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Functional organization of the cassava vein mosaic virus (CsVMV) promoter

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Key words: cassava, caulimovirus, *cis* elements, GUS, promoter, transgenic

Abstract

Cassava vein mosaic virus (CsVMV) is a pararetrovirus that infects cassava plants in Brazil. A promoter fragment isolated from CsVMV, comprising nucleotides –443 to +72, was previously shown to direct strong constitutive gene expression in transgenic plants. Here we report the functional architecture of the CsVMV promoter fragment. A series of promoter deletion mutants were fused to the coding sequence of *uidA* reporter gene and the chimeric genes were introduced into transgenic tobacco plants. Promoter activity was monitored by histochemical and quantitative assays of β -glucuronidase activity (GUS). We found that the promoter fragment is made up of different regions that confer distinct tissue-specific expression of the gene. The region encompassing nucleotides –222 to –173 contains *cis* elements that control promoter expression in green tissues and root tips. Our results indicate that a consensus *as1* element and a GATA motif located within this region are essential for promoter expression in those tissues. Expression from the CsVMV promoter in vascular elements is directed by the region encompassing nucleotides –178 to –63. Elements located between nucleotides –149 and –63 are also required to activate promoter expression in green tissues suggesting a combinatorial mode of regulation. Within the latter region, a 43 bp fragment extending from nucleotide –141 to –99 was shown to interact with a protein factor extracted from nuclei of tobacco seedlings. This fragment showed no sequence homology with other pararetrovirus promoters and hence may contain CsVMV-specific regulatory *cis* elements.

Introduction

Caulimoviruses (type member: cauliflower mosaic virus, CaMV) and badnaviruses (type member: comelina yellow mottle virus, ComYMV) are grouped together as plant pararetroviruses [34]. These viruses possess a circular double-stranded DNA genome of around 8 kb that is replicated through a process that includes a step of reverse transcription [30]. Replication is initiated by the synthesis of a terminally redundant genome-length RNA under the control of a viral promoter, also called the genomic promoter [34]. Pararetrovirus genomic promoters interact with the plant RNA polymerase II [27] and their regulation

is apparently not dependent upon any virus-encoded transcriptional factors [26]. Consequently, these promoters were isolated from pararetrovirus genomes to express foreign genes in transgenic plants [6, 25]. The 35S promoter of CaMV has been widely used in plant biotechnology and its functional organization is well described. This promoter is highly active in plant cells and directs constitutive gene expression in most tissues of transgenic plants [15, 28, 40]. The regulatory region of the 35S promoter is made up of distinct domains that are each involved in organ-specific expression [1, 3]. It has been suggested that the strength and the constitutive properties of the 35S promoter de-

rive from additive and synergistic interactions between these different domains [2, 10]. Within these promoter domains two *cis*-acting elements were characterized. The activating sequence 1, or as1, is a TGACG repeat motif extending from -82 to -64 that is the binding site for the tobacco transcription factor TGA1a [16, 19]. It was shown that this *cis* element has an important role in the control of gene expression in roots [11, 19] and functions in synergy with upstream domains to potentiate promoter activities in other tissues [1, 3, 10, 11]. Also, a GATA motif located at from -85 to -105, called activating sequence 2 (as2), interacts with nuclear proteins from tobacco leaf tissues and has biological importance for promoter activity in those tissues [20]. A promoter similar to the 35S CaMV promoter was isolated from figwort mosaic caulimovirus, FMV [35]. This promoter, referred to as the 34S promoter, also directs constitutive gene expression in transgenic plants and is of comparable strength to the CaMV 35S promoter. Sequence comparison between the two promoters in the region extending from -100 to +1 reveals 65% nucleotide homology [35]. Based on the similarity between these two promoters, it has been suggested that similar mechanisms of regulation may govern their expression.

The cassava vein mosaic virus (CsVMV) is a double-stranded DNA virus that infects cassava in Brazil [17, 21] and has previously been listed as a putative member of the genus *Caulimovirus* [14]. However, the recent molecular characterization of the CsVMV genome [4, 7] revealed that this virus has a specific genome organization not found in other caulimoviruses or badnaviruses that have been characterized. Also it was proposed that CsVMV might be representative of a new genus of double-stranded DNA plant viruses [7]. Recently, we isolated a promoter fragment from the CsVMV genome [37] that is capable of driving high levels of heterologous gene expression in different plants and in protoplasts. This promoter directs expression of a reporter gene in all organs and in different cell types of transgenic plants suggesting a constitutive activity similar to that of the CaMV 35S promoter. However, the CsVMV has limited sequence homology with the two caulimovirus promoters previously described. These facts suggest the presence of different *cis*-elements in the promoter and therefore the possibility of different regulatory mechanisms compared with the CaMV 35S promoter. To address this question, we analyzed the functional organization of the CsVMV promoter using a deletion analysis of the region upstream of the TATA box. The

effects of various deletions on promoter activity were monitored in transgenic tobacco plants using the *uidA* reporter gene. Additional data were also provided by transient expression experiments in protoplasts and by *in vitro* DNA-binding assays. We show that the constitutive pattern of expression of the CsVMV promoter is due to the presence of distinct organ specific elements. The relative importance of the different regions of the CsVMV promoter as well as its mechanisms of regulation are discussed.

Materials and methods

Promoter constructs and plasmids

The starting plasmid for this study was pILTAB:CVP2 which contains a CsVMV promoter fragment extending from +72 to -443 [37]. Due to the absence of convenient restriction sites in the CsVMV promoter fragment, polymerase chain reaction (PCR) was used to generate a set of 5'-terminal and internal deletions.

