

Analysis of *cis*-sequence of subgenomic transcript promoter from the *Figwort mosaic virus* and comparison of promoter activity with the *cauliflower mosaic virus* promoters in monocot and dicot cells

Somnath Bhattacharyya, Nrisingha Dey, Indu B. Maiti *

Molecular Plant Virology and Plant Genetic Engineering Laboratory, Tobacco and Health Research Institute, University of Kentucky, Lexington, KY 40546-0236, USA

Received 12 February 2002; received in revised form 7 June 2002; accepted 24 June 2002

Abstract

A sub-genomic transcript (Sgt) promoter was isolated from the *Figwort mosaic virus* (FMV) genomic clone. The FMV Sgt promoter was linked to heterologous coding sequences to form a chimeric gene construct. The 5'-3'-boundaries required for maximal activity and involvement of *cis*-sequences for optimal expression in plants were defined by 5'-, 3'-end deletion and internal deletion analysis of FMV Sgt promoter fragments coupled with a β -glucuronidase reporter gene in both transient protoplast expression experiments and in transgenic plants. A 301 bp FMV Sgt promoter fragment (sequence -270 to +31 from the transcription start site; TSS) provided maximum promoter activity. The TSS of the FMV Sgt promoter was determined by primer extension analysis using total RNA from transgenic plants developed for FMV Sgt promoter: *uidA* fusion gene. An activator domain located upstream of the TATA box at -70 to -100 from TSS is absolutely required for promoter activity and its function is critically position-dependent with respect to TATA box. Two sequence motifs AGATTTTAAT (coordinates -100 to -91) and GTAAGCGC (coordinates -80 to -73) were found to be essential for promoter activity. The FMV Sgt promoter is less active in monocot cells; FMV Sgt promoter expression level was about 27.5-fold higher in tobacco cells compared to that in maize cells. Comparative expression analysis of FMV Sgt promoter with *cauliflower mosaic virus* (CaMV) 35S promoter showed that the FMV Sgt promoter is about 2-fold stronger than the CaMV 35S promoter. The FMV Sgt promoter is a constitutive promoter; expression level in seedlings was in the order: root > leaf > stem.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Caulimovirus*; *Figwort mosaic virus*; Sub-genomic transcript promoter; Promoter *cis*-sequence; Transgenic plants

1. Introduction

The *Figwort mosaic virus* (FMV), a species of the genus *Caulimovirus*, belonging to the family *Caulimoviridae*, has a small circular DNA genome

* Corresponding author. Tel.: +1-859-257-3296; fax: +1-859-323-1077

E-mail address: imaiti@pop.uky.edu (I.B. Maiti).

of approximately 8 kb with 7 open reading frames (ORFs) (Richins et al., 1987). The genome organization of FMV is similar to that of *Cauliflower mosaic virus* (CaMV), the type species of the genus *Caulimovirus* (Franck et al., 1980; Gardner et al., 1981).

In general, two major transcriptional promoters are present in the *Caulimovirus* genome. One to transcribe whole genome of the virus (a full-length transcript equivalent to CaMV 35S transcript) is located in the 3' terminus of ORF VI and extending into the large intergenic region. A second promoter that is situated at the 3' terminus of ORF V and extending into the small intergenic region between ORF V and VI, transcribes only the ORF VI, (a sub-genomic transcript equivalent to CaMV 19S transcript; Odell et al., 1981).

The role of *cis*- and cognate *trans*-acting factors and their interactions in promoter function have been studied extensively in mammalian, (Birnbau et al., 2001; Laurencikiene et al., 2001) *Drosophila* (Pham and Sauer, 2000) and yeast (Pilpel et al., 2001) systems but such factors and their interactions have been analyzed for only a limited number of plant promoters because consensus *cis*-sequences in plant promoters are often not evaluated to that extent. A number of plant transcriptional promoters have been isolated from double stranded DNA viruses belonging two subgroups of plant pararetrovirus genome (Caulimoviridae family), namely *Caulimovirus* (Odell et al., 1985; Hasegawa et al., 1989; Sanger et al., 1990; Verdaguer et al., 1996; Maiti et al., 1997; Maiti and Shepherd, 1998; Dey and Maiti, 1999a,b) and *Badnavirus* (Medberry et al., 1992; Bhattacharyya-Pakrasi et al., 1993). The CaMV 35S promoter has been well characterized (Benfy and Chua, 1989; Fang et al., 1989; Odell et al., 1985; Ow et al., 1987; Benfy and Chua, 1990; Benfy et al., 1990a,b; Lam, 1994). The CaMV 35S promoter is a strong constitutive promoter, and it has been used extensively for expressing foreign genes in monocotyledonous and dicotyledonous plants (Holtorf et al., 1995; Mitsuhara et al., 1996; Wilmlink et al., 1995). The 35S promoter from CaMV is also active in microbes and *Xenopus oocytes* (Assad and Signer, 1990; Probyecky et al., 1990; Ballas et al., 1989).

Transcriptional activity of the CaMV 35S promoter is the result of combinatorial and synergistic interaction of different *cis*-elements present in the promoter sequence and the *trans*-acting nuclear binding factors (Benfy and Chua, 1990; Benfy et al., 1990a,b; Fang et al., 1989), similar to other promoters such as SV40 promoter in mammalian systems (Schirm et al., 1987; Fromental et al., 1988; Ondek et al., 1988). Two nuclear binding protein factors, known as activating sequence factor-1 and -2 (ASF-1 and ASF-2) from tobacco have been well characterized. ASF-1 binds to the activating sequence as -1 (-82 to -62) region of the 35S promoter. Two TGACG motifs within the site are essential for DNA-protein interaction (Lam et al., 1989). The as -1 motif is also found in other caulimovirus promoters like FLt promoter of FMV (Maiti et al., 1997; Sanger et al., 1990), the major transcript promoter of PCISV (Richins, 1993) and mirabilis mosaic virus (MMV) (Dey and Maiti, 1999a). The ASF-1 element seems to be mainly responsible for root specific expression.

Promoters isolated from the *Badnaviruses* are reported to be primarily active in vascular tissues (Bhattacharyya-Pakrasi et al., 1993). Promoters from the *Rice tungro bacilliform virus* (RTBV) and *Commelina yellow mottle virus* (CoYMV) direct phloem specific gene expression in transgenic plants (Bhattacharyya-Pakrasi et al., 1993; Medberry et al., 1992; Yin et al., 1997).

The CaMV 19S promoter is a weak promoter compared to the 35S promoter (Lawton et al., 1987), although the product of the gene VI is the most abundant matrix protein in infected cells. We documented in tobacco cells that sub-genomic transcript (Sgt) promoters from the MMV (Dey and Maiti, Transgenics, in press) and from the FMV (Bhattacharyya et al., this report), in contrast to the CaMV 19S promoter, are strong constitutive promoters, with strengths comparable to or greater than that of the full-length transcript promoters of caulimoviruses, including CaMV 35S promoter.

In the present study, we report the identification and characterization of the FMV Sgt promoter. A 5'-, and 3'-end promoter deletion analysis showed that a 301 bp FMV Sgt promoter fragment (sequence -270 to +31 from the transcription

start site, TSS) is sufficient for maximum promoter activity. Activator and enhancer sequences have been revealed by fine deletion analysis of the promoter sequence. Sequence motifs GTAAGCGC (coordinates –80 to –73) and AGATTTTAAT (coordinates –100 to –91) were found to be very essential for promoter activity. The strength of this promoter was evaluated in electroporated protoplasts derived from cell suspension cultures of tobacco (Xanthi) and maize (BMS), and also in transgenic tobacco plants. The expression level in dicot tobacco cells is about 27-fold higher compared to that in monocot maize cells. It is a strong constitutive promoter. Strength of the FMV Sg1 promoter is greater than that of the CaMV 35S promoter.

2. Materials and methods

2.1. Protoplasts, plants and enzymes

Isolation of protoplasts from the tobacco cell suspension cultures (Xanthi 'Brad') and electroporation of tobacco protoplasts with supercoiled DNA containing the promoter fragment fused with a β -glucuronidase (GUS)-encoding gene were done essentially as described earlier (Maiti et al., 1998; Dey and Maiti, 1999a). The maize Black Mexican Sweet suspension culture (BMS-P2-S10) was obtained from Dr K. Scheets, Oklahoma State University, Oklahoma, USA. Isolation of protoplasts from the BMS cell suspension culture and electroporation of protoplasts with supercoiled DNA containing GUS constructs were performed essentially as described by Forman et al. (1987). Electroporation was performed by using the Gene Pulser II apparatus (BioRad, CA) with the Capacitance Extender II (Model 165-2107). An aliquot of 750 μ l containing 2×10^6 protoplasts in an electroporation cuvette (0.4 cm electrode gap) was electroporated (150 V used for charging 950 μ F capacitance for 50–60 ms) with 10 μ g of supercoiled plasmid DNA containing the GUS gene. After 20 h, protoplasts were harvested for GUS assay. Relative expression levels were within $\pm 10\%$ for a given construct in this study. All

constructs were tested at least in 4 independent experiments.

