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Promoter Deletion and Comparative Expression Analysis of the *Mirabilis mosaic caulimovirus* (MMV) Sub-genomic Transcript (Sgt) Promoter in Transgenic Plants

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A sub-genomic transcript (Sgt) promoter was isolated from a genomic clone of the *Mirabilis mosaic virus* (MMV), a double-stranded DNA plant pararetrovirus belonging to the *Caulimoviridae* family. The MMV Sgt promoter fragment (genomic co-ordinates 4830 to 5840) was mapped by 5′-3′-deletion analysis to define the boundaries required for maximal promoter expression. Expression patterns of promoter fragments coupled to GUS reporter gene were evaluated both in protoplast transient expression experiments and in transgenic *Nicotiana tabacum* cv. Samsun NN plants. A 333 bp MMV Sgt promoter fragment (sequence –306 to +27 from the transcription start site, TSS) was found sufficient for strong promoter activity in protoplast transient expression experiments. The transcription start site (TSS) of the MMV Sgt promoter was determined by primer extension analysis using total RNA isolated from transgenic plants containing a MMV Sgt promoter:*uidA* fusion gene. In seedlings, the level of GUS expression was in the order of leaf ≥ root >stem. Histochemical GUS-staining of seedlings showed highest GUS activity in root tips, leaf midribs, veins and vascular tissues. The MMV Sgt promoter fragment is a strong constitutive promoter, with strength comparable to that of the MMV full-length transcript (FLt) promoter. MMV Sgt promoter also demonstrated much greater activity, 8-fold more compared to the CaMV 19S promoter and 2-fold stronger than the CaMV 35S promoter.

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INTRODUCTION

The *Mirabilis mosaic virus* (MMV) infects *Mirabilis* plant species (family *Nyctaginaceae*), a member of the *Caulimoviridae* family, and has a circular double-stranded DNA genome of about 8 Kb with four single-stranded discontinuities in the DNA, one in the alpha strand and three in the complementary strand [1]. The restriction map of the MMV genome is quite different from the other members of the genus *Caulimovirus* [1]. The MMV virus was characterized as a member of the genus *Caulimovirus* based upon the morphology of its virions and inclusion bodies [2]. Recently, MMV has been fully sequenced (GenBank nucleotide sequence database accession number AF454635), and homology analysis of its genomic DNA has shown that it is a definitive member of the genus *Caulimovirus* [Maiti, unpublished]. However, MMV is serologically distinct from the *Cauliflower mosaic virus* (CaMV), the type species of this genus [2].

Several *Caulimoviridae* genomes have been fully sequenced and characterized. These include *Cauliflower mosaic virus* (CaMV) [3], *Carnation etched ring virus* (CERV) [4], *Figwort mosaic virus* (FMV) [5], *Soybean chlorotic mottle virus* (SoCMV) [6], *Peanut chlorotic streak virus* (PClSV) [7], *Casava vein mosaic virus* (CVMV) [8], *Strawberry vein banding virus* (SVBV) [9], *Petunia vein clearing virus* (PVCV) [10], and *Mirabilis mosaic virus* (MMV) [Maiti, unpublished].

The *Caulimovirus* genome generally contains two transcriptional promoters, one for the full-length transcript and the other for the subgenomic transcript; these are equivalent to the CaMV 35S and 19S transcript respectively [6, 11, 12]. A number of strong constitutive promoters have been derived from *Caulimoviridae* family, particularly from the *Cauliflower mosaic virus* (CaMV): CaMV35S and 19S promoter [13, 14]. For constructing plant transformation vectors, genetic promoters have also been isolated from other members of this family, namely *Rice tungro bacilliform virus* (RTBV) [15], *Commelina yellow mottle virus* (CYMV) [16], *Soybean chlorotic mottle virus* (SoCMV) [6], *Figwort mosaic virus* (FMV, strain DxS) [17, 18]), FMV strain M3 [19], *Cassava vein mosaic* virus (CVMV) [20], *Peanut cholotic streak virus* (PClSV) [21] and *Mirabilis mosaic virus* (MMV) [22, 23]. Transcript promoters from the *Caulimovirus* like CaMV, FMV, PClSV, MMV and FMV are active in all plant organs [13, 18, 21-23], whereas, transcript promoters from the *Badnavirus* like CYMV and RTBV are phloem-specific [15, 16] in expressing genes in transgenic plants. The CaMV 35S promoter has been well characterized [13, 24-30] and widely used in chimeric gene constructs for heterologous gene expression in transgenic plants [31-33]. The CaMV 35S promoter is also active in bacteria [34], in yeast [35], in Hela cells [36] and in *Xenopus oocytes* [37].

In the present study, we report the identification and characterization of a subgenomic transcript (Sgt) promoter from the *Mirabilis mosaic virus* (MMV), a newly described species of the genus *Caulimovirus*. The optimal boundaries required for the maximal promoter activity were defined by 5′- and 3′- end deletion analysis of the promoter/leader region of the subgenomic transcript promoter

