

# Isolation of full-length transcript promoter from the *Strawberry vein banding virus* (SVBV) and expression analysis by protoplasts transient assays and in transgenic plants

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## Abstract

A full-length transcript (FLt) promoter was isolated from a genomic clone of *Strawberry vein banding virus* (SVBV), a double-stranded DNA plant pararetrovirus belonging to the Caulimoviridae family, and its activity was analyzed both in protoplasts and transgenic plants. The 5'–3'-boundaries required for maximal promoter activity were determined by 5'- and 3'-end deletion analysis of the SVBV promoter fused to a  $\beta$ -glucuronidase (GUS) reporter gene. A 371-bp promoter fragment (–352 to +19 from the transcription start site; TSS) was found sufficient for maximal promoter activity in a transient protoplast expression assay, and this was chosen for further analysis. Finer deletion analysis of a 90-bp sequence (coordinates –392 to –302 from TSS) of the SVBV FLt promoter revealed the presence of a negative and a positive regulatory element in this region. In gain-of-function experiment, the fusion of the putative positive regulatory elements with minimal promoter showed very little increment in promoter activity, suggesting that a combinatorial action of various cis-sequences is involved in promoter function. The transcription start site of the full-length transcript promoter was mapped to an A-residue that is located 25-bp downstream of the TATA-box. In protoplast transient expression analysis, the SVBV FLt promoter showed about six-fold higher activity in tobacco compared to maize. A quantitative GUS activity assay showed that in transgenic tobacco plants the average promoter activity was about three-fold higher in roots than in leaves, and this higher activity was due to the accumulation of more GUS specific mRNA in roots. Real-time qRT-PCR analysis and quantitative GUS activity assay showed that the relative strength of the SVBV FLt promoter was greater than the CaMV35S promoter in transgenic tobacco plants. The SVBV FLt promoter is a strong, constitutive promoter and has great application potential in expression of foreign genes in plants.

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**Keywords:** *Caulimovirus*; *Strawberry vein banding virus* (SVBV); Full-length transcript promoter; Transgenic plants

## 1. Introduction

A number of plant transcriptional promoters have been isolated from double-stranded DNA viruses belonging to subgroups of plant pararetrovirus (Caulimoviridae family), namely *Caulimovirus* [1–8] and *Badnavirus* [9,10]. In general, two major transcriptional promoters are present in the *Caulimovirus* genome. One that directs transcription of the whole genome of the virus (a full-length transcript (FLt) equivalent to CaMV 35S transcript) is located in the 3' ter-

minus of ORF VI and extends into the large intergenic region. A second promoter that is situated at the 3' terminus of ORF V and extends into the small intergenic region between ORF V and VI, transcribes only ORF VI (a subgenomic transcript equivalent to CaMV 19S transcript).

The CaMV 35S promoter has been well characterized [11–17]. It is a strong constitutive promoter, and has been used extensively for expressing foreign genes in monocotyledonous and dicotyledonous plants [18–20]. 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 with the transacting nuclear binding factors [12,14,15], similar to other promoters such as SV40 promoter in mammalian sys-

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tems [21,22]. Two nuclear binding protein factors, known as activating sequence factor –1 and –2 (ASF-1 and ASF-2) from tobacco have been well characterized [17]. The *as-1* motif is also found in other *Caulimovirus* promoters such as the full-length transcript promoters of *Figwort mosaic virus* (FMV) [3,5], *Peanut chlorotic streak virus* (PCISV) [23] and *Mirabilis mosaic virus* (MMV) [7] as well as in the sub-genomic transcript promoter of FMV [8]. The promoters isolated from *Caulimoviruses* are generally constitutive in nature. However, promoters isolated from the *Badnaviruses* are reported to be primarily active in vascular tissues [10]. Promoters from *Rice tungro bacilliform virus* (RTBV) and *Commelina yellow mottle virus* (CoYMV) direct phloem-specific gene expression in transgenic plants [9,10,24].