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were used for plant transformation. Restriction enzymes, antibiotics, components of plant tissue culture medium, RNA isolation kit were purchased from commercial sources and used according to the manufactures' specifications. Nytran membrane was obtained from Schleicher & Schuell (Keene, NH).

2.2. Construction of vectors for transient expression experiment in protoplasts

A series of FMV Sg1 promoter fragments were generated by PCR amplification to study the influence of *cis*-sequences upstream and downstream of TATA box on promoter activity. Defined FMV Sg1 promoter sequences, of indicated lengths (as in the Fig. 3A) were amplified by PCR from the FMV genomic clone (Richins et al., 1987) with appropriately designed primers to introduce an *Eco*RI site at the 5'-end and a *Hind*III site at the 3'-end of amplified products. PCR amplification was carried out for 30 cycles under the following standard conditions: denaturation (92 °C for 1 min), annealing (55 °C for 1 min), extension (68 °C for 2 min) using Platinum high fidelity *Taq*DNA polymerase from Gibco-BRL, ML (BRL Cat. No. 11304-011). Each PCR amplified fragment from 1 to 12 was restricted with *Eco*RI and *Hind*III; the restricted fragments were gel-purified and cloned into the corresponding sites of pUC119 vector and sequenced by dideoxy chain terminator method (Sanger et al., 1977) using synthetic primers. The sequence integrity of each of the FMV Sg1 promoter fragments cloned in pUC119 was verified before subcloning to a protoplast expression vector pUCPMAGUS, (Dey and Maiti, 1999a). FMV Sg1 promoter fragments were individually gel-purified from the corresponding pUC119 clone after restriction digestion with *Eco*RI and *Hind*III and subcloned into the corresponding sites of pUCPMAGUS (Dey and Maiti, 1999a). The following deletion plasmids were developed (Fig. 3A). The 5' and 3' coordinates of the promoter fragments with respect to TSS are given in

parentheses: pFS1GUS (–369 to +31) pFS2GUS (–320 to +31), pFS3GUS (–270 to +31), pFS4GUS (–220 to +31), pFS5GUS (–170 to +31), pFS6GUS (–120 to +31), pFS7GUS (–70 to +31), pFS8GUS (–369 to –70), pFS9GUS (–369 to –20), pFS10GUS (–369 to +1), pFS11GUS (–369 to +71), pFS12GUS (–369 to +101).

2.3. Construction of plasmids for internal deletion analysis of FMV Sgt promoter

The specified FMV Sgt promoter region was deleted from the full-length promoter fragment (coordinates –369 to +31 in respect of TSS) as depicted in Fig. 4A. Each of the following FMV Sgt promoter regions (coordinates –270 to –221; –270 to –171; –220 to –171; –170 to –121; –170 to –71; and –120 to –71 with respect to TSS) was subjected for internal deletion.

In PCR amplification, the region specified for internal deletion was excluded from the fragment by amplifying separately the upstream (1st half) and downstream (2nd half) regions of the desired fragment from the full-length promoter (FMV Sgt1) with appropriately designed primers to generate the fragment of following general structure: 5'-*EcoRI* – 1st half – *SmaI*–*HindIII*-3' and 5-*HincII* – 2nd half–*HindIII*-3'. The PCR amplified fragment was cloned into pUC119 or pBluescript(KS+) and DNA sequence was checked before use. Plasmid containing the first half was restricted with *SmaI* and *HindIII* and was used as a vector to insert the *HincII*/*HindIII* fragment from the plasmid containing the 2nd half of the specified region. The respective internal deletion fragment was cloned as *EcoRI*–*HindIII* fragment into the corresponding sites of pUCP-MAGUS as describe earlier. The resulting plasmids were designated as pID34, pID35, pID45, pID56, pID57, and pID67 (Fig. 4A).

2.4. Construction of plasmids to map the promoter region (coordinates –120 to –70 from TSS)

For further analysis of the promoter *cis*-sequence, the following promoter segments were generated as *EcoRI*–*HindIII* fragments by PCR

amplification using appropriately designed primers; the corresponding 5'- and 3'-end coordinates with respect to TSS shown in parentheses: 7a (–72 to +31); 7b (–80 to +31), 7c (–90 to +31), 7d (–100 to +31), 7e (–110 to +31). The respective promoter fragment was cloned as *EcoRI*–*HindIII* fragment into the corresponding sites of pUCP-MAGUS as described earlier. The resulting plasmids were designated as p7a, p7b, p7c, p7d, p7e, respectively (Fig. 3A).

2.5. Construction of plasmid pdAE to examine position effect of an AE by placing it further upstream of TATA box

A 30-bp activator element (AE, coordinates –100 to –70 with respect to TSS) was PCR amplified as a 5'-*EcoRI*–AE–*SmaI*–*HindIII*-3' fragment from the full-length promoter FMVSgt1 using appropriately designed primers. The fragment was cloned into pUC119 as an *EcoRI*–*HindIII* fragment. The resulting plasmid was named pUFSAE. A 150-bp promoter fragment (5'-end at –170) that is devoid of the AE sequence was PCR amplified from pID67 with appropriately designed primers to insert a *HincII* site at the 5'-end and a *HindIII* site at the 3'-end of the fragment. This PCR-fragment was cloned as *HincII*–*HindIII* into pUC119 to generate the plasmid pUFS5ΔAE. A ~150-bp *HincII*–*HindIII* fragment isolated from plasmid pUFS5ΔAE was inserted into the *SmaI* and *HindIII* digested vector pUFSAE. The resulting plasmid was named as pUFS5^{–120}. The promoter sequence isolated as an *EcoRI*–*HindIII* fragment from pUFS5^{–120} was cloned into the corresponding sites of pUCP-MAGUS as described earlier to generate the plasmid pdAE.

2.6. Construction of plant expression vectors with FMV Sgt promoter, plant transformation and analysis of transgenic plants

The FMV Sgt promoter fragments isolated from pFS1GUS (coordinates –369 to +31 from TSS) pFS3GUS (coordinates –270 to +31 from TSS) were cloned into the plant expression vector pKYLX71 GUS (Schardl et al., 1987; Dey and

Maiti 1999a) at its unique *Eco*RI and *Hind*III sites that flank the promoter. Resulting plant expression vectors were designated pKFS1GUS and pKFS3GUS, respectively. Constructs pKFS1GUS and pKFS3GUS were introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed with the engineered *Agrobacterium* as described earlier (Maiti et al., 1993). About 12 independent plant lines were generated for each construct. Regenerated kanamycin-resistant plants were grown under greenhouse conditions.

2.7. β -Glucuronidase assay

Fluorometric GUS assays to measure GUS activity in plant tissue or protoplast extracts and histochemical GUS staining to localize the distribution of GUS activity in plants were performed according to Jefferson et al. (1987) as described earlier (Maiti et al., 1997). Total protein content in plant extracts was determined according to the method of Bradford (1976) using BSA as a standard.

2.8. RNA extraction, Northern blot and RNA dot blot analysis

Protoplasts were transformed with individual FMV Sgt promoter constructs. After 12 h, total RNA was prepared from the transformed protoplasts by using an RNA extraction kit (Plant RNeasy kit, Cat No. 74904, QIAgen, CA) with RNase-free DNase treatment as per manufacturer's specification. For northern blot analysis, total RNA (10 μ g) was separated on 1.0% agarose gel containing 1 \times denaturing gel buffer (Ambion Inc., Austin, TX; Cat.#8676). After electrophoresis the gel was rinsed with 1X MOPS gel running buffer. RNA from the gel was transferred onto the Nytran membrane (Schleicher & Schuell; Cat.#77403) by downward movement using Ambion's transfer buffer (Ambion Inc.; Cat.#8672G). The GUS probe was made using the random primer labelling kit (Stratagene, La Jolla, CA; Cat.#300385). RNA on the membrane was fixed by UV cross-linking (Stratalinker, Stratagene) at

auto-setting. The membrane was prehybridized for 1 h at 42 °C in Northmax hybridization solution (Ambion Inc.; Cat.#8670G). Hybridization was carried out under the same conditions using ³²P-labeled GUS probe for 20 h. After hybridization the membrane was washed three times at 65 °C for 15 min each; once in low stringency washing solution (Ambion, Cat.#8673G) followed by two high stringency washings (Ambion, Cat.#8674G) and finally exposed to X-ray film.

2.8.1. RNA dot blot

An aliquot of 10 μ g of RNA was denatured with 50% deionized formamide and 7% formaldehyde at 65 °C for 15 min then chilled on ice. Denatured RNA samples were diluted with 6XSSC to 500 μ l and transferred onto the pre wet Nytran membrane in 6XSSC using a BRL hybrid-dot manifold. After RNA transfer the membrane was UV cross-linked. It was hybridized with ³²P-labeled GUS probe, washed and detected by autoradiography as described above for Northern blot.

