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Level of Expression of the Tomato *rbcS-3A* Gene Is Modulated by a Far Upstream Promoter Element in a Developmentally Regulated Manner

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By *Agrobacterium*-mediated transformation we have demonstrated that a 1.10-kilobase promoter sequence from the tomato *rbcS-3A* gene confers light-inducible and organ-specific expression upon fusion to the bacterial chloramphenicol acetyltransferase gene. A biphasic expression profile was obtained by 5′ deletion analysis of this promoter, indicating the presence of both positive and negative regulatory elements. A severe reduction in the level of expression was observed when the 5′-terminal 90 base pairs were deleted from the 1.10-kilobase promoter. DNA sequence elements responsible for light inducibility and organ specificity of the gene reside within the −374 base pairs of the proximal part of the promoter and the sequences spanning from −374 to −205 are essential for promoter function. The DNA sequences upstream from −374 modulate the level of expression in leaf tissue; this modulation is under developmental control.

INTRODUCTION

Genes encoding proteins of the photosynthetic apparatus such as chlorophyll *a/b* binding (CAB) proteins and proteins of ribulose-1,5-bisphosphate carboxylase small subunits (RBCS) exhibit light-regulated expression. For RBCS genes this light regulation has been shown to be mediated primarily at the level of transcription and involves both phytochrome and the blue-light photoreceptor (Tobin and Silverthorne, 1985; Fluhr and Chua, 1986; Kuhlemeier et al., 1987a). Furthermore, the expression of these photosynthetic genes is under developmental regulation and is commonly restricted to chloroplast-containing photosynthetic tissues (Fluhr et al., 1986).

Recent *Agrobacterium*-mediated gene transfer studies with reporter genes have demonstrated that 5′-flanking DNA sequences of RBCS genes from several plant species confer light-inducible and tissue/organ-specific expression (Facciotti et al., 1985; Morelli et al., 1985; Nagy et al., 1985; Timko et al., 1985). There have been a number of recent reports demonstrating the existence of both positive and negative *cis*-acting elements within the 5′-flanking sequences of light-regulated genes including RBCS (Kuhlemeier et al., 1987a) and chlorophyll *a/b* binding genes (Simpson et al., 1986; Nagy et al., 1987; Castresana et al., 1988). Several evolutionarily conserved elements have recently been identified in promoters of RBCS genes from pea and tomato, some of which share homology to constitutive mammalian enhancer elements and function as *cis*-acting elements (Kuhlemeier et al., 1987a, 1987b, 1988; Giuliano et al., 1988). In the pea *rbcS-3A* promoter, there is a redundancy of *cis*-acting elements that seem to control the expression level during leaf development. Recently, gel retardation and DNase I foot-printing studies have demonstrated binding of nuclear protein factors to these conserved DNA sequence elements (Green et al., 1987; Giuliano et al., 1988).

RBCS genes belong to small nuclear multigene families (Berry-Lowe et al., 1982; Broglie et al., 1983; Wimpee et al., 1983; Coruzzi et al., 1984; Dean et al., 1987; Sugita et al., 1987). In tomato, the RBCS gene family consists of five members found at three different chromosomal loci (Vallejos et al., 1986; Sugita et al., 1987), and each member has been shown to be expressed differently both with respect to the level of expression and developmental and/or organ specificity (Sugita and Gruissem, 1987). In addition, there are differences in the kinetics of mRNA accumulation or disappearance upon transfer of plants from dark to light or from light to dark, respectively, for different RBCS gene members. These observations suggest that the role of the RBCS gene family is not only to amplify expression in photosynthetic tissues but is also to display differential expression under different environmental conditions as well as at different stages in plant development.

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With the aim of elucidating mechanisms involved in the differential regulation of RBCS gene expression in tomato, we have investigated the 5′-flanking DNA sequences from the *rbcS-3A* gene, by deletion analysis and *Agrobacterium*-mediated transformation. The work presented in this paper demonstrates that a 1.10-kb 5′ promoter sequence confers light inducibility and organ specificity. Regulatory elements essential for these functions are located between −374 and −205 from the start site of transcription. DNA sequences present upstream from −1009 have a profound effect on the level of expression and this effect is modulated in a developmentally regulated manner.

RESULTS

Isolation of the Tomato *rbcS-3A* Gene Promoter and Construction of a Chimeric *rbcS-3A*-CAT Gene

To obtain the 5′-flanking sequence of the tomato *rbcS-3A* gene, a 2.2-kb EcoRI fragment containing part of the coding sequence and 1.7 kb of 5′-flanking sequence was isolated from the phage clone 20B obtained from a tomato genomic library (Pichersky et al., 1986) and cloned into the EcoRI site of plasmid pUC9. This fragment was subjected to Bal31 exonuclease digestion from the SstII site located in the coding sequence and a fragment extending from −1700 to +8 was selected, as shown in Figure 1. Subsequently, the fragment containing −1099 to +8 was obtained by HindIII digestion and was fused to the coding sequence of *Escherica coli* chloramphenicol acetyltransferase (CAT) gene equipped with the 3′ signal from *Agrobacterium tumefaciens* octopine synthase (OCS) gene in the pMH1-neo plant expression vector (Castresana et al., 1988) (Figure 1). The nucleotide sequence of this 5′-flanking fragment is shown in Figure 2.

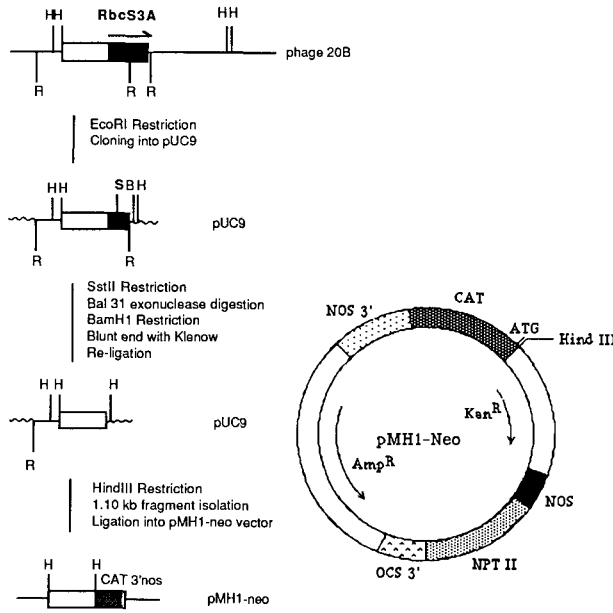

Figure 1. Cloning of the 1.10-kb 5′-Flanking Sequence of the Tomato *rbcS-3A* Gene from the Phage Clone 20B (Pichersky et al., 1986) and Construction of the 1.10-kb *rbcS-3A* Promoter-CAT Fusion in the pMH1-neo Vector (Castresana et al., 1988).

