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Maize *rbcS* Promoter Activity Depends on Sequence Elements Not Found in Dicot *rbcS* Promoters

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Although the molecular mechanisms of dicot photosynthetic gene regulation have been pursued actively, comparable studies of monocot regulation have been slow to come forth. We show here that monocot (maize and wheat) but not dicot (pea, tobacco, and Arabidopsis) ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) gene promoters are active in maize mesophyll protoplasts. The evolutionarily conserved GT and G boxes of dicot *rbcS* promoters are not essential for light-responsive expression in monocot leaf cells. Instead, at least six constitutive and light-sensitive regulatory elements are likely important for maize *rbcS* expression. Synergism between upstream and downstream promoter elements is required. Whereas in dicots, light triggers coupled leaf development and photosynthetic gene expression, in monocots, light regulation of *rbcS* is uncoupled from leaf development. Light regulation of maize *rbcS* may be divided into direct and indirect contributions mediated by different regulatory elements. Because wheat and maize *rbcS* promoters show sequence homologies and similar expression patterns in monocot and dicot leaf cells, it appears likely that monocots share conserved regulatory elements irrespective of whether they utilize the C3 or C4 pathway for carbon fixation.

INTRODUCTION

The photosynthetic genes encoding ribulose-1,5-bisphosphate carboxylase small subunit (RBCS) have been studied extensively in pea, Lemna, petunia, Arabidopsis, tomato, potato, soybean, wheat, and maize (Kuhlemeier et al., 1987; Schell, 1987; Krebbers et al., 1988; Manzara and Gruissem, 1988; Willmitzer, 1988; Dean et al., 1989a, 1989b; Meagher et al., 1989; Nelson and Langdale, 1989; Donald and Cashmore, 1990; Gilmartin et al., 1990; Silverthorne and Tobin, 1990). Using an Agrobacterium-mediated transformation system, several DNA motifs, such as the GT boxes, G box, I box, AT-1 box, and 3AF1 binding site (Dean et al., 1989b; Ueda et al., 1989; Donald and Cashmore, 1990; Gilmartin et al., 1990) have been identified and proposed to be involved in dicot *rbcS* regulation. However, the wheat *rbcS* promoter is not expressed when transformed into tobacco plants (Keith and Chua, 1986). The study of a *rbcS* promoter in the aquatic monocot *Lemna gibba* reveals no obvious homologies to the dicot *rbcS* promoters studied so far (Rolfe and Tobin, 1991). Therefore, it is possible that the molecular mechanisms underlying *rbcS* regulation differ between dicots and monocots.

We have developed a transient expression method based on protoplasts isolated from fresh tissues of maize. The assay allows the convenient dissection of the molecular mechanisms regulating the differential expression of photosynthetic genes (Sheen, 1990, 1991). We show here that maize and wheat *rbcS* promoters, but not pea, tobacco, and Arabidopsis *rbcS* promoters, are active in maize mesophyll protoplasts. In contrast, dicot but not monocot *rbcS* promoters are functional in tobacco mesophyll cells, in agreement with data from transgenic tobacco plants (Kuhlemeier et al., 1988; Poulsen and Chua, 1988; Donald and Cashmore, 1990). At least six constitutive and light-sensitive regulatory elements, distinct from the regulatory elements of dicot *rbcS* except for the I box, are potentially responsible for the differential expression of maize *rbcS* genes. Sequence homologies to the upstream and downstream promoter elements of maize *rbcS* are found in the wheat *rbcS* promoter. The utilization of different constitutive and light-sensitive elements allows the dark expression of maize *rbcS* and divides the light regulation of the gene into direct and indirect contributions.

RESULTS

Isolation of Maize *rbcS* Genomic Clones

Because the maize genome is heavily methylated and contains many repeated sequences, we constructed a

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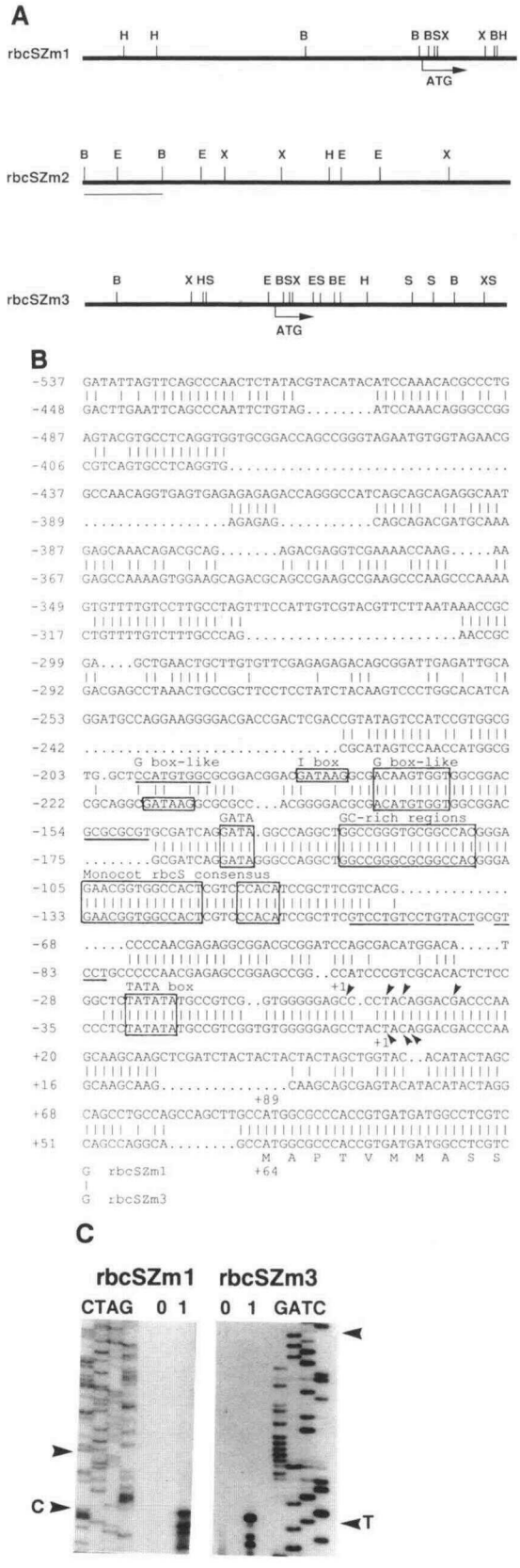

genomic library in the λ phage vector Charon 40 which can be propagated in a *recA*, *mcrA*, *mcrB Escherichia coli* strain (Dunn and Blattner, 1987). With this library, three different *rbcS* clones were obtained and mapped, as shown in Figure 1A.

Genomic DNA and cDNA analyses predict the existence of three or four members of the maize *rbcS* family (Sheen and Bogorad, 1986a). Analysis of the genomic clones and cDNA sequences (data not shown) indicates that the clones λrbcSZm1 and λrbcSZm3 correspond to the two highly expressed SS1 and SS7 cDNA clones, respectively (Sheen and Bogorad, 1986a). A previously reported maize genomic clone (Lebrun et al., 1987) and a cDNA clone (Matsuoka et al., 1987) are derived from the same locus as λrbcSZm3. The clone λrbcSZm2 represents a third locus, which requires further characterization.

Sequence Comparison of the Two Maize *rbcS* Promoters

To study the putative regulatory elements of maize *rbcS* genes, the 5′ regions of *rbcSZm1* and *rbcSZm3* were sequenced. The 5′ regions of the two genes are homologous, as shown in Figure 1B. However, many insertions, deletions, and base substitutions are also found between the conserved sequences of the two genes (Figure 1B). Transcription is initiated at multiple sites for both *rbcSZm1* and *rbcSZm3*, as shown in Figure 1C.

Monocot but Not Dicot rbcSCAT Chimeric Genes Are Highly Active in Maize Mesophyll Protoplasts

To determine the generality and molecular basis of the difference between monocot and dicot *rbcS* regulation, we

Figure 1. Analyses of Maize *rbcS* Gene Family.

(A) Maps of maize *rbcS* genomic clones. Arrowed lines represent transcripts. The line under *rbcSZm2* indicates regions that show hybridization signals with a maize *rbcS* cDNA probe (data not shown). Restriction enzymes are B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XhoI.