*Strawberry vein banding virus* (SVBV), a species of the genus *Caulimovirus*, belonging to the family Caulimoviridae, has a small circular DNA genome of approximately 8-kb with seven open reading frames (ORFs). The strawberry vein banding disease of cultivated strawberries is caused by this virus and is probably distributed world wide in cultivated strawberries [25,26]. The genome organization of SVBV is similar to that of *Cauliflower mosaic virus* (CaMV), the type species of the genus *Caulimovirus* [27,28]. Phylogenetic analysis based on amino acid sequence of protein shows a closer relationship of SVBV with CaMV, *Figwort mosaic virus* and *Carnation etched ring virus* (CERV) [26].

In the present study, we report the isolation and expression analysis of the SVBV full-length transcript promoter. A 5' and 3'-end promoter deletion analysis has been performed to show that a 371-bp SVBV FLt promoter fragment (sequence –352 to +19 from the TSS) is sufficient for maximum promoter activity. Finer deletion analysis of a 90-bp sequence (coordinates –392 to –302 from TSS) of the SVBV FLt promoter shows the presence of a putative negative and a positive regulatory element in this region. In gain-of-function experiment, fusion of positive regulatory element to the minimal promoter shows no or very little increase in promoter activity. The strength of this promoter has been evaluated both in tobacco and maize protoplast transient expression assay, and also in transgenic tobacco plants. The expression level in dicot tobacco cells is higher than that in monocot maize cells. SVBV FLt promoter is a strong constitutive promoter and its strength is greater than CaMV 35S promoter in transgenic tobacco plants.

## 2. Materials and methods

### 2.1. Isolation of SVBV promoter fragments and construction of vectors for the protoplast transient expression assay

A 951 bp segment (genomic coordinates 6460–7410) from the SVBV genome was PCR-amplified as an *EcoRI*–*Bam*HI fragment using appropriately designed primers. The

PCR-amplified fragment was gel-purified and cloned into the corresponding sites of pBS(KS+)dH (a derivative of pBS(KS+) in which the *Hind*III site is modified) to generate the plasmid pBS24. The unique *Hind*III site present in the promoter fragment (–404-bp upstream of TATA-box) in pBS24 was modified and the resulting plasmid was designated as pBS24dH. A series of defined SVBV FLt promoter fragments, of indicated lengths (as depicted in the Fig. 2A) were amplified by PCR from pBS24dH with appropriately designed primers to tailor an *Eco*RI site at the 5'-end and a *Hind*III site at the 3'-end of the amplified products. PCR amplification was carried out for 30 cycles under the following standard conditions: denaturation (94 °C for 30 s), annealing (55 °C for 30 s), extension (68 °C for 1 min) using Platinum high fidelity *Taq*DNA polymerase (Invitrogen, California, USA). Each PCR-amplified fragment from 1 to 20 was gel-purified and restricted with *Eco*RI and *Hind*III; the restricted fragments were cloned into the corresponding sites of the pUC119 vector and sequenced by dideoxy chain terminator method [29] using synthetic primers. The sequence integrity of each of the promoter fragments was verified before subcloning into a protoplast expression vector pUCPMAGUS [7]. SVBV FLt 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 [7]. The following deletion plasmids were developed (Fig. 2A). The 5' and 3' coordinates of the promoter fragments with respect to TSS are given in parentheses: pSV1GUS (–792 to +19), pSV2GUS (–742 to +19), pSV3GUS (–692 to +19), pSV4GUS (–642 to +19), pSV5GUS (–592 to +19), pSV6GUS (–542 to +19), pSV7GUS (–492 to +19), pSV8GUS (–442 to +19), pSV9GUS (–392 to +19), pSV10GUS (–352 to +19), pSV11GUS (–302 to +19), pSV12GUS (–252 to +19), pSV13GUS (–202 to +19), pSV14GUS (–152 to +19), pSV15GUS (–102 to +19), pSV16GUS (–52 to +19), pSV17GUS (–352 to +159), pSV18GUS (–352 to +109), pSV19GUS (–352 to +60) and pSV20GUS (–352 to –42).