(Left) The procedures used for the promoter cloning and the chimeric gene construction are outlined in the diagram. The 1.10-kb 5′-flanking sequence and the coding sequence of the tomato *rbcS-3A* gene in the phage clone 20B are represented by the white and black boxes, respectively. Abbreviations for the restriction sites: B, BamHI; H, HindIII; R, EcoRI; S, SstII.

(Right) The pMH1-neo vector contains a selectable marker gene for the transgenic cells and plants, which consists of the coding sequence of *E. coli* neomycin phosphotransferase II (NPT II) gene fused to the nopaline synthase (NOS) gene promoter and the 3′ end of the octopine synthase (OCS) gene from the *A. tumefaciens* Ti-plasmid. It also contains a reporter gene consisting of the coding sequence of the *E. coli* CAT gene fused to the 3′ end of *A. tumefaciens* NOS gene for testing the function of plant gene promoters. All promoter deletions tested were inserted into the HindIII site to produce chimeric-CAT genes.

Expression of the Chimeric 1.10-kb Tomato *rbcS-3A* Promoter-CAT Gene in *Nicotiana tabacum* SR1 Plants

To assess the promoter function of the 1.10-kb 5′-flanking sequence of the tomato rbcS-3A gene, the chimeric gene construct described above was transferred to the genome of *N. tabacum* SR1 plants via leaf disc transformation (Horsch et al., 1985). Upon infection of leaf discs with *Agrobacteria* harboring the co-integrate containing the chimeric 1.10-kb *rbcS-3A* promoter-CAT construct, shoot initiation took place within 2 to 3 weeks on MS medium supplemented with 2 mg/l 6-benzylaminopurine under kanamycin selection at 100 μg/ml. During the subsequent few weeks, most of these shoots further developed into larger plants with roots, upon being transferred to MS medium lacking the phytohormone but still containing 100 μg/ml kanamycin. The CAT assay analysis of protein extracts from leaves of these plants (over 15 plants analyzed) revealed significant level of CAT gene expression in about 95% of kanamycin-resistant plants of independent origins. Further kanamycin selection of these plants for the following 2 to 3 weeks resulted in death of those plants that had not exhibited CAT gene expression in earlier analyses. Death was observed more frequently in those plants with very little or no root formation. Since the prolonged kanamycin selection described above eliminated the possibility of false transformants and/or mosaic plants that might have been derived from a mixture of transformed and nontransformed cells, the formation of strong roots during

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                                                                    -1099
AAGCTTGCAAGTAATAAACCATATGATTGAGTGAATGGACTTTTTGTGCC                  -1050
                                                          -1009
AGACAGGATTTTAGCTATATAGCTTGTAGAAAATTTTAATATTTTTATTT                  -1000
AGTATTTTTCAATGTACTAAAAGAAAAAAAAAAGTATATAGTCGTTTGTT                  -950
AGTAGTGTGCGTTAATTATGATTTTCATTTACCACAAAAATTGTAATTGT                  -900
TTGATTTCGTGTGATTGCTTGGTAAATAAGTTGATTATTTCGAACGTTCT                  -850
GTTATTATCGTTGATTCTTGGTTTATTACACCAATGTGGATTGCTACGTG                  -800
ACATAGCGGTAAAACTTTTTCGTACATTTGTAATTCGTATCTAATTAGAC                  -750
AACATCAATCTTGCTTCTTGGGGTCGCTAAGAGAAAAATTCGAGAAAAAA                  -700
AATCCATTATACAGGAAACTACGGAAAATTACTTGTTCTAATTTATTTGG                  -650
TCTAAAATATAAGAAATAATAAACTACCTGATTTTTTAATTGTTTTTTAT                  -600
TGGAAATAAAAGAAAACCTTTTCATATATATAGAAACTAGGAGATGTTAT                  -550
GTTCCACACATACAAAGGATAAGAACATTTCCAAGTTGCAACCAAGGAAC                  -500
                                                    -496
AATATTTGATTTTGAACTTGAAATTACAAAAAAAATAAAATGATTTGCAT                  -450
                                                -418
GGAAACAAAAGAAAATCTGAATGTGTCTGCCCAAAGGAATGGCTCCAAAT                  -400
                                          -374
GCAAGCAAAACGGCTACAAAGTAGCAGCCAATATAAATTCAGAATGACAA                  -350
                                -322 L-Box      -312 H-Box
CAAAACAATAAACACTGACCCAAAATGAAATTAACCAACCATTTTCACTC                  -300
                    -283  I-Box                    -258  G-Box
ATCCTTACCCCTTTTAGGATGAGATAAGACTATTCTCATTCTGACACGTG                  -250
                                                  -204
GCACCCTTTCTTGTGACTTAATTAATATATCAATTATTATTATAGCTCAC                  -200
CCACCCTCCACGCCCAAATTAATGTCATTAAGATGGGGTTATAATTCTAC                  -150
TTAATAGATTCGATAAAATTCTACTTTTGAAATGTGAACAAGGGCATGAT                  -100
-98              -84  L-Box
CCAATGGTTACAAATGGGTTGGTTAATTTGTGTCCGTTAGATGGGAAAGT                  -50
                  -30
TAAAGTGAAACCTTATCATTATATATAGAGGGAGAGACTAGAAAGCAATA                  +1
  +8                                        +40
ACCCTCTTGAGTTCAAGATAAGCACTTGGTTTTCAGCAATG                           +42
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Figure 2. Nucleotide Sequence of the 1.10-kb 5′-Flanking Region of the Tomato *rbcS-3A* Gene.