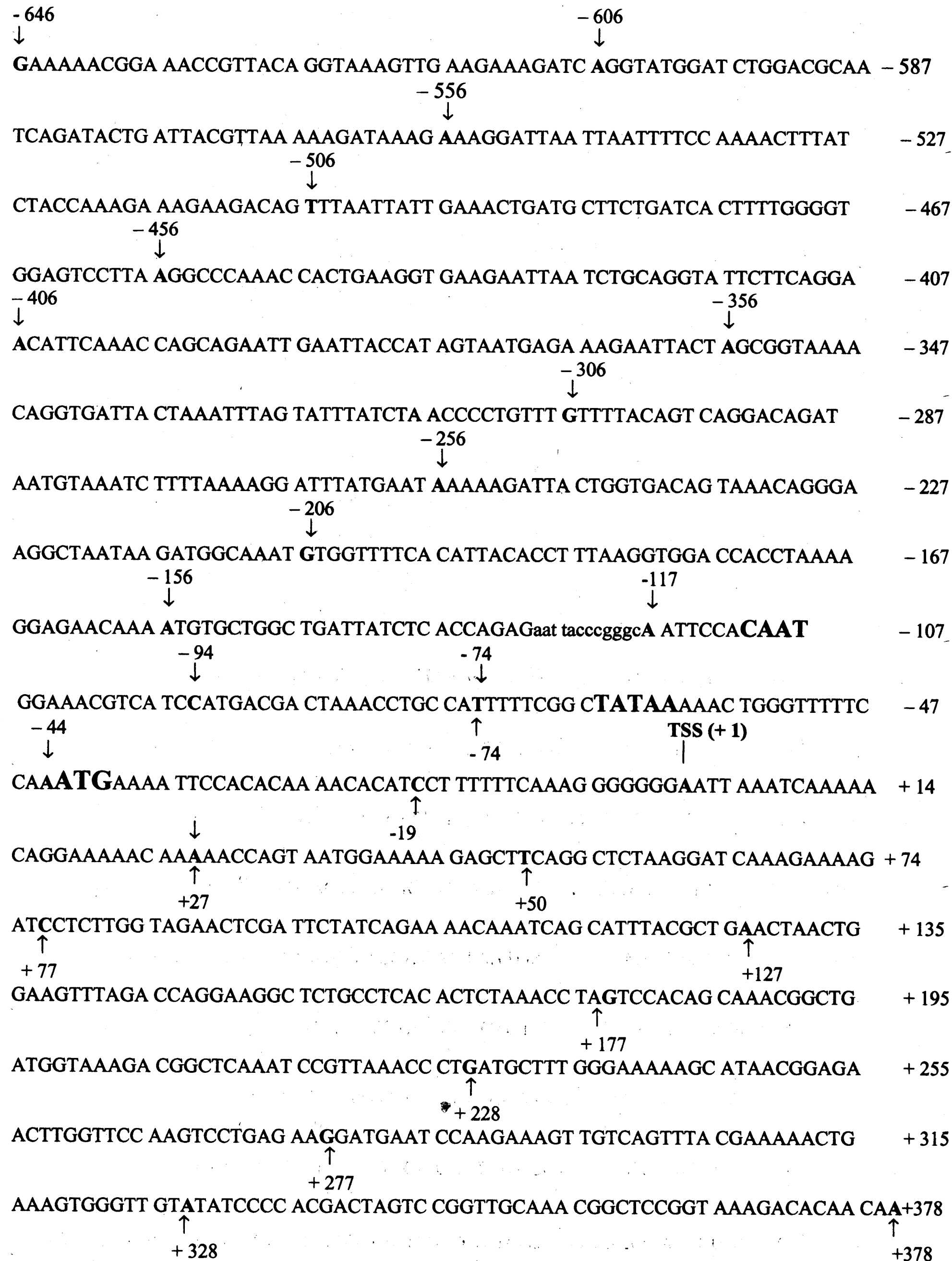

FIGURE 1
The DNA sequence of the subgenomic transcript (Sgt) promoter from the *Mirabilis mosaic virus* (MMV). The nucleotide sequence of the MMV Sgt promoter (coordinates -646 to +378 in respect to transcription start site; corresponding coordinates in MMV genome 4829 to 5840), a 1023 bp fragment includes the 3′-end of gene V, followed by the small intergenic region and 5′ portion of gene VI presented from left to right in the 5′ to 3′ direction of the transcript. Modification of promoter sequence resulted from inserting the 'EcoRI to SmaI' adapter is shown in lowercase. The end points for the 5′ or the 3′ deletion constructs are also indicated above or below the sequence respectively. The tentative regulatory sequence: the TATA-box (TATAA), the CAT box (CAAT) and the initiator ATG codon for gene VI are shown in bold. The transcriptional start site (TSS) is indicated as +1.

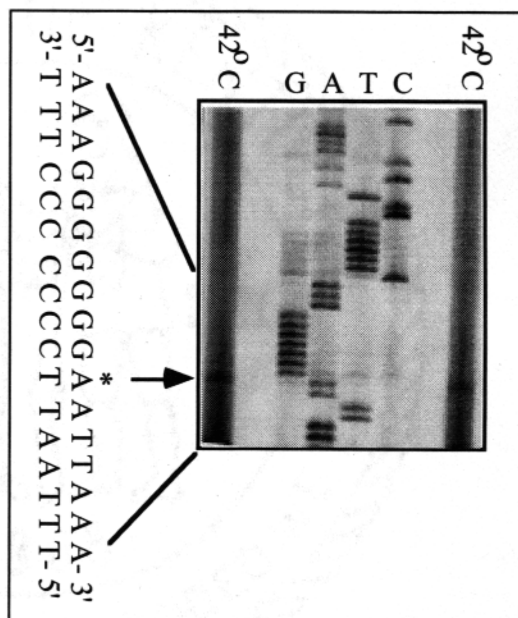

FIGURE 2
Determination of the transcriptional start site (TSS) of the MMV Sgt promoter by primer extension analysis. Primer extension was carried out as described in Materials and Methods. Primer extension reaction products were subjected to electrophoresis on denaturing polyacrylamide gel containing urea, alongside with the sequence reaction of GUS gene construct (lane G, A, T and C). The process was performed with the same labeled primer. The minus strand DNA sequence read on the gel is shown, and the transcriptional start site (A*) in the corresponding plus strand is indicated by an arrow.

of MMV both in transgenic plants and in protoplast transient expression experiments. A 333 bp MMV Sgt promoter fragment (sequence -306 to +27 from transcription start site) was found sufficient for maximal promoter activity. The strength of the MMV Sgt promoter is very compatible with the MMV FLt promoter and greater than that of the CaMV 19S and CaMV 35S promoters. This is the first report, to our knowledge, documenting the strong and constitutive expression characteristic of the MMV Sgt promoter.

MATERIALS AND METHODS

Protoplasts, Plants and Enzymes

Isolation of protoplasts from the tobacco cell suspension cultures (Xanthi 'Brad') and electroporation of protoplasts with supercoiled DNA containing promoter fragment fused with GUS gene were done essentially as described earlier [38]. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were used for plant transformation. Restriction enzymes, antibiotics, components of plant tissue culture medium, and RNA isolation kits were purchased from commercial sources. Nitrocellulose and Nytran membranes were obtained from Schleicher & Schuell, USA.

Isolation of MMV Sgt promoter and modification of its DNA sequence

A full-length genomic clone of MMV in the plasmid pMMV-B10 [1] was fully sequenced (Maiti, unpublished results), and it was used as a template for PCR reactions. A 1012 bp segment (co-ordinates 4829 to 5840 of the MMV genome) was selected for promoter deletion analysis, and it was isolated as a *Bam*HI and *Hin*dIII fragment by PCR using the following primers: (i) Forward primer: 5′-GCG GGC GGA TCC **GAA AAA CGG AAA CCG TTA**-3′ and (ii) Reverse primer: 5′-ATG CAG AAG CTT **TTG TTG TGT CTT TAC CGG**-3′. Promoter sequences in the primers are shown in bold, and restriction enzymes sites are underlined. The PCR-fragment was gel-purified using the QIAgen procedure (Valencia, CA 91355, USA). After digestion with *Bam*HI and *Hin*dIII, the PCR fragment was cloned into the corresponding sites of pBS(KS+) (Strategene, La Jolla, CA 92037, USA). We designated this plasmid as

