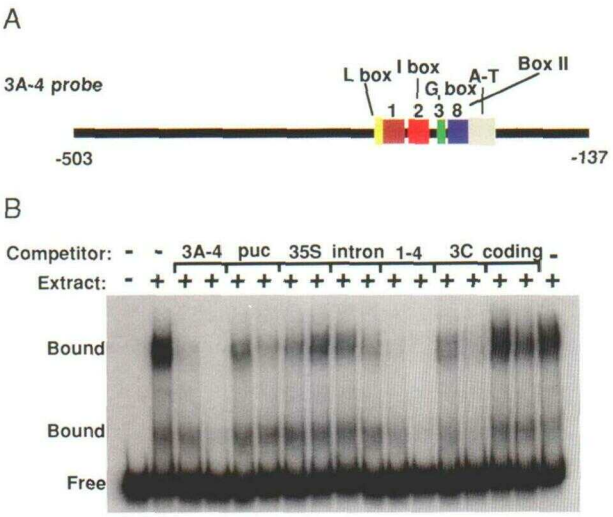

Figure 3. Binding Competition Assay Using the 3A-4 Probe and Nuclear Extract from Dark-Grown Cotyledons.

(A) The 3A-4 probe is shown.
(B) Each lane contains 1.8 μg of nuclear protein and 1 fmol of the 3A-4 probe. In each pair of competitor lanes, the left lane contains 50 fmol and the right lane contains 250 fmol of competing fragment. The competing fragments are as follows: 3A-4 is the same as the probe; puc is the 212-bp NdeI-EcoRI fragment of pUC19; 35S is the region from −416 to +9 of the 35S gene from CaMV; intron is the region from +57 to +345 of the *rbcS2* gene, including most of exon 1 and part of intron 1; 1-4 is the region from −627 to −146 of the *rbcS1* gene; 3C is the region from −320 to −138 of the *rbcS3C* gene; coding is a cDNA clone containing most of the coding region and the 3′ untranslated region of *rbcS2*.

Light-Induced Transcriptional Activation of *rbcS* Is Not Reflected by Changes in DNase I Footprinting Patterns

We have previously shown that during cotyledon development in tomato, transcription of all five *rbcS* genes is developmentally controlled and light regulated (Sugita et al., 1987; Wanner and Gruissem, 1991). Transcriptional activity of all five genes is generally increased in response to 6 hr of light in cotyledons of 7-day-old dark-grown seedlings, although the degree to which the individual genes respond to illumination is significantly different (Wanner and Gruissem, 1991). The relative increase in transcription rates ranges from less than twofold for *rbcS1* to threefold to fivefold for *rbcS2* and *3A* following illumination, relative to dark-grown control plants. In contrast, transcriptional activation of *rbcS3B* and *3C* is light dependent, and no transcription is detectable in dark-grown cotyledons. To determine whether light-induced transcriptional activation of *rbcS* genes in cotyledons is accompanied by changes in DNA-protein interactions in their

promoter regions, we compared the DNase I footprint patterns obtained with nuclear protein extracts from cotyledons of 7-day-old dark-grown seedlings and illuminated cotyledons of the same age.

Figure 4 shows examples of DNase I footprints for the 3A-4 and the 3B-2 fragments using extracts derived from 7-day-old dark-grown cotyledons and from cotyledons exposed to light for 12 hr. The 3A-4 DNA fragment spans the region from −503 to −137 of the *rbcS3A* gene and serves as an example of a promoter region from an *rbcS* gene that is transcribed in both light-grown and dark-grown cotyledons. The 3B-2 fragment spans the region from −192 to +33 of the *rbcS3B* gene and serves as an example of a promoter region from a gene that is transcribed in light-grown cotyledons but not in dark-grown cotyledons. The protein binding regions defined by DNase I footprinting assays (indicated by blank ellipses) and conserved DNA sequence motifs are diagramatically represented above each autoradiogram. From the pattern of DNA-protected regions for each fragment, we conclude that multiple binding activities interact with the fragments from each gene and that the complex(es) interacting with the 3B-2 DNA fragment are distinct from those interacting with the 3A-4 DNA fragment. Furthermore, there were no differences in the DNase I footprints produced using extracts derived from dark-grown cotyledons and from cotyledons exposed to light for 12 hr for either of the two fragments. Extracts derived from dark-grown seedlings exposed to 6 hr of light (representing the time required to reach half-maximal transcription levels for *rbcS3B* and *3C*, Wanner, 1990) or from seedlings grown under normal dark-light cycles produced footprints identical to those shown in Figure 4. Similar experiments with both strands of fragments derived from the 5′ upstream regions of all five *rbcS* genes (refer to fragments shown in Figure 6) substantiated the results obtained for 3A-4 and 3B-2. In no case were differences detected between DNase I footprints generated with extracts from dark-grown and 6-hr light-treated cotyledons (data not shown). These results are in agreement with Green et al. (1987), who found that DNase I-protected regions corresponding to GT-1 binding sites did not change when nuclear extracts derived from light-grown versus dark-adapted leaves were compared. The proportion of GT-1 (with respect to total protein) did, however, change depending on light conditions, and this has also been shown to be true for the GBF (Giuliano et al., 1988) and light-regulated nuclear factor-1 (LRF1) (Buzby et al., 1990) activities. The work we present here shows that multiple DNase I-protected sites, encompassing the promoter regions of an entire light-regulated gene family, were shown to be identical in dark-grown versus light-exposed tissues.

