The Plant Cell, Vol. 4, 185–192, February 1992 © 1992 American Society of Plant Physiologists

The Commelina Yellow Mottle Virus Promoter Is a Strong Promoter in Vascular and Reproductive Tissues

Scott L. Medberry,[a,1] B. E. L. Lockhart,[b] and Neil E. Olszewski,[a,2]

[a] Department of Plant Biology, Plant Molecular Genetics Institute, University of Minnesota, St. Paul, Minnesota 55108
[b] Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota 55108

Commelina yellow mottle virus (CoYMV) is a double-stranded DNA virus that infects the monocot *Commelina diffusa*. Although CoYMV and cauliflower mosaic virus (CaMV; another double-stranded DNA virus) probably replicate by a similar mechanism, the particle morphology and host range of CoYMV place it in a distinct group. We present evidence that a promoter fragment isolated from CoYMV confers a tissue-specific pattern of expression that is different from that conferred by the CaMV 35S promoter. When the CoYMV promoter is used to drive expression of the β-glucuronidase reporter gene in stably transformed tobacco plants, β-glucuronidase activity occurs primarily in the phloem, the phloem-associated cells, and the axial parenchyma of roots, stems, leaves, and flowers. Activity is also detected throughout the anther, with highest activity in the tapetum. In contrast, the CaMV 35S promoter is active in most cell types. The CoYMV promoter is a strong promoter, and when the activity of the CoYMV promoter is compared with that of a duplicated CaMV 35S promoter, it is 30% as active in tobacco suspension cells and up to 25% as active in maize suspension cells. These properties of the CoYMV promoter make it potentially useful for high-level expression of engineered genes in vascular cells.

INTRODUCTION

The caulimoviruses, and cauliflower mosaic virus (CaMV) in particular, are the source of several useful and widely studied promoters. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on viral encoded proteins. CaMV is the source for both the 35S and 19S promoters. These promoters direct the production of the major viral transcripts detected in infected tissues. The CaMV 19S promoter directs the production of an abundant subgenomic mRNA encoding a protein that is both a major structural component of the cytosolic inclusion bodies and a translational activator for the polycistronic 35S RNA (Bonneville et al., 1989; Gowda et al., 1989). Although the CaMV 19S promoter is a strong promoter, it is reportedly 10- to 50-fold less active than the CaMV 35S promoter (Lawton et al., 1987). The CaMV 35S promoter directs the production of a terminally redundant transcript that is 180 nucleotides greater than genome length. This transcript is believed to be both an mRNA and the template for reverse transcriptase-mediated replication of the viral genome. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (Odell et al., 1985). The strength and constitutive activity of the CaMV 35S promoter is thought to occur through the interaction of a series of discrete *cis* elements (Benfey et al., 1989).

Although the CaMV 35S promoter is from a virus that infects only dicots, it is highly active in monocots (Dekeyser et al., 1990; Terada and Shimamoto, 1990). Duplication of the −343 to −90 region of the CaMV 35S promoter produces a promoter (D35S) that is 10-fold more active in stably transformed tobacco than the unmodified CaMV 35S promoter (Kay et al., 1987); others have reported only a twofold to threefold increase in promoter activity upon similar modification (Ow et al., 1987; Fang et al., 1989).

Recently, a second group of double-stranded DNA plant viruses, the badnaviruses, have been identified. Commelina yellow mottle virus (CoYMV), the type member of the badnaviruses, infects the monocot weed *Commelina diffusa* (Lockhart, 1990) and, like CaMV (Guilley et al., 1983; Hull and Covey, 1983; Pfeiffer and Hohn, 1983), has properties that suggest that it is a pararetrovirus (Medberry et al., 1990). Purified virion DNA has a site-specific discontinuity adjacent to a $tRNA^{Met}$ binding site and a site-specific discontinuity on the other strand next to a polypurine-rich region. The transcribed strand contains an open reading frame capable of encoding a protein of 216 kD with regions of sequence similarity to viral coat protein, protease, reverse transcriptase, and RNase H. The only known CoYMV transcript is abundant, of greater than genome length, and terminally redundant.

[1] Current address: Plant Gene Expression Center, United States Department of Agriculture, 800 Buchanan Street, Albany, CA 94720.
[2] To whom correspondence should be addressed.

These features are consistent with replication from the CoYMV transcript using a viral encoded reverse transcriptase.