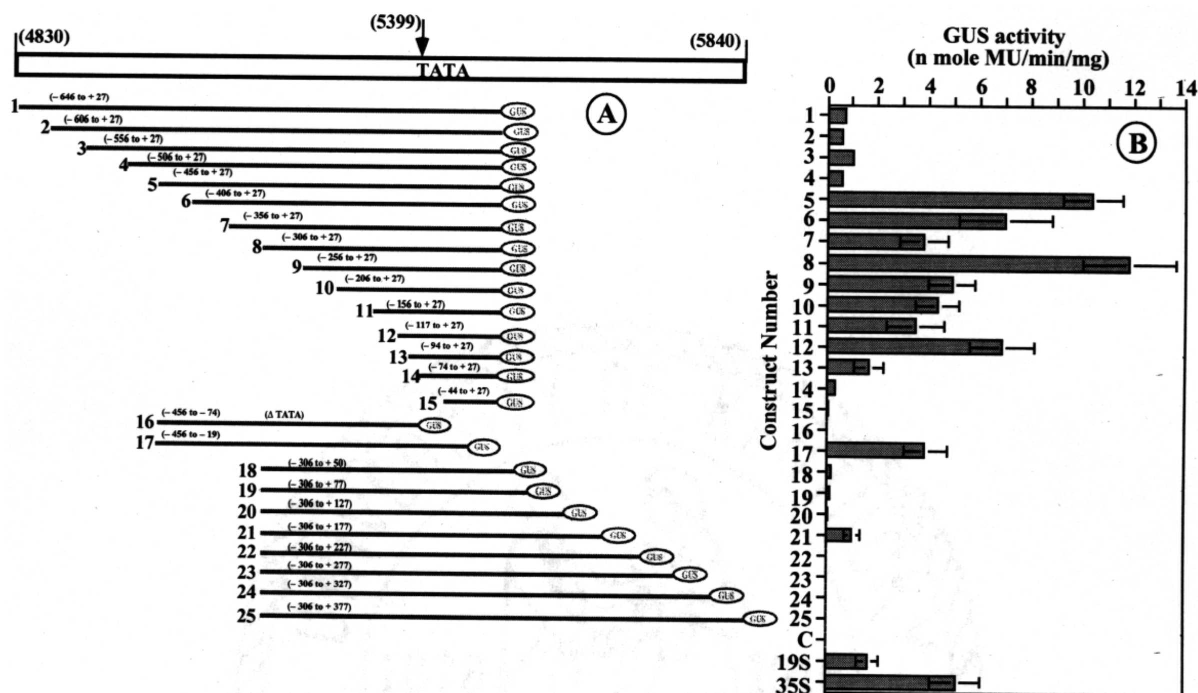

FIGURE 3
A and B. (A) A schematic map of the GUS constructs (number 1 to 25) developed for the deletion analysis of the MMV Sgt promoter. The coordinates of the relative deleted promoter fragments are given in parenthesis. The end points for the 5′ or the 3′ deletion constructs are also indicated above or below the sequence, respectively, in Figure 1. At the top, the relative position of the TATA box and the MMV genomic coordinates are shown. (B) MMV Sgt promoter expression analysis in protoplast transient expression assay using GUS reporter gene. Soluble protein extracts (5 μg) from transformed protoplasts were used for GUS assay. Each construct was assayed at least three times in three independent experiments. The average GUS activity is presented in the histogram. Error bars show a 95% confidence interval of the means. The statistical (one-way analysis of variance, ANOVA) analysis showed an extremely significant P value of < 0.001. C, untransformed control, extract from untransformed protoplast; 19S, 35S, GUS gene is directed by the CaMV 19S and 35S promoter, respectively.

pBSMS1011.

Modification of EcoRI site to a SmaI site:

The pBSMS1011 has an *Eco*RI site at the MMV genome coordinate 5345. This *Eco*RI site located 52 bp upstream of the TATA sequence was modified to insert a *Sma*I site using two primers with the following sequences: forward primer: 5′-AATTACCCGGGC-3′ and reverse primer: 5′-AATTGCCCGGGT-3′. These two primers were mixed in a 1:1 molar ratio and allowed to anneal by slow cooling (2°C/ min) from 94° C to 24° C under a programmed PCR cycle. The plasmid pBSMS1011 was digested with *Eco*RI and ligated with the '*Eco*RI to *Sma*I' adapter, followed by transformation. The resulting plasmid was designated as pBSMS1011DE. This change inserted 12 additional nucleotides (5′-AATTACCCGGGC-3′) into the promoter sequence (Fig. 1).

Construction of vectors for transient expression of genes in protoplasts

A series of promoter fragments included in constructing the plant transformation vector with the subgenomic promoter of MMV were designed to study the influence of upstream and downstream sequence with respect to the TATA box toward the promoter activity. The defined MMV Sgt promoter sequence of length, as indicated (in Fig. 3A) was amplified by PCR using pBSMS1011DE as template and appropriately designed primers to introduce an *Eco*RI site at the 5′-end and a *Hin*dIII site at the 3′-end of amplified products. PCR amplification was carried out for 31 cycles under the following standard conditions: denaturation (92° C for 1 min), annealing (55° C for 1 min), and extension (72° C for 2 min) using ELONGASE enzyme mix (recombinant high fidelity *Taq*DNA polymerase mix with proof reading 3′-5′ exonuclease activity) from Gibco-BRL, Maryland, USA. Each PCR amplified promoter fragment from 1 to 25 was restricted with *Eco*RI and *Hin*dIII; the restricted fragment was gel-purified and cloned into the corresponding sites of pUC119 vector and sequenced by the dideoxy chain terminator method [39] using synthetic primers.

Subcloning of the MMV Sgt promoter fragments from pUC119 into pUCPMAGUS vector

The sequence of each of the MMV Sgt promoter fragments cloned in pUC119 was verified before subcloning to pUCPMAGUS, a protoplast expression vector [22]. The MMV Sgt promoter fragments were individually gel-purified from the corresponding pUC119 clone after restriction digestion with *Eco*RI and *Hin*dIII and subcloned into the corresponding sites of pUCPMAGUS [22]. The following promoter deletion plasmids were developed (Fig. 3). The 5′ and 3′ co-ordinates of the promoter fragment with respect to the transcription start site (TSS) are given in parenthesis: pPMS1GUS (-646 to +27), pPMS2GUS (-606 to +27), pPMS3GUS (-556 to +27), pPMS4GUS (-506 to +27), pPMS5GUS (-456 to +27), pPMS6GUS (-406 to +27), pPMS7GUS (-356 to +27), pPMS8GUS (-306 to +27), pPMS9GUS (-256 to +27), pPMS10GUS (-206 to +27), pPMS11GUS (-156 to +27), pPMS12GUS (-117 to +27), pPMS13GUS (-94 to +27), pPMS14GUS (-74 to +27), pPMS15GUS (-44 to +27), pPMS16GUS (-456 to –74), pPMS17GUS (-456 to –19), pPMS18GUS (-306 to +50), pPMS19GUS (-306 to +77), pPMS20GUS (-306 to +127), pPMS21GUS (-306 to +177), pPMS22GUS (-306 to 227), pPMS23GUS (-306 to 277), pPMS24GUS (-306 to +327) and pPMS25GUS (-306 to +377).

Construction of vectors with MMV Sgt promoter fragments for expression of genes in plants

The MMV Sgt promoter fragments from constructs pPMS5GUS (-456 to +27), pPMS8GUS (-306 to +27) and pPMS17GUS (-456 to -19) were isolated by *Eco*RI and *Hin*dIII digestion followed by gel purification and cloned into the plant expression vector pKYLX71 [40] individually at its unique restriction *Eco*RI and *Hin*dIII sites that flank the promoter. The following plant gene expression vectors were developed: pKMS5, pKMS8 and pKMS17. These plant gene expression vectors have multiple cloning sites (MCS): *5′-Hin*dIII-*Bam*HI-*Xho*I-*Sst*I-*Xba*I-

3′) with the following unique sites: *Hin*dIII, *Xho*I, *Sst*I and *Xba*I. The reporter GUS gene from pBSGUS as an *Xho*I-*Sst*I fragment was inserted into these pKYLX-based expression vectors separately, and the resulting plasmids were designated as pKMS5GUS, pKMS8GUS and pKMS17GUS.