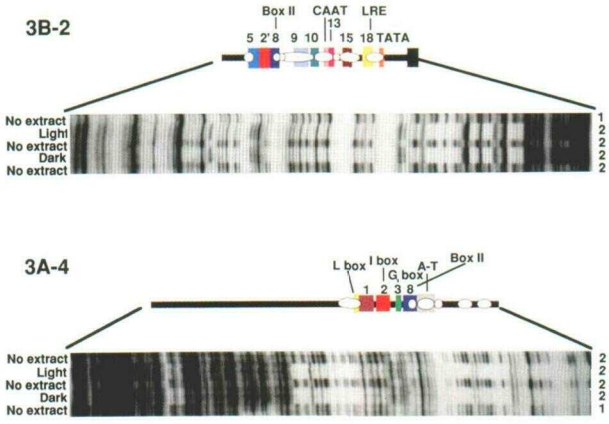

Figure 4. Comparison of DNase I Footprinting Patterns Defined by Extracts Derived from Dark-Grown Cotyledons Versus Cotyledons Exposed to Light for 12 Hr.

The 3B-2 probe, extending from −192 to +33 in the *rbcS3B* gene, and the 3A-4 probe, extending from −503 to −137 in the *rbcS3A* gene, are shown diagramatically. Colored boxes indicate conserved sequence motifs, and ellipses designate protected regions observed on the corresponding autoradiogram. Lanes containing nuclear extract (Light, Dark) and control lanes (No extract) are indicated to the left of the 3B-2 and the 3A-4 autoradiograms. Lanes designated dark contain 11.2 μg of nuclear extract derived from cotyledons of 7-day-old dark-grown seedlings. Lanes designated light contain 14.4 μg of nuclear extract from cotyledons of dark-grown seedlings exposed to light for 12 hr. All lanes contain 0.75 fmol of labeled probe. The final DNase I concentrations in milligrams per milliliter are indicated to the right of each autoradiogram.

Organ-Specific Mobility-Shift Patterns Are the Result of Differences in DNA-Protein Interactions

We have shown in the accompanying report that the transcription of the *rbcS* genes in tomato shows organ-specific and developmental regulation (Wanner and Gruissem, 1991). For example, all *rbcS* genes are actively transcribed in leaves and illuminated cotyledons, but their relative transcriptional activities vary. In green tomato fruit, only *rbcS1* and *2* are transcribed at high levels, and transcription of *rbcS3A*, *3B*, and *3C* is not detectable. During fruit development and ripening, transcription of *rbcS1* and *2* is inactivated. No transcription is detected for any of the genes in roots, an organ that is not photosynthetically active. As shown in Figure 2, the mobility-shift assays indicate that organ-specific DNA-protein complexes may be formed with the *rbcS* promoter fragments and DNA binding proteins from leaves, cotyledons, young fruit, mature fruit, and root. Organ-specific DNA-protein interactions, therefore, could be a critical factor in the regulation of the differential transcription activity of the tomato *rbcS* genes in these organs. To investigate this possibility in detail, we used DNase I footprinting analysis

to determine the extent to which organ-specific complex formation was the result of differences in specific DNA-protein interactions.

A DNase I footprinting experiment using the 3A-4 fragment and extracts from various organs of tomato is shown in Figure 5. The data indicate that multiple regions were protected by extracts from leaves, cotyledons, and young fruit, whereas a single region was protected by extracts from mature fruit and roots. Furthermore, with the exception of mature fruit and roots, the composite pattern of DNase I protection differed among extracts from different organs, although specific components of a given binding pattern were sometimes maintained among extracts. For example, regions A, D, and G were protected by cotyledon and leaf extracts, region E was protected by leaf, young fruit, and cotyledon extracts, and C was protected by leaf, young fruit, mature fruit, and root extracts. Organ-specific DNase I-protected regions were also present, specifically regions F and H for cotyledon extracts and region B for fruit extracts. These data, together with the results of the mobility-shift assay presented in Figure 2, imply that different relative concentrations of DNA binding activities are present in each extract that result in the formation of distinct binding complexes between the 3A-4 fragment and extracts from different organs.