Although badnaviruses and caulimoviruses share a common method of replication, differences in many other properties indicate that they are discrete groups. All known caulimoviruses infect only dicots, whereas some badnaviruses infect monocots (Lockhart, 1990; Hay et al., 1991). Caulimovirus virions are icosahedral and 50 nm in diameter, whereas badnavirus virions are bacilliform with dimensions of 30 by 130 nm. Caulimoviruses and badnaviruses are transmitted by different insect vectors. The coat protein and replicase of the caulimoviruses are produced as distinct proteins, whereas in the badnaviruses these are believed to be encoded by a single open reading frame whose product is then processed to produce the mature proteins.

To determine whether the CoYMV promoter has properties that are similar to or different from those of caulimovirus promoters, we determined the strength and tissue specificity of this promoter. We compared the strength of the CoYMV promoter in maize and tobacco suspension cells to that of the D35S promoter. Transgenic tobacco plants containing the CoYMV promoter fragment driving β-glucuronidase (GUS) expression were analyzed to determine the tissue specificity of promoter activity.

RESULTS

Analysis of CoYMV Promoter Expression in Maize and Tobacco Suspension Cells

A 1-kb CoYMV promoter fragment was isolated from the full-size genomic clone pCoYMV89 (Medberry et al., 1990). The promoter fragment encompasses the region from −1026 to +12 relative to the 5′ end of the CoYMV transcript. This fragment was subcloned into the transient introduction plasmids pMON755 and pMON772, where it drove the expression of the GUS or luciferase (LUC) genes, respectively.

The relative strength of the CoYMV promoter fragment was determined using an experimental design similar to that of Callis et al. (1987). Two plasmids were transiently cointroduced into the cells by microprojectile bombardment. Use of the two-plasmid system allows for the normalization of variation in promoter activity due to differences in plasmid introduction efficiency and differences between extract preparations. In all cointroductions, one plasmid contained a reporter gene whose expression was driven by the D35S promoter (normalizing gene) and the other plasmid contained a reporter gene whose expression was driven by the CoYMV, D35S, or no promoter (test gene). GUS and LUC were used as reporters, and the activity of each promoter was tested using both reporters. Test gene activity was calculated both as a ratio of GUS activity to LUC activity and as the percentage of the activity observed when the D35S promoter was driving the same reporter gene. The relative strength of the CoYMV promoter was compared to the D35S promoter because it is one of the strongest available promoters (Kay et al., 1987) and hence should be a good indicator of the usefulness of the CoYMV promoter for driving high-level gene expression.

In tobacco suspension cells, as shown in Table 1, the CoYMV promoter was 30% as active as the D35S promoter. The strength of the CoYMV promoter relative to the D35S promoter was similar regardless of which reporter gene was used with each promoter (30 versus 34%). The CoYMV-GUS gene produced 43-fold more GUS activity than the promoterless GUS gene, and the CoYMV-LUC gene produced 670-fold more LUC activity than the promoterless gene. The amount of reporter gene product produced by cells containing either the promoterless GUS or promoterless LUC gene was indistinguishable from that of untransformed cells.

In maize suspension cells, the CoYMV-LUC gene was 27% as active as the D35-LUC gene (Table 1). However, the CoYMV-GUS gene was only 8% as active as the D35S-GUS gene. The reason for this difference remains unclear. The CoYMV-GUS gene produced 25-fold more GUS activity than a promoterless GUS gene, and the CoYMV-LUC gene produced 50-fold more LUC activity than a promoterless gene.

Analysis of the Tissue Specificity of CoYMV Promoter Activity in Transgenic Tobacco Seedlings

To determine the cell-specific expression patterns of the CoYMV promoter, stably transformed tobacco lines containing the CoYMV-GUS gene were produced and GUS activity was localized by histochemical staining. In addition, tobacco plants containing either the promoterless GUS gene or an unenhanced 35S-GUS gene were produced and similarly analyzed. The expression of each gene was analyzed in at least 15 independent transformants. The GUS expression patterns of the various constructs were determined in T_0 flowers and in T_1 plants. The histochemical staining shown in Figure 1 is representative of the staining patterns observed in plants that exhibited high to moderate levels of GUS activity.

In seedlings containing the CoYMV-GUS gene, intense GUS staining was observed in the vascular tissue of the roots and leaves, whereas less intense staining occurred in the vascular tissue of the stem (Figure 1A). Although the amount of staining varied between different transgenic lines, within a seedling the stem always stained less than the roots or leaves. The expression pattern in leaves varied with the position of the leaf on the plant (Figure 1A). In addition to vascular expression, the cotyledonary leaves also exhibited high levels of expression in nonvascular tissues, especially mesophyll cells. The first true leaves exhibited lower levels of nonvascular expression while vascular expression remained constant. Nonvascular expression became further restricted in each successive leaf (data not shown). In the roots and stems of seedlings, the tissue specificity and strength of expression of the 35S-GUS gene were indistinguishable from