(B) Sequence comparison of the 5′ regions of *rbcSZm1* and *rbcSZm3*. Short, vertical lines indicate matched nucleotides. Arrows indicate transcription initiation sites. Dots indicate deletions. Boxed regions represent the putative regulatory elements shared by both genes. Gene-specific elements are underlined. The most upstream transcription initiation sites are numbered as +1.

(C) Multiple transcription initiation sites of *rbcSZm1* and *rbcSZm3*. The transcription initiation sites of *rbcSZm1* and *rbcSZm3* were mapped by primer extension. About 1 μg of the poly(A)$^+$ mRNA isolated from greening maize leaves was used per reaction (lanes 1). About 20 μg of tRNA was used for control (lanes 0). DNA sequences generated by the same ^{32}P-labeled primers are shown in parallel. Lower arrows indicate the most upstream transcription initiation sites (C and T). Upper arrows indicate the location of the TATA boxes.

analyzed maize, wheat, pea, tobacco, and Arabidopsis chimeric genes by transient expression in maize (Sheen, 1990, 1991) and tobacco mesophyll protoplasts (Harkins et al., 1990). These chimeric genes were constructed by fusing various *rbcS* promoters to the chloramphenicol acetyltransferase (CAT) gene at the translation initiation site. A construct containing the promoter of the 35S RNA of cauliflower mosaic virus (35S) and the β-glucuronidase (GUS) gene was used as an internal control (Sheen, 1990, 1991). The dicot *rbcS* promoters used here have been shown previously to be active in transgenic tobacco plants (Kuhlemeier et al., 1988; Poulsen and Chua, 1988; Donald and Cashmore, 1990).

As shown in Figure 2, the rbcSCAT chimeric genes with monocot but not dicot *rbcS* promoters direct a high level of CAT activity in maize mesophyll protoplasts. In contrast, dicot but not monocot rbcSCAT chimeric genes show CAT activity in tobacco mesophyll protoplasts. Maize *rbcS* promoters are more active than the wheat promoter in maize cells, whereas the tobacco *rbcS* promoter shows highest expression in tobacco cells.

Neither the Upstream nor Downstream Promoter Elements of Pea *rbcS* Can Substitute Functionally for the Corresponding Elements of Maize *rbcS*

To explore the differences between monocot and dicot *rbcS* promoters, we constructed and analyzed maize and pea hybrid promoters. We define the upstream promoter

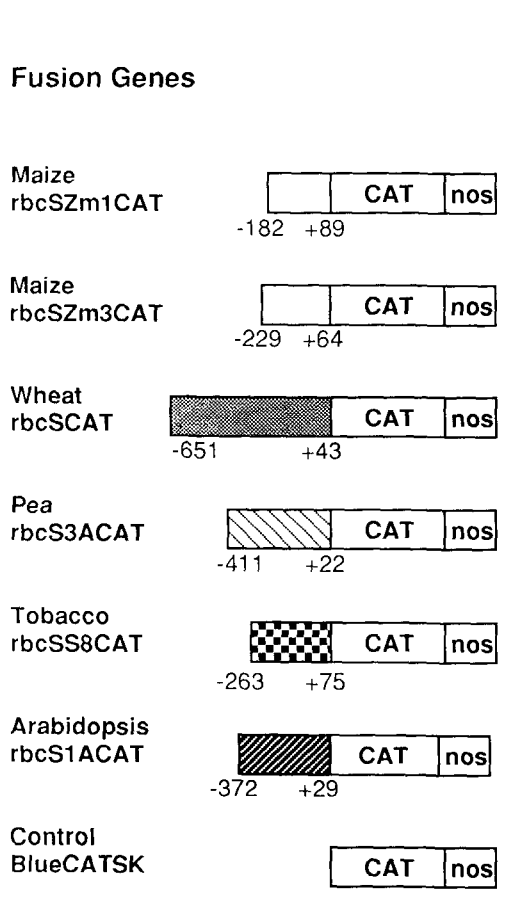

Figure 2. Transient Expression of Monocot and Dicot rbcSCAT Chimeric Genes in Maize and Tobacco Mesophyll Protoplasts.

Plasmids carrying various rbcSCAT chimeric genes (10 μg/kb, in equal molar ratio) were electroporated into 10^5 maize or tobacco mesophyll protoplasts. Different batches of protoplasts were used for each experiment. About 20 μg of 35SGUS was coelectroporated as an internal control. CAT assays were performed with cell extracts prepared from 2.5×10^4 protoplasts for 90 min. Relative CAT activity was normalized to the expression level of rbcSZm1CAT in maize cells and to the expression level of rbcSS8CAT in tobacco cells. GUS assays were performed with cell extracts prepared from 10^3 protoplasts for 90 min. Relative GUS activity was the direct fluorescence reading divided by 10.

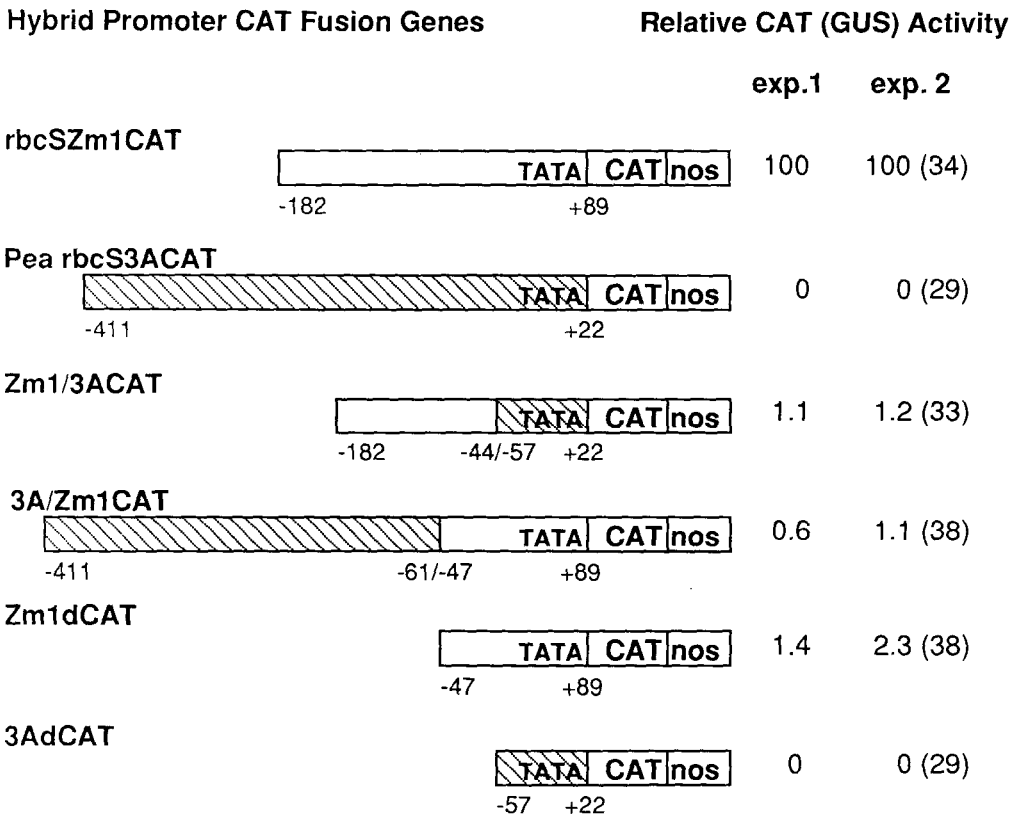

Figure 3. Analysis of Pea and Maize *rbcS* Hybrid Promoters.

Electroporation and CAT and GUS assays were performed as described in the legend to Figure 2. Relative CAT activity was normalized to the expression level of the wild-type rbcSZm1CAT.

elements to be the sequences upstream of the TATA box of the pea (−411 to −61) and maize (−182 to −44) *rbcS* promoters. We define the downstream promoter elements as containing the TATA box and downstream sequences of the pea (−57 to +22) and maize (−47 to +89) *rbcS* promoters. Although the pea promoter showed little activity in maize cells, it is not clear whether its downstream elements, which contain the TATA box, can cooperate with maize *rbcS* upstream elements or whether its upstream elements can function in the presence of maize *rbcS* downstream elements.