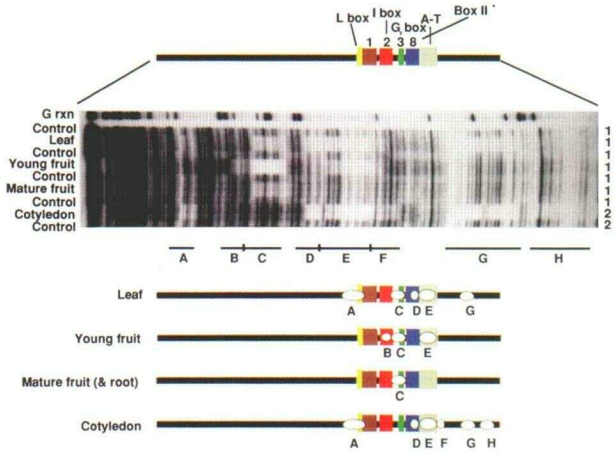

Figure 5. Comparison of DNase I Protection Patterns Defined by Extracts from Various Organs of Tomato.

All lanes contain 0.75 fmol of the 3A-4 probe, except the Maxam and Gilbert G reaction lane (G rxn). The nuclear extract used is indicated to the left of each lane, where Control designates lanes containing no extract. The amount of extract in each lane is as follows: Leaf, 4.5 μg; Young fruit, 4.5 μg; Mature fruit, 10.1 μg; Cotyledon, 11.2 μg. A similar protection to that shown for mature fruit was obtained using 8.1 μg of root extract (data not shown). The final concentration of DNase I in milligrams per milliliter is indicated to the right of each lane. The protected regions defined by each extract are subdivided based on comparisons between lanes and are represented by ellipses in the diagram below the autoradiogram.

DNase I footprinting experiments were performed to define organ-specific differences in binding patterns, using DNA fragments derived (predominantly) from the 5′ upstream regions of the five tomato *rbcS* genes. To simplify the interpretation, the results are shown diagramatically in Figure 6, where conserved DNA sequences are depicted as boxes, protected regions are designated by ellipses, and the fragments used for footprinting are indicated at the bottom of each panel. If the binding patterns defined by extracts from different organs are compared for any given gene, it becomes apparent that the results derived from the 3A-4 footprint are generally applicable in the sense that organ-specific expression of *rbcS* is reflected by differences in the DNase I protection pattern for all five genes. Furthermore, pronounced organ-specific differences in binding activities were evident when comparing the binding patterns defined by extracts from organs in which *rbcS* is expressed (leaves, cotyledons, and young fruit) versus binding patterns defined by extracts from organs in which *rbcS* is not expressed (mature fruit and roots) (Figure 6). A general loss of transcription factors in roots and mature fruit does not account for this result, as other genes are actively transcribed in these organs. Furthermore, because both extracts contain at least one factor that interacts with sequence 3 (G-box) (see Figures 5 and 6) and our root extract contains a factor, presumed to be ASF-1 (Lam et al., 1989), that interacts with the appropriate sequence in the CaMV 35S promoter (data not shown), the absence of *rbcS*-related binding activities in these extracts is not artifactual. We conclude that fewer *rbcS*-related binding activities are present or are capable of interacting in organs where *rbcS* is not expressed versus organs where *rbcS* is expressed and that developmental inactivation of *rbcS* during tomato fruit ripening is reflected at the level of DNA-protein interaction.

Assuming that in vivo DNA-protein interactions are faithfully reproduced in vitro, our data provide a basis from which we can address specific questions concerning how transcriptional control is mediated at the molecular level for the five *rbcS* genes of tomato. The fidelity of the in vitro footprinting data has been verified by in vivo footprinting of a portion of the *rbcS3B* 5′ upstream region in cotyledons of tomato (P. Carrasco, T. Manzara, and W. Gruissem, unpublished data). These data indicate that all in vitro-protected regions are also detected in vivo but that certain in vivo protections are not detected in the in vitro system. Coupled with the fact that use of cloned fragments for footprint analysis could result in the disruption of binding sites or protein-protein interactions, we conclude that our data may underrepresent the true number of DNA binding sites present in vivo. Therefore, correlations between in vivo protein binding and transcription may exist in addition to those that are apparent from the in vitro analysis. Accordingly, the relationship of specific