Table 1. Comparison of Promoter Activity in Tobacco and Maize Suspension Cells

Test Gene	Normalizing Gene	Average GUS/LUC Activity[a]	Error[b]	Activity as a % of D35S[c]
Tobacco				
D35S-GUS	D35S-LUC	0.822	0.216	100.0
-GUS[d]	D35S-LUC	0.006	0.005	0.6[e]
-LUC[d]	D35S-GUS	1600	1370	0.1[f]
CoYMV-GUS	D35S-LUC	0.254	0.058	30.9[e]
CoYMV-LUC	D35S-GUS	2.40	0.633	34.2[f]
Maize				
D35S-GUS	D35S-LUC	2.65	0.380	100.0
-GUS[d]	D35S-LUC	0.008	0.003	0.2[e]
-LUC[d]	D35S-GUS	490	509	0.5[f]
CoYMV-GUS	D35S-LUC	0.203	0.082	7.6[e]
CoYMV-LUC	D35S-GUS	9.91	4.27	26.8[f]

[a] Average of four experiments × 10,000.
[b] SD of the ratios in the column labeled "Average GUS/LUC Activity."
[c] Activity of the test promoter as a percentage of D35S activity.
[d] Indicates a promoterless test gene.
[e] Calculated as 100 × the ratio of activity in this row divided by the ratio of D35S-GUS to D35S-LUC activity.
[f] Calculated as 100 × the ratio of D35S-GUS to D35S-LUC activity divided by the ratio of activity in this row.

that of the CoYMV-GUS gene. Although both genes were active in most cells of seedlings leaves, differences in the levels of expression were observed. In mesophyll and epidermal cells, the 35S-GUS gene was more highly expressed than the CoYMV-GUS gene, but in the vascular tissue this situation was reversed. No GUS activity was detected in transgenic plants containing the promoterless gene (data not shown).

Analysis of the Tissue Specificity of CoYMV Promoter Activity in Transgenic Tobacco Plants

Unlike what was observed in seedlings, in more mature plants the expression of the CoYMV-GUS gene exhibited much greater tissue specificity than did the 35S-GUS gene. In mature plants transformed with the CoYMV-GUS gene, intense GUS staining occurred in the phloem and phloem-associated tissue of roots, stems, and petioles (see Figures 1B to 1D). Staining also occurred in the axial parenchyma cells adjacent to the xylary elements. Although under dark-field illumination the staining of these parenchyma cells appeared similar to that of phloem and phloem-associated tissues (Figure 1C), we believe that the CoYMV promoter is less active in these parenchyma cells than in phloem and phloem-associated tissues because, when viewed under bright-field illumination, these parenchyma cells always appeared less intensely stained (data not shown). Occasional staining of cortex cells adjacent to either the vascular tissues or lateral roots was detected. Even after prolonged incubation with high substrate concentrations, no detectable GUS activity was observed in the epidermal or undifferentiated parenchyma cells except for light staining in cells located adjacent to the vascular tissues. In contrast, staining due to expression of the 35S-GUS gene was detected in all cell types in stems and petioles, with the most intense staining occurring in the vascular tissue (data not shown). In root tissue, the 35S-GUS gene expression was similar to the expression observed for the CoYMV-GUS gene. The observed tissue specificity of the 35S-GUS gene is consistent with previous reports (Jefferson et al., 1987; Benfey et al., 1989).

The leaves of plants transformed with the CoYMV-GUS gene exhibited GUS activity in the vascular tissue. Examination of transverse sections of leaves indicated that the staining was in the vascular bundles, especially phloem or phloem-associated cells. Occasionally, lighter staining was observed in guard cells and epidermal cells that were directly adjacent to the vascular bundles (lefthand side of Figure 1F). No expression was observed in the mesophyll cells or in trichomes. The vascular-specific expression pattern of the CoYMV-GUS gene is seen in Figure 1E, where the staining highlights the reticulate vein pattern of the leaf. In contrast, the 35S-GUS gene expression occurred in all of the major cell types of the leaves, including trichomes, and epidermal and mesophyll cells, as well as in the vascular bundles (data not shown).