As shown in Figure 3, neither upstream nor downstream promoter elements of pea *rbcS* can functionally replace those of maize *rbcS* in maize mesophyll protoplasts. The results suggest that the GT box and G box included in the upstream promoter elements of pea *rbcS* may not function in maize mesophyll cells (Donald and Cashmore, 1990; Gilmartin et al., 1990), and that the activity of upstream regulatory elements of maize *rbcS* requires specific downstream promoter elements besides the TATA box.

Expression of Maize *rbcS* in Mesophyll Cells Is Superimposed with Upstream Silencers and Post-Transcriptional Control

In C4 plants such as maize, *rbcS* mRNA usually does not accumulate in greening and green mesophyll cells that are adjacent to bundle sheath cells (Sheen and Bogorad, 1986a; Nelson and Langdale, 1989). However, in etiolated mesophyll cells and green mesophyll cells that are not close to bundle sheath cells, some *rbcS* mRNA has been found (Sheen and Bogorad, 1986a; Langdale et al., 1988a, 1988b). It has been proposed that C3-pattern gene expression in C4 mesophyll cells is a default pattern overridden by cell position and light signals (Sheen and Bogorad, 1985; Langdale et al., 1988b; Nelson and Langdale, 1989). However, the molecular mechanisms for C4-pattern gene expression are unknown.

We have shown that both maize (C4) and wheat (C3) *rbcS* promoters are active in maize mesophyll protoplasts by transient expression (Figure 2). Because all of the maize

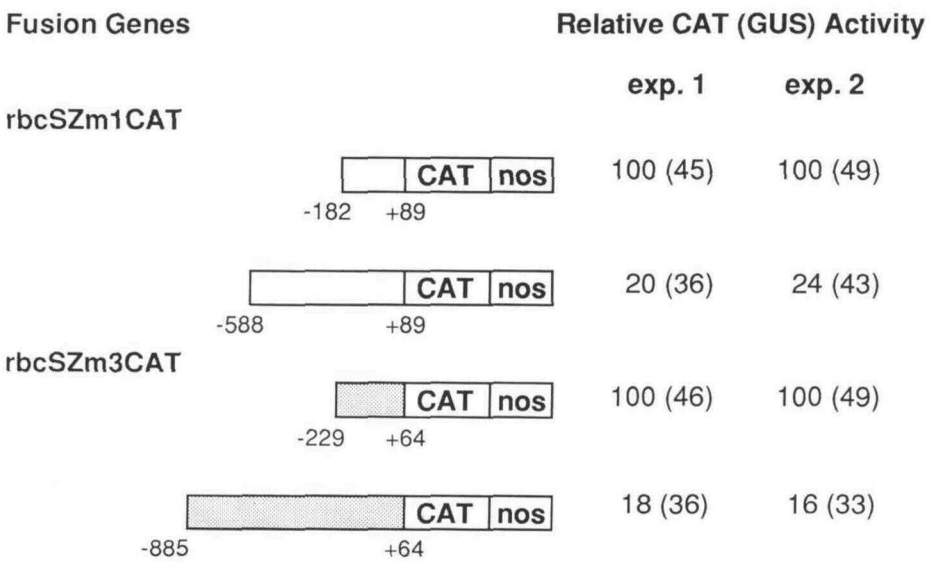

Figure 4. Upstream Silencers Inhibit the Activity of Maize *rbcS* Promoters.

Electroporation and CAT and GUS assays were performed as described in the legend to Figure 2. Relative CAT activity was normalized to the expression levels of the shorter constructs of rbcSZm1CAT and rbcSZm3CAT, respectively.

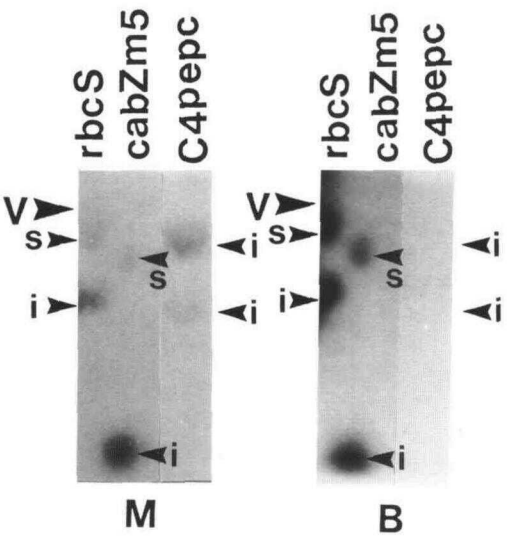

Figure 5. Nuclear Run-On Transcription of Maize Bundle Sheath (B) and Mesophyll (M) Cells.

Plasmid DNA (2 μg) containing *rbcS*, *cabZm5*, and *C4pepc* cDNA was double digested with EcoRI and HindIII (1-kb insert), EcoRI and BamHI (0.15-kb insert), and EcoRI and XbaI (2.3- and 1-kb inserts), respectively. Large arrows indicate the position of vector DNA (V) (2.3 kb) free from inserts after separation by agarose gel electrophoresis and blotting (data not shown). No signal was detected at the vector region. Small arrows indicate the location of DNA inserts (i) and incompletely digested supercoiled plasmid DNA (s). The shadow at the left edge of the maize bundle sheath cell dot blot was from the overexposure of rRNA signals (data not shown).

rbcSCAT constructs studied so far contained less than 300 bp of the 5′ regions, we made and analyzed additional constructs including further upstream sequences. In Figure 4, we show that the addition of the sequences, −588 to −183 from *rbcSZm1* and −885 to −230 from *rbcSZm3*, inhibits the expression of both maize rbcSCAT chimeric genes about fourfold in mesophyll protoplasts. Upstream silencers are usually not found in other maize photosynthetic gene promoters assayed in mesophyll protoplasts (Sheen, 1990, 1991; A.R. Schäffner and J. Sheen, unpublished results). Unlike the tomato *rbcS-3A* (Ueda et al., 1989), Lemna *SSU5B* (Rolfe and Tobin, 1991), and tobacco chlorophyll *a/b*-binding protein gene *cabE* (Castresana et al., 1988) promoters, the addition of the sequences upstream of −588 and −885 of *rbcSZm1* and *rbcSZm3*, respectively, does not restore the promoter activity (data not shown).

Despite the presence of upstream silencer activity, substantial amounts of maize *rbcS* promoter activity are still found in mesophyll protoplasts (Figure 4). To see whether *rbcS* promoters are active in mesophyll cell nuclei of intact tissues, we carried out nuclear run-on experiments with nuclei isolated from mesophyll and bundle sheath cells of fresh tissues. As shown in Figure 5, maize *rbcS* mRNAs

are detected in mesophyll as well as bundle sheath cell nuclei, albeit at a lower level. In contrast, the transcriptional activity of the C4 phosphoenolpyruvate carboxylase gene (*C4pepc*) is only detected in mesophyll but not bundle sheath cell nuclei (Figure 5). The transcriptional activity of the chlorophyll *a/b*-binding protein gene *cabZm5* is detected in both cell types as is its steady-state mRNA (Figure 5) (Sheen and Bogorad, 1986b). Therefore, the expression of the maize *rbcS* promoter in mesophyll protoplasts most likely reflects its physiological activity in plants.