2.9. Determination of TSS of FMV Sgt promoter and DNA sequencing

The transcriptional start site was determined by primer extension analysis as described earlier (Dey and Maiti 1999a). Sequencing reactions were carried out according to Sanger et al. (1977) using SEQUENASE Version 2.0, USB. Products of sequencing reactions and the primer extension product were run side by side on the same sequencing gel (Sambrook et al., 1989). Automated DNA sequencing was done with an Applied Biosystem ABI Prism 310 Genetic Analyzer (Perkin Elmer) using ABI Prism Dye terminator cycle sequencing ready reaction kit containing Ampli Taq DNA polymerase as described earlier (Dey and Maiti 1999a).

3. Results and discussion

3.1. Structure and sequence analysis of FMV Sgt promoter

The FMV Sgt promoter was isolated from a genomic clone (FMV, strain DxS; Richins et al.,

1987). Nucleotide sequence of the FMV Sgt promoter (FMV genomic coordinates 4963–5432) is shown in Fig. 1. The TSS (TTCTAAA, start site in bold letter, FMV genomic coordinates 5332) was determined by primer extension analysis.

The FMV Sgt promoter sequence does not contain a consensus eukaryotic regulatory sequence such as TATATAA or CAAT box that are present in full-length transcript promoters of caulimoviruses. It has a TATA-like element (TATAAA) at position –42 to –46. The TATA box of MMV Sgt promoter has a similar sequence (TATAAA) instead of consensus TATA box (Dey and Maiti, in press). Deletion analysis presented in a following section indicates that TATAAA sequence of FMV Sgt promoter functions as a TATA element. In FMV Sgt promoter, a transcriptional enhancer element (TGACG) similar to the core sequence of the *as*–1 motif (Lam, 1994) is

present at 21 bp upstream of TATA element. However, it is not in duplicate as is usually present in full-length transcript promoters of FMV, CaMV and MMV (Odell et al., 1985; Maiti et al., 1997 and Dey and Maiti, 1999a). In addition, there are several direct repetitive sequences AGAATTA (marked as 1a, 1b, 1c, 1d, and 1e); AAAAACG (denoted as 2a, 2b, 2c); AGATT (denoted as 3a, 3b, 3c) and ACAAATT (denoted as 4a and 4b) are not present in other caulimovirus promoters but they may have some regulatory function. More work will be needed to evaluate their regulatory role, if any, in promoter function.

The FMV Sgt promoter has a high AT content (66%). DNA sequence alignment (ClustalW multiple sequence alignment program) analysis of FMV Sgt promoter sequence with that of full-length transcript promoters from FMV, PCISV, MMV and CaMV showed about 25–34% sequence identity. DNA sequence identity of FMV Sgt

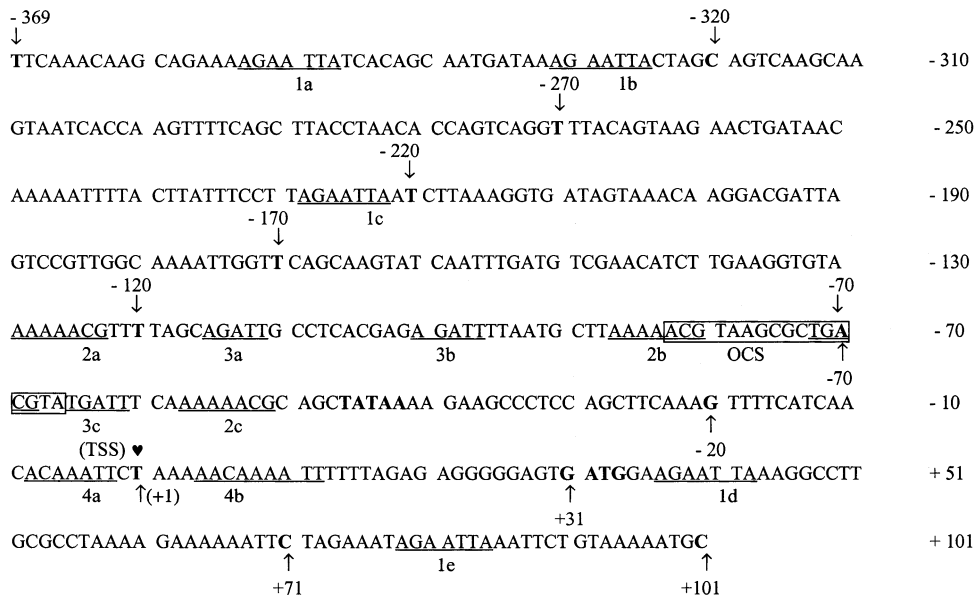


Fig. 1. The DNA sequence of the sub-genomic transcript (Sgt) promoter of the FMV. A 470 bp fragment (–369 to +101 with respect to the TSS; corresponding coordinates in FMV genome 4963–5432) includes the 3'-end of FMV ORF V (–369 to –84 from TSS; corresponding FMV coordinates 4963–5248) followed by the small intergenic region (–83 to +31 in respect of TSS) and the 5' portion of the FMV ORF VI (coordinates +32 to +101 from TSS) presented from left to right in the 5' to 3' direction. The TSS is indicated (♥) as +1. An arrow above or below the sequence indicates the end point of the 5' or the 3' deletion fragment, respectively. The tentative TATA box, and the ATG codon of FMV ORF VI are shown in bold. All repeat sequence domains (designated 1a, 1b, 1c, 1d, 1e; 2a, 2b, 2c; 3a, 3b, 3c; 4a, 4b) are underlined. The region homologous to the OCS element containing as–1 like sequence TGACG shown in box.

promoter with that of the CaMV 19S promoter, MMV Sgt promoter and PCISV Sgt promoter is 55, 54, and 32%, respectively.

3.2. Transcription start of FMV Sgt promoter

The TSS of the FMV Sgt promoter was determined by primer extension analysis with total RNA isolated from transgenic tobacco seedlings (homozygous with respect to the Kan^R marker gene, R2 progeny) developed for the construct pKFS3GUS. A major extension product was detected and mapped to a T residue that is located at 46-nucleotides downstream of the TATA element (Fig. 2). Most likely it represents the 5'-end of the FMV Sgt transcript in this context. Sequence comparisons of the TSS with other cauli-

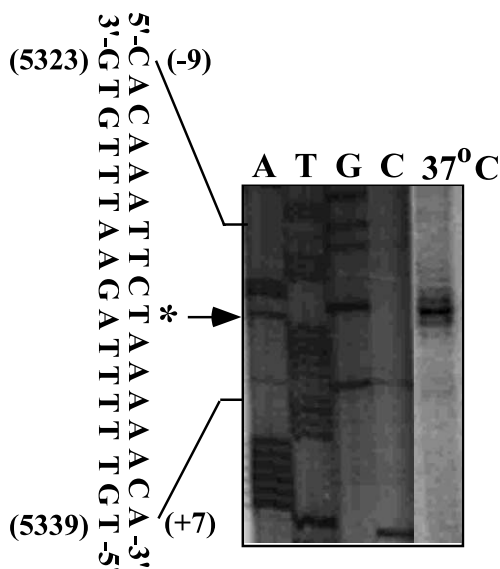


Fig. 2. Determination of the transcriptional start site (TSS) of the FMV Sgt promoter by primer extension analysis. Primer extension was carried out as described in Section 2; annealing was done at 37 °C. Primer extension reaction products were subjected to electrophoresis on denaturing polyacrylamide gel containing urea alongside with the sequence reaction of GUS gene construct (lane A, T, G and C) performed with the same labeled primer. The minus strand DNA sequence read on the gel is shown and the transcriptional start site (T*) in the corresponding plus strand is indicated by an arrow. Numbers in parentheses correspond to the FMV Sgt promoter coordinates -9 to +7 with respect to TSS (corresponding nucleotide coordinates 5323–5339 of the FMV genome, Richins et al., 1987).

movirus promoters showed little overall sequence homology.