In flowers of plants containing the CoYMV-GUS gene, GUS activity was observed primarily in the vascular bundles of the sepals, petals, filament, and pistil (Figure 1G). Intense staining was observed in the receptacle of the flower bud and was probably due to the large amount of vascular tissue present in this region. In the ovary, with the exception of the hilum and

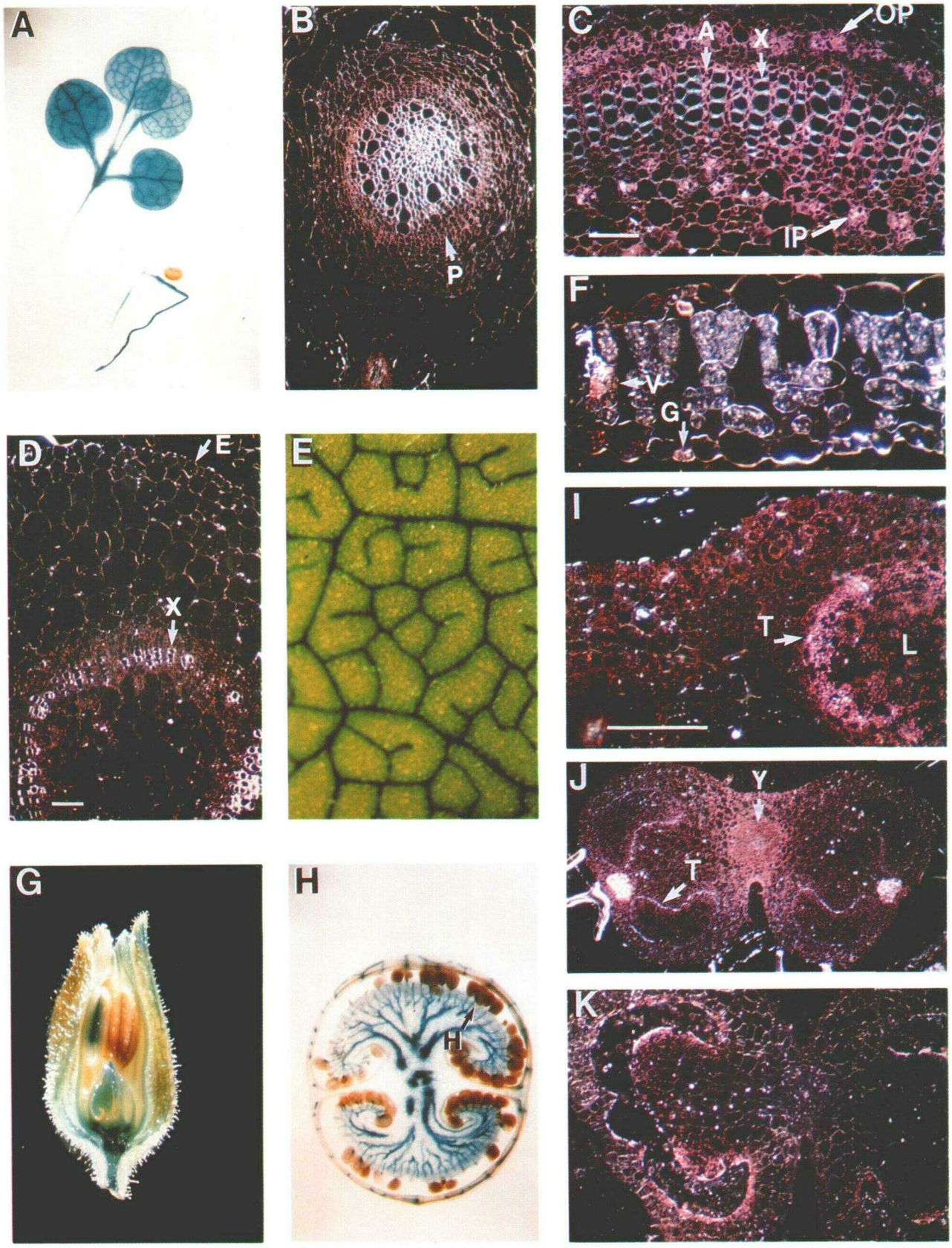

Figure 1. Histochemical Localization of GUS Activity in Transgenic Tobacco Plants Containing the CoYMV-GUS Gene.

testa tissue that is adjacent to vascular tissue, GUS expression was observed only in vascular tissue (Figure 1H). Although weak expression was detected in the developing embryo, similar expression was detected in the flowers of plants containing the promoterless GUS gene.