Nucleotides are numbered with the cap site designated as +1 (arrow). The evolutionarily conserved elements including TATA and CAAT boxes are underlined. The 3′ end point of the promoter (+8) and 5′ end points of the promoter deletions analyzed are also indicated (filled circles). The ATG codon (underlined) is located at +40.

the 1 month of extended selection period was used to identify transgenic plants for all the transformation works described in this paper.

Variation in the level of expression among different transgenic plants was relatively small when leaves of about the same age and size were analyzed; up to three-fold difference was observed. DNA gel blot analysis revealed single copy integration of the introduced chimeric gene construct in most of the transgenic plants but multiple integration up to 10 copies was also observed in some plants (data not shown). However, the level of CAT gene expression was not correlated with the copy number of integrated genes. To obtain a relative comparison of the promoter strength of the tomato *rbcS-3A* gene, the 450-bp promoter sequence derived from cauliflower mosaic virus (CaMV) 35S gene was fused to the CAT gene in the pMH1-vector and CAT gene expression was analyzed. The level of CAT gene expression driven by the 1.10-kb 5′-flanking sequence of the tomato *rbcS-3A* gene in leaf tissues was found to be about 50% to 70% of that driven by the 450 bp of CaMV35S gene promoter, as shown in Figure 3.

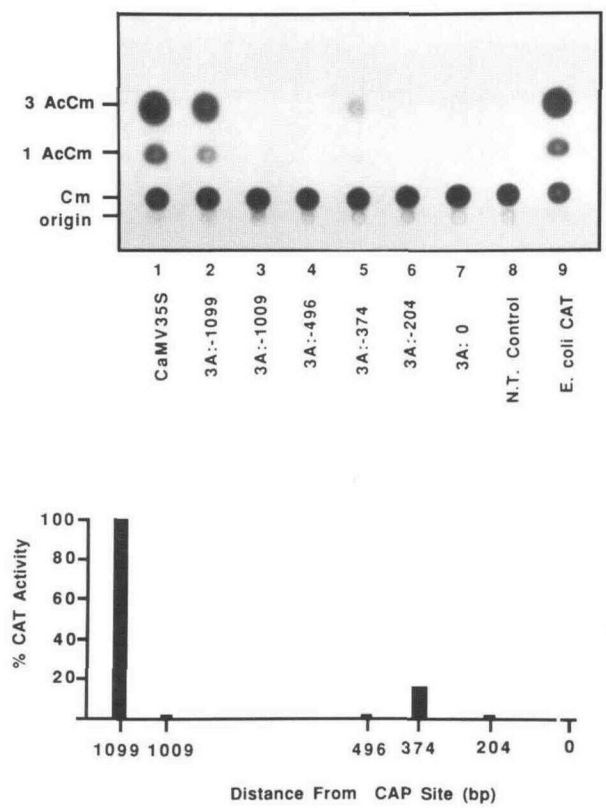

Figure 3. 5′ Deletion Analysis of the Tomato *rbcS-3A* Promoter.

(Top) CAT enzyme assays of transgenic plants containing various *rbcS-3A* promoter 5′ deletion-CAT constructs. Protein extracts (150 μg) of mature leaf tissues were assayed for CAT activities. A representative example is shown: lane 1, −450 CaMV35S promoter-CAT construct; lanes 2 to 6, various tomato *rbcS-3A* promoter 5′ deletion-CAT constructs (The 5′ end points of promoter deletions with respect to the cap site are indicated.); lane 7, promoter-less CAT construct of the pMH1-neo vector; lane 8, nontransformed control plants; lane 9, positive control with the purified *E. coli* CAT enzyme. Abbreviations: Cm, unreacted substrate (^{14}C)chloramphenicol; 1 AcCm, 1-acetylchloramphenicol; 3 AcCm, 3-acetylchloramphenicol.

(Bottom) Comparison of the levels of CAT activities expressed by the *rbcS-3A* 5′ promoter deletions. The 5′ end point and the promoter strength assigned to each deletion are indicated. The data represent the average CAT activity determined for 10 independent transgenic plants, and are expressed as a relative percentage of the activity determined for the wild-type promoter (deletion −1099).

5′ Deletion Analysis

Several 5′ deletion constructs were produced by utilizing restriction sites located within the 1.10-kb promoter fragment. These promoter deletions were fused to the CAT coding sequence in the pMH1-neo vector and transferred to SR1 plants. Upon analysis of leaf tissues from transgenic plants for CAT enzymatic activity, a pronounced biphasic profile for CAT gene expression was observed with the 5′ deletion promoter constructions (Figure 3). Deletion of 90 bp from the 5′ end of the promoter resulted in a drastic reduction in the CAT gene expression to about 5% of the level exhibited by the −1099 promoter. Further deletion to −496 did not cause any change in the reduced level of expression. However, when the deletion was extended to −374, a significant increase (about 15% to 25% of that of the −1099 promoter) in the level of CAT gene expression was observed. When the 5′ deletion was extended to −204, the level of CAT gene expression was almost undetectable, and this level was similar to the background level found in nontransgenic control plants or the transgenic plants containing the promoterless CAT gene of the pMH1-neo vector. The samples of leaf tissue assayed in the 5′ deletion analysis were 10 cm to 15 cm long, 6 cm to 8 cm wide, and dark green.

Light-Inducible Expressions of the −1099 and −374 *rbcS-3A* Promoter-CAT genes

To determine the light inducibility of the CAT gene expression observed with the −1099 and −374 tomato *rbcS-3A* promoters, total RNA was prepared from leaf tissues from transgenic plants, which were growing either in the light or dark, and the RNA was examined by RNA gel blot analysis. The plants were grown under a 14-hr light/10-hr dark photoperiod at 24°C, transferred to the dark at 24°C for 4 days, and subsequently placed for 24 hr under continuous white light at 24°C. Figure 4 shows that the increase in the level of CAT gene transcripts during the light induction period was observed for both −1099 and −374 promoter-CAT constructs, whereas no CAT transcripts were detected for the −204 promoter-CAT construct.