3.3. 5'- and 3'-end deletion analysis of FMV Sgt promoter

In order to define the boundaries required for maximal promoter activity and to analyze the influence of *cis*-sequences upstream and downstream of the TATA element on promoter activity, a 470-bp FMV Sgt promoter fragment (coordinates -369 to +101 with respect to TSS) was subjected to 5'- and 3'-end deletion analysis. Twelve promoter fragments generated by PCR amplification were cloned into the protoplast expression vector pUCPMAGUS as described in Section 2. A schematic map of deletion constructs is shown in Fig. 3A. The 5'- and 3'-end points of each fragment are indicated in parentheses. Promoter fragments containing the GUS reporter gene were introduced into tobacco protoplasts for transient expression assays. Results of the expression analysis of the FMV Sgt promoter are shown in Fig. 3B. The expression level of construct 1 (pFS1GUS, coordinates -369 to +31 from TSS) was 7.02 ± 1.01 nmol MU/min/(mg protein), and this value was considered to represent 100% promoter activity for comparison with other end deletion and internal deletion constructs presented here and later sections. The expression level of deletion constructs 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 was 135, 169.5, 123.4, 85.5, 70.8, 3.3, 0, 5.1, 100, 4.1, and 5.1%, respectively, relative to construct 1. Our results showed that a deletion of ~50 bp (-369 to -321) from the 5'-end in construct 2, resulted in a 35% increase in promoter activity. Further deletion by ~100 bp from the 5'-end resulted in a 69% increase in promoter activity. Construct 3 (pFS3GUS, coordinates -270 to +31 from TSS) gave maximum promoter activity. This data suggests that the upstream promoter sequence between -369 and -271 from TSS, in this context, may not be essential for maximal promoter activity. However, internal deletion analysis described later shows that expression may depend on the combinatorial organization of promoter sequences. Successive deletion of ~150, ~200, 250 bp from the 5'-end in construct

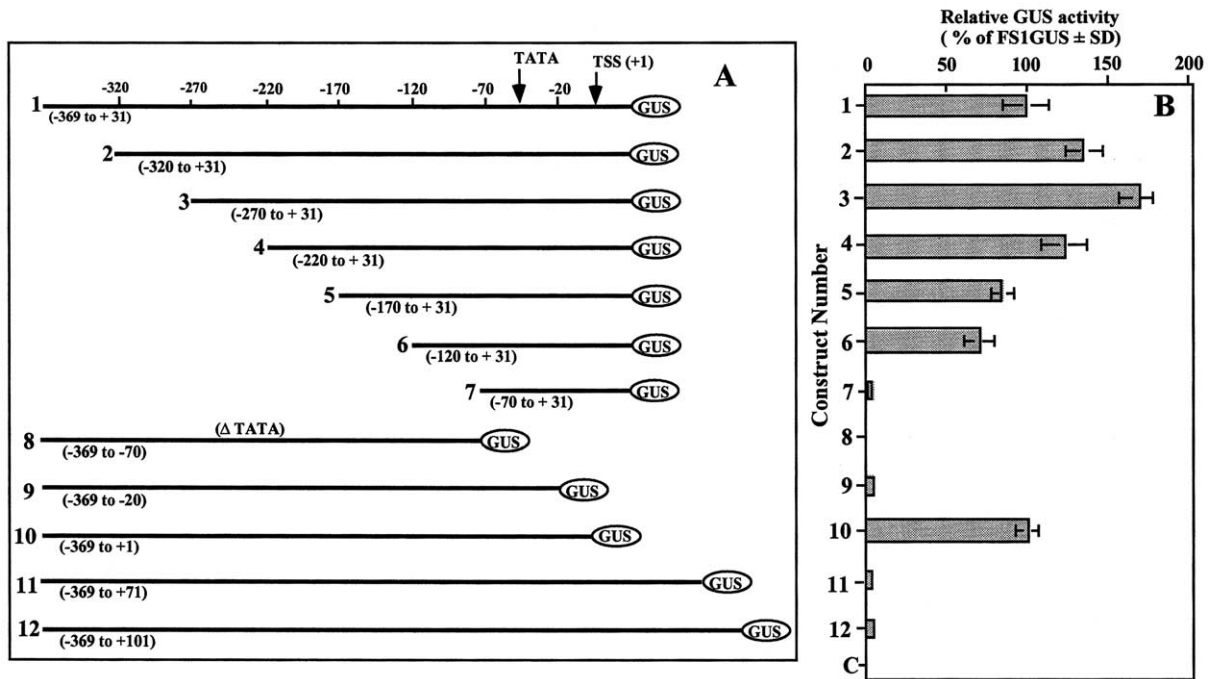


Fig. 3. (A) A schematic map of the GUS constructs (number 1–12) developed for the deletion analysis of FMV Sgt promoter. The 5′- and 3′-end coordinates of the relative deletion fragments are given in parentheses. The end points for the 5′- or the 3′-end deletion constructs are also indicated above or below the sequence respectively in Fig. 1. At the top, the relative position of the TATA box, TSS (+1) and the FMV Sgt promoter coordinates are shown. (B) FMV 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 four independent experiments. The average GUS activity (as % of FS1GUS) with standard deviation is presented in the histogram. Error bars show the 95% confidence intervals of the means. Statistical (one-way analysis of variance, ANOVA) analysis showed an extremely significant *P* value of < 0.001. (C) Untransformed control, extract from protoplasts.

4, 5, and 6 respectively, reduced the promoter activity to 123.4, 85.5, and 70.8% respectively, compared to the maximal activity (169.5%) obtained with construct 3. Further deletion of ~50 bp (coordinates –120 to –71 with respect to TSS) in construct 7 (pFS7GUS, coordinates –70 to +31 from TSS) drastically reduced the promoter activity to 3.3% suggesting the importance of the *cis*-sequence present in this region for promoter activity. This TATA-upstream region (coordinates –120 to –71 from TSS) was chosen for more detailed analysis as described in a later section.

Construct 8 (pFS8GUS, coordinates –369 to –70 from TSS) that is devoid of the TATA element showed no or below the detection level of promoter activity. This demonstrates the importance of the TATA element for FMV Sgt promoter func-

tion, although the FMV Sgt TATA box sequence (TATAAA) does not share the same consensus as that of the FLt promoter TATA sequence (TA-TATAA) present in other caulimovirus including CaMV, PCISV. The MMV Sgt promoter has a similar TATAAA sequence that was also shown to function as a TATA element for the MMV Sgt promoter (Dey and Maiti, *in press*). However, no CAAT-like sequence was detected in FMV Sgt sequence in contrast to MMV Sgt promoter (Dey and Maiti, *in press*).

The 3′-end deletion construct 9 (pFS9GUS, coordinates –369 to –20 from TSS), showed much less activity (5.1%) compared to construct 10 (pFS10GUS, coordinates –369 to +1) that gave 100% activity, suggesting the importance of the TATA downstream *cis*-sequence (–21 to +1

from TSS) for FMV Sgt promoter function. Deletion constructs 11 (pFS11GUS, –369 to +71) and 12 (pFS12GUS, –369 to +101) with extended untranslated leader sequences showed strongly decreased promoter activity, 4–5% as compared to construct 1 (pFS1GUS, coordinate –369 to +31). In this context, the extended leader sequence (coordinates +32 to +101 from TSS) has significant inhibitory effect on promoter function. In the case of caulimovirus FLt promoters, which have 25–30 nt longer untranslated leader sequences compared to that of Sgt promoters, extended leader sequence is required for maximum promoter activity (Mandy et al., 1993; Dey and Maiti, 1999a). In contrast, for the FMV Sgt promoter (this study) and MMV Sgt promoter (Dey and Maiti, in press) containing extended leader sequences (beyond +31 for FSgt, and +27 for MSgt promoter) were found to reduce promoter activity.

The transcript level in construct 11 (coordinates –369 to +71) and 12 (coordinates –369 to +101) containing longer leader sequence was relatively less (Fig. 5A) and the promoter activity was reduced probably through its effect of longer untranslated leader sequence on transcription and subsequent translation. It may have some implication on the formation of secondary structure of transcripts. In this context, the inclusion of 5'-end portion of gene VI (37- and 70-nt in construct 11 and 12, respectively) reduced the FMV Sgt promoter activity whereas MMV Sgt showed better activity when fused with the 5'-end portion of gene VI (Dey and Maiti, in press). More work will be needed to evaluate the regulatory role, if any, of untranslated leader sequence derived from gene VI sequence in promoter function. The functional mechanism of various caulimovirus Sgt promoters may differ within the wild type caulimoviruses.

3.4. Internal deletion analysis of FMV Sgt promoter

The 5'- and 3'-end deletion analysis of FMV Sgt promoter suggests a regulatory role of the *cis*-region present in between coordinates –270 to –221 and –220 to –171 with respect to TSS

(compare the expression level of construct 3, 4, and 5; Fig. 3B). In order to elucidate whether these domains can function independently or in combination with other regions, internal deletion analysis was carried out as described below. Internal deletion promoter fragments were generated and cloned into the protoplast expression vector pUCPMAGUS (Dey and Maiti, 1999a) as described in Section 2. A general scheme of the internal deletion and results are shown in Fig. 4A and B, respectively. Construct ID34 with an internal deletion of ~50-bp located between the 5'-ends of construct 3 and 4 (coordinates –270 to –221) showed about 94.5% promoter activity compared to pFS1GUS (coordinates –369 to +31 from TSS) giving 100% activity. Construct ID35 with an internal deletion of ~100-bp sequence between 5'-ends of construct 3 and 5 gave 164% promoter activity. In construct ID45, deletion of 50-bp sequence located between 5'-ends of construct 4 and 5 (coordinates –220 to –171 from TSS) resulted in a 164% increase of promoter activity. Taking these data together, it indicates that a 100-bp sequence (coordinates –270 to –171 from TSS) that is essential for maximal promoter activity shown in construct 3 (coordinates –270 to +31 from TSS, Fig. 3), may not be essential in this context (compare construct 1 with construct ID35 and ID45) for maximal promoter activity when it is replaced with the upstream sequence of coordinates –369 to –271 from TSS. As mentioned in the previous section, our results suggest that FMV Sgt promoter function may be optimized by different combinatorial organizations of *cis*-sequence domains located upstream of the TATA elements.