Light Regulation of Maize *rbcS* Can Be Divided into Direct and Indirect Components

Although expressed in the dark at a low level, the expression of *rbcS* is induced dramatically during greening of etiolated maize leaves and declines in green leaves (Nelson et al., 1984; Sheen and Bogorad, 1986a). The molecular mechanisms underlying the differential expression of *rbcS* associated with illumination and chloroplast development are not known. Using mesophyll protoplasts isolated from etiolated, greening, and green leaves, we found that the expression patterns of rbcSZm1CAT and rbcSZm3CAT in mesophyll protoplasts resembled those of *rbcS* found in plants, as shown in Figure 6. The expression of both rbcSZm1CAT and rbcSZm3CAT is lowest in etiolated cells cultured in the dark and is much higher in greening and green cells cultured under light. However, illuminated etiolated protoplasts give CAT activity similar to illuminated greening protoplasts (Figure 6). Because chloroplast development is inhibited in protoplasts (data not shown), these results suggest that light has an immediate effect on *rbcS* expression that is independent of the presence

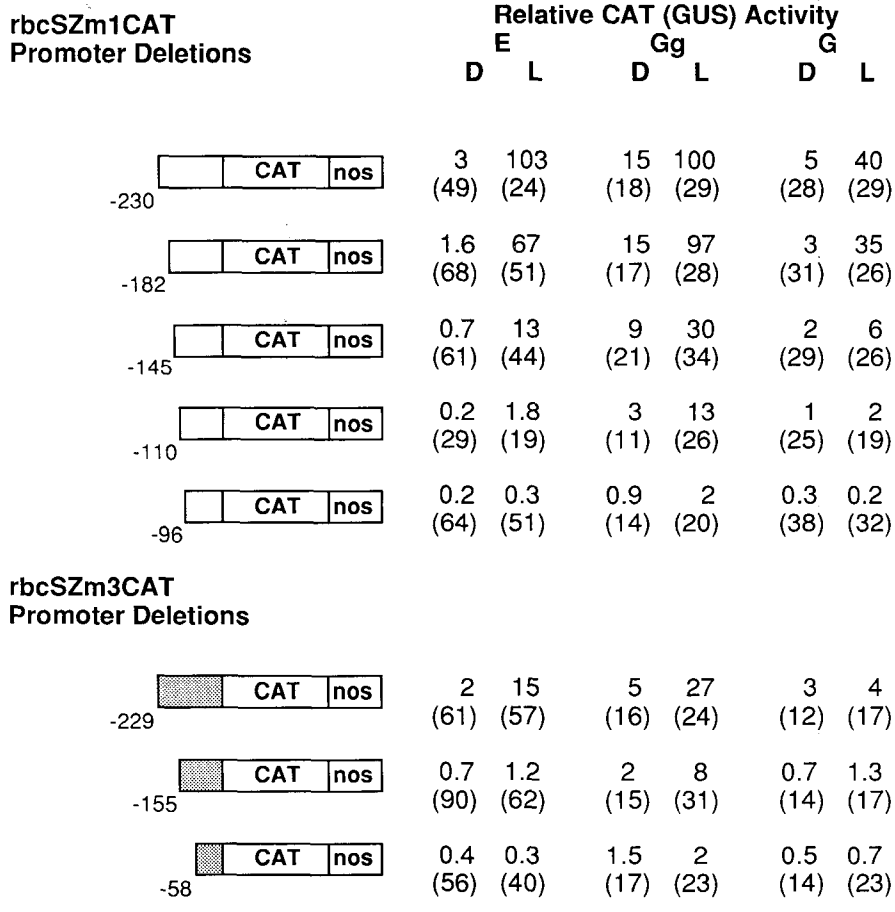

Figure 6. Light Regulation of Maize *rbcS* Promoters.

Protoplasts were isolated from etiolated (E), greening (Gg), and green (G) maize leaves of similar age. Electroporation was performed with 2×10^5 protoplasts. Each electroporated sample was divided into two parts (10^5 each) and cultured either under light (L) or in the dark (D). CAT and GUS assays were performed as described in the legend to Figure 2. Representative results are shown here with internal controls. Relative CAT activity was normalized to the highest expression level of rbcSZm1CAT in greening protoplasts.

and development of chloroplasts. Thus, transient expression in maize mesophyll protoplasts can serve as a convenient system for the study of light regulation of cereal monocot genes.

The effect of chloroplast developmental stages on *rbcS* expression is only obvious when etiolated and greening protoplasts are both cultured in the dark. In the absence of light, *rbcS* expression is much higher in greening than in etiolated cells (Figure 6). Light regulation of *rbcS* is much reduced in green protoplasts compared with etiolated and greening protoplasts (Figure 6).

To dissect the sequences important for *rbcS* expression at various chloroplast developmental stages, we carried out deletion analyses. As shown in Figure 6, multiple *cis*-regulatory elements located between −182 and −96 and between −229 and −58 are important for the expression of rbcSZm1CAT and rbcSZm3CAT, respectively. The light inducibility of rbcSZm1CAT is higher than that of rbcSZm3CAT. The deletion of sequences between −145 and −111 affects light regulation of rbcSZm1CAT more dramatically in etiolated protoplasts than in greening protoplasts, where higher dark expression is observed (Figure 6). The results also suggest that the increased activity of downstream promoter elements (downstream of −96 and −58 for rbcSZm1CAT and rbcSZm3CAT, respectively) are likely responsible for the higher CAT activity found in greening than in etiolated cells cultured in the dark (Figure 6).

Upstream Regulatory Sequences of Maize *rbcS* Are a Mosaic of Constitutive and Light-Sensitive Elements

To locate the regulatory elements more precisely, detailed deletion analyses were carried out. As shown in Figure 7, the sequences important for rbcSZm1CAT expression in greening protoplasts are located between −182 and −174, −152 and −146, −123 and −111, and −110 and −97. Two G box-like sequences (Giuliano et al., 1988; Donald and Cashmore, 1990) located between −198 and −190 and −170 and −162 (Figure 1B) are not essential, whereas the deletion of the sequences containing an I box (−179 GATAAG −174) (Giuliano et al., 1988) reduces light expression 2.5-fold (Figure 7). The deletion of the I box in rbcSZm3CAT has a similar effect (data not shown). The sequences between −152 and −146 and between −123 and −111 contain two types of GC-rich elements. The former are unique to *rbcSZm1*. Their deletion results in further decline of the promoter activity both in the dark and light (Figure 7). Although not obvious in greening protoplasts (possibly because of higher dark expression), the sequence between −123 and −111 probably contains a light-sensitive element assayed in etiolated protoplasts, where dark expression is low (Figure 6 and data not shown). The deletion of sequences between −110 and −97 abolishes *rbcSZm1* expression (Figure 7). These regulatory elements are also essential for *rbcSZm1* expression in etiolated and green protoplasts (data not shown).

To determine the specific role of some of the regulatory elements, site-directed mutageneses were performed. As shown in Figure 7, the mutation of the *rbcSZm1*-specific element CGCGCGTGCG (mutant 1) reduces CAT activity in both dark and light. The mutation of sequences between −104 and −90 (mutant 7) has a profound effect on rbcSZm1CAT expression even in the presence of other regulatory elements. The double mutation shows a combined effect (Figure 7). Because the effect of mutant 7 is striking, we analyzed the sequences further by finer mutational analysis. Two separable activities were found within the sequences between −104 and −92 (Figure 7). Mutant 7A (AACGGT to TCTAGA) reduces both dark and light expression, whereas mutant 7B (GGCCACT to TCTAGAA) decreases light expression (Figure 7). Although reproducible, the reason that the dark activity of mutant 7 is higher than that of mutant 7A is unclear and requires further investigation. These mutations also have a profound effect on *rbcSZm1* expression in etiolated and green protoplasts (data not shown).

In this study, we have defined light-sensitive elements as sequences that affect light expression but not dark expression, such as I box and 7B; and constitutive elements as sequences which affect both light and dark expression, such as GC-rich regions and 7A. In etiolated cells, the conserved GC-rich region of maize *rbcSZm1* promoter (Figures 1B and 6 and data not shown) is also involved in light regulation. Therefore, the upstream regulatory sequences of maize *rbcSZm1* are composed of a mosaic of constitutive and light-sensitive elements.