### 2.2. Constructs made for finer deletion analysis of SVBV FLt promoter

For a finer deletion analysis of the 90-bp region (coordinates –392 to –302, relative to TSS) following nine promoter fragments were generated by PCR-amplification from the full-length SVBV promoter (pBS24dH) with appropriately designed primers (Fig. 3A). The corresponding 5' and 3'-end coordinates of the promoter fragments relative to TSS are given in parentheses: pSV9a (–382 to +19), pSV9b (–372 to +19), pSV9c (–362 to +19), pSV10a (–342 to +19), pSV10b (–332 to +19), pSV10c (–322 to +19) and pSV10d (–312 to +19). All these promoter fragments were digested with *Eco*RI and *Hind*III and cloned into corresponding sites of the protoplast expression vector as described earlier. The sequence integrity of each of the promoter frag-

ments was verified before subcloning into a protoplast expression vector.

### 2.3. Construction of plasmids for gain-of-function experiment

For gain-of-function experiment, the following cis-regions (coordinates –352 to –333, –352 to –302, –352 to –252, –352 to –202 relative to TSS) were individually inserted upstream of the minimal promoter, pSV15GUS (coordinates –102 to +19 relative to TSS). The cis-regions were separately PCR-amplified from the SVBV FLt promoter to generate fragments of the following general structure: 5'-EcoRI–cis-region–SmaI–HindIII–3'. The fragments were then digested with EcoRI and HindIII and cloned into the corresponding site of pUC119. The SVBV minimal promoter was PCR-amplified as HincII–HindIII fragment and inserted into the SmaI and HindIII site of pUC119 plasmids containing the upstream (cis-region) fragment. The SVBV minimal promoter with respective cis-region was then isolated as EcoRI–HindIII fragment and cloned into the corresponding site of the protoplast expression vector as described earlier. The resulting constructs were designated as pSV15a, pSV15b, pSV15c and pSv15d (Fig. 3B). The sequence integrity of each of the promoter fragments was verified.

### 2.4. Construction of plasmids for internal deletion analysis of SVBV FLt promoter

The specified promoter region was deleted from the full-length promoter fragment pSV10 (–352 to +19) as depicted in Fig. 3C. In PCR amplification, the region specified for internal deletion (ID) was excluded by separately amplifying the upstream (first half) and downstream (second half) regions of the desired fragment from the full-length promoter with appropriately designed primers, and fragments of following general structure 5'-EcoRI–1st half–SmaI–HindIII–3' and 5'-HincII–2nd half–HindIII–3' were generated. The PCR-amplified upstream fragment (first half) was cloned into the EcoRI and HindIII site of pUC119 and the resulting plasmid was restricted with SmaI and HindIII to insert the downstream HincII–HindIII fragment. The respective internal deletion fragments were then isolated as EcoRI–HindIII fragments and cloned into the corresponding site of the protoplast expression vector. The resulting plasmids were designated as ID11–12, ID12–13, ID13–14, ID14–15 and ID15–16 (Fig. 3C).

### 2.5. Protoplast isolation and electroporation

Isolation of protoplasts from tobacco cell suspension cultures (*Nicotiana tabacum* L. cv. Xanthi 'Brad') and electroporation of tobacco protoplasts with supercoiled plasmid DNA containing the promoter fragment fused with a glucuronidase (GUS)-encoding gene were done essentially as

described earlier [7]. Isolation of protoplasts from maize cell suspension cultures (*Zea mays* cv. Black Mexican Sweet, BMS-P2-S10) and electroporation of protoplasts with supercoiled plasmid DNA containing GUS constructs were performed essentially as described [30,31]. Electroporation was performed by using the Gene Pulser II apparatus (BioRad, USA) with the Capacitance Extender II (Model 165–2107). An aliquot of 750  $\mu$ l containing  $2 \times 10^6$  protoplasts in an electroporation cuvette (0.4 cm electrode gap) was electroporated (150 V used for charging 850  $\mu$ F capacitance for 15–20 ms) with 20  $\mu$ g of supercoiled plasmid DNA containing the GUS gene and 60  $\mu$ g sheared, non-denatured salmon sperm DNA as carrier. After 20–22 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 in at least three independent experiments.