Isolation of CaMV 19S promoter, and construction of vectors with the CaMV 19S and 35S promoters for transient and stable expression of genes in plant cells

A 990 bp *Sal*I to *Sst*I fragment containing the CaMV 19S promoter was isolated from pCaMV10, a full-length genomic clone of the CaMV strain CM1841 [3]. This *Sal*1 to *Sst*I restricted fragment, corresponding to the CaMV genomic coordinates 4833 to 5822, was gel-purified and cloned into the corresponding sites of pBS(KS+). The resulting plasmid was designated as pBSCaMV (4833-5822). An internal *Eco*RI site in the pBSCaMV(4833-5822) corresponding to the CaMV genomic coordinate 5646 was modified to *Sma*I site using the synthetic adaptor (as described in earlier section), and the modified plasmid was designated pBSCaMV(4833-5822)DE. Using this clone as a template, a 412 bp CaMV 19S promoter fragment (CaMV coordinates 5380 to 5773) was isolated by PCR with the designed primers. The forward primer, 5′-CAAGAATTC GTTAAC**AAGCT-GCAGAAAGGAATTACC**-3′, contains *Eco*RI and *Hpa*I sites (underlined) and CaMV sequence (shown in bold). The reverse primer, 5′-CTTAAGCTT**GCTTGGAGGTCT-GATTTT**-3′, has a *Hin*dIII site (underlined) and CaMV sequence (indicated in bold). The PCR-amplified promoter fragment has *Eco*RI and *Hpa*I sites at the 5′-end and a *Hin*dIII site at the 3′-end to facilitate cloning. The fragment has the general structure 5′- *Eco*RI-*Hpa*I-promoter sequence *Sma*I-TATA-promoter sequence-*Hin*dIII-3′.

The PCR amplified CaMV 19S promoter fragment (412 bp) was cloned into the corresponding *Eco*RI-*Hin*dIII sites of the vector pUCPMAGUS [22] for the transient expression in protoplasts, and also into the corresponding sites of the vector pKYLXGUS for stable transgene expression, as discussed above. The resulting expression vectors were named pPCaSGUS and pKCaSGUS, respectively.

Similarly, the CaMV 35S promoter (-940 to +27 from TSS; corresponding to the CaMV genomic coordinates 6493 to 7459), was cloned as an *Eco*RI-*Hin*dIII fragment into the corresponding sites of the transient expression vector (pUCPMAGUS) for the expression of GUS gene in protoplasts. The resulting plasmid was named pPCa35S-GUS. The GUS reporter gene was inserted as an *Xho*I-*Sst*I fragment into the corresponding sites of PKYLX71 [40]. In the resulting plant expression vector pKCa35S-GUS, the GUS reporter gene is directed by the CaMV 35S promoter (coordinates -940 to +27 from TSS).

Plant transformation and analysis of transgenic plants

The constructs pKMS5GUS, pKMS8GUS and pKMS17GUS; developed for expressing GUS gene in plants, were introduced into *Agrobacterium tumefaciens* strain C58CI: pGV3850 by triparental mating, and tobacco (*Nicotiana tabacum* cv. Samsun NN) was transformed with the engineered *Agrobacterium*, as described earlier [41]. Regenerated kanamycin-resistant plants were grown under green house conditions. On

average, twelve to fourteen independent plant lines were raised for each construct.

β-Glucuronidase (GUS) assay

Fluorometric GUS assays to measure GUS activity in plant tissue or protoplasts extracts and histochemical GUS staining to localize the distribution of GUS activity in plants were performed according to Jefferson et al., [42], as described earlier [18]. Protein in plant extract was determined according to the method of Bradford [43], using BSA as a standard.

RNA extraction, RNA dot blot and Northern blot analysis

Total RNA was prepared from transformed protoplasts containing individual MMV Sgt promoter construct by extracting with guanidine thiocyanate [44] solution using an Ambion RNA extraction kit (RNAqueous), as described earlier [22]. The RNA dot blot and northern blot analysis were performed using a ^{32}P-labeled GUS-probe essentially, as described previously [22].

Determination of transcriptional start site (TSS) of MMV Sgt promoter and DNA sequencing

The transcriptional start site was determined by primer extension analysis. The extension product was separated on a 7.5% polyacrylamide gel containing 7M-urea [45]. Sequencing reactions were carried out according to Sanger et al., [39], using Sequenase Version 2.0, USB, as described earlier [22].

Automated DNA sequencing was done with an Applied Biosystem ABI Prism 310 Genetic Analyzed (Perkin Elmer), using ABI Prism Dye terminator cycle sequencing ready reaction kit containing Ampli Taq DNA polymerase, as described earlier [22].

RESULTS AND DISCUSSION

The MMV Sgt promoter sequence and structure

Mirabilis Mosaic virus (MMV) has a double-stranded circular DNA genome of 8 Kb [1]. The sequence of the MMV Sgt promoter is shown (Fig. 1). This MMV Sgt promoter sequence contains several regulatory domains found in other caulimovirus promoters: the TATAA sequence (coordinates –65 to –61 from TSS) and the CAAT sequence (coordinates –110 to –70 from TSS) located 41 bp upstream of the TATA box. In the MMV Sgt promoter sequence, an '*as*-1' like enhancer element (TGACG; coordinates –90 to –88 from TSS) and an '*as*-2' like motif (GATT; coordinates –145 to –142 from TSS) are located at the 22 bp and 76 bp upstream of the TATA box, respectively. The MMV Sgt promoter has only one copy of an '*as*-I' like or '*as*-2' like motif; whereas, duplicated copies are present in full-length transcript promoters of CaMV, FMV and MMV [13, 18, 22]. In addition, several direct repetitive sequences are present in the MMV Sgt promoter. These are: TCAGGA (-412 to –407 and –297 to –292), GAATTAC (-386 to –380 and –364 to –358), GGTGA (-244 to –240 and –344 to –340), CC(A/T)TTTTTC (–77 to –69 and –19 to –11) and AAACA (-28 to –24, +12 to +16, and +21 to +25) (Fig. 1). They may have some regulatory function. More work will be needed to evaluate their regulatory role, if any, in promoter function. An *Eco*RI sites located at 48 bp upstream of TATA sequence was modified to a *Sma*I site

using a *Sma*I adaptor. This change inserted 12 additional nucleotides (5′-AATTACC-CGGGC-3′), as shown in lowercase (Fig. 1), into the promoter sequence but did not affect promoter activity. In *Caulimovirus*, both subgenomic and full-length transcripts promoters share the same 3′-ends by using the same poly (A) signal.

Determination of transcriptional start site

The transcriptional start site (TSS) of the MMV Sgt promoter was determined by primer extension analysis using total RNA isolated from transgenic plants developed with the construct pKMS5GUS (Fig. 2). The major extension product was detected and mapped to an adenine residue located 63 nucleotide downstream of the TATA box in the MMV Sgt sequence and, most likely, it represents the 5′-end of the MMV Sgt transcript (Fig. 2). The location of the TSS reported for other caulimoviruses: CaMV 35S [46], FMV34S [47], FMV FLt [18], PClSV FLt [7] and MMV FLt [22] is at the 32, 37, 45, 29 and 24 nucleotides downstream of respective TATA boxes. The transcription start site of the MMV Sgt promoter shows no sequence homology with that of other caulimovirus promoter.