Synergism between Upstream and Downstream Promoter Elements Is Required for Maize *rbcS* Expression

To test whether a synergism between upstream and downstream elements is required for *rbcSZm1* expression, we fused the *rbcSZm1* upstream elements to the downstream elements of the 35S promoter functional in maize cells (Sheen, 1991). The hybrid promoters were assayed in etiolated protoplasts where light regulation is easiest to detect. As shown in Figure 8A, although the downstream elements of the 35S promoter can substitute partially for those of the *rbcSZm1* promoter, the combined activity of Zm1/35Shyb1 and the *rbcSZm1* downstream promoter (from −47 to +89) is still 12-fold lower than the activity of the native promoter. Thus, the activity of upstream regulatory elements of the maize *rbcS* promoter requires specific downstream promoter elements. Unlike the downstream promoter elements of pea *rbcS*, which show light regulation alone or when fused to the heat shock enhancer (Morelli et al., 1985; Kuhlemeier et al., 1989), similar elements of maize *rbcSZm1* show low light-independent activity (Figure 8A). To determine whether the sequences

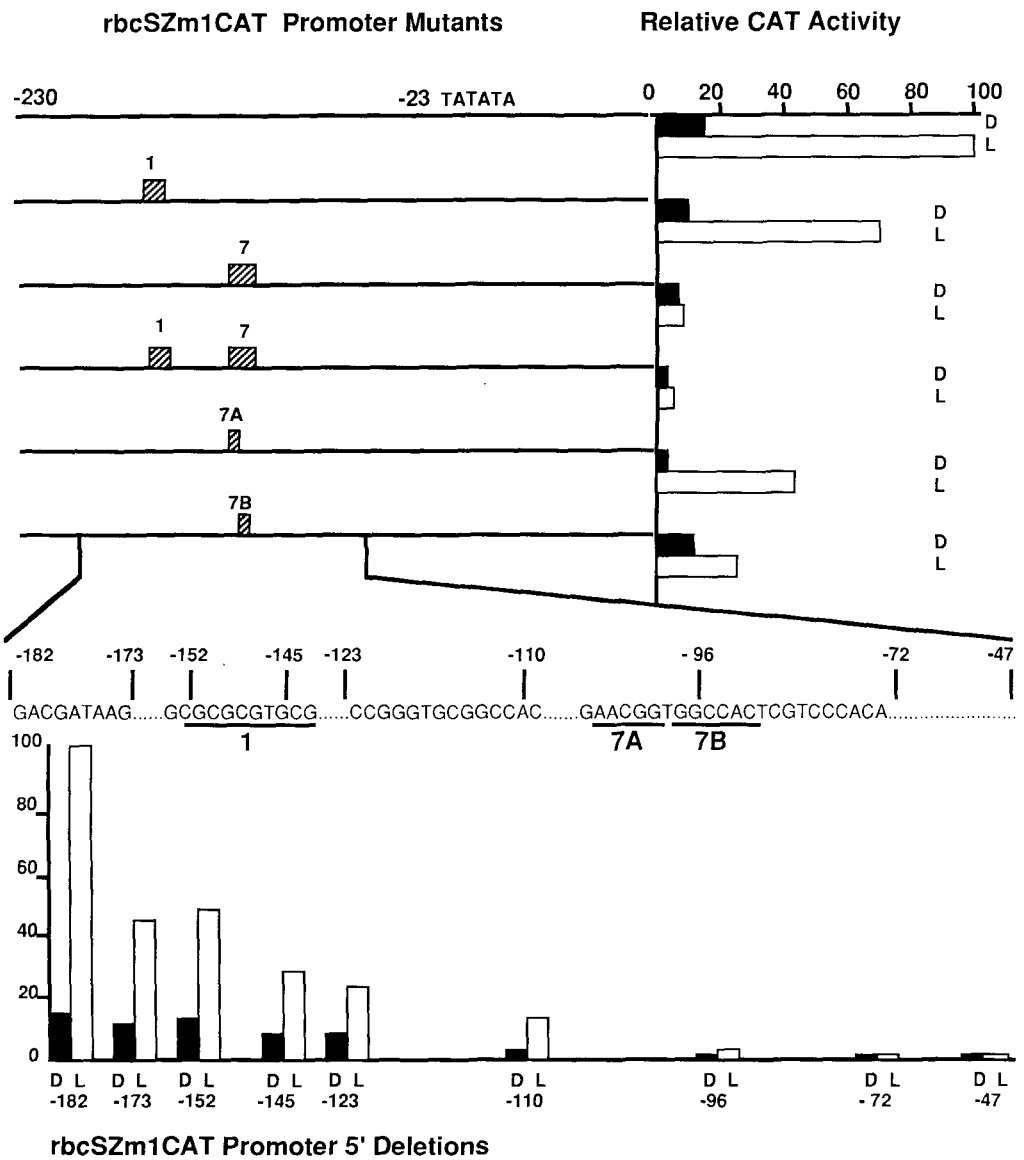

Figure 7. Mutation and Deletion Analyses of *rbcSZm1* Promoters.

Electroporation was done with 2×10^5 greening protoplasts. The locations of mutated sequences are marked as hatched boxes. The sequences of putative regulatory elements are shown. The underlined sequence between −153 and −144 was mutated to TCTAGATATC in mutant 1. The sequence between −104 and −90 was mutated to TTCTAGACTGATATC in mutant 7. The underlined sequence between −104 and −99 was mutated to TCTAGA in mutant 7A. The underlined sequence between −98 and −92 was mutated to TCTAGAA in mutant 7B. Relative CAT activity was normalized to the expression level of the wild-type rbcSZm1CAT. The data represent the average of two independent experiments with less than 10% variation.

between +1 and +89 are important for *rbcSZm1* expression, we fused the *rbcSZm1* promoter sequences (−182 to +1) to a TATA-free 35S promoter (−25 to +1). This fusion directs more than half of the native *rbcSZm1* promoter activity, as shown in Figure 8B. Therefore, sequences located downstream of the transcription initiation site are not essential for *rbcSZm1* expression.

Differential Light Regulation by Common Elements in Different DNA Contexts

We have shown that sequences common to both maize *rbcS* promoters are essential for light regulation (Figures 1B, 6, 7, and 8A). However, light induction is much higher for rbcSZm1CAT than for rbcSZm3CAT (Figure 6). To

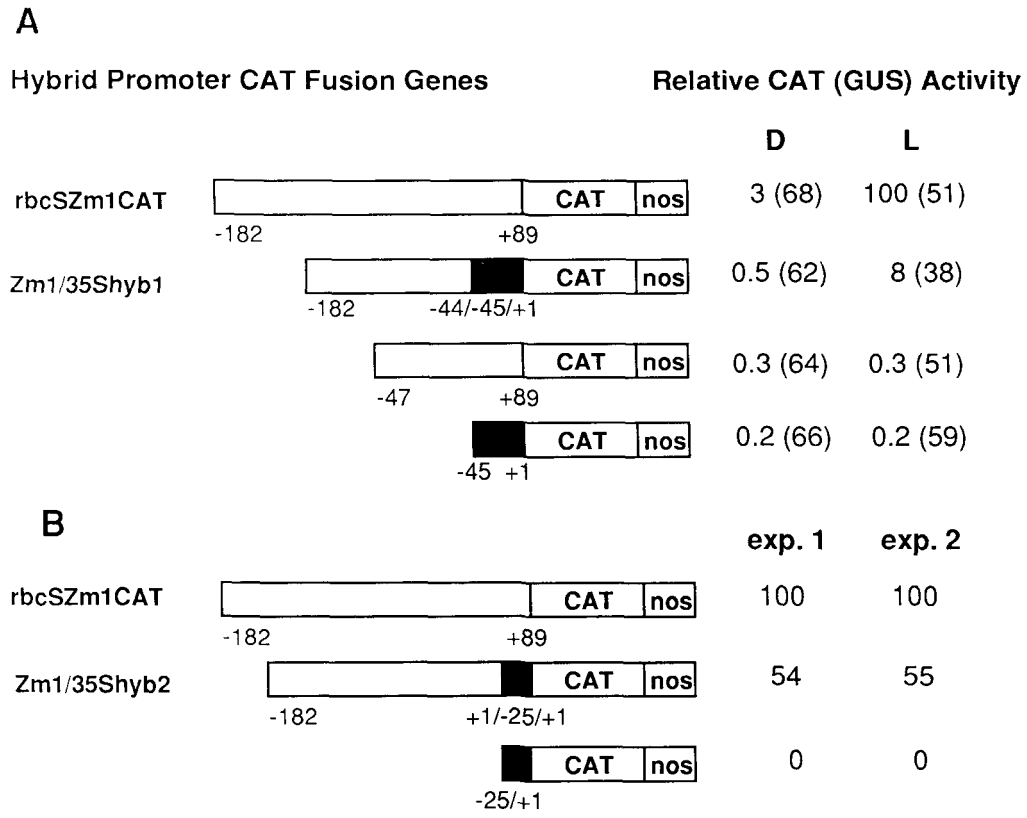

Figure 8. Analyses of *rbcSZm1* and *35S* Hybrid Promoters.