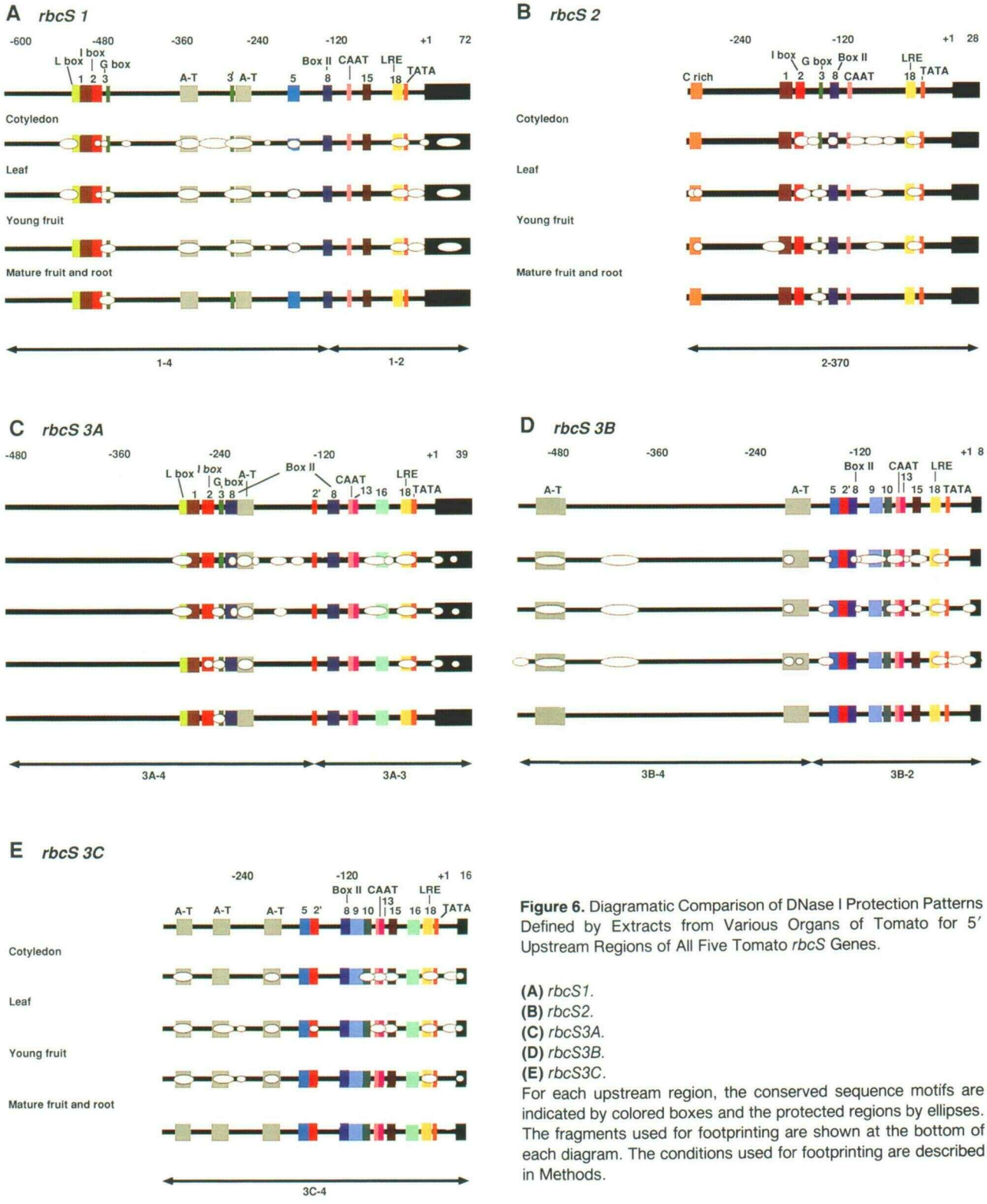

Figure 6. Diagramatic Comparison of DNase I Protection Patterns Defined by Extracts from Various Organs of Tomato for 5′ Upstream Regions of All Five Tomato *rbcS* Genes.

(A) *rbcS1.*
(B) *rbcS2.*
(C) *rbcS3A.*
(D) *rbcS3B.*
(E) *rbcS3C.*

For each upstream region, the conserved sequence motifs are indicated by colored boxes and the protected regions by ellipses. The fragments used for footprinting are shown at the bottom of each diagram. The conditions used for footprinting are described in Methods.

conserved DNA sequences and associated binding activities to differential, light-regulated, organ-specific, and developmentally regulated transcription of *rbcS* is discussed below.

DISCUSSION

DNase I footprint analysis of the promoter regions from all members of the tomato *rbcS* gene family establishes correlative relationships between DNA-protein interactions and the developmental, organ-specific, and differential transcriptional regulation of these genes. The gene-specific spatial arrangements and modular organization of conserved DNA sequence motifs in each promoter suggest that they are most likely required for the developmental and organ-specific differences in the temporal control of *rbcS* transcription in tomato. The DNase I footprint analysis demonstrates that several of the identified DNA sequence motifs interact with proteins, but only a few of these motifs have been identified as *cis*-acting regulatory elements for *rbcS* genes in other plants (Gilmartin et al., 1990). In the following, we discuss characterized sequence motifs in the context of their putative function and their ability to interact with tomato nuclear proteins. The precise coordinates and DNA sequences of all DNase I-protected regions shown in Figures 4, 5, and 6A to 6E will be reported elsewhere (T. Manzara, P. Carrasco, and W. Gruissem, unpublished data; P. Carrasco, T. Manzara, and W. Gruissem, unpublished data).