### 2.6. Construction of plant expression vectors with SVBV FLt promoter and plant transformation

The SVBV FLt promoter fragment isolated from pSV10GUS (coordinates –352 to +19 relative to the TSS) was cloned into the plant expression vector pKYLX71 GUS [32] at its unique EcoRI and HindIII sites that flank the promoter. The resulting plant expression vector was designated pKSV10GUS. The construct was introduced into *Agrobacterium tumefaciens* strain C58C1:pGV3850 by triparental mating. Tobacco plants (*N. tabacum* cv Sam-sun NN) were transformed with the engineered *Agrobacterium* as described earlier [33]. About twelve independent plant lines were generated for this construct. Regenerated kanamycin-resistant plants were grown under greenhouse conditions.

### 2.7. $\beta$ -Glucuronidase (GUS) assay

Fluorometric GUS assays to measure GUS activity in protoplast extracts or plant tissue, and histochemical GUS staining to localize the distribution of GUS activity in plants were performed according to Jefferson et al. [34] as described earlier by Maiti et al. [5]. Total protein content in plant extracts was determined according to the method of Bradford [35] using BSA as a standard.

### 2.8. Analysis of transgenic plants: isolation of RNA and RT-PCR analysis

Total cellular RNA from electroporated protoplasts, or transgenic tobacco seedlings expressing the constructs pKSV10GUS and pKCaMV35SGUS [7] was isolated using the RNeasy plant mini kit (Qiagen, Chatsworth, USA). The total RNA (2  $\mu$ g) was treated with RNase free DNase (Sigma, USA) as per manufacturer's instructions. Superscript™ First Strand Synthesis System for RT-PCR (Invitrogen, USA) was used for synthesis of first-strand cDNA from the DNase treated RNA in a total volume of

20  $\mu$ l following the manufacturer's instructions. For the no-reverse-transcriptase control, an individual reaction was performed in parallel without addition of reverse transcriptase. A 1/20 volume (1  $\mu$ l) of the RT reaction was used in the subsequent PCR reaction with appropriately designed forward and reverse primers to detect the full-length GUS transcript. The PCR reaction was performed in a total volume of 25  $\mu$ l for 30 cycles (94 °C for 30s, 55 °C for 30s, 68 °C for 2 min). As a negative control, each primer pair was tested against DNase-treated RNA to confirm cDNA dependence of amplification. PCR products were displayed on an ethidium bromide-stained agarose gel.

### 2.9. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The expression level of GUS mRNA in protoplasts electroporated with plasmids pSV10GUS and pCaMV35SGUS and plants developed for plasmids pKSV10GUS and pKCaMV35SGUS was evaluated by real-time quantitative RT-PCR [36]. A 150-bp fragment was amplified from the full-length transcript using appropriately designed sequence-specific primers. A plasmid containing the full-length GUS cDNA was used as an external control. Serial dilution ( $10^8$ – $10^3$  copies/ $\mu$ l) of the control plasmid was used to generate a standard curve. A house-keeping gene ( $\alpha$ -tubulin), which is present at a constant amount in all samples, was used as an internal control to correct for any minor variation in samples. PCR amplification was performed in DNA Engine Opticon™2 System for continuous fluorescence detection (MJ Research Inc., USA) in a total volume of 20  $\mu$ l containing 1  $\mu$ l of cDNA, gene-specific primers using the DyNamo™ SYBR Green qPCR kit (MJ Research Inc., USA). Each PCR reaction was performed in triplicate using the following conditions: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 78 °C for 1 s, plate read, 35 cycles followed by 5 min extension at 72 °C. Copy number of the target samples was calculated using the standard curve generated for the plasmid standard by Opticon Monitor™ software. Melting curve analysis [37] was done to characterize the amplified products by slowly raising the temperature (0.2 °C/s) from 65 to 95 °C with fluorescence data collected at 0.2 °C intervals.