(A) Synergism between upstream and downstream promoter elements of *rbcSZm1*. Electroporation was performed with 2×10^5 etiolated protoplasts. Representative data with internal controls are shown. Relative CAT activity was normalized to the expression level of the wild-type rbcSZm1CAT. CAT and GUS assays were performed as described in the legend to Figure 2.
(B) Sequences upstream of the transcription initiation site are sufficient for *rbcSZm1* expression. Electroporation was performed with 1×10^5 etiolated protoplasts. Transfected protoplasts were cultured under light. Expression was normalized to that of rbcSZm1CAT.

locate *rbcSZm3* promoter sequences that might inhibit the activity of light-sensitive elements, we constructed and analyzed hybrid promoters of *rbcSZm1* and *rbcSZm3*. As shown in Figure 9, a fusion (Zm1/Zm3hyb1) containing the upstream regulatory elements of *rbcSZm1* (−182 to −44) and the downstream promoter elements of *rbcSZm3* (−58 to +64) shows light regulation similar to the *rbcSZm1* promoter. This result suggests that the downstream promoter elements of *rbcSZm3* (−58 to +64) are functionally equivalent to those of *rbcSZm1* (−43 to +89). The 5′ untranslated sequences of *rbcSZm3*, which are divergent from these of *rbcSZm1* (Figure 1B), do not seem to affect light regulation. On the other hand, the fusion (Zm1/Zm3hyb2) containing the GTCCT repeats (−102 to −81), which are unique to *rbcSZm3* and located between the upstream and TATA elements, is fourfold less light inducible than the fusion without the GTCCT repeats (Figure 9).

A summary of putative regulatory elements of maize and other monocot *rbcS* is shown in Figure 10. Including gene-specific elements, each maize *rbcS* is modulated by at least six regulatory elements.

DISCUSSION

Maize *rbcS* Expression Requires Stringent Interactions between Upstream and Downstream Promoter Elements That Are Distinct from Those of Dicot *rbcS*

The molecular bases for the differences in the regulation of monocot and dicot *rbcS* have been investigated. Our data show that maize *rbcS* expression requires a stringent interaction between upstream and downstream promoter

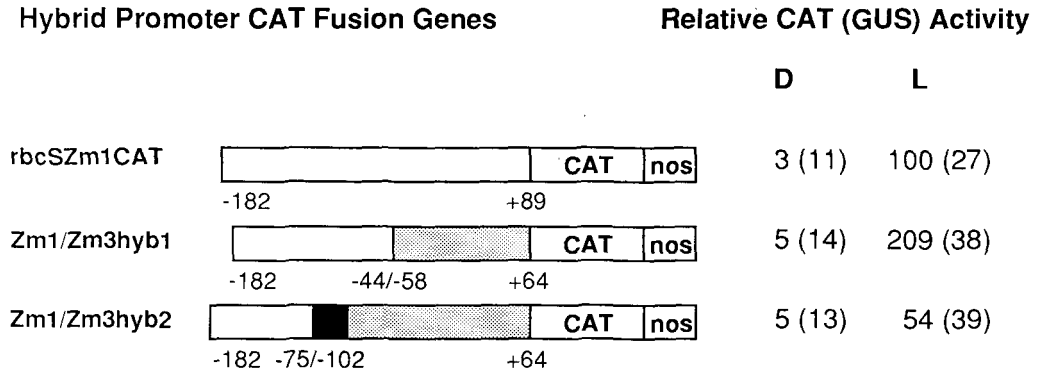

Figure 9. Differential Light Regulation by Common Elements in Different DNA Contexts.

Electroporation was performed with 2×10^5 etiolated protoplasts. The black box indicates the location of the GTCCT repeats. Representative data with internal controls are shown. Relative CAT activity was normalized to the expression level of the wild-type rbcSZm1CAT. CAT and GUS assays were performed as described in the legend to Figure 2.

elements. Neither upstream nor downstream promoter elements of pea *rbcS* can replace functionally the cognate maize elements. The 35S downstream promoter elements (+45 to +1) are somewhat more effective, but still 12-fold less active than the authentic maize sequences. The specific downstream promoter elements, deduced from sequence comparison and hybrid promoter analysis (Figures 1B, 8A, 8B, and 9), are most likely located between the TATA box and the transcription initiation site. The sequences in this region are conserved among several monocot *rbcS* genes (Figure 10). Presumably, a specific TATA-binding factor or a distinct factor binding to sequences downstream of the TATA box is essential for maize *rbcS* promoter activity. A requirement for specific downstream promoter sequences has also been shown for the mouse immunoglobulin gene (Garcia et al., 1986) and the Drosophila alcohol dehydrogenase gene (Fisher and Maniatis, 1988).

The deletion and mutational analyses show that the upstream regulatory sequences of maize *rbcS* are a mosaic of constitutive and light-sensitive elements that show no striking sequence homologies to those of dicot *rbcS* genes except the I box (Donald and Cashmore, 1990; Gilmartin et al., 1990). The constitutive element GAACGGT is conserved among monocot *rbcS* promoters (Figure 10). The study of a Lemna *rbcS* promoter also identifies constitutive as well as phytochrome-regulated domains (Rolfe and Tobin, 1991). Constitutive elements have not been demonstrated directly in native dicot *rbcS* promoters analyzed so far because of their low dark expression (Gilmartin et al., 1990). However, constitutive elements should be present in some petunia, tomato, and amaranth *rbcS* promoters that are active in the dark (Manzara and Gruissem, 1988; Dean et al., 1989b; Berry et al., 1990). Their identity awaits functional analysis of these promoters. In addition, the synthetic tetramers of the 3AF1 binding site of pea *rbcS-3A* can cooperate with the 35S promoter sequences to direct constitutive activity in transgenic tobacco leaves (Lam et al., 1990).

Our results indicate that the I box, GC-rich region, and part of the monocot *rbcS* consensus are most likely involved in light regulation (Figures 6, 7, and 10). The I box is present in most *rbcS* and *cab* promoters (Giuliano et al., 1988; Gilmartin et al., 1990). Its importance for photosynthetic gene regulation has been proposed for tobacco *cabE* (Castresana et al., 1988, Schindler and Cashmore, 1990), petunia *cab22R* (Gidoni et al., 1989), Arabidopsis *rbcS-1A* (Donald and Cashmore, 1990), and Lemna *SSUB5* (Buzby et al., 1990). However, the I box was not sufficient for a DNA binding activity in Lemna (Buzby et al., 1990). The pea *rbcS-3A* promoter contains the I box but is inactive in maize cells. Thus, other sequences are likely required for the function of I box. Another light-sensitive element contains a conserved GGCCACT motif (Figures 7 and 10). Whether its function requires other elements, such as the conserved CCACA motif located downstream, awaits further analysis (Figure 10). The G box (Giuliano et al., 1988; Donald and Cashmore, 1990) and GT boxes (Manzara and Gruissem, 1988; Dean et al., 1989b; Gilmartin et al., 1990; Schindler and Cashmore, 1990), conserved in many dicot photosynthetic gene promoters, are most likely not important for *rbcS* expression in monocot leaf cells (Rolfe and Tobin, 1991; this study). Recently, it has been shown that the mutation of a GT box in an oat phytochrome promoter does not affect promoter expression in bombarded rice leaves (Bruce and Quail, 1990). The "GT" motif found to be essential for rice phytochrome promoter activity is distinct from that of pea *rbcS-3A* (Green et al., 1988; Gilmartin et al., 1990; Dehesh et al., 1990).