In the anther, unlike other floral organs, the expression of the CoYMV-GUS gene occurred in both nonvascular and vascular tissue, and this expression was developmentally regulated (Figures 1I to 1K). During the early stages of anther development, nearly all cells of the anther exhibited GUS expression. Highest levels of activity were seen in tapetal cells and in vascular tissues. As the anther matured and the tapetal cells started to disintegrate, activity became more localized to the tapetum and adjacent cells. In the dark-field microscopy shown in Figures 1J and 1K, the GUS staining of the tapetum is so intense that the normally pink crystals appear as a white line surrounding the locules. When the anther sections were viewed with bright-field illumination, tapetal cells were stained more intensely than any other cells (data not shown). Pollen, both in the anther and after extrusion from the anther, exhibited GUS activity. This GUS activity probably originated from the tapetum, which was disintegrating at the time the pollen was analyzed, rather than from the pollen, because all of the pollen grains of plants that were hemizygous for the CoYMV-GUS gene exhibited GUS activity (data not shown). In comparison, expression of the 35S-GUS gene in floral tissue was restricted to vascular tissue (data not shown).

DISCUSSION

Our results indicated that the CoYMV promoter is a strong promoter in tobacco and maize suspension cells. Although the CoYMV promoter is 30% as strong as the D35S promoter in tobacco and up to 25% as strong in maize, the CoYMV promoter is likely to be as strong or stronger than the unmodified CaMV 35S promoter because the D35S promoter is up to 10-fold more active than the unmodified CaMV 35S promoter (Kay et al., 1987). The CaMV 35S promoter is active in most cell types of tobacco. In contrast, the activity of the CoYMV promoter is restricted mainly to the vascular tissue of stems, petioles, leaves, and flowers with little or no activity detectable in the nonvascular tissues of older organs; only in the anthers are exceptions to this expression pattern observed. The CoYMV promoter is active throughout the anther and is particularly active in the tapetum. The anther is one of the few organs in the plant in which the CaMV 35S promoter has very little activity.

The differences in the activities of the CoYMV and CaMV 35S promoters in reproductive tissues might account for differences in the seed transmissibility of these viruses. Seed transmission has not been demonstrated for any caulimovirus (Hull, 1984) and the CaMV 35S promoter is not active in the germline, whereas 10% of seed from CoYMV-infected plants gives rise to virus-infected progeny (B.E.L. Lockhart, unpublished data) and the promoter is active throughout the anther, suggesting that the lack of caulimovirus seed transmission may be due to the lack of promoter activity in the germline. Tempering this argument is the observation that the tissue specificity of a promoter can vary from species to species. The CaMV 35S promoter exhibits different tissue specificities in tobacco, rice, and petunia (Benfey and Chua, 1989; Terada and Shimamoto, 1990). It seems likely that the tissue specificity of the CoYMV promoter differs between species because there is no detectable CoYMV promoter activity in the mesophyll of mature tobacco leaves, but CoYMV particles are present in both mesophyll and vascular tissues of infected Commelina plants (B.E.L. Lockhart, unpublished data).

Although we have observed differences in the tissue specificity of the CoYMV and CaMV 35S promoters in aerial portions of the plant, they have nearly identical patterns of expression in the roots of transgenic tobacco plants. Both promoters are active primarily in the axial parenchyma,

Figure 1. (continued).

(A) Bright-field view of axenically grown seedling.
(B) Transverse mature root section viewed with dark-field microscopy. The crystalline indigo dye precipitate that is blue in bright-field microscopy appears pink under these conditions.
(C) Dark-field view of transverse petiole section.
(D) Dark-field view of transverse stem section.
(E) Bright-field close-up of leaf tissue.
(F) Dark-field view of transverse mature leaf section.
(G) Bright-field view of a flower split axially and then stained for GUS activity. On one of the two anthers, there is no cut surface for the GUS substrate to penetrate.
(H) Bright-field view of a handcut transverse section of a seed pod.
(I) Dark-field microscopy of a tetralocular anther with tapetal cells intact.
(J) Dark-field view of a tetralocular anther with tapetal cells collapsed.
(K) Dark-field view of a bilocular anther.
Bars = 0.1 mm. Scale is the same in **(B)**, **(C)**, **(J)**, and **(K)**. Scale is the same in **(F)** and **(I)**. P, phloem; OP, outer phloem; IP, inner phloem; X, xylem; A, axial parenchyma; E, epidermis; V, vascular bundle; G, guard cell; H, hilum; T, tapetum; L, locule; Y, anther vascular bundle.

Promoter	as-1 Sequence	Distance from TATA	TATA Sequence
CaMV	CAC**TGACG**TAAGGGA**TGACG**CAC	34	CTC**TATATAA**GCA
CoYMV	TGA---**T**--C-TT-------GCG	176	-CT---**T**------
FMV	GTA-**T**---A-C-CAG-----ACA	22	A-----**T**---AG-

Figure 2. Comparison of Sequence and Spacing of *cis* Elements Present in the CoYMV, CaMV 35S, and FMV 34S Promoters.