Transient expression analysis of MMV Sgt promoter deletion constructs in tobacco protoplasts

A deletion analysis scheme of the MMV Sgt promoter is shown in Fig. 3A. A series of 5′- and 3′- end-deleted promoter fragments (total of 25 fragments) were included to map the optimal boundaries required for maximal expression from the promoter/leader region and also to analyze the influence of the upstream and downstream cis-sequences with respect to the TATA box. The designed deletion promoter fragments 1 to 25 (Fig. 3A) were amplified by PCR and cloned into the expressing sites of vector pUCPMAGUS, as described in the Methods section. Results of the expression analysis of the MMV Sgt promoter are shown in Fig. 3B. In transient expression assay, the construct 8 (pPMS8GUS) that contains the promoter fragment (coordinate –306 to +27 from TSS) gave maximum activity in protoplasts. The expression levels of 5′ deletion constructs 1, 2, 3 and 4 was 6%, 5%, 9% and 5% respectively relative to the construct 8. This suggests that the upstream sequence region (coordinates –646 to –455 from TSS) may contain repressor elements. However, in this context, to obtain maximal promoter activity this region (coordinates -646 to - 455 from TSS) is not essential. The 5′ deletion construct 5, 6 and 7 showed 88%, 60% and 32% of maximal activity, respectively. In construct 7, 5′ deletion of 50 bp of sequence (–406 to –356 from TSS) reduced the promoter activity by 46% relative to construct 6 (compared construct 6 and 7) and by 63% relative to construct 5 (compare construct 5 and 7). These deletions results clearly showed the importance of this region (-406 to –357 from TSS) in overall promoter activity. Two direct repeats sequence GAATTC (coordinates –386 to –380, and –364 to –358 from TSS) in this region may be important, but more work is needed to evaluate the importance of this cis-sequence. Although, construct 8 with promoter sequence (–306 to +27 from TSS) showed maximum activity in protoplast transient expression experiments, the construct 5 with promoter coordinates (-406 to +27 from TSS) exhibited more activity in stable expression assay in transgenic plants, documented in latter section. Construct 9, 10 and 11 gave 42%, 37% and 29% of maximal

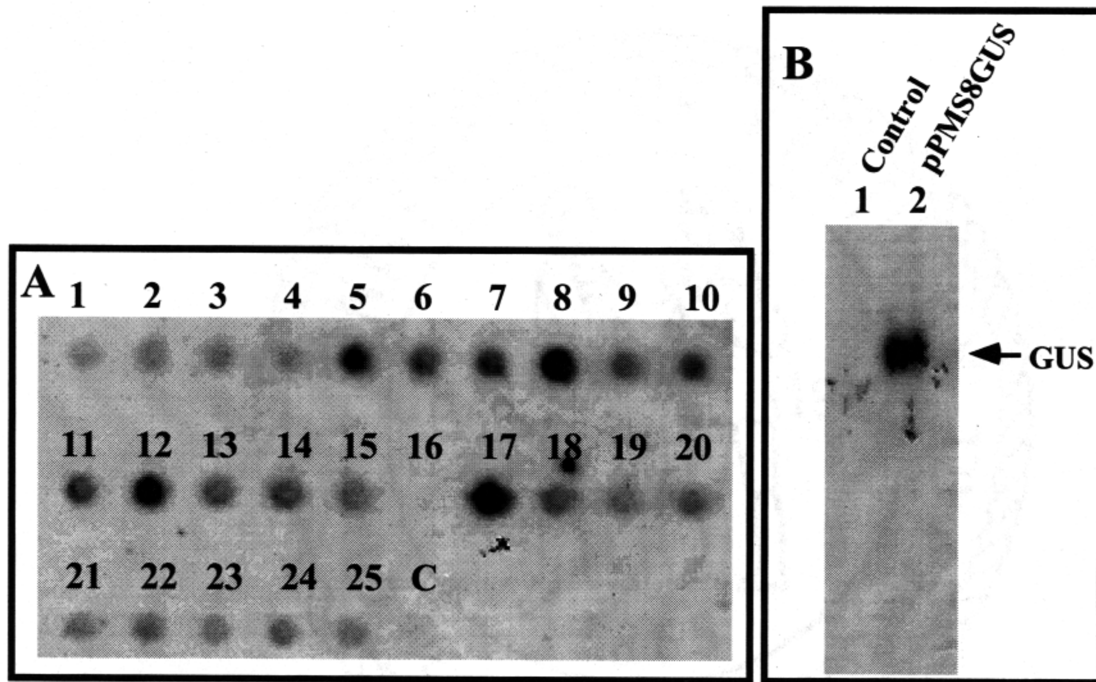

FIGURE 4
A and B: (A) RNA dot-blot analysis of total RNA (10 µg) obtained from transformed protoplasts with construct No 1 to 25 as indicated in Figure 3. (B). Northern blot analysis of total RNA (10 µg) obtained from pPMS8GUS with ^{32}P labeled GUS as probe (lane 2) and RNA obtained from untransformed Samsun NN plant (lane 1).

activity compared to construct 8, demonstrating the importance of cis sequences between –306 to –255 from TSS, as deletion of this stretch reduced maximal promoter activity by 58% (compare construct 9 with construct 8). Construct 12, containing CAAT sequence (coordinates –110 to –107 from TSS), showed 58% of construct 8 activity. However, further deletion of 22 bp (coordinates –117 to –93 from TSS) in construct 13 (which contains 'as-1' motif) reduced promoter activity to 14% of that of construct 8, and to 24% of that of construct 12, suggesting the importance of the CAAT box in this region for promoter function. The 5′ deletion construct 13 that contains the 'as-1' like motif, construct 14 that contains the TATA like element, and construct 15 lacking TATA region, showed 14%, 3% and 1% of maximal activity (compare with construct 8). This demonstrates the requirement of further additional TATA upstream sequence for full promoter activity. The 3′ deletion-construct 16 (promoter coordinates –456 to –74 from TSS), devoid of TATA box showed no appreciable promoter activity, suggesting the importance of TATAA sequence in the MMV Sgt promoter function. Although, the MMV Sgt promoter does not contain eukaryotic consensus regulatory sequence TATATAA, this result indicates that TATAAA sequence in MMV Sgt promoter functions as a TATA box. The 3′ deletion construct 17 (-456 to –19 in respect to TSS) showed about 33% of maximal promoter activity. In this