### 2.10. Determination of transcription start site of the SVBV FLt promoter by 5'-RACE analysis

The transcription start site of the SVBV FLt promoter was determined by 5'-rapid amplification of cDNA ends (5'-RACE). Total RNA was isolated from transgenic tobacco seedlings developed for the construct pKSV10GUS as described earlier. RACE was carried out using the 5'-RACE System 2 (Invitrogen, California, USA) as per the manufacturer's instructions. A gene specific primer was used for the synthesis of first-strand cDNA. For amplification of the dC-tailed first-strand cDNA, two nested

gene-specific antisense primers were used in the subsequent PCR reactions. The 5'-RACE products were cloned in pGEM-T Easy (Promega, USA) and 10 positive clones were sequenced to determine the transcription start site.

## 3. Results and discussion

### 3.1. Structure and sequence analysis of the SVBV FLt promoter

The SVBV FLt promoter was isolated from a genomic clone of SVBV:pSVBV-E3 [26] as described in Section 2. The complete DNA sequence of SVBV has been reported ([26], Genbank accession number X97304). Nucleotide sequence of the SVBV FLt promoter (SVBV genomic coordinates 6460–7410) is shown in Fig. 1. The SVBV FLt promoter sequence contains several consensus eukaryotic regulatory domains such as a TATA-box, and a CAAT box-like sequence that are present in full-length transcript promoters of other *Caulimoviruses* [5,7]. The TATA-box is present 33-bp upstream of the transcription start site. Deletion analysis presented in a following section indicates that the TATA-box sequence of the SVBV promoter is essential for its activity. A CAAT box-like sequence is present 22-bp upstream of the TATA-box and a PolyA signal (AATAAA) is located 98-bp downstream of TATA-box. The OCS-element, a transcriptional enhancer, with a 20 bp consensus sequence: TGACG(T/C)AAG(C/G)(G/A)(A/C)T(G/T)ACG(T/C)(A/C)(A/C), has been shown to be a functional component of a number of plant promoters including *Caulimovirus* promoters [38]. In the SVBV FLt promoter, a 20-nt sequence present 92-bp upstream of the TATA-box (coordinates –144 to –125 relative to the TSS) shows strong homology (75%) to the OCS-sequences. In different promoters, the position of the OCS-element varies from 27 to 146-bp upstream of respective TATA-box [38]. The SVBV FLt promoter does not contain the consensus as-1 motif (TGACG) that is found in other *Caulimovirus* promoters such as CaMV, FMV and MMV [5,7,8,17]; however as-1 like sequences (TGAAG, TGACC with a single mismatch indicated in bold) are present at positions –144 to –140 and –132 to –128 from the TSS which may have some regulatory function in promoter activity; more work will be needed to evaluate their exact role in promoter function. In the SVBV FLt promoter, a transcriptional enhancer element (GATG/A) similar to the core sequence of the as-2 motif [17] is present at positions –140 to –137, –261 to –258 and –289 to –286 upstream from the TSS. The as-2 motif is found in full-length transcript promoters of FMV, MMV and CaMV [5,7,17]. In addition, there are several transcription factor binding-domains present in SVBV FLt promoter such as the maize DOF transcription factor recognition core sequence (AAAG) [39] at positions –314 to 311, –221 to –118 and –196 to –193 from the TSS and the E-box (CANNTG) [40] at position –339 to