Construct ID56 that has an internal deletion of ~50-bp (coordinates –170 to –121) located between 5'-ends of construct 5 and 6, showed 134.7% promoter activity. This result suggests that this sequence is not critical for promoter function in this context. However, further downstream of this, a deletion encompassing ~100 bp (coordinates –170 to –71) in construct ID57, or of 50 bp (coordinates –120 to –71) in construct ID67, resulted in a drastic reduction of promoter activity to 2.4 or 2.7%, respectively compared to construct 1, representing 100% activity. Internal deletion

analysis of the FMV Sgt promoter with constructs ID56, ID57 and ID67 (Fig. 4A and B), clearly showed the presence of an AE, a ~50 bp *cis*-sequence (coordinates –120 to –70 from TSS) located upstream of the TATA element, and it is absolutely required for promoter function. This AE domain was analyzed further as shown below.

3.5. Finer deletion analysis of *cis*-domain (coordinates –120 to –70 from TSS)

A detailed deletion analysis was conducted in order to dissect further the positive regulatory sequence present in the AE (coordinates –120 to –70 from TSS). A schematic deletion map and results are shown in Fig. 4A and C, respectively. Construct 7 (coordinates –70 to +31) gave 3.3% of promoter activity compared to FS1GUS representing 100% promoter activity. In construct 7a (coordinate –72 to +31), extension of 5'-end with two more nt gave only 4% of the relative activity but with eight additional nts at the 5'-end as in construct 7b (coordinates –80 to +31 from TSS), the promoter activity increased to 89.3% indicating the importance of this 8-nt *cis*-sequence (GTAAGCGC, coordinates –80 to –73 from TSS; Fig. 1) for promoter function. Construct 7c (coordinates –90 to +31), containing a 5'-end with 10 additional nts, gave 94.9% promoter activity. Further extension of 5'-end with 10 more nt in construct 7d (coordinates –100 to +31 from TSS) increased the promoter activity to 196.6%, compared to the represented value of pFS1GUS (100%). Thus, this 10-nt sequence (AGATTTTAAT; coordinates –100 to –91 from TSS) is essential in enhancing FMV Sgt promoter activity. Construct 7e (coordinates –110 to +31 from TSS) with the 5'-end extended by 10 additional nt, gave 106.8% promoter activity. Our results show that the presence of two *cis*-acting domains within the 30-bp promoter sequence (coordinates –100 to –70 from TSS) is absolutely required for promoter activity.

A 17-nt motif with strong homology with the activator sequence OCS element (Ellis et al., 1993) is present in the FMV Sgt promoter (coordinates –82 to –66 from TSS). The *as*–1 element found in CaMV 35S promoter is also present in this

region (TGACGTAT, coordinates –72 to –65 from TSS). Our results showed that the inclusion of the *as*–1 sequence in construct 7a (coordinate –72 to +31 from TSS) was not sufficient to enhance promoter activity. However, inclusion of sequence further upstream in constructs 7b and 7d, was shown to increase promoter activity. The FMV AE is located within 35-nt upstream of the TATA element. It has been reported that the AE is present within 10-nt upstream of the TATA box for the RTBV promoter (He et al., 2000), the α -amylase gene promoter (Willmott et al., 1998) and the parsley 4CL1 promoter (Neustaedter et al., 1999). The nucleotide sequence in the AE-domain of the α -amylase promoter (–50GATCA-CATCCCCCT-36), the parsley 4CL1 promoter (5'-TCCCCATTTACCCCT-3') and the RTBV promoter (–70GTAAGAGTGTGTAATGAC-CAG TGTGCCCTGGACTC-35) is pyrimidine-rich, whereas that in FMV Sgt promoter (–100AGATTTTAATGCTTAAAAACG-TAAGCGCTGACG TA-66) lacks such a profile.

3.6. Position dependency of AE

The AE domain (coordinates –100 to –70 from TSS) of the FMV Sgt promoter was placed 50-bp further upstream, at –170 from TSS in construct pdAE; the promoter activity was drastically reduced and this indicates that the functional activity of this AE sequence is position-dependent. We speculate that this *cis*-domain of the FMV Sgt promoter may bind its cognate *trans*-acting factor(s) that may also interact with TATA-binding elements such that further displacement of the AE domain may prevent complex formation. More work is needed to gain insight in the analysis of the AE in the FMV Sgt promoter. It has been reported for other plant promoters that transcription factors that bind to TATA-upstream sequences also interact with the TFIIA–TBP–TATA transcriptional pre-initiation complex (Le Gourierrec et al., 1999) and increases in distance from the initiation complex with respect to the TATA box reduces its functional activity (Roberts et al., 1995; He et al., 2000).

3.7. RNA dot blot and Northern analysis

The relative strengths of the FMV Sgt promoter fragments fused with GUS reporter gene were determined by hybridization analysis of total RNA. Total RNA was isolated from protoplasts 12 h after transfection. RNA dot blot analysis was performed using ^{32}P -labeled GUS coding sequence as probe, for each 5'- and 3'-end deletion construct (No. 1–12 as described in Fig. 3A) as well as internal deletion constructs (ID34, ID35, ID45, ID56, ID57, and ID67 as described in Fig. 4A). Results are depicted in Fig. 5A. Transcript levels of these constructs showed very good agreement with the promoter expression activity using GUS reporter gene activities. The highest signal was obtained for 5'-end deletion construct 3 and the minimum signal was obtained from construct 8, which is devoid of the TATA element.

RNA dot blot analysis of internal deletion constructs showed a low signal for construct ID56 and ID67 but higher signal for constructs ID34, ID35, ID45 and ID56 (Fig. 5A). The relative intensity of each blot is in good correlation with GUS activity obtained for the corresponding construct.

Northern analysis of total RNA isolated from transgenic tobacco seedlings (R1 progeny, 2nd generation) developed for the construct pKFS3GUS and pK35SGUS showed expected size of GUS transcript (2100-nt) (Fig. 5B)

3.8. Comparative expression analysis of FMV Sgt promoter in monocot and dicot protoplasts

Comparative expression analysis of the FMV Sgt promoter with CaMV 35S promoter was performed in protoplast transient expression experiments using GUS as a reporter gene. Protoplasts were isolated from suspension cell cultures established for monocot (maize BMS culture) and dicot (tobacco Xanthi) plants as described in Section 2. Results are shown in Fig. 6. The expression level of FMV Sgt promoter is about 27-fold higher in tobacco as compared to that with maize cells. The FMV Sgt promoter is about 2-fold greater in strength than that of the CaMV 35S promoter. However it is interesting to note that the

CaMV 35S promoter gives higher expression in maize protoplasts compared to that of the FMV Sgt promoter.

3.9. Expression analysis of FMV Sgt promoter in transgenic plants

A number of independent tobacco transgenic lines were developed for the constructs pKFS1GUS and pKFS3GUS. Comparative expression of GUS activity in different plant tissues (roots and leaves) of seedlings (R1 progeny, 2nd generation) was carried out for plants developed for FMV Sgt promoter analysis and CaMV 35S promoter. In transgenic plants harboring the FMV Sgt promoter fusion constructs, distribution of GUS activity was about 5-fold more in roots than in leaves (Fig. 7A). Histochemical staining of whole seedlings developed for pKFS3GUS showed intense GUS staining in root tissue compared to leaf tissue (Fig. 7B). The expression level of CaMV 35S promoter in root tissue is about 4-fold higher than that seen in leaves (Fig. 7A). It has been reported that the full-length transcript promoter from FMV (Maiti et al., 1997), PCISV (Maiti and Shepherd, 1998) and MMV (Dey and Maiti, 1999b) showed about 2-fold more activity in roots compared to leaves. The FMV Sgt promoter showed more activity in roots compared to other caulimovirus analyzed in this context.

3.9.1. FMV Sgt promoter activity in flower organs

FMV Sgt promoter activity was also measured in flower organs from ten independent homozygous lines (in respect of Kan^R marker gene), developed for the construct pKS3GUS. Flower samples were collected 1 day before anthesis. The promoter activity was found to be relatively low in flowers compared to roots and leaves of plants, with a pattern of expression ranked follows: calyx > filament > styles > pedicel > ovary > stigma. Similar patterns have been observed for the full-length transcript promoter of CaMV 35S promoter (An et al., 1988), FMV (Maiti et al., 1997) and MMV (Dey and Maiti, 1999b).