L-Box

The L-box sequence 5′-AATTAACCAA (L-box; Giuliano et al., 1988) is found in *rbcS* promoters from several plants, but no function or DNA binding protein is currently known for this sequence. Identical L-box sequences are present in tomato *rbcS1* and *3A* and are protected by protein(s) from cotyledon and leaf extracts, which we term L-box binding factor. This factor either is not present or does not bind in nuclear extracts from fruit, indicating that it may be involved in light regulation or cell type-specific expression of *rbcS1* and *3A*.

I-Box, GATA Motif, and Motif 2

The sequence 5′-GGATGAGATAAGATTA (motif 2; Manzara and Gruissem, 1988) is found in the 5′ upstream regions of *rbcS1*, *2*, and *3A* from tomato, as well as several *rbcS* genes from other species. Motif 2 contains the sequence 5′-GATGAGATA, which is similar to the sequence 5′-GATGTGATA found in the *as-2* site of the CaMV 35S promoter. The *as-2* site has been shown to direct leaf-specific expression in transgenic tobacco plants and to bind ASF-2 from tobacco leaf nuclear extracts (Lam and Chua, 1989). Consistent with the possibility of leaf-specific function, motif 2 of tomato *rbcS1* and *2* is protected by leaf and cotyledon nuclear extracts. In contrast, motif 2 in *rbcS3A* is protected only by fruit nuclear extracts. It is possible that this difference in DNase I protection could be related to differences in the sequence of motif 2 in *rbcS3A*.

Also contained in motif 2 is the sequence 5′-GATAAG (GATA-box; indicated by 2′ in Figure 6), which has been designated I-box by Giuliano et al. (1988). Two of these I-box sequences from the Arabidopsis *rbcS1A* gene are necessary for high-level expression of the *adh* reporter gene in transgenic tobacco (Donald and Cashmore, 1990). Previous experiments have shown that a similar sequence in the tobacco *cab-E* promoter (5′-AGTAGATATAGATACTCAAGGATAAGG) interacts with the protein factor GA-1 from tobacco leaf nuclear extracts. This binding is competed by the *as-2* binding site sequence of the CaMV 35S promoter (Schindler and Cashmore, 1990), indicating that ASF-2 and GA-1 may be related or are different proteins both capable of binding to this sequence. Although several motif 2′ sequences are found in the promoter regions of the tomato *rbcS* genes, we did not detect protein binding to these sequences except in the *rbcS3C* gene using leaf extract.

G-Box (Motif 3)

In tomato, immediately 5′ to motif 2 is the sequence 5′-CACGTGGC, designated motif 3 in Figure 6 and G-box by Giuliano et al. (1988). This motif is apparently present only in the *rbcS1*, *2*, and *3A* promoter regions and is protected by all nuclear extracts except cotyledons. The Arabidopsis *rbcS1A* G-box sequence is critical for high-level expression of the *adh* reporter gene in leaves of transgenic tobacco plants (Donald and Cashmore, 1990) and acts as an upstream activating sequence in yeast (Donald et al., 1990). G-box-related sequences have recently been identified in several other genes for proteins of unrelated functions (reviewed in Gilmartin et al., 1990), and genes for several G-box binding factors have been cloned (Oeda et al., 1991; Weisshaar et al., 1991; I. Meier and W. Gruissem, unpublished data). The fact that we observe protection of the G-box sequence in the *rbcS1*, *2*, and *3A* promoter regions in red fruit and root, where these genes are not transcribed (Wanner and Gruissem, 1991), suggests that binding of the G-box binding factor alone is not sufficient to activate transcription.

Light Regulatory Sequences

DNA sequence motifs that may be required for light regulation of *rbcS* transcription include motif 8 (box II; Green

et al., 1987, 1988), which binds GT-1, motif 18 (LRE; Grob and Stüber, 1987), and motif 15. Motif 15 is related to an *rbcS* promoter sequence from *Lemna gibba* that binds the LRF-1 protein, whose concentration in nuclear extracts is light dependent (Buzby et al., 1990). Except for the box II sequence from the pea *rbcS3A* promoter, which confers light-regulated expression on the CaMV 35S −90 promoter in transgenic tobacco (Lam and Chua, 1990), the significance of box II, LRE, and motif 15 in light regulation of *rbcS* genes has not been demonstrated experimentally.

In tomato and other solanaceous plants, the *rbcS* LRE sequence (motif 18) is closely linked to the TATA box (Manzara and Gruissem, 1988). Our DNase I protection assays have shown that this sequence interacts with protein(s) in nuclear extracts from organs that transcribe *rbcS*. This protection, however, is also observed for *rbcS3B* and *3C* with nuclear extracts from dark-grown cotyledons and for *rbcS3A*, *3B*, and *3C* with nuclear extracts from green fruit, where these genes are not transcribed. If LRE in tomato is required for light regulation, it may act by either modification of the LRE binding protein (LREF) or interaction of LREF with other proteins. Interaction of LREF with other proteins appears likely considering the extent of the DNase I-protected sequences that span the LRE/TATA region. Our results currently do not distinguish LREF from TFIID, the TATA binding factor, or interaction of LREF with TFIID.