Deletions from the 5' end of the promoter were obtained by PCR amplification. A common reverse primer P1' (Table 1) which hybridizes at the 3' end of the promoter was paired with CsVMV-specific primers P2, P3, P4, P5 and P6 (Table 1) to generate five promoter fragments designated B, C, D, E and F. These fragments have a common 3' terminus at position +72 and their 5'-end points at positions -330, -222, -178, -112 and -63, respectively (Figure 1). A full-length promoter fragment (A fragment) was also re-synthesized using the primer P1 and P1' (Table 1). PCR reactions were carried out with 100 ng of pILTAB:CVP2, 2.5 U of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD) and standard concentrations of primers, MgCl₂ and dNTP. Twenty cycles (94 °C, 30 s; 56 °C, 30 s, 72 °C, 30 s) of amplification were performed and were followed by 5 min of final elongation at 72 °C. Each of the five amplified DNA fragments was digested by *Bam*HI and *Eco*RI and ligated into the same sites of a plasmid containing the coding sequence of the *uidA* gene (coding for β -glucuronidase) linked to the 3' polyadenylation signal of the nopaline synthase gene. The resulting plasmids were named pA, pB, pC, pD, pE, pF according to the promoter deletion they carry (Figure 1).

The internal promoter deletions were constructed through a two-step process. First, PCR reactions were performed as described above to generate a set of 3'-end deletions of the CsVMV promoter. The P1 sense

Table 1. Oligonucleotides used to generate CsVMV promoter fragments by PCR amplification. Primers contain CsVMV promoter sequences in sense (+) or reverse orientation (-). Coordinates of the primers relative to the transcription start site are noted. P1' in association with P2 to P6 were used to create 5' terminal deletions of the CsVMV promoter as described in Materials and Methods. Similarly, P1 in association with P2' to P7' were used for 3' end deletions. P1 and P1' contain, respectively, a *Xba*I site and a *Eco*RI site at their 5' ends while other primers encode *Bam*HI site. The restriction sites are indicated by bold letters. P7 and P8 as well as the reverse primers P8' and P9' were used to generate probes and competitor fragments for gel retardation assays (see Materials and methods for details).

Name	Primer sequence (5' to 3')	Position	Orientation
P1	GCTCTAGACCAGAAGGTAATTATCCAAG	-443/ - 423	+
P2	TATGGATCCTATGTTCAAAAATGAAG	-330/ - 312	+
P3	AAAGGATCCTGAAGACGTAAGCACTG	-222/ - 206	+
P4	AGAGGATCCGGTCGGTGATTGTGAA	-178 - /163	+
P5	AAAGGATCCTTATCACAAAGGAATC	-112/ - 95	+
P6	TATGGATCCGTGTCATTTTGCCTTG	-63/ - 43	+
P7	GAGACATAGAGAGGACACATGT	-161/ - 142	+
P8	AAGGTGGAAAATGTAAGGGCG	-141/ - 121	+
P1'	CGGAATTCAAACTTACAAATTTCTCTAAG	+72/ + 50	-
P2'	TAAGGATCCTTTCCGCCCTTACATT	-116/ - 132	-
P3'	CATGGATCCTCTATGTCTCTTTTAC	-149/ - 168	-
P4'	ACAGGATCCGACCTTATCTTCT	-173/ - 187	-
P5'	ACCGGATCCTCTTCTTTTCATTGTTCT	-182/ - 199	-
P6'	TCAGGATCCTTTTCTTCGCCTGGT	-228/ - 243	-
P7'	ATAGGATCCATATGTGCCGCATA	-334/ - 348	-
P8'	CCTTTGTGATAAGGTTACTTTCC	-99/ - 121	-
P9'	GACACGGAAAAATATAAAAGG	-56/ - 76	-

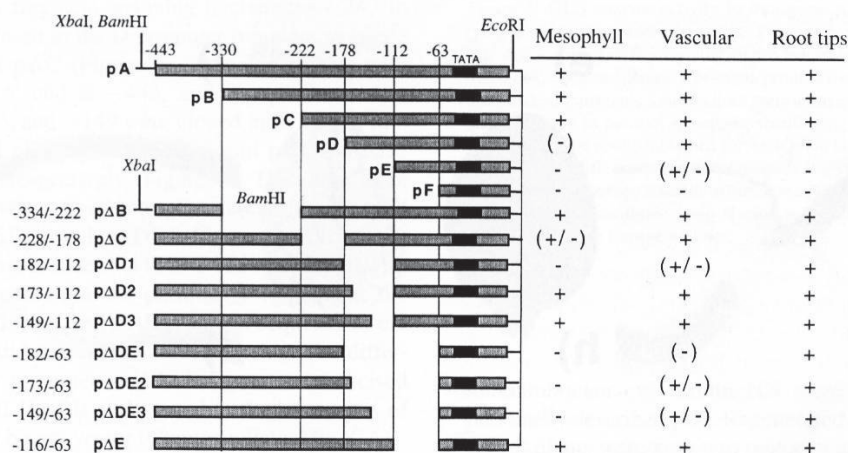


Figure 1. Schematic representation of the deleted CsVMV promoter:uidA fusion genes and a summary of histochemical detection of GUS activity in transgenic tobacco plants. The names of the different plasmids and the end points of the 5' and internal deletions are indicated. Internal deletions are designated by symbol Δ. The construct pA contains a full-length CsVMV promoter. All promoters deleted from the 5' end have a *Bam*HI site at their 5' ends. Internal deletions were created by *Bam*HI ligation of the promoters truncated from the 5' end with those truncated from the 3' end (see Materials and methods). The data summarize the observations performed on ten different transgenic R1 tobacco plants for each promoter construct. The transgenic plants were analyzed for GUS expression in mesophyll cells, vascular tissues and root tips. GUS expression is scaled in each tissue according to 4 levels. +: no visible difference with the full length promoter; (+/-): lower staining than with the full length promoter; (-): very low expression; -: no detectable staining.