The sequence of the CaMV 35S promoter as-1 and TATA regions is shown with the TGACG and TATA sequences in bold. Conserved nucleotides in the CoYMV and the FMV 34S promoters are indicated by dashes (–), whereas different nucleotides are indicated by the appropriate character.

phloem tissue, and cortex cells adjacent to lateral root junctions. These similarities in expression could be due in part to the presence of similar *cis*-acting sequences. The CaMV 35S promoter contains a *cis*-acting multifunctional activation sequence, as-1 (Lam and Chua, 1989). The as-1 sequence is in part responsible for CaMV 35S promoter activity in the root, and it also appears to function as a nonspecific enhancer for other *cis* elements. A sequence similar to an inverted as-1 is located between −205 and −227 in the CoYMV promoter. Figure 2 contains an alignment of this region of the CoYMV promoter with the CaMV 35S as-1 site and a similar site that is present in the figwort mosaic virus (FMV) 34S promoter (Sanger et al., 1990). The strongest similarity between these regions is seen in the TGACG direct repeat of the as-1 site, where the CoYMV and FMV promoters differ from the CaMV 35S promoter by only one base. The distance between the TGACG repeats is identical in both promoters. Mutational analysis indicates that the TGACG repeat is important for as-1 function (Lam et al., 1989). The TGACG sequence also occurs in the octopine synthase (Bouchez et al., 1989) and hexamer motif (Tabata et al., 1991) classes of *cis* elements. Whereas the distance separating the TGACG sequence and the TATA box is much greater in the CoYMV promoter than for the FMV 34S or CaMV 35S promoters, it is within the range of distances reported for octopine synthase and hexamer motif class elements.

The differences in the tissue specificity of the CoYMV and CaMV 35S promoters suggest that they may contain different *cis* sequences. In addition to the as-1 site, the CaMV 35S promoter also contains a CA-rich region that binds nuclear factors and a GATA region that is important for expression in green tissues (Lam and Chua, 1989). We cannot detect similarities between the CoYMV promoter and the CA-rich or GATA regions of the CaMV 35S promoter. Furthermore, the CaMV 35S promoter has several proposed CCAAT boxes (Ow et al., 1987), whereas the CoYMV promoter contains no readily apparent CCAAT-like box.

METHODS

Isolation of a Commelina Yellow Mottle Virus Promoter Fragment

The commelina yellow mottle virus (CoYMV) promoter was isolated as a 2.5-kb fragment from genomic clone pCoYMV89 (Medberry et al., 1990) spanning from a SalI site in the polylinker (adjacent to the ClaI site at 6329 in the genome) to a BamHI site at 1347. This fragment was subcloned into pUC119, and a StuI site was introduced at +12 relative to the mapped 5′ end of the transcript using the site-directed mutagenesis procedure described by Kunkel et al. (1987).

Stable Transformation and Analysis of Tobacco Plants

Binary vectors containing the CoYMV–β-glucuronidase (GUS) reporter gene were constructed by subcloning the SalI to StuI CoYMV promoter fragment into both pBI101 and pOCA101 (Jefferson et al., 1987; N.E. Olszewski, unpublished data). The binary vector pOCA101 is a derivative of pOCA28 (Medberry et al., 1990), in which the polylinker has been replaced by the promoterless GUS gene contained on a HindIII-EcoRI fragment from pBI101. The 35S-GUS gene containing binary vectors used were pBI121 and pOCA121 (constructed similar to pOCA101 except using the HindIII-EcoRI fragment from pBI121). These binary vectors were then introduced into the *Agrobacterium tumefaciens* strain AgI1 (Lazo et al., 1991) by electroporation. Transformation of tobacco was by the leaf disc method of Horsch et al. (1989).

Histochemical staining for GUS activity was done essentially as described by Jefferson et al. (1987) except for floral tissue, which was stained according to Koltunow et al. (1990), including 10 mM β-mercaptoethanol to reduce tissue browning. Stained tissue was cleared of pigment with two 1-hr washes in 70% ethanol followed by washes with 95% ethanol until tissue was cleared. For ultramicrotome sections, the embedding resin London Resin (LR) White was infiltrated into the cleared tissue by a 1-hr incubation in 3:1 LR White/95% ethanol followed by three incubations with LR White, one of which was overnight, the other two for 1 hr each. The tissue was transferred to a Beem capsule with fresh LR White and hardened 24 hr at 60°C. Sections 7-μm thick were cut on a microtome (model No. MT2-B; Sorvall Instruments Division, Newton, CT), and the sections were mounted on a slide with Permount. The slides were viewed using a microscope (model No. AH-2; Olympus Corporation of America, New Hyde Park, NY) set for dark-field illumination.