Although a functional relationship of the box II sequence to light regulation has been demonstrated (Lam and Chua, 1990), no differences have been detected in GT-1 binding to box II with nuclear extracts from light-adapted or dark-adapted pea leaves (Green et al., 1987). We observed DNase I protection of a portion of the tomato box II sequence with leaf and/or cotyledon extracts in *rbcS2*, *3A*, and *3B*, but it does not appear that a typical box II binding activity is present in our nuclear extracts. This is consistent with results from earlier experiments in which a tomato leaf nuclear extract failed to protect the pea *rbcS-3.6* box II sequence (Giuliano et al., 1988). It is possible, however, that a GT-1 binding activity is present in tomato and that the context of the consensus sequence is critical for binding because cotyledon/leaf extracts protect some, but not all, GT-1 core sequences (5′-GGTTAA) present in the tomato *rbcS* promoter regions.

The tomato *rbcS1*, *3B*, and *3C* promoter regions contain a DNA sequence (motif 15; 5′-AGATGAGG) that is related to the putative light-regulatory DNA sequence 5′-CGGATAGATGGCAGACGATAAGA from the *SSU5B* gene of *L. gibba* (Buzby et al., 1990). In *L. gibba*, this sequence is protected by a DNA binding protein termed LRF-1, which is present in higher concentrations in nuclear extracts from light-grown plants than in extracts from dark-adapted plants. DNA motif 15 in tomato is protected with nuclear extracts from cotyledons only in *rbcS3B* and *3C*, which is consistent with the strictly light-dependent transcriptional activation of these two genes (Wanner and Gruissem, 1991). We have not detected any differences in DNase I protection of motif 15 in *rbcS3B* and *3C* with nuclear extracts from dark-grown and light-grown cotyledons, but in vivo methylation interference experiments demonstrate that the methylation patterns around motif 15 differ between dark-grown and light-grown tomato cotyledons (P. Carrasco, T. Manzara, and W. Gruissem, unpublished data). Therefore, motif 15 may constitute a site that participates in the light-dependent transcription of *rbcS3B* and *3C* but not *rbcS1*.

TATA-Box Sequence

The TATA-box sequence of tomato *rbcS* genes has the consensus 5′-(C/T)TATATA(T/A)A, and binding activities for this sequence are present in all extracts derived from organs that transcribe *rbcS*. The TATA-box sequence is immediately adjacent to the LRE (motif 18) in all tomato *rbcS* genes. Because both sequences show protection against DNase I, it is difficult to distinguish from our results whether a single DNA binding factor encompasses both LRE-TATA sequences or whether there is a unique binding factor for each sequence. The extent of the DNase I-protected regions differs with nuclear extracts from different organs, suggesting that distinct proteins specific to this region may exist in different organs. It is possible that these differences in DNase I protection may result from binding of specific isoforms of TFIID to the TATA-box because at least two distinct clones for TFIID were recently isolated from Arabidopsis, both of which possess a helix-loop-helix motif (Gasch et al., 1990).

A/T-Rich and G/C-Rich DNA Sequences

We have identified several A/T-rich DNA sequences that bind nuclear proteins in the 5′ upstream regions of the tomato *rbcS* genes (see Figure 6). These sequences are similar to other A/T-rich DNA sequence elements in the promoter regions of a number of plant genes (Jofuku et al., 1987; Jensen et al., 1988; Bustos et al., 1989; Datta and Cashmore, 1989; Jordano et al., 1989; Jacobsen et al., 1990). A β-phaseolin A/T-rich DNA sequence has been shown to enhance expression of a minimal 35S promoter fused to the β-glucuronidase gene in transgenic tobacco plants (Bustos et al., 1989). Two proteins isolated from soybean that bind to A/T-rich DNA sequences appear to be related to high mobility group proteins (Jacobsen et al., 1990). A pea nuclear factor termed AT-1 can be reversibly phosphorylated and binds the sequence 5′-AATATTTTTATT of the tomato *rbcS3A* gene only in the nonphosphorylated form in vitro (Datta and Cashmore, 1989). Because the AT-1 binding site is located upstream of the *rbcS3A* promoter region examined in this study, it is unclear whether the AT-1 factor is contained in tomato

nuclear extracts. Although the function of the A/T-rich DNA sequences in the tomato *rbcS* promoter regions is unknown, it is interesting to note that, with some exceptions, they generally bind proteins in nuclear extracts from those organs in which *rbcS* genes are transcribed.