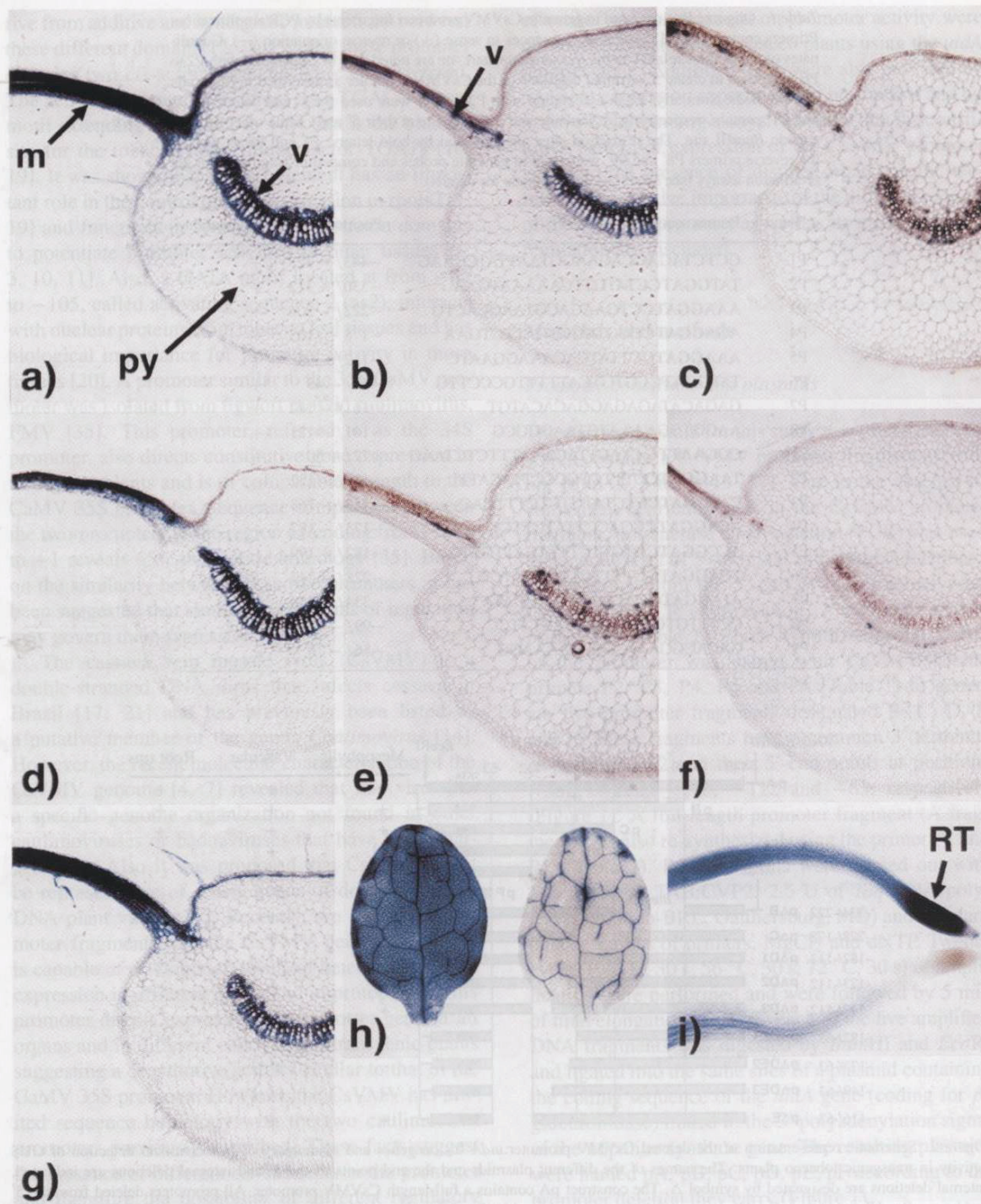


Figure 2. Histochemical localization of GUS expression in transgenic tobacco plants containing deleted CsVMV promoters: *uidA* fusion gene constructs. Pictures presented, with the exception of h and i, are cross-sections of young expanded leaves from 5-week old transgenic R1 tobacco plants. a. Construct pB. This expression profile in leaf was also observed with plants containing the construct pC. As with the full-length CsVMV promoter, strong GUS expression was detected in photosynthetic tissues (palisade and spongy mesophyll) as well as in the phloem cells and in the xylem parenchyma of the midrib. b. Construct pD. Strong staining was localized in the lateral secondary veins, in the phloem tissue and in the xylem parenchyma of the midrib while no or weak expression was detected in the mesophyll tissues. c. Construct pE. Secondary lateral veins as well as the vascular elements of the midrib exhibited a weak staining. d. Construct pΔC. Strong staining was detected in the vascular tissues of the midrib and in the lateral veins. We noticed a decrease in GUS expression in photosynthetic tissues as shown on the picture by a moderate staining in spongy mesophyll cells. e. Construct pΔD1. The same GUS expression pattern was observed with the constructs pΔDE2 and pΔDE3. Moderate staining is visualized in the lateral veins and in the phloem cells of the midrib. f. Construct pΔDE1. No expression was detectable with the exceptions of few phloem cells that exhibited a very weak staining. g. Construct pΔD2. Plants carrying the deletion constructs pΔD3 and pΔE displayed similar GUS expression pattern. GUS expression was detected in the mesophyll and vascular tissues and was in the same range of intensity as what we observed with the undeleted promoter. h. Leaves from 10-day old transgenic seedlings carrying pB construct (left) or pD construct (right). i. Roots from transgenic tobacco plants containing the pC promoter construct (top) or pD promoter construct (bottom). m, mesophyll; py, parenchyma, v, vascular tissue.