Construction of Transient Introduction Plasmids

The plant expression vectors pMON755, pMON772, and derivatives were used in the studies to determine the strength of the CoYMV promoter by transient expression. The plasmids used in tobacco suspension cells were pMON755, where the D35S promoter drives transcription of the GUS gene followed by the nopaline synthase 3′ polyadenylation signal, and pMON772, which is similar to pMON755 except that the luciferase (LUC) reporter gene replaces the GUS gene. Derivatives of these plasmids in which the D35S promoter was

either deleted or replaced with the CoYMV promoter were constructed using standard techniques. For experiments to determine the strength of the CoYMV promoter in maize suspension cells, plasmids similar to those described above were used, except that a fragment containing the maize alcohol dehydrogenase-1 first intron (from pMON19616, in which the StuI site had been removed by partial Bal 31 digestion) was introduced at a BglII site located between the promoter and the reporter gene of each plasmid. The intron was included because it is reported to increase expression in maize (Callis et al., 1987).

Biolistic Transformation and Analysis of Promoter Strength

For transient expression experiments, a particle gun (model No. PDS 1000; Du Pont) was used to introduce the various DNA constructs into tobacco TXD or maize BMS suspension cell lines. Cells at mid log growth phase were collected by filtration onto 5.5-cm filter paper (grade 363; Baxter Healthcare Corp., Miami, FL) to form a thin layer of cells. The filters were then transferred to Petri dishes containing another filter paper soaked in 1 mL of culture medium. Introduction of supercoiled plasmid DNA into the cells was carried out essentially as described by Gordon-Kamm et al. (1990), except that 12.5 μg of the plasmid containing the normalizing gene and 12.5 μg of the plasmid containing the test gene were coprecipitated with the tungsten particles. Each particle preparation was used for three independent introductions onto separate Petri dishes. After introduction of the plasmids, 1 mL of culture medium was added to each Petri dish and the dish was incubated for 48 hr. For protein extraction, the cells from the three Petri dishes were collected, frozen in liquid nitrogen, and ground in a mortar with a pestle. Following grinding, 0.7 mL of extraction buffer (0.1 M KPO_4, pH 7.8, 1 mM EDTA, 10 mM DTT, 0.8 mM phenylmethylsulfonyl fluoride, 5% glycerol) was added to the cell homogenate and it was allowed to thaw. Following thawing, it was ground further and subjected to centrifugation at 33,000*g* for 5 min at 4°C. The supernatant was transferred to a new tube and stored at −80°C until assayed. GUS assays were performed essentially as described by Jefferson et al. (1987) using 4-methylumbelliferyl β-D-glucuronide as a substrate. A fluorometer (model No. TKO 100; Hoefer Scientific Instruments, San Francisco, CA) was used to determine the amount of 4-methylumbelliferone produced during 30-, 60-, and 90-min incubations. LUC activity was quantitated by injecting 0.3 mL of LUC assay buffer (25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg/mL BSA) and 0.1 mL of 0.65 mM luciferin into 20 μL of cell extract and using a luminometer (model No. LB9501; Berthold, Nashua, NH) to record the total light units produced in the first 10 sec after injection.

ACKNOWLEDGMENTS

We thank Monsanto (Chesterfield, MO) and Glenn Austin for assistance with the transient assay system, including plasmids (pMON755, pMON772, and pMON19616 containing the maize ADH1 first intron), protocols, and the oligonucleotide for mutagenesis. Access to the PDS 1000 and facilities for its use were kindly provided by David A. Somers as part of a grant from the Midwest Plant Biotechnology Consortium (West Lafayette, IN), USDA Subgrant No. 593-0009-04. This work was supported by Grant No. IN-13-30-11 from the American Cancer Society (Atlanta, GA) to N.E.O. and USDA Grant No. 90-37262-5373 to N.E.O. and B.E.L.L.

Received December 18, 1991; accepted December 31, 1991.

REFERENCES

Benfey, P.N., and Chua, N.-H. (1989). Regulated genes in transgenic plants. Science **244,** 174–181.

Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8,** 2195–2202.

Bonneville, J.M., Sanfaçon, H., Fütterer, J., and Hohn, T. (1989). Posttranscriptional trans-activation in cauliflower mosaic virus. Cell **59,** 1135–1143.

Bouchez, D., Tokuhisa, J.G., Llewellyn, D.J., Dennis, E.S., and Ellis, J.G. (1989). The ocs-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J. **8,** 4197–4204.

Callis, J., Fromm, M., and Walbot, V. (1987). Introns increase gene expression in cultured maize cells. Genes Dev. **1,** 1183–1200.