We have identified a G/C-rich sequence contained within motif 5 of the *rbcS1* gene (5′-TTAAATAGAGGGCGTAA) that binds a protein in nuclear extracts from leaves, cotyledons, and young fruit. The consensus for motif 5 was originally defined as 5′-TTAGTTTGGGAANTTT(G/A)A, which is included in the *rbcS3B* and *3C* promoter regions (Manzara and Gruissem, 1988). The G/C-rich portion of the motif is not conserved in *rbcS3B* and *3C*, however, and no binding was observed to these sequences (Figure 6). A G/C-rich sequence motif was recently identified in the promoter region of the *cab-E* gene of *Nicotiana plumbaginifolia* that binds the factor GC-1 (Schindler and Cashmore, 1990). It is likely that a binding activity similar to GC-1 also binds to the G/C-rich portion of motif 5 of *rbcS1*, but functional studies will be required to establish this relationship.

The Role of DNA-Protein Interactions in the Developmental Regulation of *rbcS* Transcription

Collectively, our analysis of the five tomato *rbcS* promoter regions demonstrates that the temporal and quantitative regulation of *rbcS* transcription is correlated with complex and organ-specific changes in DNA-protein interactions, suggesting that these changes most likely contribute to alterations in the transcription pattern of the *rbcS* multigene family. This complexity, detected by DNase I protection in vitro and methylation interference in vivo (P. Carrasco, T. Manzara, and W. Gruissem, unpublished data) is not surprising, considering the diversity of intracellular and extracellular signals that affect the transcription of *rbcS* genes in plants. Our results demonstrate that DNA-protein interactions in the *rbcS* promoter regions are limited in roots and ripe fruit, in which transcription is not detectable for any of the five genes. The loss of DNA-protein interactions in photosynthetically inactive organs suggests that *rbcS* transcription activity is positively regulated in leaves and green fruit by a complex interaction of proteins with different *cis*-acting *rbcS* promoter sequences. Our DNase I footprint analysis of promoter regions with nuclear extracts from dark-grown cotyledons and green fruit, in which the transcription activity of *rbcS* genes is differentially regulated, also indicates that a specific pattern of DNA-protein interactions is required but not sufficient for transcription activity. The fact that we detected sequence-specific DNA-protein interactions in the *rbcS3B* and *3C* promoter regions with nuclear proteins from dark-grown cotyledons suggests that their transcriptional activation requires additional protein modification (e.g., phosphorylation) or protein-protein interactions that are not detectable in the DNase I footprint assay. Such modifications or protein-protein interactions may also fail to occur in young fruit, where *rbcS3A*, *3B*, and *3C* are not transcribed, although their promoter regions can specifically interact with DNA binding proteins at this developmental stage.

The different organization of conserved DNA sequence motifs in the *rbcS* promoter regions is most likely an important element in the differential control of transcriptional activity of the genes, but the significance of specific spatial arrangements of *cis*-acting elements is currently not well understood. The spatial contexts of different DNA sequence motifs are likely to be important in establishing functional DNA-protein interactions, especially in cases where protein-protein interactions of the cognate DNA binding proteins may be required to stabilize DNA-protein complexes. Protein-protein interactions that affect qualitative and quantitative levels of transcription activity are now well established (reviewed in Johnson and McKnight, 1989). The fact that we have characterized the complex pattern of DNA-protein interactions for all five tomato *rbcS* genes will allow further investigation into the role of protein-protein interactions regulating these genes. The *rbcS* gene family in plants will, therefore, serve as a good model system to dissect the contributions of general and organ-/cell-specific DNA binding proteins to the transcriptional control of the individual genes.

METHODS

Plant Material

For 7-day-old cotyledons and for roots, VFNT cherry LA1221 or red cherry seeds were germinated in aluminum foil-covered metal pans in a dark room. For cotyledons exposed to light, foil was replaced with plastic wrap, and seedlings were placed in a growth chamber in continuous light for 6 or 12 hr. Light-grown cotyledons were germinated in a growth chamber under a 14-hr light/10-hr dark cycle. Cotyledons (along with approximately the upper one-quarter of the hypocotyl) were harvested using a razor blade. Root tissue was also derived from dark-grown seedlings and consisted of the seedling roots plus the lower one-quarter of the hypocotyl.

Both leaf and fruit tissues were harvested between 10:00 AM and noon from greenhouse-grown tomato plants and were immediately frozen in liquid nitrogen.

Preparation of Nuclear Extracts from Tomato

Frozen tissue was ground with dry ice prior to homogenization and fresh tissue was used directly. All procedures were carried out at 4°C. Fifty-gram batches of tissue were homogenized in a Waring Blender fitted with four razor blades in a volume of approximately 300 mL of homogenization buffer consisting of 250 mM sucrose, 10 mM NaCl, 25 mM Pipes, pH 7.0, 5 mM EDTA,

0.15 mM spermine, 0.5 mM spermidine, 20 mM β-mercaptoethanol, 0.1% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenates were filtered through four layers of cheesecloth, then through three layers of Nitex nylon mesh of pore size 300, 100, and 50 μM. Nuclei were collected by centrifugation at 4225*g* for 20 min at 4°C, then were gently resuspended and washed four times with homogenization buffer with subsequent centrifugations at 1912*g* for 10 min, then at 1464*g* for 10, 8, and 6 min. After washing, nuclei were resuspended in a minimum volume of freezing buffer consisting of 100 mM NaCl, 50 mM Hepes, pH 7.6, 5 mM $MgCl_2$, 10 mM KCl, 50% glycerol, 1 mM DTT, 0.5 μg/mL leupeptin, and 50 μg/mL antipain. Nuclei were frozen in liquid nitrogen and stored at −80°C until use.