primer (Table 1) which hybridizes at the 5' end of the promoter was paired with each of 6 specific CsVMV reverse primers, P2' to P7' (Table 1), to generate six truncated promoters with a common 5' end at position -443 and 3' end points spanning from position -116 to -334. Internal deletions were engineered by cloning the different 3' end truncated promoter fragments upstream of the 5' end deleted promoter constructs (Figure 1). Accordingly, a 3' deleted promoter fragment encompassing nucleotides -443 to -334 was digested by *Bam*HI and *Xba*I and ligated to the same sites in the pC plasmid. The resulting plasmid, named pΔB (Figure 1) contains an internal deletion from nucleotides -334 to -222 (Figure 1). Similarly, a fragment spanning nucleotides -443 to -228 was fused to the D promoter fragment to create the plasmid pΔC (Figure 1). Three fragments with a common 5' end at -443, and 3' ends located at -182, -173, and -149 were cloned individually into the plasmid pE to create the plasmid pΔD1, pΔD2 and pΔD3, respectively (Figure 1). The same three fragments were cloned into pF to create the plasmid pΔDE1, pΔDE2 and pΔDE3 (Figure 1). A fragment containing nucleotides -443 to -116 was cloned with the same method into the plasmid pF to generate the plasmid pΔE (Figure 1). All promoter sequences were verified by dideoxynucleotide sequencing. The different CsVMV promoter-*uidA* fusion genes were excised by *Xba*I and *Hind*III and ligated to the same sites of the pBin 19 binary vector (Clontech, Palo Alto, CA).

Transgenic tobacco plants

The pBin 19 derivatives carrying the deleted promoter constructs were transferred by electroporation into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformations of *Nico-*

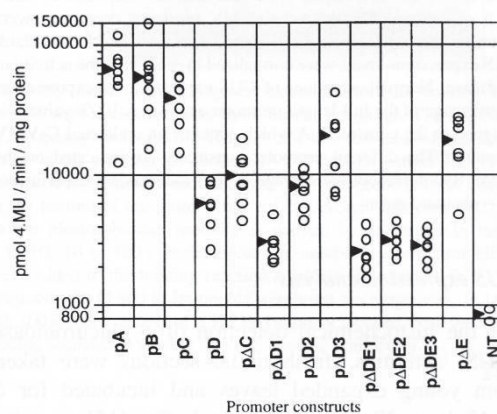


Figure 3. GUS enzyme activity in transgenic tobacco leaves carrying CsVMV promoter constructs. Proteins were extracted from leaf disks collected from young expanded leaves of 5 week old transgenic tobacco plants. For each promoter construct, 6 to 10 independent transgenic tobacco lines were assayed for GUS activity. Data are given as pmol of 4-methylumbelliferone per mg of protein per minute. The results obtained for each plant is represented by an open dot. The different promoter constructs analyzed in these assays are indicated. Average level of expression is indicated by a vertical arrowhead. A logarithmic scale is used to accommodate the large variation between transgenic lines.

tiana tabacum cv. Xanthi NN were performed as previously described [13]. Regenerated kanamycin resistant plants were grown to maturity in a greenhouse and allowed to self-fertilize. R1 seeds were germinated on Murashige and Skoog (MS) culture medium [24] with 100 mg/l kanamycin and transferred to soil in a greenhouse. Between 10 and 20 independent transgenic lines were produced for each construct. Ten independent R1 lines for each promoter construct were analyzed for this study.

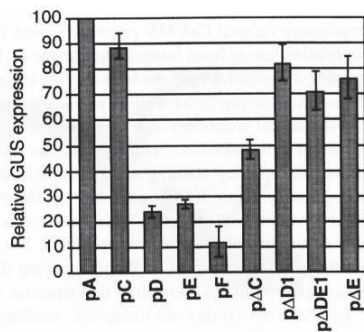


Figure 4. Transient GUS expression of CsVMV:*uidA* gene constructs in BY-2 protoplasts. Electroporated protoplasts prepared from BY-2 cell suspensions were analyzed for GUS activity after 24 h of culture. The deleted CsVMV promoter constructs were co-transfected with a luciferase plasmid used as an internal standard. GUS expressions levels were normalized in relation to the activity of luciferase. Normalized values of GUS expression are expressed as a percentage of the full-length promoter activity. A 100% value was assigned to the construct pA which contains an undeleted CsVMV promoter. The different promoter constructs are indicated on the figure. Bars represent the average of four independent experiments $\pm$ standard errors.

GUS expression analysis

For the histochemical detection of β -glucuronidase (GUS) activities, fresh tissue sections were taken from young expanded leaves and incubated for 6 to 12 h at 37 °C in reaction buffer [15] containing 1 mM 5-bromo-4-chloro-3-indolylglucuronide (X-gluc), 100 mM sodium phosphate buffer pH 7, 2 mM potassium ferrocyanide and potassium ferricyanide, and 0.1% Triton X-100. For the GUS analysis of young R1 seedlings, whole plantlets were collected around one week after germination and immersed in GUS buffer [15]. After vacuum infiltration, incubation was carried out overnight at 37 °C. Samples were cleared by several washes in 70% ethanol. Quantitative GUS analyses using the substrate 4 methylumbelliferone- β -S glucuronide (MUG) were performed as described by Jefferson *et al.* [15].

Protoplast isolation, treatment and culture

Protoplasts from BY-2 tobacco suspension cells were prepared essentially as described by Watanabe *et al.* [39]. A 30 μ g portion of plasmid DNA with 30 μ g of a 35S-luciferase construct [28], which served as internal standard, was introduced into protoplasts as previously described [37]. Protoplast were collected for protein extraction after 24 h of culture at 25 °C. The MUG and LUC assays were performed on the

protoplast protein extracts as described [37]. Results were expressed as a ratio between the GUS activity of the CsVMV promoter construct and the LUC activity of the internal control.