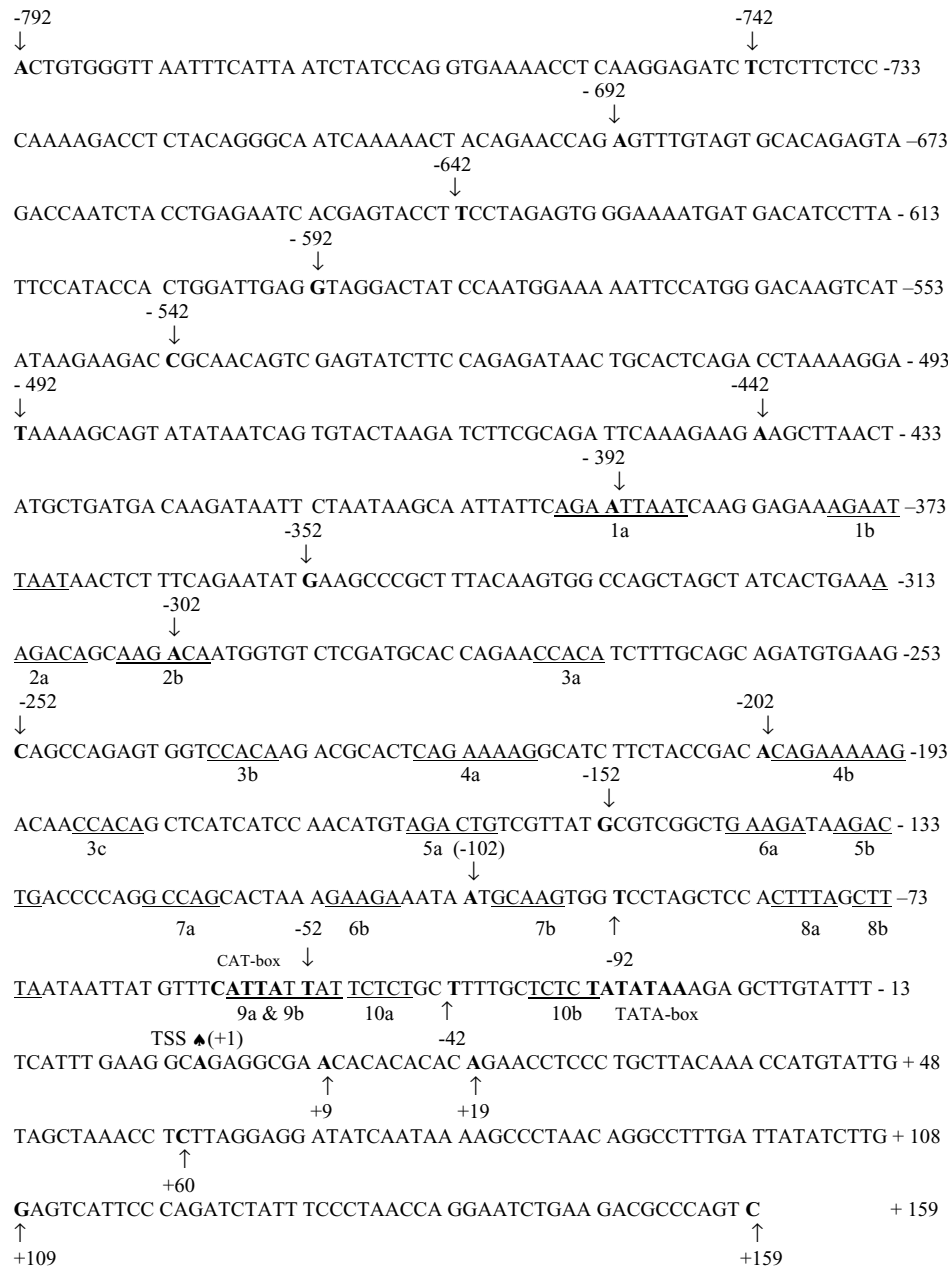


Fig. 1. The DNA sequence of the full-length transcript (FLt) promoter of the *Strawberry vein banding virus* (SVBV). A 951-bp fragment (–792 to +159) with respect to the transcription start site, TSS; corresponding coordinates in SVBV genome 6460–7410) is presented from left to right in the 5' to 3' 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 TATA box is shown in bold. All repeat sequence domains (designated 1a, 1b, 1c; 2a, 2b; 3a, 3b, etc.) are underlined.