Organ-Specific and Developmental Expression of Chimeric *rbcS-3A*-CAT Genes

To characterize the organ/tissue specificity of the CAT gene expression driven by the −1099 and −374 tomato *rbcS-3A* gene promoters, various organs were analyzed for CAT activity. The level of expression was reduced with the −374 promoter as compared with the −1099 promoter (Figure 3), and significant expression was only observed for leaf tissues (Figure 5). The size of these leaves assayed was similar to that described for the 5′ deletion study, and

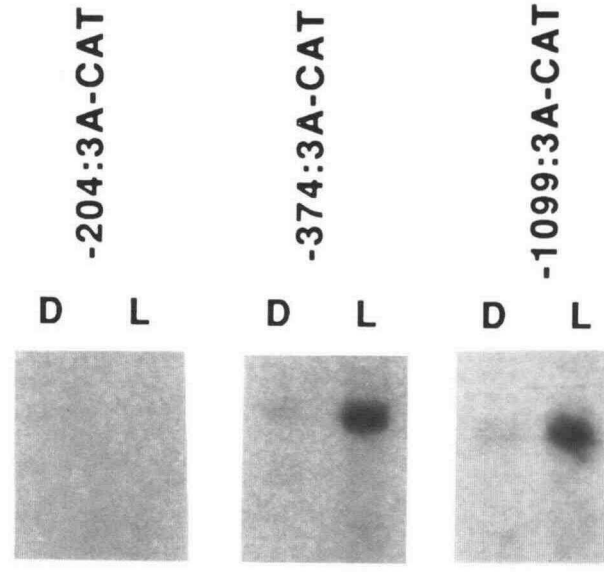

Figure 4. Light-Inducible Expression of the Chimeric Tomato *rbcS-3A* Promoter-CAT Genes.

Total RNA were isolated from leaves of transgenic plants containing the chimeric *rbcS-3A*-CAT constructs indicated, after growing in the dark for 4 days (D) and at the end of the subsequent growth for 24 hr under continuous light (L). RNA (20 μg) were fractionated on a 1.5% agarose-formaldehyde gel and blotted onto a nitrocellulose filter. The filter was hybridized to a nick-translated probe prepared from the coding sequence of the *E. coli* CAT gene at 42°C in the presence of 50% formamide and, subsequently, washed at 62°C in 1 × SSC and 0.1% SDS.

we designated them as mature leaves in this paper. In juvenile (immature) leaves, which were 1.5 cm to 2.0 cm long, 0.6 cm to 0.8 cm wide, pale green, and present at the apex of plants, a significant reduction in the level of CAT gene expression was observed for both chimeric gene constructs. However, this reduction in expression in juvenile leaf tissue was much more pronounced for the −1099 promoter construct than for the −374 promoter construct. With the −1099 promoter construct, the level of CAT gene expression was reduced in juvenile leaf tissue to about 10% of that in mature leaves (Figure 6). Although a fivefold to sixfold difference in the level of expression was observed in mature leaves between the −1099 and −374 constructs, there was little difference in the level of expression in juvenile leaves between the two constructs (Figure 6).

To assess other components of the photosynthetic apparatus in the leaf tissue at these two developmental stages, levels of RBCS and ribulose-1,5-bisphosphate carboxylase large subunit (RBCL) gene transcripts were examined by RNA gel blot analysis. The levels of RBCS and RBCL mRNA in the juvenile leaves described above were