Probes and competitors

DNA fragments used as probes in binding assays were generated by PCR using 200 ng of pILTAB:CVP2 plasmid containing the full-length CsVMV promoter [37], *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and its appropriate buffer, specific primers, and dNTPs as standard concentrations. A DNA fragment encompassing -161 to -56 was obtained using the primers P7 and P9' (Table 1) after 20 cycles of amplification performed with the following temperature profile: 30 s at 94 °C; 30 s at 58 °C; 45 s at 72 °C. The PCR mixture was extracted with phenol/chloroform and the DNA was ethanol-precipitated. Amplified DNA was subjected to electrophoresis through on a 3% Biogel (Bio 101, La Jolla, CA) and the probe fragment was eluted using the Mermaid kit (Bio 101). Purified DNA was end-labeled with [γ -³²P] ATP using T4 polynucleotide kinase (NEB, Beverly, MA). The probe was purified from unincorporated nucleotides by two successive rounds of ethanol precipitation in the presence of ammonium acetate.

Specific DNA competitors were produced by PCR using *Pfu* polymerase and the conditions described above. In addition to the -161/-56 fragment, three subsets encompassing -161 to -99 (obtained with P7 and P8' primers, Table 1), -141 to -56 (obtained with P8 and P9' primers) and -141 to -99 (obtained with P8 and P8' primers) were used as competitors. DNA fragments were gel purified using the Mermaid kit (Bio 101). A 557 bp DNA fragment isolated by *EcoRI* and *EcoRV* digestion of the coding region of the *uidA* gene was used as a non-specific competitor DNA. This fragment contains *uidA* nucleotides from the ATG position to the first *EcoRV* site in the sequence.

Preparation of tobacco nuclear extracts

A protein nuclear extract was prepared from 2-week old tobacco (*N. tabacum* cv. Xanthi NN) seedlings grown in growth chambers (16 h day light cycle). All purification steps were carried out at 4 °C. Nuclei were isolated following the protocol described by Giuliano *et al.* [12] except that benzamidine was omitted from the extraction buffer. The isolated nuclei were then resuspended (50 μ l/g of starting tissue) in lysis buffer

containing 20 mM HEPES pH 8, 0.4 mM EDTA, 0.5 mM DTT, 420 mM NaCl, 25% (v/v) glycerol, 0.1 mM PMSF, 2 μ g/l leupeptine, 3 μ g/l pepstatine and 40 μ g/l antipain. The suspension of nuclei was homogenized by pipeting up and down and incubated on ice for 1 h (with occasional mixing). The resulting extract was centrifuged at $100\,000 \times g$ for 1 h. The supernatant was collected and dialyzed for 2 h against 2 liters of dialysis buffer containing 20 mM HEPES pH 8, 50 mM KCl, 0.4 mM EDTA, 20% glycerol, 0.1 mM PMSF and 0.5 mM DTT. The dialysate was centrifuged at $12\,000 \times g$ for 10 min to remove insoluble material and the supernatant was aliquoted and stored at -80°C . Protein concentrations of nuclear extracts were determined using the Bradford assay (BioRad, Hercules, CA).

Gel shift assays

Protein-DNA binding reactions were carried out on ice in 25 μ l of $1 \times$ binding buffer (20 mM HEPES pH 7.8, 50 mM KCl, 1 mM MgCl_2 , 0.05% Nonidet 40, 1 mM DTT and 10% glycerol) containing 2 μ g of poly-dI-dC as non-specific competitor and an adequate amount of nuclear extract protein (amounts of nuclear extract are noted in the legend to Figure 5). The mixture was pre-incubated for 10 min on ice before addition of 10 000 cpm of the labeled probe. The reaction was allowed to proceed on ice for an additional 25 min.

For competition assays, competitor DNAs were included in the reaction before addition of the labeled probe. The amount of competitor used for each assay is indicated in Figure 5. Reaction mixtures were subjected to electrophoresis through a 5% non-denaturing polyacrylamide gel in $1 \times$ TGE buffer, pH 8.5, at 130 V for 3 h at 4°C . Gels were fixed in 10% methanol, 10% acetic acid, dried and exposed overnight on X-ray films with an intensifying screen.

Results

Expression patterns of deleted CsVMV promoter constructs in transgenic tobacco plants

The CsVMV promoter was mutated by progressive 5' deletions and by internal deletions as described in Materials and methods. Expression patterns of the different promoter constructs were analyzed in transgenic R1 tobacco plants using histochemical staining of GUS activity. The presence of an intact promoter:*uidA*

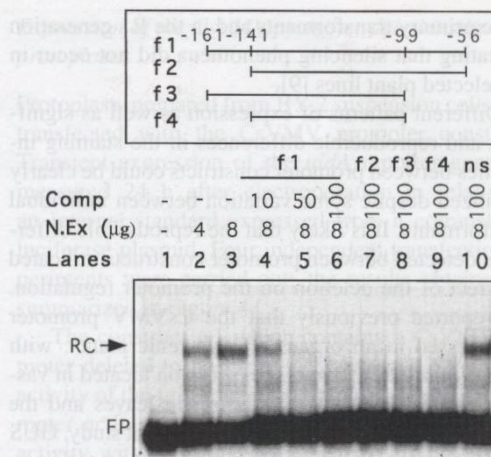


Figure 5. Interaction of a fragment extending from nucleotide -161 to -56 with nuclear extracts from tobacco seedlings. The fragment f1, spanning nucleotides -161 to -56, was end-labeled and incubated in the presence or absence (-) of nuclear extract (N.ex) as indicated in Materials and methods. The free probe (FP) is visible at the bottom of the figure while a DNA/protein complex (RC) of slower electrophoretic mobility is marked by an arrow. In lanes 4 to 10, 10 to 100 $\times$ molar excess of unlabeled competitor DNA were added to the binding reaction mixtures. In addition to the f1 fragment, f2, f3 and f4 fragments were used as competitors. In lane 10, 100 $\times$ molar excess of a 557 bp fragment isolated from the *uidA* coding sequence was used as a non-specific competitor.

gene cassette was confirmed by PCR and/or Southern analysis for each transgenic plant line used in this study (data not shown). For each promoter construct, we obtained between 10 to 20 transgenic plant lines. GUS expression patterns observed between plants containing the same promoter construct were similar while we observed variations in the level of transgene expression among individual transformants. The plants tested in this study contained between 1 and 5 copies of the *uidA* fusion gene (data not shown). The copy number did not affect the characteristic pattern of expression observed with each construct and there was no clear correlation between the copy number and the apparent intensity of the staining. For each promoter construct, we always obtained a small number of transgenic plants for which the expression of the transgene was not detected despite its presence as shown by Southern blotting, suggesting a possible silencing phenomenon [29]. Data obtained from these plants were not taken into account for the current deletion analysis. However, in the lines selected for this study, transgene expression was stable during plant development and the pattern of GUS staining was similar

in the primary transformants and in the R1 generation indicating that silencing phenomena did not occur in the selected plant lines [9].