334 and –99 to –93 from the TSS. The DOF transcription factors are involved in signal response and/or tissue specific gene expression in plants [39]. The E-box has been shown to be involved in both tissue-specific and developmental control of the b-phaseolin gene [40]. In addition, several direct repetitive sequences such as AGAATTAAT (denoted as 1a and 1b), AAGACA (denoted as 2a and 2b), CCACA (denoted as 3a, 3b, and 3c), CAGAAAG (denoted as 4a and 4b), AACTG (denoted as 5a and 5b), GAAGA (denoted as 6a and 6b), GC(C/A)AG (denoted as 7a and 7b), CTTTA

(denoted as 8a, and 8b) and ATTAT (denoted as 9a and 9b), TCTCT (denoted as 10a and 10b), are present in the SVBV FLt promoter (Fig. 1). These repetitive sequences may have some regulatory function. However, more work is needed to evaluate their regulatory role, if any, in promoter function. The A+T content of the SVBV FLt promoter sequence is about 60%. Multiple DNA sequence alignment analysis (MacVector CustalW program) of the SVBV FLt promoter with that of full-length transcript promoters from CaMV, PCISV, FMV, MMV and CVMV showed sequence

identities of 42, 25, 25, 33 and 23%, respectively. The DNA sequence identity of the SVBV FLt promoter with the subgenomic transcript promoters from CaMV, PCISV, FMV, MMV is 27, 30, 28 and 25%, respectively. There is very limited sequence homology between the SVBV FLt promoter and other *Caulimovirus* promoters, although they are functionally analogous.

### 3.2. Transcription start of the SVBV FLt promoter

The transcription start site of the SVBV FLt promoter was determined by 5'-RACE using total RNA isolated from pKSV10GUS-transformed R1 tobacco plants followed by cloning and sequencing of the amplified PCR products. The transcription start site was mapped to an adenine (A) residue located 25-nucleotides downstream of the TATA element (Fig. 1). Sequence comparisons of the transcription start site with other *Caulimovirus* promoters showed little overall sequence homology.

### 3.3. 5'- and 3'-End deletion analysis of SVBV FLt promoter

In order to define the boundaries required for maximal promoter activity and the influence of cis-acting sequences

present upstream and downstream of TATA-box, a 951-bp SVBV FLt promoter fragment (coordinates 6460–7410 of the SVBV genome) was subjected to 5'- and 3'-end deletion analysis. Twenty promoter fragments were generated by PCR amplification and cloned into the protoplast expression vector pUCPMAGUS as described in Section 2. A schematic map of deletion constructs is shown in Fig. 2A. The 5'- and 3'-end points of each fragment with respect to the transcription start sites are indicated in parentheses. Promoter fragments fused to the GUS reporter gene were introduced into tobacco protoplasts for transient expression assay. Results of the expression analysis are shown in Fig. 2B. The expression level of construct 1 (pSV1GUS, coordinates -792 to +19 from TSS) was 0.15 nmole MU/(min mg protein), and this value was considered to represent full (100%) promoter activity for comparison with the following deletion constructs. The 5'-end deletion constructs 2, 3, 4 and 5 (with deleted 50, 100, 150 and 200-bp, respectively, from the 5'-end of construct 1) showed a two- to three-fold increase in promoter activity. Further deletion of 50-bp in construct 6 decreased the promoter activity (compare construct 5 and 6). Further successive deletion of 50-bp up to 150-bp from the 5'-end of construct 6 resulted in a 3- to 3.5-fold increase in promoter activity (see constructs 7–9) compared to that of construct 1.