The present investigation documents that an AE is present in the FMV Sgt promoter; located upstream of the TATA element, that is absolutely

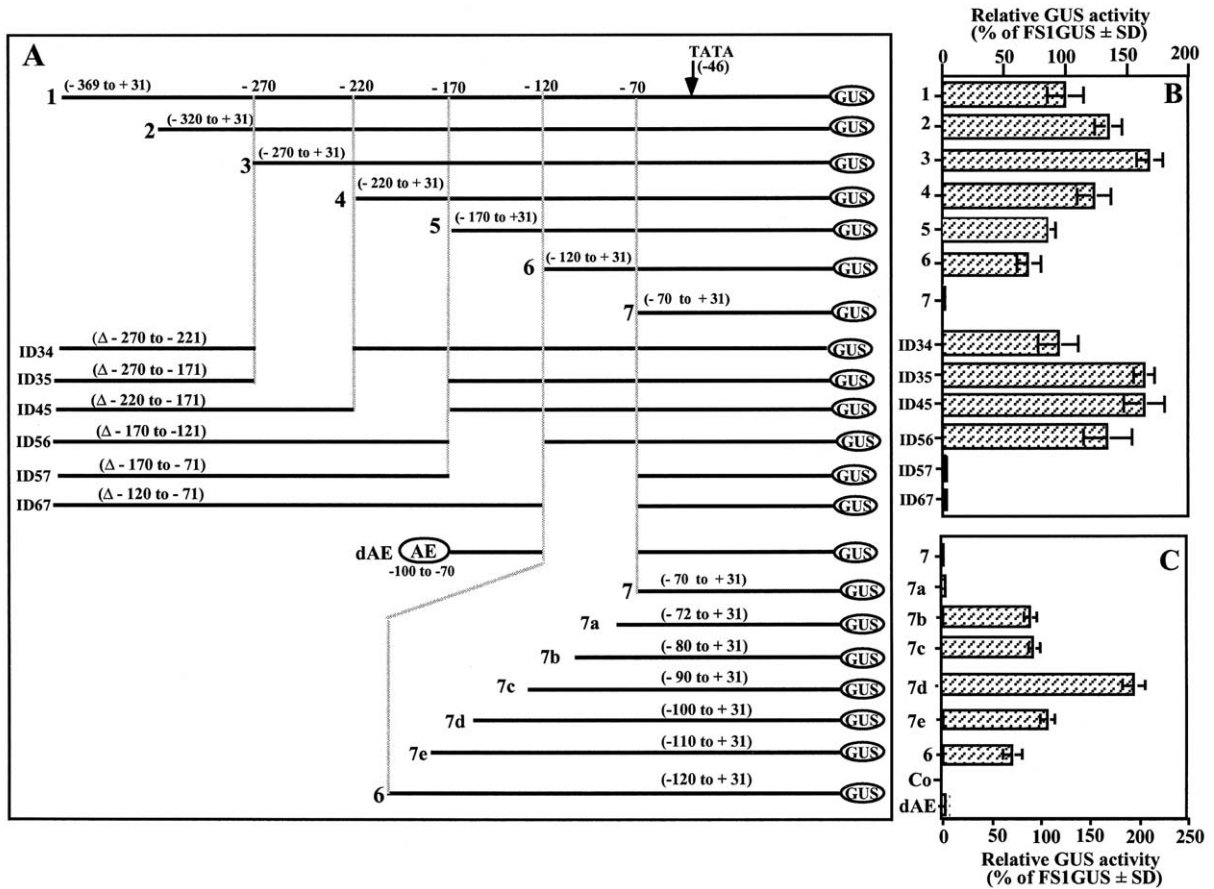


Fig. 4. (A) A schematic representation of the internal deletion constructs and 5'-end deletion constructs of FMV Sgt promoter used to map the AE. At the top, the relative position of the TATA box, and the coordinates with respect to TSS of FMV Sgt promoter are shown. The 5'- and 3'-end coordinates of the relative deletion fragments are given in parentheses (construct number 1–7) as pointed out in Fig. 3A. Constructs developed for internal deletion of FMV Sgt promoter are denoted as ID34, ID35, ID45, ID56, ID57, and ID67. Deleted regions marked with lighter vertical lines and promoter coordinates of deleted regions in parenthesis are shown. In the bottom part of (A), constructs denoted as 7a, 7b, 7c, 7d, 7e were generated to map the promoter region -120 to -70 from TSS. This region is located in between the 5'-ends of constructs 6 and 7 as pointed out by lighter vertical line. (B) Expression analysis of FMV Sgt promoter fragments in protoplast transient expression assay using a GUS reporter gene. Soluble protein extracts ($5 \mu\text{g}$) from transformed protoplasts were used for GUS assays as described in Section 2. Construct number 1–7 shown in Fig. 3 are taken for comparisons with internal deletion constructs ID34, ID35, ID45, ID56, ID57, ID67. Each construct was assayed at least three times in four independent experiments. The average GUS activity (as % of FS1GUS) with standard division is presented in the histogram. Error bars show the 95% confidence intervals of the means. The statistical (one-way ANOVA) analysis showed an extremely significant P value of <0.001 . (C) Expression analysis of FMV Sgt promoter fragments in construct 7a, 7b, 7c, 7d, 7e in protoplast transient expression assay using GUS reporter gene. For comparison, level of expression for construct 7 and 6 are also shown. Expression levels of the internal deletion construct (pdAE) with activator sequence (promoter coordinates -100 to -71 from TSS) placed upstream of -170 ; and control (Co), extract from untransformed protoplast are shown. Each construct was assayed at least three times in four independent experiments. The average GUS activity (as % of FS1GUS) with standard division is presented in the histogram. The statistical (one-way ANOVA) analysis showed an extremely significant P value of <0.001 .

essential for promoter function. The function of the AE is critically dependent on its position relative to the promoter TATA box. The FMV

Sgt promoter is constitutive in nature and can direct expression of genes in transgenic plants. The expression level in roots is higher than that of

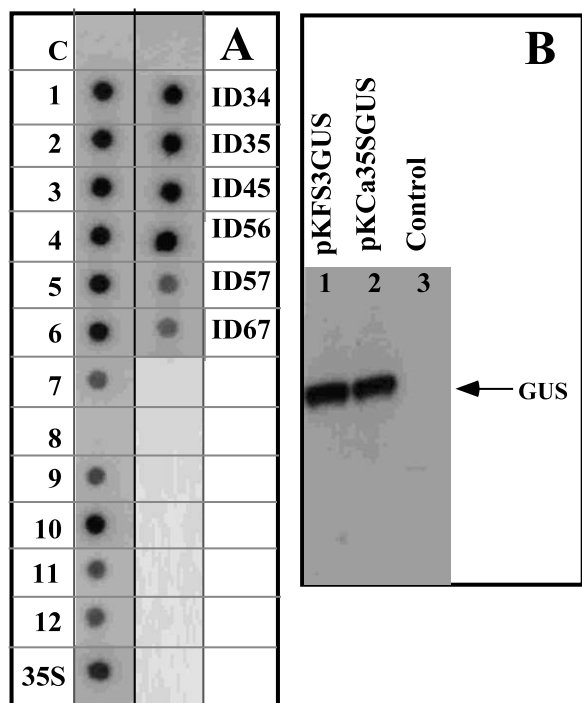


Fig. 5. (A) RNA dot blot analysis of total RNA (10 µg) obtained from transformed protoplasts with end deletion construct No. 1–12 and internal deletion constructs (ID34, ID35, ID45, ID56, ID57, ID67) as indicated in Fig. 3A and Fig. 4A, respectively. Protoplasts transformed with plasmid (pBsK3GUS) containing the GUS gene with no promoter (Vector control, C) and with (pCa35SGUS) with the GUS gene under 35S promoter (Positive control, 35S) are shown. (B) Northern blot analysis of total RNA (10 µg) obtained from plants developed for pKFS3GUS (lane 1), pKCa35SGUS (lane 2) and untransformed Samsun NN plant (lane 3). Blots were hybridized with ^{32}P -labeled GUS as described in Section 2.

other caulimovirus promoters. The strength of the FMV SgT promoter is dependent on the types of cells used for analysis. The promoter activity is greater than that of CaMV 35S promoter and very much comparable to FMV FLt promoter reported earlier (Maiti et al., 1997) in dicot tobacco, whereas FMV SgT promoter is less active compare to 35S promoter in monocot maize cells. We are interested in further studies to analyze the molecular interactions between different *cis*-sequence and the cognate *trans*-elements involved in FMV SgT promoter function and its regulation.