Dekeyser, R.A., Claes, B., De Rycke, R.M.U., Habets, M.E., and Van Montagu, M.C. (1990). Transient gene expression in intact and organized rice tissues. Plant Cell **2,** 591–602.

Fang, R.-X., Nagy, F., Sivasubramaniam, S., and Chua, N.-H. (1989). Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell **1,** 141–150.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W.G., O'Brien J.V., Chambers, S.A., Adams, W.R., Jr., Willetts, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., and Lemaux, P.G. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell **2,** 603–618.

Gowda, S., Wu, F.C., Scholthof, H.B., and Shepherd, R.J. (1989). Gene VI of figwort mosaic virus (caulimovirus group) functions in posttranslational expression of genes on the full-length RNA transcript. Proc. Natl. Acad. Sci. USA **86,** 9203–9207.

Guilley, H., Richards, K.E., and Jonard, G. (1983). Observations concerning the discontinuous DNAs of cauliflower mosaic virus. EMBO J. **2,** 277–282.

Hay, J.M., Jones, M.C., Blakebrough, M.L., Dasgupta, I., Davies, J.W., and Hull, R. (1991). An analysis of the sequence of an infectious clone of rice tungro bacilliform virus, a plant pararetrovirus. Nucl. Acids Res. **19,** 2615–2621.

Horsch, R.B., Fry, J., Hoffman, N., Neidermeyer, J., Rogers, S.G., and Fraley, R.T. (1989). Leaf disc transformation. In Plant Molecular Biology Manual, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. A5/1–A5/9.

Hull, R. (1984). Caulimovirus group. No. 295. In Descriptions of Plant Viruses. (Kew, Surrey, England: Commonwealth Mycology Institute/Association for Applied Biology).

Hull, R., and Covey, S.N. (1983). Does cauliflower mosaic virus replicate by reverse transcription? Trends Biol. Sci. **8,** 119–121.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Kay, R., Chan, A., Daly, M., and McPherson, J. (1987). Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236,** 1299–1302.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M., and Goldberg, R.B. (1990). Different temporal and spatial gene expression patterns occur during anther development. Plant Cell **2,** 1201–1224.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154,** 367–382.

Lam, E., and Chua, N.-H. (1989). ASF-2: A factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in *Cab* promoters. Plant Cell **1,** 1147–1156.

Lam, E., Benfey, P.N., Gilmartin, P.M., Fang, R., and Chua, N.-H. (1989). Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc. Natl. Acad. Sci. USA **86,** 7890–7894.

Lawton, M.A., Tierney, M.A., Nakamura, I., Anderson, E., Komeda, Y., Dubé, P., Hoffman, N., Farley, R.T., and Beachy, R.N. (1987). Expression of a soybean β-conclycinin gene under the control of the cauliflower mosaic virus 35S and 19S promoters in transformed petunia tissues. Plant Mol. Biol. **9,** 315–324.

Lazo, G.R., Stein, P.A., and Ludwig, R.A. (1991). A transformation competent *Arabidopsis* genomic library in Agrobacterium. *Bio/Technology* **9,** 963–967.

Lockhart, B.E.L. (1990). Evidence for a double-stranded circular genome in a second group of plant viruses. Phytopathology **80,** 127–131.

Medberry, S.L., Lockhart, B.E.L., and Olszewski, N.E. (1990). Properties of *Commelina* yellow mottle virus's complete DNA sequence, genomic discontinuities and transcript suggest that it is a pararetrovirus. Nucl. Acids Res. **18,** 5505–5513.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* **313,** 810–812.

Ow, D.W., Jacobs, J.D., and Howell, S.H. (1987). Functional regions of the cauliflower mosaic virus 35S promoter determined by the use of the firefly luciferase gene as a reporter of promoter activity. Proc. Natl. Acad. Sci. USA. **84,** 4870–4874.

Pfeiffer, P., and Hohn, T. (1983). Involvement of reverse transcription in the replication of cauliflower mosaic virus: A detailed model and test of some aspects. Cell **33,** 781–789.

Sanger, M., Daubert, S., and Goodman, R.M. (1990). Characteristics of a strong promoter from figwort mosaic virus: Comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine synthase promoter. Plant Mol. Biol. **14,** 433–443.

Tabata, T., Nakayama, T., Mikami, K., and Iwabuchi, M. (1991). HBP-1a and HBP-1b: Leucine zipper-type transcription factors of wheat. EMBO J. **10,** 1459–1467.

Terada, R., and Shimamoto, K. (1990). Expression of CaMV 35S-GUS gene in transgenic rice plants. Mol. Gen. Genet. **220,** 389–392.