Different patterns of expression as well as significant and reproducible differences in the staining intensities between promoter constructs could be clearly visualized despite some variation between individual transformants. It is likely that the reproducible differences detected between promoter constructs indicated an effect of the deletion on the promoter regulation. We reported previously that the CsVMV promoter is expressed in all organs of transgenic plants, with regions of highest promoter expression located in vascular tissues, mesophyll cells of the leaves and the root tips [37]. Accordingly, for the present study, GUS activity was analyzed in these three different tissues (Figure 1).

5'-end deletions

GUS staining in transgenic plants that contained the promoter constructs deleted to -330 and -222 (constructs pB and pC) occurred in the same pattern and was in the same range of intensity as what was observed with the full length promoter (Figures 1 and 2a). Further deletion of the promoter to -178 (construct pD) caused an important change in the GUS expression pattern (Figure 1). In leaf cross-sections of most of the plants carrying the pD construct, a strong staining restricted to the vascular tissues was observed (Figure 2). GUS staining was absent in the mesophyll cells of most of the plants. However, three plants lines out of ten presented a low staining in these cells. In all plants transformed with the pD construct, the root tips did not exhibit GUS staining (Figure 2i) while this tissue was intensely stained in plants containing the pC promoter construct. GUS activity from the promoter construct pE, deleted to -112, was restricted to the vascular tissues (Figures 1 and 2c). The intensity of the expression was very low and long incubation times were required to detect a blue precipitate in these tissues. The construct pF did not display any detectable expression (Figure 1).

Internal deletions

Internal deletion of the region from -334 to -222 (promoter Δ B) did not affect the general expression pattern of the CsVMV promoter (Figure 1). A significant decrease in GUS accumulation was visualized in the spongy and palisade mesophyll cells of the plants transformed with p Δ C (Figures 1 and 2d). However the deletion did not suppress expression in vascular

elements and root tips, which exhibited a strong staining. Transgenic plants containing p Δ D1 (deletion from -182 to -112) displayed a weak GUS staining restricted to the phloem cells (Figures 1 and 2e). Additionally, GUS expression was also observed in the root tips. The internal deletion encompassing -182 to -63 (p Δ DE1) had a profound effect on promoter activity (Figure 1). Of the 10 independent transgenic plants tested, 8 did not have any detectable GUS activity in the leaves or in stems. Very pale blue staining localized in the phloem cells was visualized in two plants after prolonged incubation (Figure 2f). In contrast, strong staining was revealed in the root tip as well as weaker staining in the vascular elements of the root. The construct p Δ E displayed a constitutive pattern of expression similar to the undeleted promoter (Figure 1 and 2g).

These results emphasized the importance of the (-182/-112) region for constitutive expression of the CsVMV promoter. We observed that the deletion p Δ D1 suppressed promoter activity in the mesophyll tissue and also diminished vascular GUS expression (Figure 2e). Consequently, we made the constructs p Δ D2 and p Δ D3 to investigate this region in greater details (Figure 1). The construct p Δ D2, deleted from nucleotides -173 to -112, displayed a profile of expression similar to the full length CsVMV promoter (Figure 2g). This result suggested that the addition of 9 nucleotides at the 5' end point of the (-182/-112) deletion could restore the full expression pattern altered with the construct Δ D1. No significant difference was detected between the construct p Δ D2 and p Δ D3.

In contrast, addition of the sequences -182/-173 and -182/-149 to p Δ DE1 to make p Δ DE2 and p Δ DE3, respectively, did not lead to a restoration of promoter activity (Figures 1 and 2e), suggesting that elements located between -112 and -63 might influence promoter activity.

Histochemical GUS analysis in young seedlings

A histochemical GUS analysis on 10-day old R1 seedlings was carried out in order to analyze the expression pattern of the deleted promoter at earlier stages of development. The deleted promoter constructs directed specific expression patterns that were similar to those observed in adult plants. As in expanded leaves of adult plants, the constructs pD, pE and p Δ D1 conferred a vascular-specific pattern of expression in cotyledonary leaves (Figure 2h). Sim-

ilarly, the pB and pC constructs which were active in both mesophyll and vascular tissues in tobacco plants had the same constitutive expression pattern in seedlings (Figure 2h). These results suggested that specific expression patterns observed with the different deletions in transgenic plants are not influenced by the developmental stage of the plant.

GUS activity in leaf extracts of transgenic plants

GUS activities in protein extracts prepared from leaf tissues were measured using a fluorometric assay [15]. The samples were collected from interveinal tissue of young expanded leaves from 5-week old transgenic tobacco plants. Consequently, the GUS activity levels reflected mainly the promoter expression in mesophyll tissue. As shown in Figure 3, the values of GUS activities in transgenic lines carrying a specific promoter construct varied by a maximal factor of 17. We did not observe a clear correlation between levels of expression and transgene copy number (not shown) as plants with the same copy number exhibited large differences in levels of GUS activity.