At present, research on higher plants has been focused on the more tractable dicots, tobacco, petunia, tomato, and Arabidopsis. However, it is clear that many differences

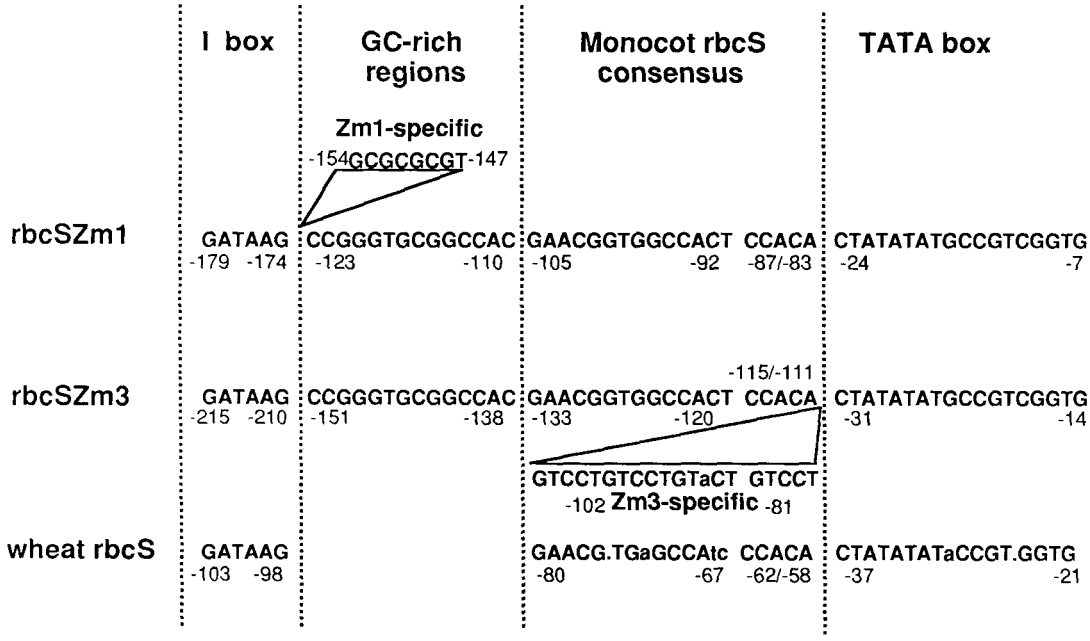

Figure 10. A Summary Diagram of Putative Maize *rbcS* Regulatory Elements.

Sequences associated with the triangles are gene-specific elements. Sequence homologies found in wheat *rbcS* promoters are also shown. Lowercase letters indicate unmatched nucleotides. Dots represent deletions. Numbers are relative to the most upstream transcription initiation sites.

can be found between monocots and dicots in the molecular bases of transcriptional regulation, RNA splicing, and developmental patterns (Poethig, 1984; Keith and Chua, 1986; Mullet, 1988; Willmitzer, 1988; this study). The development of techniques for transient expression in protoplasts isolated from fresh tissues (Sheen, 1990, 1991), for tissue transient expression (Sanford, 1988; Klein et al., 1989; Bruce and Quail, 1990; Dekeyser et al., 1990; Rolfe and Tobin, 1991), and for transgenic maize (Fromm et al., 1990; Gordon-Kamm et al., 1990) and rice (Shimamoto et al., 1989) should facilitate our understanding of these regulatory mechanisms.

Light Regulation of Maize *rbcS* Is Uncoupled from Leaf Development and Can Be Divided into Direct and Indirect Contributions

To understand light-dependent photosynthetic gene regulation in maize leaves, we developed a transient expression assay with etiolated, greening, and green mesophyll protoplasts (Sheen, 1990, 1991). Unlike dicot leaves, in which light triggers coupled leaf development and photosynthetic gene expression (Chory et al., 1989), maize leaves can differentiate fully in the dark with a low level of *rbcS* expression (Nelson et al., 1984; Sheen and Bogorad, 1986a). Our data show that light has two effects on *rbcS* expression. The direct effect is chloroplast independent and occurs immediately in etiolated protoplasts. In this phase, continuous illumination but not the existence of the chloroplast is required. The I box, the GC-rich region, and part of the monocot *rbcS* consensus (Figure 10) are most likely responsible for this activity. Similar light regulation controlled by phytochrome has recently been reported with a Lemna *rbcS* promoter in bombarded etiolated fronds (Rolfe and Tobin, 1991). The indirect effect is associated with light-triggered chloroplast development. The *rbcS* expression associated with chloroplasts does not require continuous illumination. Downstream promoter elements are important for this activity (Figure 6). Both types of light regulation are much reduced in green leaves. We are currently analyzing the photoreceptor and signal transduction pathways that are involved in light regulation of maize photosynthetic genes by transient assay.

In dicots, light-inducible expression of photosynthetic genes has been shown in dark-grown, undeveloped primary leaves and dark-adapted green true leaves (Fluhr and Chua, 1986; Fluhr et al., 1986; Schäfer and Briggs, 1986; Manzara and Gruissem, 1988; Nagy et al., 1988; Chory et al., 1989; Dean et al., 1989b). Although light induction of photosynthetic gene expression is found in etiolated leaves of both dicots and monocots, their response kinetics and developmental status are quite different (Tobin and Silverthorne, 1985; Fluhr et al., 1986;

Schäfer and Briggs, 1986; Lissemore and Quail, 1988; Nagy et al., 1988; Lam et al., 1989; Rolfe and Tobin, 1991). It is unclear whether a similar response occurs at different developmental stages of etiolated leaves in monocots because a mixed cell population at various developmental stages is usually used. To avoid these complications, the results presented in this study were obtained from experiments conducted with differentiated nondividing leaf cells (Sharman, 1942; Poethig, 1984; Martineau and Taylor, 1985; Sheen and Bogorad, 1985; Nelson and Langdale, 1989). Studies with developing seedlings, continuous leaf sections, and regenerating seedlings of maize indicate that photosynthetic gene expression is mostly confined to differentiated nondividing leaf cells in monocots (Sharman, 1942; Nelson et al., 1984; Poethig, 1984; Martineau and Taylor, 1985; Aoyagi and Bassham, 1986; Nelson and Langdale, 1989; Loza-Tavera et al., 1990).

Maize *rbcS* Expression in Mesophyll Cells Is a Default Mechanism Superimposed with Upstream Silencers and Post-Transcriptional Control

In greening and green maize leaves, *rbcS* mRNA accumulates in bundle sheath but not mesophyll cells. However, the molecular mechanisms underlying this C4-pattern gene expression are mostly unknown. Several lines of evidence suggest that the default monocot transcriptional regulation of *rbcS* is maintained in maize mesophyll cells with C4-specific modifications. It is well established that monocot and dicot plants diverged long before C4 evolution (Moore, 1982; Meagher et al., 1989). The coding and promoter sequences of monocot *rbcS* genes, regardless of the use of a C3 or C4 carbon fixation pathway, are more similar to each other than to those of dicot *rbcS* genes (Dean et al., 1989b; Meagher et al., 1989; this study). We report here that monocot *rbcS* promoters, independent of their use of C3 or C4 photosynthetic pathways, are active in monocot cells but not in dicot cells. In contrast, dicot but not monocot *rbcS* promoters are expressed in C3 dicot cells (Keith and Chua, 1986; this study). In a C4 dicot plant *Flaveria*, a C3 dicot *rbcS* promoter (petunia) is active after transformation (Martineau et al., 1989). The upstream silencers of maize *rbcS* revealed in protoplast transient expression might be important for mesophyll-specific repression. It is also well documented that the C3-pattern expression of *rbcS* is found in some mesophyll cells of maize (Sheen and Bogorad, 1986a; Langdale et al., 1988a, 1988b; Nelson and Langdale, 1989).