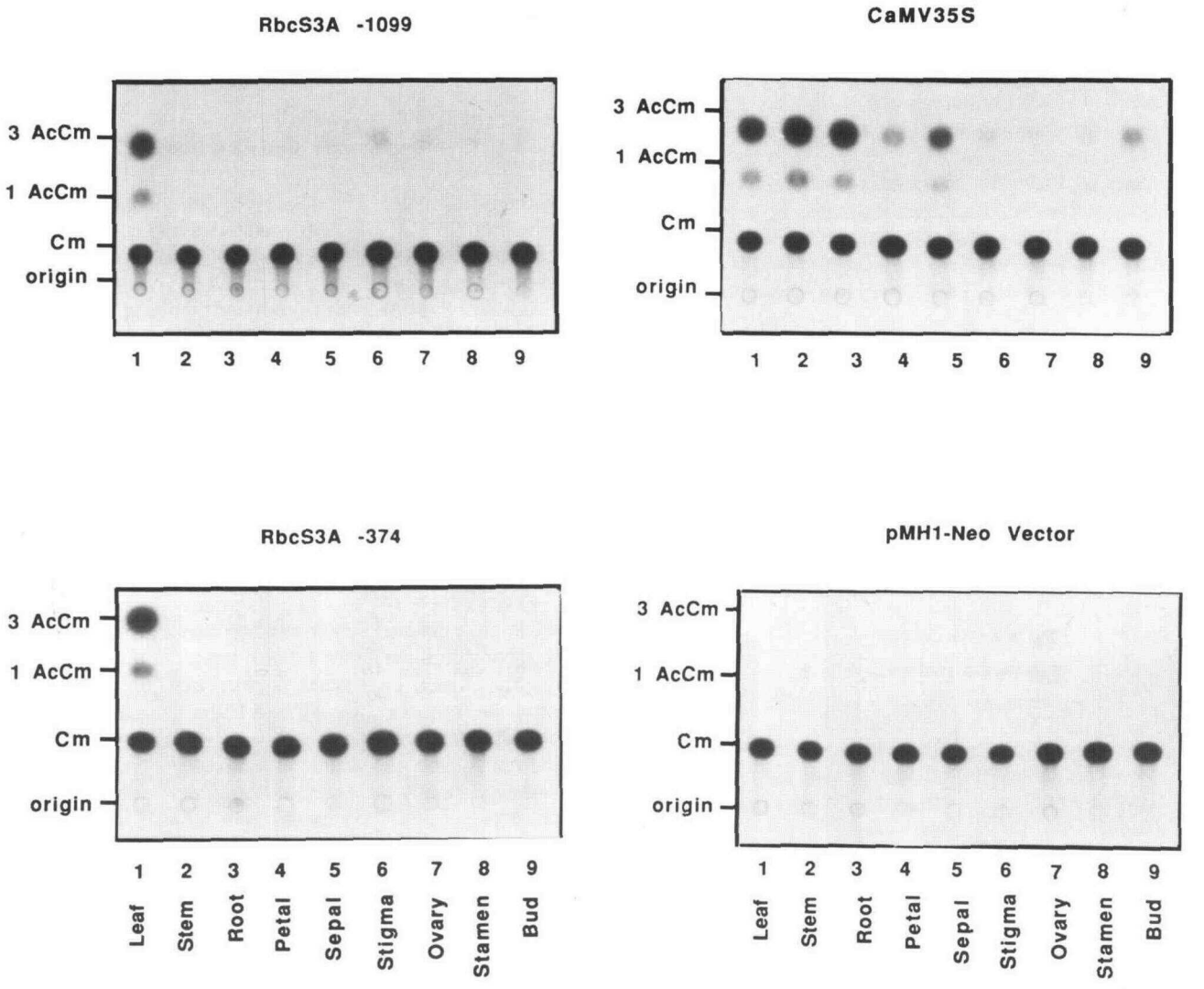

Figure 5. Organ Specificity of the Chimeric Tomato *rbcS-3A* Promoter-CAT Gene Expression.

CAT enzymatic activity was determined in various organs in transgenic plants containing the −1099 *rbcS-3A* promoter-CAT, −374 *rbcS-3A* promoter-CAT, −450 CaMV35S promoter-CAT, and promoterless CAT (pMH1-neo vector) constructs. Organs examined were mature leaves (lane 1), stems (lane 2), roots (lane 3), petals (lane 4), sepals (lane 5), stigmas (lane 6), ovaries (lane 7), stamens (lane 8), and flower buds (lane 9). Protein extracts (150 μg) were assayed for the −1099 *rbcS-3A* promoter-CAT. −450 CaMV35S promoter-CAT, and pMH1-neo vector constructs, whereas 250 μg of protein extracts were assayed for the −374 *rbcS-3A* promoter-CAT construct to obtain a higher level of detection. Abbreviations are as in Figure 3.

about 33% and 10%, respectively, of those in the mature leaves (Figure 7). In addition, the chlorophyll content in the juvenile leaves was found to be about 20% to 30% of that in the mature leaves (Figure 7). Similar analysis for the 450-bp CaMV35S-CAT construct revealed a relatively constitutive mode of expression in the various organs examined, although reduced or very low levels of expression were found in floral organs with the assay conditions used in this work (Figure 5). There was no significant difference in the expression level in mature and juvenile leaves with the 450-bp CaMV35S promoter construct (Figure 6).

To examine gene activity at an early stage of plant development, 4-week-old, light-grown seedlings were analyzed for CAT gene expression. These seedlings contained cotyledons with one or two leaves. The level of CAT gene expression in these seedlings was similar for the −1099 and −374 promoter constructs, reaching only 2.5% to 3% of the level exhibited by the −1099 construct in mature leaves (Figure 6). The level of CAT gene expression was

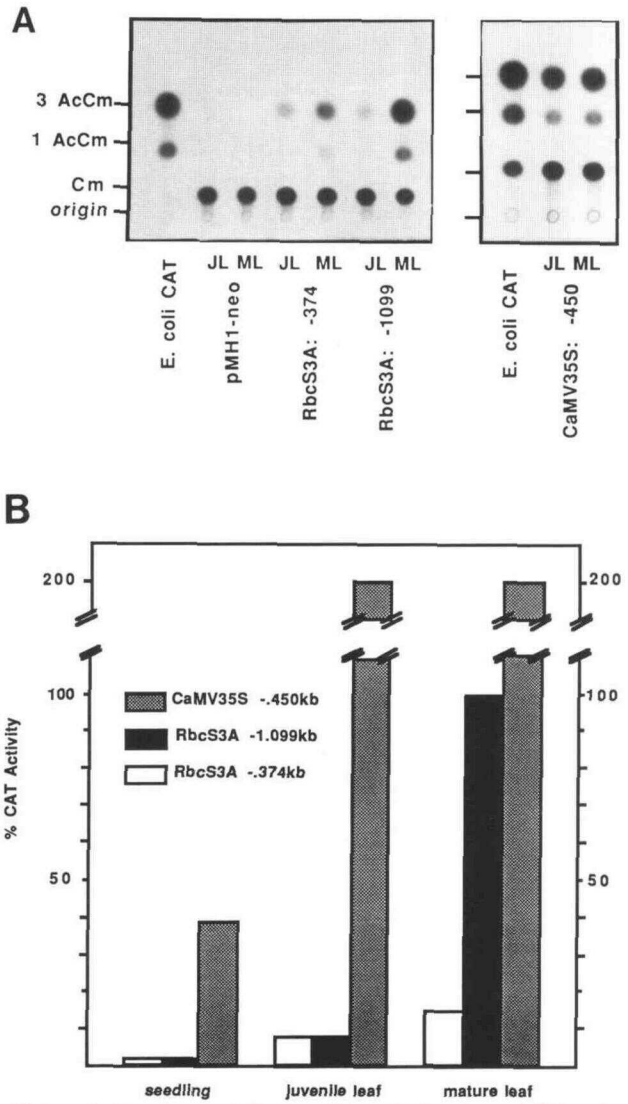

Figure 6. Developmentally Regulated Expression of Chimeric Tomato *rbcS-3A* Promoter-CAT Genes.

(A) Expression of chimeric tomato *rbcS-3A* promoter-CAT genes in juvenile (immature) and mature leaf tissues. Protein extracts (150 μg) from juvenile (JL) and mature (ML) leaves from transgenic plants containing the −1099 *rbcS-3A* promoter-CAT, the −374 *rbcS-3A* promoter-CAT, and the −450 CaMV35S promoter-CAT constructs were assayed for CAT enzymatic activities. Protein extracts from transgenic plants containing the promoterless CAT gene of the pMH1-neo vector and the purified *E. coli* CAT enzyme were used as negative and positive controls, respectively. A representative example is shown.