A significant decrease in level of GUS expression was detected in transgenic plants containing the pD promoter construct. A low level of GUS activity was also measured with the internal deletion pΔC confirming the importance of the region extending from -222 to -178. This result was consistent with the histochemical analysis since pD and pΔC displayed low or no staining in mesophyll cells of transgenic plants. Similarly, low levels of GUS activity were measured for the constructs pΔD1, pΔDE1, pΔDE2 and pΔDE3 which had vascular-specific profiles of expression in leaves of transgenic plants. The average level of activity measured with the construct pΔD2 was higher than with the pΔD1 construct confirming that the region from -182 to -173 is involved in promoter activation. However, the pΔD3 activity levels were in the higher range while the construct pΔD2 gave moderate levels of expression. This difference was not detected using the histochemical assay (Figure 2g). As observed with the histochemical assay, the high expression level measured with the construct pΔD3 was abolished by deletion of the region spanning -112 to -63 (construct pΔDE3). The construct pΔE retained a high level of GUS activity suggesting that the promoter expression in green tissues is weakly affected by this deletion.

Expression of the CsVMV promoter deletions in protoplasts

Protoplasts prepared from BY-2 suspension cells were transfected with the CsVMV promoter constructs. Transient expression of the *uidA* reporter gene was measured 24 h after electroporation in relation to an internal standard expressed from a cotransfected luciferase plasmid. Four independent transfection experiments were carried out; the results obtained are summarized in Figure 4.

The construct pC which contains a CsVMV promoter deleted to position -222 retained 88% of the activity of the full-length promoter fragment. The promoter activity dropped sharply to only 24% of full activity with a further deletion to -178. Constructs pD and pE had almost the same expression level while a second drop of activity was observed with a deletion extending to -63. The construct pF with 12% of the full promoter activity was just above background level. An internal deletion from -228 to -178 (construct pΔC) decreased expression by more than 50%. Surprisingly the constructs pΔD1 and pΔDE1 which gave very low expression in transgenic plants allowed high levels of expression in protoplasts. The (-222/-178) region plays a critical role for promoter activity in protoplast while the region from -178 to -63 appears to be of lower importance for expression of the promoter in protoplasts than in plants.

DNA-protein interactions in the -161 to -56 region

A labeled DNA fragment encompassing -161 to -56 was used to detect specific binding with nuclear protein extracts prepared from tobacco seedlings. Interaction of the (-161/-56) probe with the nuclear extract generated a single complex of slower mobility than unbound probe (Figure 5). The intensity of the retarded band increased with increasing amount of nuclear extract in the reaction. The formation of the complex was reduced or abolished by addition of an excess of unlabeled probe whereas addition in the reaction of an unrelated DNA fragment had no effect (Figure 5). This result suggested that one or more DNA-binding proteins in the tobacco nuclear extract interact with *cis*-elements within the (-161/-56) promoter region.

To map more precisely the binding site of this interaction, we performed competition assays with sub-fragments of the probe. The complex was eliminated when overlapping sub fragments spanning -161 to -99, -141 to -56 or -141 to -99 were added to the reactions at 100-fold molar excess in the assay.

This suggested that a binding site for a protein factor is located between -141 and -99.

Discussion

This study showed that the CsVMV promoter has a complex structure made of discrete domains that exercise distinct influences on patterns of tissue specific expression. Our results suggest that the constitutive activity of the promoter is the result of additive and synergistic interactions between different promoter elements. The functional organization of the CsVMV promoter as defined by the deletion analysis is summarized in Figure 6.

Promoter expression in green tissues is directed by the region extending from -222 to -173. In this region, we identified a consensus sequence as1 [19] at -219/-203 as well as a GATA sequence at position -183/-180. The construct p Δ D1 (deletion of the region from -182 to -112) exhibited a low level of expression (Figure 2e) and showed that as1 element on its own cannot activate gene expression in mesophyll tissue. As suggested when we analyzed the expression patterns of the construct p Δ D1 and p Δ D2, the sequence extending from -182 to -173, which contains a GATA motif, is essential for promoter activity in green tissue. However, the specific role of this GATA region independent of the as1 element cannot be assessed based on expression of the constructs used in this study. Consequently, two hypotheses are presented: either the GATA region, on its own, controls promoter expression in green tissues, or the GATA region and the as1 element act together to control the CsVMV promoter activity in these tissues. Published data reported that a GATA motif in the CaMV promoter, referred to as the activating sequence 2, as2 [20], is also involved in leaf expression. Moreover, gene expression in leaf tissues controlled by the GATA region in the CaMV promoter was dependent on sequences located within the -90 to -46 region (which contains as1 element). Similar interactions may control expression of the CsVMV promoter in green tissues. However, the GATA motif identified in the CsVMV promoter is not identical with the as2 element of the CaMV promoter. We found a greater sequence homology with a GATA box identified in the rice tungro bacilliform badnavirus promoter [5, 42] which plays an important role in the activation of that promoter [43] and with a box I element (GATAAPu)

found in several light and circadian-clock-regulated promoters [8, 36].

A low level of GUS expression was observed in mesophyll cells of plants transformed with the promoter construct p Δ C which deleted the as1 and GATA regions (Figure 2d). It is unlikely that this residual GUS staining in green tissue is due to additional minor elements located in the region from -443 to -228. Indeed, this region in the context of the deletion p Δ D1 did not direct any GUS expression in mesophyll cells (Figure 2e). The GUS staining in mesophyll cells of plants containing p Δ C is due either to diffusion of the dye from intensely colored vascular tissues or to the new arrangement of sequence created by the deletion that could be favorable to gene expression in the mesophyll cells.

The results of the studies in transgenic plants showed that expression of the CsVMV promoter in vascular tissues is directed by the region encompassing -178 to -63 and is not dependent upon the presence of as1 or the GATA region (as shown by the construct pD, Figure 2b). The latter elements may have a moderate effect on vascular expression as suggested by the construct p Δ DE2 and p Δ DE3 (Figure 2e) but is clearly not required for full promoter activity in vascular tissues. The expression pattern displayed by construct pE showed that a vascular domain is located in the -112/-63 region. We detected in this region a 22 nucleotide sequence containing a CTTATC repeat previously identified in the ComYMV promoter [23]. However, the results obtained showed that additional elements located between -178 to -112 are responsible for the strong vascular expression (Figure 6). Within the latter region, important elements may be contained between -149 to -116 as suggested by comparing the expression pattern of p Δ DE3 and p Δ E.