Nuclear run-on experiments show that bundle sheath-specific genes, such as *rbcS*, phosphoribulose kinase gene, C4 malic enzyme gene, and 3-3 (Sheen and Bogorad, 1987; J. Sheen, unpublished results), are transcribed in mesophyll nuclei, which agrees with the result of promoter analysis in maize mesophyll protoplasts (Sheen, 1990; J. Sheen, unpublished results). Moreover, the bundle sheath-specific chloroplast gene encoding the RBC large subunit is also actively transcribed in chloroplasts of mesophyll cells (J. Sheen, unpublished results). Therefore, post-transcriptional regulation is likely an important mechanism for the differential accumulation of bundle sheath-specific mRNAs in C4 plants. Recently, other examples of post-transcriptional regulation have been found in higher plants. For instance, Lemna *SSU5B* mRNA does not accumulate but is transcribed in roots (Silverthorne and Tobin, 1990). Light regulation of chloroplast genes (Gruissem et al., 1988), a pea ferredoxin I gene (Elliott et al., 1989), and amaranth (a C4 dicot) *rbcS* expression (Berry et al., 1990) is controlled at the post-transcriptional level.

In summary, our data suggest that the transcription of *rbcS* in maize reflects a default C3-type mechanism subjected to upstream silencer and post-transcriptional regulation. Further studies of *rbcS* regulation in mesophyll and bundle sheath cells by tissue transient expression (Dekeyser et al., 1990) and stable transformation in maize (Fromm et al., 1990; Gordon-Kamm et al., 1990) should reveal the precise mechanisms for C4-pattern gene expression.

METHODS

Plant Material

Experiments with maize were carried out with a hybrid line FR9^{cms} × FR37 (Illinois Foundation Seed, Champaign, IL). Growth conditions for maize seedlings have been described (Sheen, 1991). Tobacco plants (*Nicotiana tabacum* W38) were grown aseptically in MS medium (Murashige and Skoog, 1962).

Library Construction and Screening

Experiments were performed as described previously (Sheen, 1991) except that the λ vector Charon 40 was used for genomic library construction (Dunn and Blattner, 1987).

DNA and RNA Analyses

DNA sequencing and primer extension with RNA were performed as described (Sheen, 1991). The sequences of the primers are 5′-AGGCCATCATCACGGTGGGCGCCAT-3′ (25-mer) for *rbcSZm1* and 5′-TGTATGTACTCGCTGCTT-3′ (18-mer) for *rbcSZm3*. The *rbcSZm3* primer is gene specific, whereas the *rbcSZm1* primer is not. Analyzing RNA with an *rbcSZm1*-specific primer was unsuccessful, probably because of the repetitive nature of the sequences (Figure 1B and data not shown). Only the primer extension products of *rbcSZm1* are shown in Figure 1C.

Construction of BlueCAT Vectors

The EcoRI and NcoI sites in the CAT coding region were mutated from GAATTC to GAATTt and from CCATGG to CtATGG, respectively, without changing the amino acid coding sequence. Site-directed mutagenesis was done by Kunkel's method (Kunkel, 1985; Sheen, 1990). The TaqI fragment containing mutated CAT was subcloned into the SalI sites in the 35SCAT plasmid (Fromm et al., 1986; Sheen, 1991) to acquire the 3′ nopaline synthase (*nos*) gene sequences. The NcoI-CATnos fragment was transferred to the pBluescript KS M13$^+$ (Stratagene) between the newly inserted NcoI site (replacing RcoRV) and EcoRI or ClaI sites to create BlueCATSK and BlueCATKS.

Polymerase Chain Reaction (PCR) Technique

PCR was carried out as described by Sheen (1991). About 2 μg of genomic DNA isolated from wheat, pea, and tobacco was used as template per reaction. About 0.2 μg of Arabidopsis genomic DNA was used per reaction. The sequences of the primers are 5′-CCTGGATCCGGTGACTCAAAAAAGAAGAGCCGC-3′ and 5′-CACGGCGGGGGCCATGGTATTTGTATTATC-3′ from the wheat *rbcS* promoter (Broglie et al., 1983), 5′-CTCGGATCCAAA-AGCTTGGACAGGAACAAATGTTAC-3′ and 5′-TCCCCATGGT-TCTCACTTCTGTATGAATTG-3′ from the pea *rbcS-3A* promoter (Kuhlemeier et al., 1988), 5′-ATCTAGGATGAGATAAGATTACTG-3′ and 5′-AAGAACTGAGGAAGCCATGGTTAATTACACTTA-3′ from the tobacco *rbcS-8B* promoter (Poulsen and Chua, 1988), and 5′-TATGAAAGCTCTATAGTAAGTAAA-3′ and 5′-CCATGG-TCTTCTTTACTCTTTGTGTGACTG-3′ from the Arabidopsis *rbcS-1A* promoter (Krebbers et al., 1988). All PCR fragments were cloned into pBluescript KS M13$^+$ at the EcoRV site by blunt-end ligation. The orientation of the fragments was verified by restriction enzyme analysis (data not shown).

Construction of Chimeric Genes

All promoter fragments were fused to CAT at the NcoI site in BlueCATSK except the 35SCAT and 35SGUS (Sheen, 1990, 1991). The numbering refers to the most upstream transcription initiation site. All fusion promoters were made by blunt-end ligation. Promoter deletions were generated by Bal31 digestion (Sheen, 1990). Site-specific mutants were created by Kunkel's method (Kunkel, 1985; Sheen, 1990). Constructs were verified by restriction enzyme analysis and DNA sequencing.

Protoplast Transient Expression

Protoplast isolation, electroporation, and culture were the same as described by Sheen (1991). Cellulases were purchased from Karlan (Torrance, CA). Maize greening protoplasts (16- to 20-hr illumination) were used for most experiments unless specified. All constructs were assayed at least three times with different batches of protoplasts to ensure the consistency. The reproducibility of the maize protoplast transient expression assay has been vigorously demonstrated (Sheen, 1990). Representative results with internal controls from the same batch of protoplasts are usually shown. The CAT and GUS assays were performed as described (Jefferson, 1987; Seed and Sheen, 1988; Sheen, 1990, 1991). Because different assays were used to measure CAT and GUS activities and the GUS assay showed higher fluctuation among repeated samples (Sheen, 1990), the result of the CAT assay was not standardized with GUS activity, which was used as a positive internal control.

Nuclear Run-On Transcription

Bundle sheath strands and mesophyll cells were isolated as described by Sheen and Bogorad (1985, 1987). Nuclei were released from purified bundle sheath strands and mesophyll cells by grinding gently for 30 sec with a Polytron (speed 3 to 4) in a cold solution containing 3% Ficoll (Sigma), 6% Dextran 40 (Pharmacia), 0.4 M sucrose, 20 mM Hepes (pH 6.6), 10 mM NaCl, 5 mM EDTA, 1 mM spermidine, 0.15 mM spermine, and 50 mM β-mercaptoethanol (Chappell and Hahlbrock, 1986). After the addition of 0.2% Triton X-100, the mix was filtered through a 20-μm nylon mesh before being loaded on the top of a 4-mL 20% Percoll gradient (Pharmacia) made in the same grinding solution in a 15-mL centrifuge tube. Nuclei were pelleted through the Percoll gradient (3 min, 4000 rpm, 4°C) and washed twice with cold nuclei resuspension buffer (20 mM Hepes [pH 7.9], 5 mM $MgCl_2$, 20% glycerol, and 10 mM β-mercaptoethanol). The final nuclei pellet was resuspended in nuclei suspension buffer with 50% glycerol at 10^8 per milliliter and stored at −80°C. In vitro transcription was carried out in 100 μL with 5×10^6 nuclei, 100 units of RNasin (Promega, Madison, WI), 0.5 mM ATP, GTP, and CTP, 50 mM KCl, and 150 μCi of ^{32}P-UTP at 30°C for 30 min. After the reaction, 20 μg of tRNA and 10 units of RNase-free DNase (Worthington, Freehold, NJ) were added and incubated at 37°C for 10 min. Before phenol/chloroform extraction, 10 mM EDTA, 1% SDS, and 10 μg of proteinase K (Boehringer Mannheim) were added and incubated at 37°C for 15 min. Labeled RNA was precipitated with 200 μL of isopropyl alcohol and 2 M NH_4 acetate twice. About 3×10^6 cpm labeled RNA was used in 3 mL of hybridization buffer (5% SDS, 100 μg/mL calf thymus DNA, 100 μg/mL poly(A), 100 mM sodium phosphate, and 1 mM EDTA). Plasmid DNA (2 μg) was digested to separate the inserts and vector before being blotted onto GeneScreen-*Plus* (Du Pont-New England Nuclear) for hybridization.

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