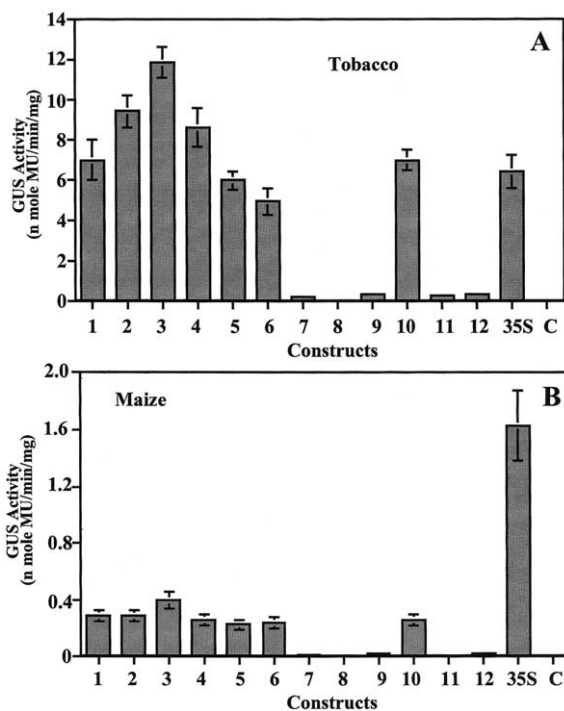


Fig. 6. (A) Comparative expression analysis of FMV SgT promoter with CaMV 35S promoters in a protoplast transient expression experiment using dicot tobacco cells (A) and monocot maize cells (B). GUS constructs with FMV SgT promoter fragments (1–12 as described in Fig. 3A) and with CaMV 35S promoters (pCa35SGUS) 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 the 95% confidence intervals on the means. The statistical ANOVA analysis showed a P value < 0.001 , this is considered to be extremely significant.

Acknowledgements

We are very much indebted to Kentucky State THRI for facilities and support. This work was supported by the KY state THRI grant 5-41132 to IBM. Authors would like to thank Dr K. Scheets for providing the BMS cell culture, Drs Deane Falcone, Arthur Hunt and the two anonymous reviewers for critical reading and helpful comments in improving the manuscript.

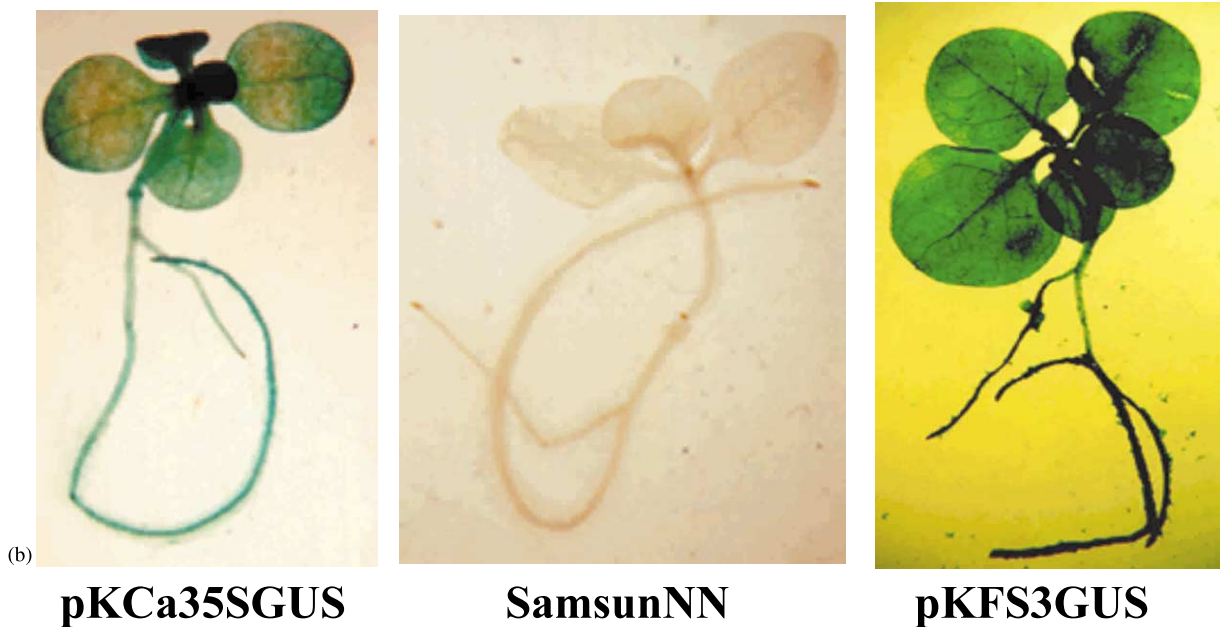
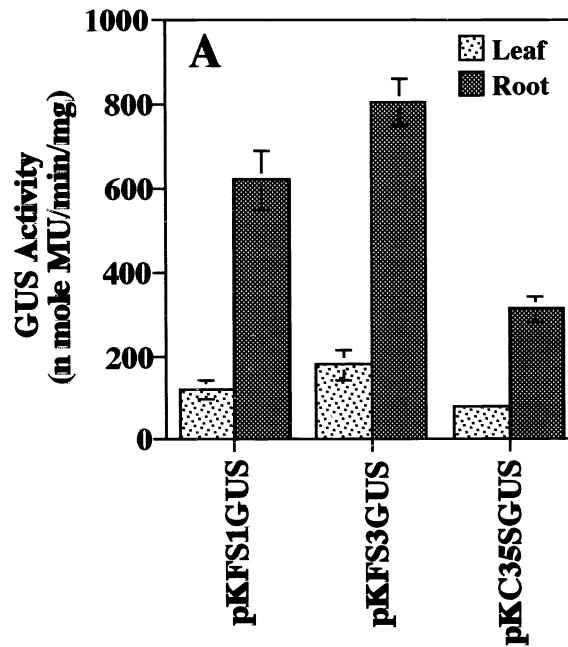


Fig. 7. Expression of FSgt promoter and CaMV35S promoter in transgenic plants. (A) The promoter activity FMV Sgt promoter in GUS-constructs pKFS1GUS, pKF3GUS and CaMV35S promoter in GUS-constructs pKCa35SGUS respectively were evaluated. The promoter activity was measured in 4 week old seedlings (R1 progeny) grown aseptically on an MS-agar medium in presence of kanamycin (200 mg/l) and 3% sucrose. Soluble protein extract from the roots and leaves of 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. Untransformed control (Control), tissue extract from wild-type *N. tabacum* cv. Samsun NN. (B) Histochemical localization of GUS activity in transgenic tobacco seedlings (R1 progeny, 2nd generation, 28 day old) developed for the following constructs: pKFS3GUS, pKCa35SGUS (Dey and Maiti, in press).