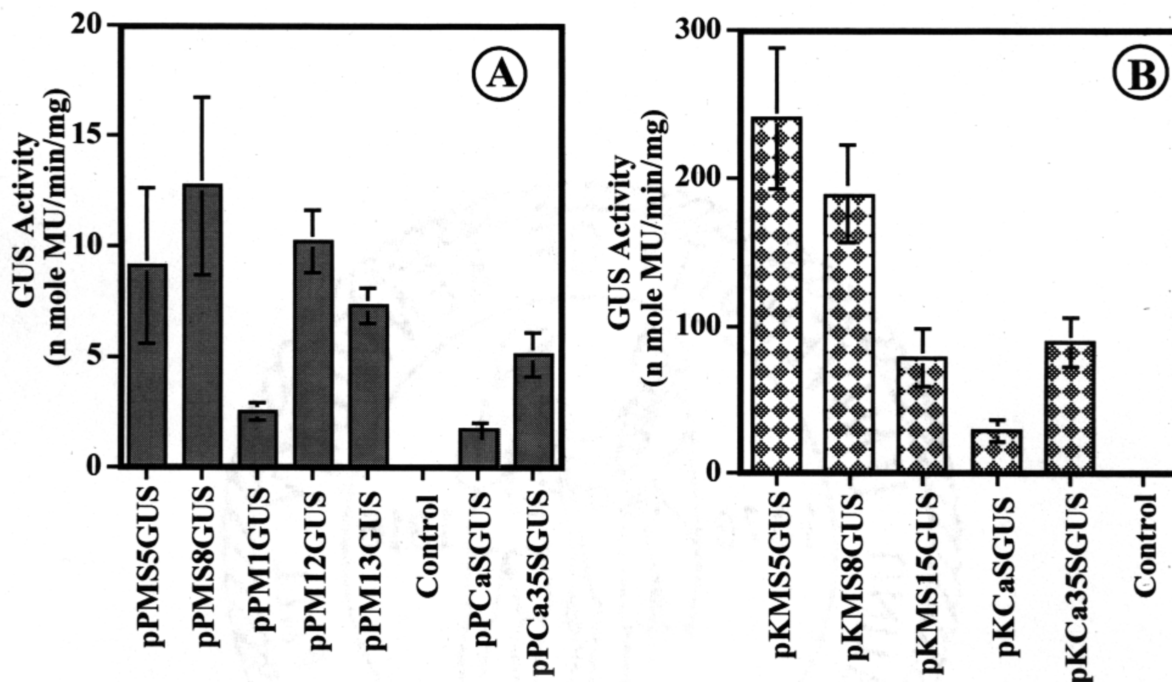

FIGURE 5
A and B. (A) Comparative expression analysis of the MMV Sgt promoter with the MMV FLt promoters and CaMV promoters (35S and 19S) in (A) transient expression in protoplasts and (B) stable expression in transgenic plants.
(A) GUS constructs: pPMS5GUS, pPMS8GUS with the MMV Sgt promoter; pPM1GUS, pPM12GUS and pPM13GUS with MMV FLt promoter, as described earlier [22]; and pPCaSGUS and pPCa35SGUS with CaMV 19S and 35S promoters, respectively, were assayed in protoplast transient expression experiments. Each construct was assayed at least three times in three independent experiments. The average GUS activity is presented in the histogram. Error bars show a 95% confidence interval on the means. The statistical ANOVA analysis showed a P value < 0.001; this is considered to be extremely significant.
(B) The MMV Sgt promoter (GUS- constructs pKMS5GUS, pKM8GUS and pKMS17GUS) and CaMV 19 S and 35S promoter (GUS constructs pKCaSGUS and pKCa35SGUS respectively) were compared. The promoter activity was measured in four-week-old seedlings (R1 progeny) grown aseptically on an MS-agar medium in the presence of kanamycin (200 mg/ liter) and 3% sucrose. Soluble protein extract from the whole seedlings were used for the GUS assay. The data are means of five independent experiments for each construct; eight to ten independent transgenic lines developed for each construct were assayed. The average GUS activity is presented for each construct in the histogram, with standard deviation from the mean indicated by an error bar. Error bars show a 95% confidence interval on the means. The statistical ANOVA analysis indicated that the P value < 0.001 means extremely significant. Untransformed control (Control), tissue extract from wild-type *N. tabacum* cv. SamsunNN.

context, construct 17 may produce transcripts with different TSS. The constructs 18, 19, 20, 21, 22, 23, 24 and 25, with successively extended 3′ leader sequence, gave very less activity (2%, 1%. 0.7%, 9%, 0.25%, .3%, 0.4%, and 0.3%, respectively) of full promoter activity. These results suggest that, in this context, the longer leader sequence +50 to +378 has a significant inhibitory effect on promoter function. In the case of the FLt promoter from FMV and MMV, an extended leader sequence is required for maximum promoter activity [18, 22]. Thus, a 320 bp MMV Sgt promoter/leader fragment, sequence –306

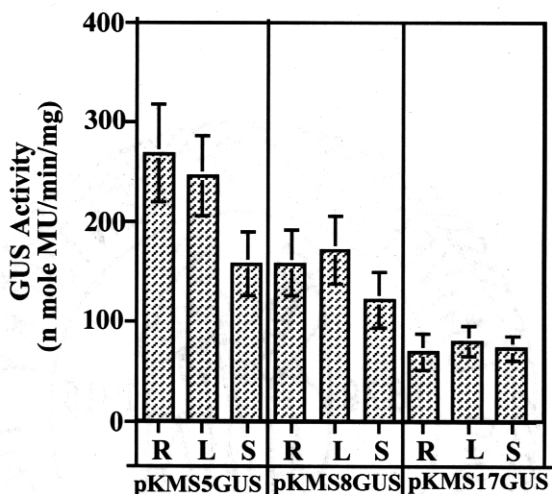

FIGURE 6
Expression of MMV Sgt promoters in various parts (roots, R; leaves, L; stems, S) of four-week-old seedlings developed for pKMS5GUS, pKMS8GUS and pKMS17GUS. GUS activity was measured fluorometrically using soluble protein extract (5 μg) from roots, stems and leaves of seedlings. The presented value in the histogram, with standard deviation indicated by an error bar, is the average of six samplings from each of the eight independent lines developed for each construct. Error bar shows a 95% confidence interval on the means. The statistical ANOVA analysis showed that a P value <0.001 means extremely significant.

to +27 from TSS, was found to be sufficient for maximal GUS expression.

The relative strengths of the MMV Sgt promoter fragments with the GUS reporter gene were evaluated by hybridization analysis of total RNA. Total RNA extracted from the transformed protoplasts with each of the constructs (No. 1 to 25, as described in Fig. 3A) was used for RNA dot-blot analysis (Fig. 4A). The P^{32}-labeled GUS coding sequence was used as a probe. Construct 8 gave the highest signal and was followed by construct 5, 6, 7, 9, 10 and 17. A minimum signal was obtained from construct 16 that is devoid of TATA-box (Fig. 4A). The relative transcript level obtained with these constructs, in general, is in good agreement with the GUS activity. The level of transcript in construct 18 to 25 containing longer leader sequence (+50 to +378) was relatively less, and promoter function was reduced probably through its effect of longer untranslated leader sequence on transcription and subsequent translation.

Northern analysis of total RNA isolated from tobacco protoplasts transformed with construct 8 showed a single discrete band corresponding to GUS transcripts of the expected size (2100 nt) (Fig. 4B).

Stable Expression analysis of MMV Sgt promoter in transgenic plants

Ten to twelve independent primary transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) lines (R_0 progeny) were developed for each of these constructs (pKMS5GUS, pKMS8GUS and pKMS17GUS) and grown under greenhouse condition. Leaf extracts from these R_0 plants were used for fluorometric GUS assays. Analysis of these lines from R_0 progeny showed that the GUS expression level in transgenic plant lines obtained from pKMS5GUS construct is maximum followed by plant lines obtained from construct pKMS8GUS (82% of pKMS5GUS activity) and pKMS17GUS (48% of pKMS5GUS activity), (data not presented for R_0 plants). Seeds were collected from self-fertilized independent Ro lines. Segregation analysis for the marker gene (Kan^R) was performed. About 8 to 9 individual R_1 transgenic lines, showing the expected segregation ratio (Kan^R : Kan^S = 3:1) for the marker Kan^R for each construct, were further analyzed. Whole seedling extracts were used for fluorometric GUS assays. The GUS activity in R_1 transgenic plants (Fig. 5B) is, however, 5 to 8 times