(B) Comparison of the levels of CAT enzymatic activities in seedlings, juvenile, and mature leaf tissues of transgenic plants. For the juvenile and mature leaves, the data represent the average CAT activity determined for seven independent transgenic plants for each construct. For seedlings, CAT activities were determined for the protein extracts that had been pooled from 75 to 100 seedlings. CAT activities are expressed as a relative percentage of the activity determined for the −1099 tomato *rbcS-3A* promoter in mature leaf tissues.

also reduced significantly in seedlings containing −450 bp CaMV35S construct, being about 20% of that in mature or juvenile leaves.

Role of DNA Sequences Spanning from −374 to −205

In the 5′ deletion study, deletion of DNA sequences spanning from −374 to −205 resulted in complete loss of promoter function. To assess the essentiality of these sequences for the full-length *rbcS-3A* promoter, an internal deletion of this region was produced by fusing to the −204 truncated promoter a 3′ deletion fragment spanning from −1099 to −360 (Figure 8, lane 5). The promoter containing this internal deletion (from −359 to −205) was inactive. Subsequently, the −374 to −205 fragment was fused back to the −204 truncated promoter (Figure 8, lane 6). This fusion essentially reconstituted the −374 truncated promoter, although a 24-bp plasmid polylinker sequence was introduced at the site of fusion as a result of the cloning procedures. This fusion promoter showed 70% of the activity of that exhibited by the intact −374 promoter.

Finally, the −374 to −205 fragment was inserted into the internal deletion construct described above, essentially to reconstitute the entire 1.10-kb promoter (Figure 8, lane 4). This fusion introduced another addition of a 37-bp polylinker sequence as well as a 15-bp duplication of DNA sequence spanning from −374 to −360. This construct did not exhibit significant promoter function. Its activity was significantly less than either of the −374 truncated promoters (lane 4 compared with lanes 6 and 7) and drastically less than the full-length promoter (lane 4 compared with lane 3). These results taken together indicate that the DNA sequence elements present within the −374 to −205 region perform an essential function for the tomato *rbcS-3A* promoter. Furthermore, whereas disruption of sequences in the immediate vicinity of −204 has relatively little effect on the truncated −374 promoter, disruption of these sequences and/or those in the vicinity of −374 has a pronounced negative effect on the activity of the full-length promoter.

Sequence Analysis of the Tomato *rbcS-3A* Promoter

Our previous sequence analysis of the tomato *rbcS-3A* promoter has revealed several DNA sequence elements that are evolutionarily conserved among many RBCS gene promoters from a wide variety of plant species. Many of these elements are localized within the −374 to −205 region of the tomato *rbcS-3A* promoter (Figure 2). In addition to the TATA and CAAT boxes found in many eukaryotic gene promoters transcribed by RNA polymerase II, we have identified four elements which we designated as L, I, G, and H boxes, localized at −322, −283, −258, and −312, respectively. The L box sequence is also present as an inverted repeat at −84. Conservation of

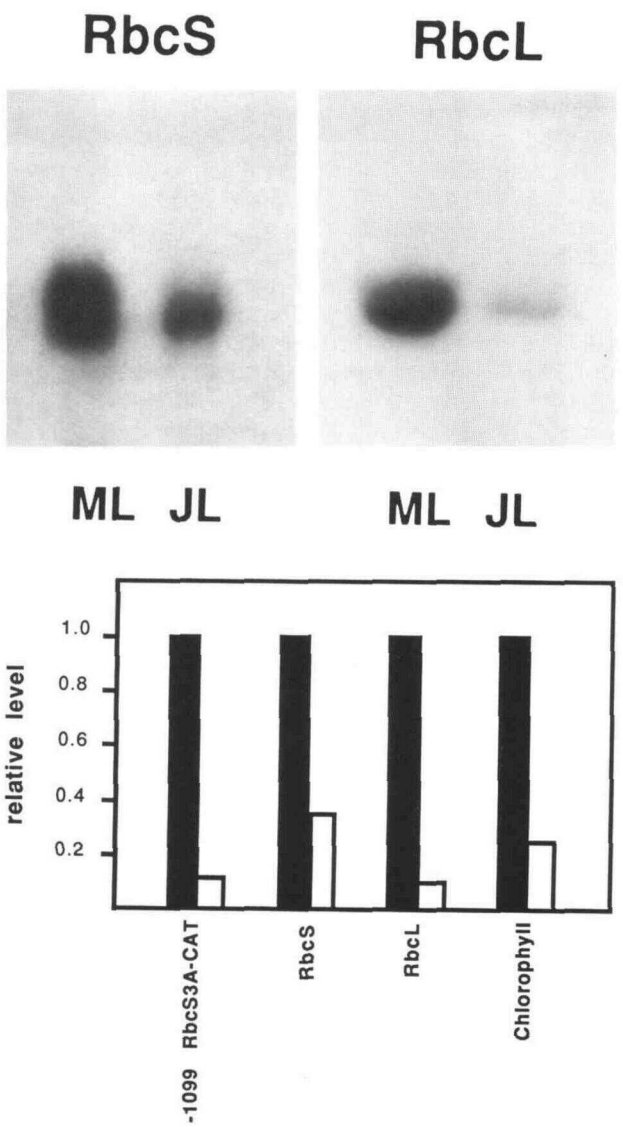

Figure 7. Comparison of the RBCS and RBCL Transcript Levels and the Chlorophyll Contents in Mature and Juvenile Leaf Tissues in Tobacco Plants.

(Top) The total RBCS and RBCL RNA levels were examined by RNA gel blot analysis using a pea RBCS cDNA (Coruzzi et al., 1983) and a spinach RBCL genomic clone (pJZA 4) (Erion et al., 1981) as probes, respectively. The hybridizations were carried out at 42°C in the presence of 50% formamide and blots were washed at 55°C in 2 × SSC and 0.1% SDS. The sizes of the bands hybridized in RBCS and RBCL blots were 0.9 kb and 1.8 kb, respectively.