Nuclear extracts were prepared by thawing nuclei on ice and lysing by adjusting the NaCl concentration to 0.47 M with lysing buffer containing 2.5 M NaCl, 50 mM Hepes, pH 7.6, 5 mM $MgCl_2$, 10 mM KCl, 20% glycerol, 1 mM DTT, 0.5 μg/mL leupeptin, and 50 μg/mL antipain, and then rocking at 4°C for 30 min. Chromatin was pelleted by centrifugation in an Eppendorf microcentrifuge for 15 min, and the supernatant containing nuclear proteins was dialyzed for 3 to 4 hr against a buffer consisting of 20 mM Hepes, pH 7.6, 40 mM NaCl, 0.2 mM EDTA, 20% glycerol, and 1 mM DTT. Nuclear extracts were concentrated using Amicon Centricon 10 devices, frozen on liquid nitrogen, and stored at −80°C. Protein concentrations were determined using the Bio-Rad protein assay kit.

Fragment Labeling Reactions

CsCl-purified plasmid DNA was linearized by cutting at one end of the insert with either EcoRI or HindIII. The staggered ends were filled in using Sequenase 2.0 enzyme in the presence of the appropriate ^{32}P-labeled/unlabeled nucleotides. Following extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation, insert DNA was released from vector DNA by digestion with either PstI or SstI enzyme. Insert DNA was purified by polyacrylamide gel electrophoresis, eluted, and ethanol precipitated. The labeled fragments were resuspended in 10 mM Tris, pH 8.0, 20 mM NaCl, and 1 mM EDTA, and stored at 4°C.

Mobility-Shift Assay

The standard mobility-shift reaction was done in a volume of 10 μL using 1 fmol of ^{32}P-labeled DNA fragment, 1.5 μg of poly(dI-dC), and 0.2 to 1.8 μg of nuclear protein in 18 mM Hepes, pH 7.6, 37 mM NaCl, 1 mM EDTA, 18% glycerol, 0.9 mM DTT, and 0.5 mM Tris, pH 8.0. Binding was for 45 min at room temperature. Reactions were loaded without addition of dyes onto 4% polyacrylamide nondenaturing gels having a ratio of 29:1 acrylamide to bisacrylamide in 10 mM Tris, pH 7.5, and 1 mM EDTA. Gels were run at approximately 10 V/cm with recirculation of buffer. For the binding competition assays, 50 to 250 fmol of unlabeled fragment were included in the reaction, and the final concentrations were 16 mM Hepes, pH 7.6, 35 mM NaCl, 1.1 mM EDTA, 16% glycerol, 0.8 mM DTT, and 1.5 mM Tris, pH 8.0.

DNase I Footprinting Reactions

The reactions for the DNase I footprinting were exactly the same as those for the mobility-shift assay except for the fact that the footprinting reactions contained 4.5 to 14.4 μg of nuclear protein and 0.5 to 1 fmol of ^{32}P-labeled fragment. Binding was for 30 min at room temperature. Following the binding reaction, 1 μL of DNase I solution was added to achieve a final DNase I concentration of 1 to 2 μg/mL and was incubated for 10 sec at room temperature. The reaction was stopped by the addition of 90 μL of 6.25 mM EDTA, 0.125% SDS, 0.375 M sodium acetate, and 62.5 μg/mL tRNA (Green et al., 1988). Reactions were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated. Pellets were resuspended in 95% deionized formamide containing 10 mM EDTA, pH 8.0, 0.025% xylene cyanol, and 0.025% bromophenol blue, boiled for 3 min, and loaded onto either 4% or 6% denaturing acrylamide gels in 1/2 × Tris, borate, EDTA buffer containing 44 mM Tris base, 44 mM boric acid, and 0.1 mM EDTA, pH 8.0.

ACKNOWLEDGMENTS

We thank Drs. Jonathon Narita, Leslie Wanner, and Jill Deikman for helpful discussions, and Drs. Barbara Kloeckener, Iris Meier, and Leslie Wanner for critical reading of the manuscript. We also thank Ben Greene for construction of the plasmids. P.C. was funded by the Consellena Educacio i Cienca Generalitat Valenciana. This research was supported by National Science Foundation (NSF) Grant DMB-8905202 to W.G. and by the NSF Center for Plant Development.

Received September 26, 1991; accepted October 29, 1991.

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