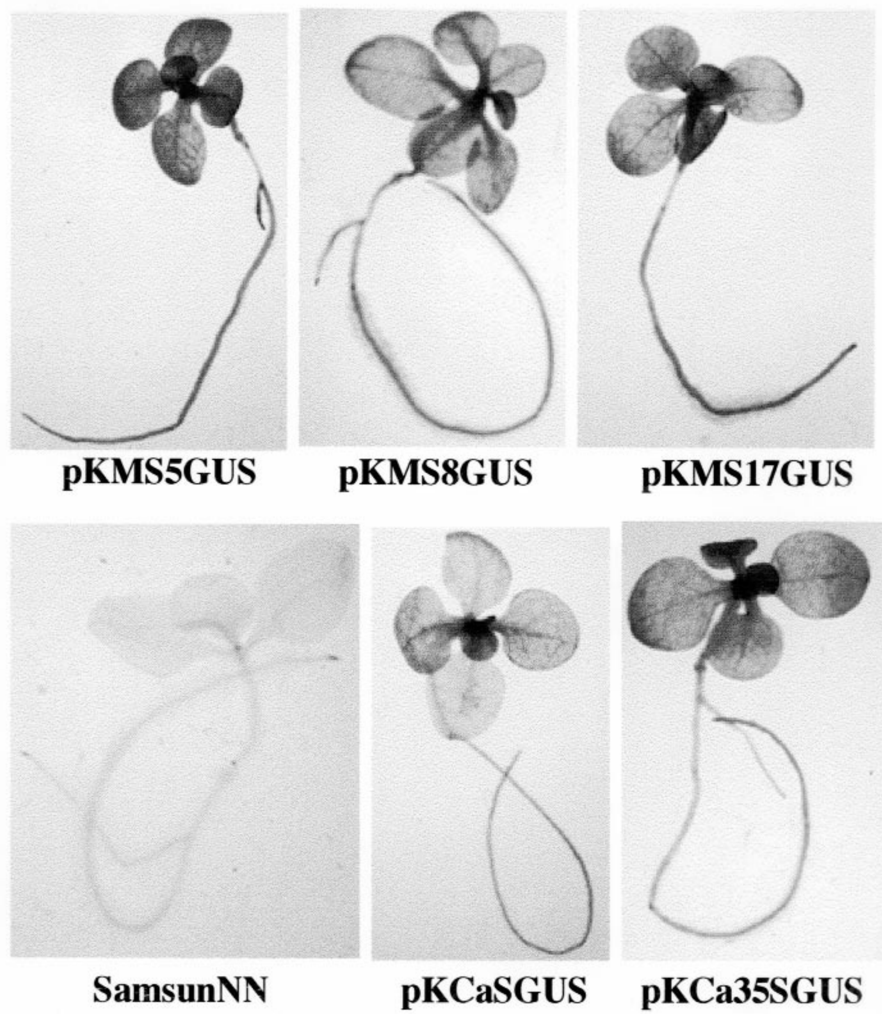

FIGURE 7
Histochemical assay of GUS expression in transgenic tobacco (*N. tabacum* cv. Samsun NN) seedlings (R1 progeny, 24-day old) developed for the following constructs: pKMS5GUS, pKMS8GUS, pKMS17GUS, pKCaSGUS (19S-GUS) and pKCa35SGUS (35S-GUS). These data were derived from pools of transformed lines with best expressing independent lines shown representing each construct. Untransformed control is shown wild type *N. tabacum* Samsun NN. See Color Plate VIII at back of issue.

higher than the GUS activity obtained in R_0 plants. Transgenic plants (R1 progeny) developed for construct pKMS5GUS showed highest activity followed by pKMS8GUS (-306 to + 27, 58% of pKMS5GUS) and pKMS17GUS (-456 to –19, 32% of pKMS5GUS). Histochemical GUS staining was carried out with whole seedlings separately from these three constructs showed comparable intensity of GUS activity (Fig. 7).

It is noted that in transient protoplast expression experiments the GUS expression

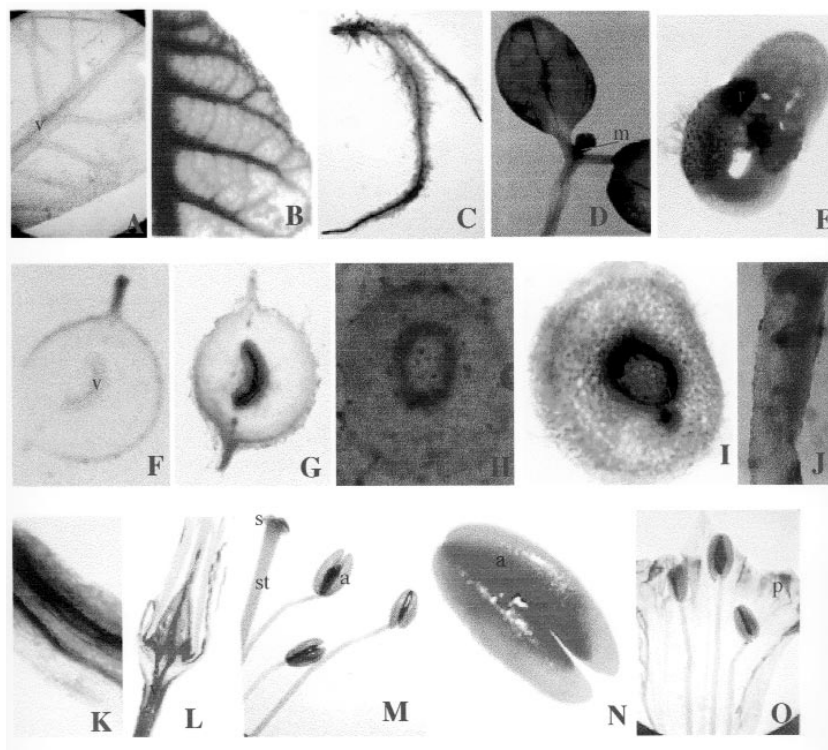

FIGURE 8
Histochemical localization of GUS activity in developing transgenic tobacco plants expressing the GUS reporter gene directed by MMV Sgt promoter. All sections are at 15X magnification. A. Samsun NN tobacco plant (non-transformed) as control; note no GUS staining. B. Matured leaf section from thirteen-weeks-old plants (R1 progeny) developed for the construct pKMS5GUS; note more GUS staining in vascular tissues (v), midrib and veins. C. Root from four-week-old seedlings (pKMS5GUS, R1 progeny) showing intense staining at the tip and in vascular (v) tissue. D. Top portion of ten-day old seedling (pKMS5GUS, R1 progeny); most GUS activity localized in leaves and apical meristematic (m) region. E. Transgenic tobacco seedling (pKMS5GUS, R1 progeny) at day 7 after imbibition, grown axenically on agar plate; GUS activity is localized primarily in root tips, root hairs and in the lower hypocotyls. F and G. Transverse cross section of petiole from control non-transformed Samsun NN (F), no GUS staining; and from four week-old seedlings (pKMS5GUS, R1 progeny), GUS staining is most intense in the vascular (v) cells (G). H, I, J and K. Transverse cross section (H and I) and longitudinal cross section (J and K) of stem from four week old control seedlings non-transformed Samsun NN (H and J respectively), note no GUS activity; and from four week old transformed seedlings (pKMD5GUS, R1 progeny); GUS activity localized mostly in vascular (v) tissues (I and K). L. Transverse section of tobacco flower pedicel and ovary; M. Stigma (s) and style (St); N. Anther (a); and O. The petal (p) and anther (a) in flower tissues display GUS staining. See Color Plate IX at back of issue.

level of the construct pPM8GUS (-306 to +27) was highest followed by constructs pPMS5GUS (-456 to +27, 89% of pPMS8GUS) and pPMS17GUS (-456 to –19, 52% of pPMS8GUS) (Fig. 3B). Whereas, in transgenic plants assay, the construct pKMS5GUS (–456 to +27) showed highest activity followed by construct pKMS8GUS (-306 to +27, 82% of pKMS5GUS) and pKMS17GUS (–456 to –19, 48% of pKMS5GUS) (Fig. 5B). The appropriate transacting factors needed for maximal pro-

moter function in construct 5 may be limited in protoplasts as compared to whole plants and this may be a reason, construct 5 gave less activity in protoplasts.