(Bottom) Comparison of the relative amounts of the RBCS and RBCL gene transcripts and chlorophylls in mature (black bars) and juvenile leaves (white bars) in tobacco plants. For each comparison, the data are expressed as a relative percentage of the levels exhibited in mature leaves. The total RBCS and RBCL RNA levels were quantitated by spectrophotometrical scanning of autoradiographic signals. Chlorophyll contents in leaf tissues were determined by the method described by Arnon (1949). The ratio of the levels of CAT enzymatic activities exhibited by the chimeric tomato −1099 *rbcS-3A* promoter-CAT construct in these two leaf tissues is also shown.

these four elements in RBCS gene promoters from various plant species is intriguing since DNA sequences surrounding them have diverged extensively during evolution. The G box is a strongly conserved element and is present in 14 different RBCS genes from seven different dicotyledonous plants that have been analyzed (Giuliano et al., 1988). Recently, we have demonstrated the binding of organ-specific nuclear protein factor(s) to the G box element by gel retardation assay and DNase I footprinting (Giuliano et al., 1988). The H box shares a high degree of homology to the "Box III" element, which has been identified in a pea RBCS gene promoter and shown to have regulatory functions in gene expression (Green et al., 1987; Kuhlemeier et al., 1987a, 1988).

The 5′ deletion studies described demonstrated that the 122-bp sequence spanning from −496 to −375 had a negative effect on the positive regulatory element located within −374 and −205. Sequence comparison of this AT-rich (67%) region with the similar region of the tomato *rbcS-1* gene promoter revealed a conserved DNA sequence, CAA(A/G)GGAATGG(C/-)TC, at −418 (Figure 2). This sequence is located at approximately the same distance from the LHIG box cluster in both *rbcS-3A* and *rbcS-1* gene promoters.

DISCUSSION

We have shown in this paper that the 1.10-kb 5′-flanking DNA sequences of the tomato *rbcS-3A* gene confers organ-specific and light-inducible expression to the chimeric CAT gene in *N. tabacum* SR1 plants. Similar observations have been made for other RBCS genes (Facciotti et al., 1985; Morelli et al., 1985; Nagy et al., 1985; Timko et al., 1985). Our deletion analysis has revealed a pronounced biphasic profile indicating that the tomato *rbcS-3A* promoter consists of multiple regulatory elements. Similarly, complex profiles have been reported for the 5′-flanking promoter sequences of a tobacco chlorophyll *a/b* binding gene (Castresana et al., 1988) and a soybean leghemoglobin *lbc3* gene (Stougaard et al., 1987). DNA sequence elements sufficient to confer the organ specificity and light inducibility of the tomato *rbcS-3A* gene reside within −374 bp of the start site of transcription. The DNA sequence elements present within −374 and −205 are essential for promoter function. Deletion of the sequences spanning from −359 to −205 from the full-length promoter results in complete loss of promoter activity.

Furthermore, no activity is regained when a full-length promoter is reconstituted containing modifications in the immediate vicinity of −374 and −204, indicating that a sequence element at one of these sites is required for full promoter activity. Evolutionarily conserved DNA sequence elements within the −374 to −205 region of the promoter may play an essential role in the light-inducible and/or organ-specific expression of the tomato *rbcS-3A* gene. Our recent demonstration of specific binding of a nuclear factor from tomato leaves to the G box (Giuliano et al., 1988) supports this hypothesis.

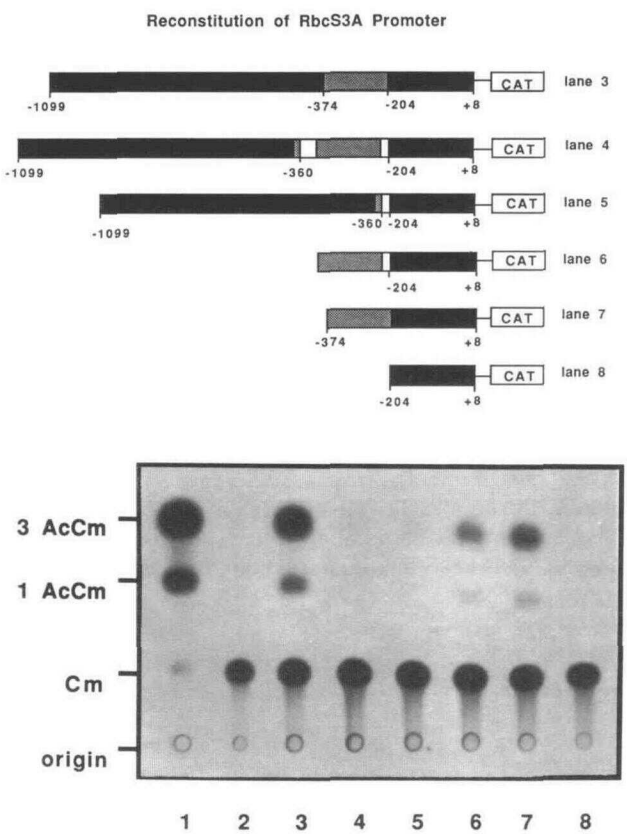

Figure 8. Reconstitution of the Tomato *rbcS-3A* Promoter.

(Top) Diagrammatic representation of the reconstituted promoters (lanes 4, 5, and 6): lane 3, the intact −1099 *rbcS-3A* promoter-CAT construct; lane 4, the reconstituted −1099 *rbcS-3A* promoter-CAT construct; lane 5, internal deletion of −360 to −205; lane 6, the reconstituted −374 *rbcS-3A* promoter-CAT construct; lane 7, the intact −374 *rbcS-3A* promoter-CAT construct; lane 8, the intact −204 *rbcS-3A* promoter-CAT construct. The dotted and white boxes represent the −374 to −205 fragment of the *rbcS-3A* promoter and the *E. coli* plasmid polylinker sequences, respectively. The duplicated 15 bp sequence (spanning from −374 to −360) is also shown as a dotted box in the lanes 4 and 5.

(Bottom) A representative example of the CAT enzymatic assays for the constructs diagrammed above. A positive control with the purified *E. coli* CAT enzyme (lane 1) and a negative control with the promoterless CAT construct of the pMH1-neo vector (lane 2) are also included. Protein extracts (150 μg) from mature leaf tissues were assayed for CAT enzymatic activities.

The 5′-flanking DNA sequences present upstream from −374 mediate high levels of expression. Mechanisms involved in controlling the expression level are of a complex nature, as evident from the deletion profile. This promoter region contains sequences that impose both positive and negative influences upon the downstream domain. Such elements, referred to as enhancers and silencers, respectively, have been identified in promoters of several other light-regulated genes including RBCS and chlorophyll *a/b* binding genes (Simpson et al., 1986; Kuhlemeier et al., 1987a, 1987b; Nagy et al., 1987; Castresana et al., 1988).