In vitro binding assays performed with the region -161 to -56 revealed a specific interaction with nuclear protein. We detected a single retarded band in these assays, the formation of which was efficiently disrupted by competition with a 43 nucleotide fragment extending from -141 to -99. This was somewhat surprising since regions from -149 to -112 and from -112 to -63 play an active role in expression of the promoter. It is possible that certain binding activities are not detected due to a low concentration of transcriptional factors in our nuclear extract, to a lower affinity for the binding site, or because cooperative binding with other factors is required.

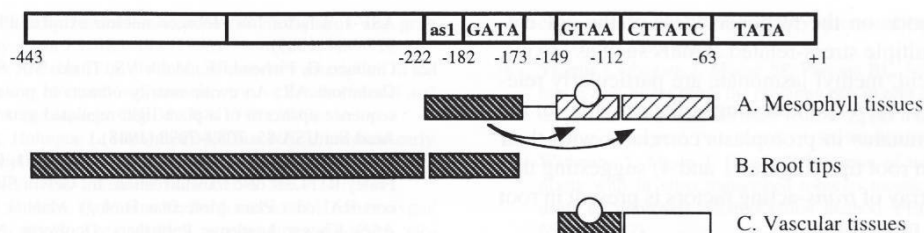


Figure 6. Summary of the CsVMV promoter regions required for *uidA* gene expression in various tissues of transgenic tobacco plants. Coordinates relative to the transcription start site indicate the position of promoter domains as defined in this study. Promoter domains required for expression in mesophyll tissues (A), in root tips (B) and in vascular tissue (C) were identified and are indicated below the map. A. An essential promoter region for expression in mesophyll tissues (hatched grey box) is located between -222 and -173. This region contains a consensus *as1* element [19] and a GATA motif. The hatched boxes from -149 to -112 and from -112 to -63 represent promoter regions that could not specify mesophyll expression by themselves but that were required in combination with the upstream hatched grey box to direct expression in green tissues. These putative synergistic interactions are represented in the figure by the arrows. B. Two promoter domains (hatched grey boxes) from -443 to -222 and from -222 to -178 could direct gene expression in root tips. Our data suggest that these two domains have a redundant function. C. The region extending from -149 to -112 (hatched grey box), characterized by a GTAA repeat, was responsible for high level of gene expression in vascular tissues. The region extending from -112 to -63 (open box) was also able to specify vascular expression but could not direct high level of gene expression. This region contains a CTTATC repeat previously identified within the ComYMV promoter sequence [23]. Formation of a complex between the promoter region extending from -141 to -99 and a protein factor present in tobacco nuclear extract is indicated by an open circle.

More detailed examination of the sequence of this 43 nt fragment revealed the presence of a GTAA repeat located at -129 to -113. The upstream GTAA motif of the latter repeat resembles the core-sequence of the P-box identified in the zein gene promoters [38]. The *tefl* box [32] located in the -100 region of the EF-1a gene promoters contains a GTAA repeat with similarities to the GTAA box of the CsVMV promoter. Determination of the role of the GTAA repeat in the CsVMV promoter will require further study.

Deletion of regions of the CsVMV promoter that are important for expression in vascular tissues, as in the construct p Δ DE3, suppressed gene expression in green tissues. Expression in the mesophyll tissue was restored by the addition of nucleotides -419 to -116 (construct p Δ E) or by the addition of nucleotides -112 to -63 (construct p Δ D3). These results could be explained by an effect due to spacing between the 'mesophyll domain' (namely, the *as1* element and the GATA motif) and the TATA box in construct p Δ DE3. By contrast, the promoter is active when at least 49 nucleotides are present between the GATA element (-182 to -173) and nucleotide -63.

In the CaMV 35S promoter, the *as1* and GATA motifs are located between -64 and -105 [20], much closer to the TATA box than in the CsVMV promoter. By analogy, the shorter distance between the GATA region and TATA box created by the p Δ DE2 and p Δ DE3 internal deletions would not be expected to prevent the activity of the *as1* and GATA *cis* ele-

ments. The data may suggest that additional *cis*-acting elements contained in the region -149/-112 and in the region -112/-63 are required for promoter activity in green tissues (Figure 6). Elements contained in these regions may act in synergy with the 'mesophyll domain' of the CsVMV promoter to promote gene expression in green tissues.

The CsVMV promoter includes an important regulatory domain for expression in root tips between -222 and -178 (Figure 2i). It is likely that the *as1* element contained in this region is involved in this function, based on analogy with the role of the *as1* element of the 35S CaMV promoter in root expression [1, 11, 19]. However the construct p Δ C, which lacks the *as1* element, is strongly expressed in root tip, and indicates that other promoter-elements are also involved in root tip expression (Figure 6).

In BY2 protoplasts, CsVMV promoter activity appeared to be controlled by the region encompassing -222 to -178 that contains the *as1* consensus sequence. Similar results were obtained with leaf mesophyll protoplasts (not shown). It was surprising to find that expression from the promoter is independent of sequences from -182 to -63. Discrepancies of results between protoplast-based transient assays and transgenic plants were also observed for the 35S CaMV promoter [10, 18]. Protoplasts are in highly stressed physiological state [33] that could be responsible of activation or inactivation of various *trans*-acting factors interacting with the promoter. In this regard,

several reports on the responsiveness of the *as1* element to multiple stress-related signals such as auxins, salicylic acid, methyl jasmonate are particularly relevant [22, 31, 41, 44]. Interestingly, the activities of the promoter mutants in protoplasts correlates with their activities in root tips (Figures 1 and 4) suggesting that a similar array of *trans*-acting factors is present in root tips and in protoplasts.

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