References

- An, G., Costa, M.A., Mitra, A., Ha, S.-B., Marton, L., 1988. Organ-specific and developmental regulation of the nopaline synthase promoter in transgenic tobacco plants. *Plant Physiol.* 88, 547–552.
- Assad, F.F., Signer, E.R., 1990. *Cauliflower mosaic virus* P35S promoter activity in *Escherichia coli*. *Mol. Gen. Genet.* 223, 517–520.
- Ballas, N., Shimshon, B., Hermona, S., Abraham, L., 1989. Efficient functioning of plant promoters and polyadenylated sites in *Xenopus oocytes*. *Nucleic Acids Res.* 17, 7891–7904.
- Benfy, P.N., 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.
- Benfy, P.N., Chua, N.H., 1990. The *Cauliflower mosaic virus* 35S promoter: combinatorial regulation of transcription in plants. *Science* 250, 959–966.
- Benfy, P.N., Ren, L., Chua, N.H., 1990a. Combinatorial and synergistic properties of CaMV 35S enhancer subdomains. *EMBO J.* 9, 1685–1696.
- Benfy, P.N., Ren, L., Chua, N.H., 1990b. Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. *EMBO J.* 9, 1677–1684.
- Bhattacharyya-Pakrasi, M., Pen, J., Elmer, J.S., Laco, G., Shen, P., Kaniewska, M.B., Kononowicz, H., Wen, F., Hodges, T.K., Beachy, R.N., 1993. Specificity of a promoter from the *Rice tungro bacilliform virus* for expression in phloem tissues. *Plant J.* 4, 71–79.
- Birnbaum, K., Benfy, P.N., Shasha, D.E., 2001. *cis*-Element/transcription factor analysis (*cis*/tif): a method for discovering transcription factor/*cis* element relationships. *Genome Res.* 11, 1567–1573.
- Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248–254.
- Dey, N., Maiti, I.B., 1999a. Structure and promoter/leader deletion analysis of *Mirabilis mosaic virus* (MMV) full length transcript promoter in transgenic plants. *Plant Mol. Biol.* 40, 771–782.
- Dey, N., Maiti, I.B., 1999b. Further characterization and expression analysis of *Mirabilis mosaic virus* (MMV) full-length transcript promoter with single and double enhancer domains in transgenic plants. *Transgenics* 3, 61–70.
- Dey, N., Maiti, I.B. Promoter deletion and comparative expression analysis of the *Mirabilis mosaic caulimovirus* (MMV) sub-genomic transcript (Sgt) promoter in transgenic plants. *Transgenics*, in press.
- Ellis, J.G., Tokuhisa, J.G., Llewellyn, D.J., Bouchez, D., Singh, K., Dennis, E.S., Peacock, W.J., 1993. Does the OCS-element occur as a functional component of the promoters of plant genes. *Plant J.* 4, 433–443.
- Fang, R.X., Nagy, F., Sivasubramaniam, S., Chua, N.H., 1989. Multiple *cis* regulatory elements for maximal expression of the *Cauliflower mosaic virus* 35S promoter in transgenic plants. *Plant Cell* 1, 142–150.
- Forman, M., Callis, J., Taylor, L.P., Walbot, V., 1987. Electroporation of DNA and RNA into plant protoplasts. *Methods Enzymol.* 153, 351–366.
- Franck, A., Guiley, H., Jonard, G., Richards, K., Hirth, L., 1980. Nucleotide sequence of *Cauliflower mosaic virus* DNA. *Cell* 21, 285–294.
- Fromental, C., Kanno, M., Nomiyama, H., Chambon, P., 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. *Cell* 1, 141–150.
- Gardner, R.C., Howarth, A., Hahn, P., Brown-Leudi, M., Shepherd, R.J., Messing, J., 1981. The complete nucleotide sequence of an infectious clone of *Cauliflower mosaic virus* by M13mp7 shotgun sequencing. *Nucleic Acids Res.* 9, 2871–2888.
- Hasegawa, A., Verver, J., Shimada, A., Saito, M., Goldbach, R., Vankamen, A., Miki, K., Kameya-Iwaki, M., Hibi, T., 1989. The complete sequence of Soybean chlorotic mottle virus DNA and the identification of a novel promoter. *Nucleic Acids Res.* 17, 9993–10013.
- He, X., Hohn, T., Futterer, J., 2000. Transcriptional activation of the *Rice tungro bacilliform virus* gene is critically dependent on an activator element located immediately upstream of the TATA box. *J. Biol. Chem.* 275, 11799–11808.
- Holtorf, S., Apel, K., Bohlman, H., 1995. Comparison of different constitutive and inducible promoters for the over expression of transgenes in *Arabidopsis thaliana*. *Plant Mol. Biol.* 29, 637–646.
- Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusion: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907.
- Lam, E., 1994. Analysis of tissue specific elements in the CaMV35S promoter. In: Nover, L. (Ed.), *Results and Problems in Cell Differentiation, Plant Promoter and Transcription Factors*, vol. 20. Verlag, Berlin/Heidelberg, pp. 181–196.
- Lam, E., Benfy, P.N., Gilmartin, P.M., Fang, R.X., Chua, N.H., 1989. Site specific mutation alter in vitro factor binding and change the promoter expression pattern in plants. *Proc. Natl. Acad. Sci. USA* 86, 7890–7894.
- Laurencikiene, J., Deveikaite, V., Severinson, E., 2001. HS1 enhancer regulation of germline ϵ and γ 2b promoters in murine B lymphocytes: evidence for specific promoter-enhancer interaction. *J. Immunol.* 167, 3257–3265.
- Lawton, M.A., Tierney, M.A., Nakamura, I., Anderson, E., Komeda, Y., Dube, P., Hoffman, N., Fraley, R.T., Beachy, R.N., 1987. Expression of a soybean β -cinglycinin gene under the control of the *Cauliflower mosaic virus* 35S and 19S promoters in transformed petunia tissues. *Plant Mol. Biol.* 9, 315–324.
- Le Gourierrec, J., Li, Y.F., Zhou, D.X., 1999. Transcriptional activation by *Arabidopsis* GT-1 may be through interaction with TFIIA-TBP-TATA complex. *Plant J.* 18, 663–668.
- Maiti, I.B., Shepherd, R.J., 1998. Isolation and expression analysis of peanut chlorotic streak caulimovirus (PCISV)

- full-length transcript (FLt) promoter in transgenic plants. *Biochem. Biophys. Res. Commun.* 244, 440–444.
- Maiti, I.B., Murphy, J.F., Shaw, J.G., Hunt, A.G., 1993. Plants that express a potyvirus proteinase genes are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* 90, 6110–6114.
- Maiti, I.B., Gowda, S., Kierman, J., Ghosh, S.K., Shepherd, R.J., 1997. Promoter/leader analysis and plant expression vectors with the *Figwort mosaic virus* (FMV) full-length transcript (FLt) promoter containing single and double enhancer domains. *Transgen. Res.* 6, 143–156.
- Maiti, I.B., Richins, R.D., Shepherd, R.J., 1998. Gene expression regulated by gene VI of caulimovirus: transactivation of downstream genes of transcripts by gene VI of peanut chlorotic streak virus in transgenic tobacco. *Virus Res.* 57, 113–124.
- Mandy, J., Day, D., Ashurst, J.L., Mathias, S.F., Watts, J.W., Michel, T., Wilson, A., Dixon, R.A., 1993. Plant viral leaders influence expression of a reporter gene in tobacco. *Plant Mol. Biol.* 23, 97–109.
- Medberry, S.L., Lockhart, B.E.L., Olszewski, N.E., 1992. The *Commelina yellow mottle virus* promoter is a strong promoter in vascular and reproductive tissues. *Plant Cell* 4, 185–192.
- Mitsuhashi, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y., Ohashi, Y., 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* 37, 49–59.
- Neustaedter, D.A., Lee, S.P., Douglas, C.J., 1999. A novel parsley 4CL1 *cis* element is required for developmentally regulated expression and protein–DNA complex. *Plant J.* 18, 77–88.
- Odell, J.T., Dudley, R.K., Howel, S.H., 1981. Structure of the 19S RNA transcript encoded by the *Cauliflower mosaic virus* genome. *Virology* 111, 377–385.
- Odell, J.T., Nagy, F., Chua, N.-H., 1985. Identification of DNA sequences required for activity of the *Cauliflower mosaic virus* 35S promoter. *Nature* 313, 810–812.
- Ondek, B., Gloss, L., Herr, W., 1988. The SV40 enhancer contains two distinct levels of organization. *Nature* 333, 40–45.
- Ow, D.W., Jacobs, J.D., Howell, S.H., 1987. Functional regions of the *Cauliflower mosaic virus* 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. *Proc. Natl. Acad. Sci. USA* 84, 4870–4874.
- Pham, A.D., Sauer, F., 2000. Ubiquitin-activating/conjugating activity of TAF_{II}250, a mediator of activation of gene expression in *Drosophila*. *Science* 289, 2357–2360.
- Pilpel, Y., Sudarsanam, Y., Church, M.G., 2001. Identifying regulatory networks by combinatorial analysis of promoter elements. *Nat. Genet.* 29, 153–159.
- Probjecky, N., Rosenberg, G.H., Dinter-Gottlieb, G., Kaufer, N.F., 1990. Expression of the β glucuronidase gene under the control of the CaMV 35S promoter in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* 220, 314–316.
- Richins, R.D., 1993. Organization and expression of the Peanut chlorotic streak virus genome. Ph.D. dissertation, University of Kentucky, Lexington, KY (for the PCISV genomic sequence: DNA EMBL Data Library GenBank Accession Number U13988).
- Richins, R.D., Scholthof, H.B., Shepherd, R.J., 1987. Sequence of *Figwort mosaic virus* DNA (caulimovirus group). *Nucleic Acids Res.* 15, 8451–8466.
- Roberts, S.G., Choy, B., Walker, S.S., Lin, Y.S., Green, M.R., 1995. A role for activator mediated TFIIB recruitment in diverse aspects of transcriptional regulation. *Curr. Biol.* 5, 508–516.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, M., Daubert, S., 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.
- Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain terminator inhibitor. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- Schardl, C.L., Byrd, A.D., Benzion, G., Altschuler, M.A., Hilderbrand, D.F., Hunt, A.G., 1987. Design and construction of a versatile system for the expression of foreign genes in plants. *Gene* 61, 1–11.
- Schirm, S., Jiricny, J., Schaffner, W., 1987. The SV40 enhancer can be dissected into multiple segments each with a different cell type specificity. *Genes Dev.* 1, 65–74.
- Verdaguer, B., de Kochko, A., Beachy, R.N., Fauquet, C., 1996. Isolation and expression in transgenic tobacco and rice plants of the cassava nein mosaic virus (CVMV) promoter. *Plant Mol. Biol.* 31, 1129–1139.
- Wilmink, A., Van de Ven, B.C.E., Dons, J.J.M., 1995. Activity of constitutive promoter in various species from the Liliaceae. *Plant Mol. Biol.* 28, 949–955.
- Willmott, R.L., Rushton, P.J., Hooley, R., Lazarus, C.M., 1998. DNase I footprinting suggest the involvement of at least three types of transcription factors in the regulation of α -Amy2/A by gibberellin. *Plant Mol. Biol.* 38, 817–825.
- Yin, Y., Chen, L., Beachy, R., 1997. Promoter elements required for phloem-specific gene expression from the RTBV promoter in rice. *Plant J.* 12, 1179–1188.