The drastic reduction in promoter activity by the removal of the 90 bp from the 5′ end of the −1.10-kb promoter suggests the presence of such an enhancer element in this upstream region. The modulation of transcription levels by the sequences present upstream from −374 are under developmental regulation. During the development of leaves, the enhancement of expression level by the upstream sequences is only visible in mature leaves. In juvenile (immature) leaves, the absence of these sequences had no effect on the level of expression. In addition, the presence of the upstream sequences had no significant effect on the level of expression in the 4-week-old seedlings. The transcriptional activities of the endogenous RBCS and RBCL genes in juvenile leaves are significantly lower than those in the mature leaves, and chlorophyll content exhibits a similar reduction. It seems likely that the modulation of expression level is established via interaction of *trans*-acting factor(s) with *cis*-elements prespresent in this upstream region and that the activities of these *trans*-acting factors are under developmental control.

In a pea RBCS gene two conserved DNA elements (Boxes II and III) are repeated in the upstream region of the promoter, and Kuhlemeier et al. (1988) have recently implicated these repeated elements in regulation of gene expression during leaf development. Whether sequences related to these elements play a similar role in modulating the developmentally regulated expression of the tomato *rbcS-3A* gene remains to be determined.

METHODS

Promoter DNA Sequencing and Construction of Chimeric Gene Constructs

Isolation of the tomato (*Lycopersicon esculentum*) *rbcS-3A* gene from the genomic library has been described previously (Pichersky et al., 1986). The nucleotide sequence of the *rbcS-3A* promoter was determined by the method of Maxam and Gilbert (1980). For construction of chimeric genes, cloning of DNA fragments was performed according to standard procedures described by Maniatis et al. (1982). Plasmid DNA was prepared using *Escherichia coli* HB101, JM83, and XL-1 strains, and pUC 9 and 19 as host bacteria and plasmid vectors, respectively, according to Birnboim and Doly (1979). The final promoter fragments to be tested were fused to the coding sequence of *E. coli* chloramphenicol acetyltransferase (CAT) gene by cloning them into the pMH1-neo vector (Castresana et al., 1988) in its unique HindIII site.

Agrobacterium-Mediated Gene Transfer and Regeneration of Transgenic Plants

Intermediate pMH1-neo vectors containing different chimeric gene constructs were mobilized into *Agrobacterium tumefaciens* harboring the Ti-plasmid pGV3850 (Zambryski et al., 1983) by the two-step mating method as described by Lichtenstein and Draper (1985). The chimeric gene constructs were transferred to *Nicotiana tabacum* SR1 cells via leaf-disc transformation by *Agrobacteria* (Horsch et al., 1985). Shoot regeneration was induced in the *Agrobacterium*-infected leaf discs cultured on a semisolid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 6-benzylaminopurine, 2% (w/v) sucrose, and 0.8% (w/v) agar, pH 5.8, at 24°C under a 14-hr light/10-hr dark photoperiod in a growth chamber. After 3 to 6 days, the discs were transferred onto the same medium but containing 500 μg/ml cefotaxime (Behring Diagnostics) and 100 μg/ml kanamycin sulfate to suppress the bacterial growth and to impose selection of transgenic cells, respectively. Rooting of shoots and further development into small plantlets were achieved by transferring kanamycin-resistant shoots onto a semisolid MS medium supplemented with 1% (w/v) sucrose but lacking phytohormones. Kanamycin sulfate at concentration of 100 μg/ml was maintained in the medium throughout this regeneration period. Rooted transgenic plants were grown in Magenta boxes (Magenta Corp.) on the above medium at least for 1 month before being transferred to soil to ensure their resistance to kanamycin.

Biochemical Analyses of Transgenic Plants

Transgenic plants were analyzed for CAT gene expression by CAT enzyme assay after they had been transferred to soil and grown in the greenhouse. For comparison of expression level with different promoter constructs, leaves of 10 cm to 15 cm in length and 6 cm to 8 cm in width from transgenic plants at similar developmental stages were analyzed. We designated the leaves described above as "mature" leaves and those that are 1.5 cm to 2.0 cm long, 0.6 cm to 0.8 cm wide, and present at the apex of plants as "juvenile" (immature) leaves in this paper. Five to 15 independent transgenic plants were analyzed for each construct.

For the analysis of CAT enzyme activity in seedlings, F1 seeds obtained from selfing of transgenic plants were germinated on a semisolid MS medium (half-strength) supplemented with 200 μg/ml to 250 μg/ml kanamycin sulfate at 24°C under a 14-hr light/10-hr dark photoperiod in a growth chamber. Growth of nontransgenic seedlings was inhibited during the 2 to 3 weeks after germination and their cotyledons appeared white. On the other hand, transgenic seedlings grew rapidly on this medium and formed green cotyledons and leaves. CAT enzymatic activity was assayed according to the procedures described by Malmberg et al. (1985). Levels of CAT activities were quantitated according to the procedures described by Timko et al. (1985).

For DNA gel blot analysis (Southern, 1975) genomic DNA from transgenic plants was isolated according to the procedures described by Dellaporta et al. (1983). Total RNA was prepared from leaf tissues according to the procedures described by Castresana et al., (1988). For RNA gel blot analysis, 20 μg of total RNA was denatured with formamide (6.4% final concentration) and formaldehyde (50% final concentration) in 20 mM MOP (pH 7.0) buffer, fractionated in 1.5% agarose-formaldehyde gels containing 20 mM MOP (pH 7.0), and blotted onto nitrocellulose filters. Specific probes were labeled with ^{32}P-dCTP by nick translation.

Conditions used for the hybridization and the washing of filters are described in the figure legends. To quantitate relative levels of RBCS and RBCL gene transcripts, autoradiograms were scanned with a Joyce Loebl densitometer and mRNA levels were determined by peak area measurements. Chlorophyll contents in tobacco leaf tissues were determined according to the procedures described by Arnon (1949).

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