Comparative expression analysis of MMV Sgt promoter with MMV FLt, CaMV 35S and CaMV19S promoters

In pPCaSGUS or pKCaSGUS, the GUS reporter gene is directed by the CaMV subgenomic transcript promoter sequence (corresponding to CaMV genomic coordinates 5380 to 5773).

The MMV Sgt promoter constructs pKMS5GUS, pKMS8GUS and pKMS17GUS were compared with the CaMV promoters (19S and 35S), and three MMV FLt promoter constructs pKM1GUS, pKM12GUS and pKM13GUS developed earlier [22] both in protoplast assay (Fig. 5A) and transgenic plant expression analysis (Fig. 5B). In protoplast transient expression assays, the MMVSgt promoter fragments in constructs pPMS5GUS and pPMS8GUS showed more activity (5 and 7 fold, respectively), as compared to pPCaSGUS, and about 2 fold greater activity than CaMV 35S promoter (Fig. 5A). MMV Sgt promoter in pPMS5GUS and pPMS8GUS showed very comparable activity with MMV FLt promoter in pPM12GUS and pPM13GUS (Fig. 5A), suggesting that MMV Sgt promoter is comparable in strength to or greater than that of the MMV FLt promoter. The CaMV 19S is a weak promoter, as compared to the 35S promoter [14]. This suggests that the MMV Sgt promoter may have a different functional mechanism, as compared to the CaMV 19S promoter.

For stable transformation assays, a number of independent transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) lines were generated. Flurometric GUS assays were carried out with whole seedling (RI progeny) extracts. The results of transgenic plant analysis are shown in Fig. 5B. In the protoplast assay, the GUS expression with construct pPMS8GUS was highest. Three MMV Sgt promoter fragments analyzed in transgenic plants showed strong GUS expression compared to both CaMV 19S and 35S promoter. The level of expression of GUS reporter gene in pKMS5GUS (construct giving maximum GUS expression) is about 8 fold more than the CaMV 19S promoter and 2 fold more than the CaMV 35S promoter (Fig. 5B).

Constitutive expression of MMVSgt promoter in different parts of transgenic seedlings

The MMV Sgt promoter activity was examined in various tissues during seedling development. Transgenic seedlings (R1 progeny) were aseptically grown on an MS-agar medium in presence of kanamycin (240 μg/ml) supplemented with 3% sucrose. Seedlings from the independent lines showing segregation ratio (Kan^s: Kan^r =1:3) for the Kan^r gene were selected for further analysis. Eight independent lines for each construct were examined. The relative expression of the GUS reporter gene in 28-day old (4 weeks) seedlings (R1progeny, 2nd generation) transformed with pKMS5GUS, pKMS8GUS and pKMS15GUS were monitored by fluorometric GUS assay and by histochemical staining. A relative level of GUS activity in roots, leaves and stems is shown in Fig. 6. On average, GUS activity was slightly more in roots than in leaves followed by stems in seedlings developed for pKMS5GUS. Seedlings developed with pKM8GUS showed little more activity in leaves than in roots followed by

stems. Seedlings developed for construct pKMS17GUS showed more uniform GUS activity in different parts of seedlings, although slightly more activity in leaves followed by stems and roots (Fig. 6). The full-length transcript promoter (FLt) from FMV [18] PClSV [21] and MMV [22] showed about 2-fold more activity in roots compared to leaves. The expression level of CaMV 35S promoter in root tissue is about 4-fold higher than in leaves [30]. The CaMV 35S promoter contains two '*as*-1' motif TGACGA responsible for root specific expression and two '*as*-2' motif (GATA) for leaf specific expression [30]. The MMV Sgt promoter has only one unit of '*as*-1' like or '*as*-2' like motif. Further studies will be necessary to evaluate the impact of these or other cis- sequence, if any, on MMV Sgt promoter function. Different expression patterns in these promoter fragments may be due to the presence or absence of specific *cis*-sequence and their cognate interacting factors involved in promoter function. This suggests that caulimovirus promoters may have different functional mechanism.

The relative intensity of histochemical GUS staining of the young seedlings developed for these constructs pKMS5GUS and pKMS8GUS, pKMS17GUS showed strong promoter activities compared to pKCaSGUS (with CaMV19S promoter) and pKCa35SGUS (with CaMV 35S promoter) (Fig. 7).

Histochemical localization of GUS activity in transgenic plants

The MMV Sgt promoter activity was measured in various tissues during seedling (R1 progeny, second generation) development. The level of intensity of GUS activity was measured by histochemical staining of hand-cut fresh tissue sections of various organs of transgenic plants developed for the construct pKMS5GUS shown in Figure 8. Strong GUS activity was detected in vascular tissues in midrib and lateral secondary veins of matured leaves (Fig. 8B), in young leaves and in the apical meristem region of young seedlings (Fig 8D). Cross section of stems (Fig. 8I) and petioles (Fig. 8G) showed intense staining of the phloem cells. Strong GUS accumulation was detected in vascular tissues of roots and root tips (Fig. 8C). The non-transformed tobacco showed no GUS staining in mature leaves (Fig. 8A), in root tissues (data not shown) or in cross sections of stems (Fig 8H), and petioles (Fig. 8F). Histochemical GUS staining of different floral tissues was performed. The petal (corolla) portion of the flower showed light GUS staining (Fig. 8O). Anther containing pollen grains exhibited intense GUS activity (Fig. 8N). The stigma and style portion of the flower showed much less GUS staining (Fig. 8M). The longitudinal cross-section of the flower pedicel and ovary (6 days after opening of the flower) showed intense staining of the pedicel and the basal vascular part of the ovary (Fig. 8L). Differential GUS staining in various floral organs may be due to tissue specific expression of MMV Sgt promoter. Similar tissue specific expression pattern was documented for FLt promoter from CaMV [30], FMV [18], PClSV [21] and MMV [22, 23].

Our studies indicated that the MMV Sgt promoter is a strong constitutive promoter able to direct foreign gene expression in heterologous systems including transgenic plants at a greater level than that of both the CaMV35S and CaMV 19S promoters. There is a very limited sequence homology between the MMV Sgt promoter and other caulimovirus promoters, although they are func-

tionally analogous. For metabolic engineering, expression of multiple genes in a single cell will be necessary. The use of different promoters with non-homologous sequences may be useful in order to avoid genetic instability due to recombination between identical promoter sequences.

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