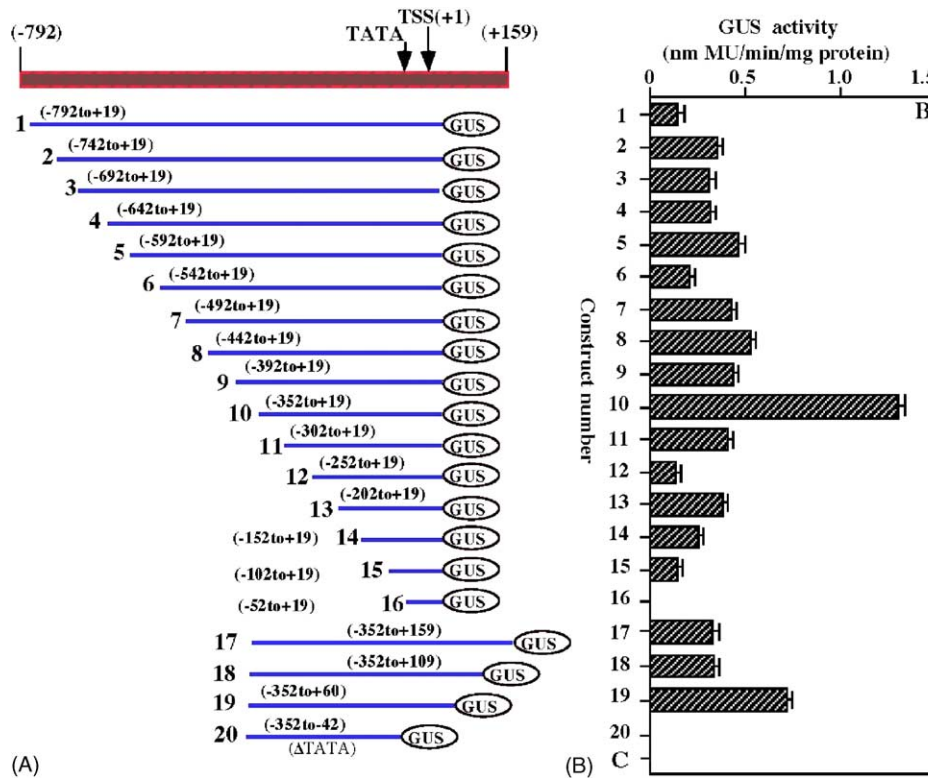


Fig. 2. (A) A schematic map of the GUS constructs (number 1–20) developed for the deletion analysis of SVBV FLt promoter. The 5'- and 3'-end coordinates of the relative deletion fragments are given in parentheses. The end points for the 5'- or the 3'-end deletion constructs are also indicated above or below the sequence, respectively in Fig. 1. At the top, the relative position of the TATA box, transcription start site (TSS, +1) and the SVBV FLt promoter coordinates are shown. (B) SVBV FLt promoter expression analysis in the protoplast transient expression assay using GUS reporter gene. Each construct was assayed at least three times in four independent experiments. The average GUS activity (nm MU/(min mg protein)) with standard deviation is presented. 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  $<0.001$ .

Construct 10 was generated by further deletion of 40-bp from the 5'-end of construct 9. Construct 10 (pSV10GUS, coordinates -352 to +19 from TSS) gave maximum promoter activity and it was about nine-fold higher than construct 1. This data suggests that the upstream promoter sequence between -792 and -353 from TSS, in this context, may not be essential for maximal promoter activity. However, successive deletion of ~50, 100, 150, 200 and 250-bp from the 5'-end of construct 10, (as in construct 11–15, respectively), markedly reduced the promoter activity compared to construct 10 (coordinates -352 to +19 from the TSS) that gave maximal activity. Further deletion of ~50-bp as in construct 16 (pSV16GUS, coordinates -52 to +19 from the TSS) resulted in the loss/below detection level of promoter activity. These results indicate that the cis-sequences present in the TATA-upstream region are indispensable for SVBV FLt promoter activity.

The 3'-end deletion construct 20 (coordinates -352 to -42 upstream of the TSS) that is devoid of the TATA box showed no or below detection level of GUS activity. This demonstrates the importance of the TATA element in SVBV FLt promoter function. The 3' deletion constructs 17 (coordinates -352 to +159), 18 (coordinates -352 to +109), and 19 (coordinates -352 to +60) with extended leader sequences showed marked decrease in promoter activity, compared to construct 10 (coordinate -352 to +19) that showed maximal activity. This suggests that, in this context, the extended leader sequence (coordinates +20 to +159 downstream of the TSS) may have an inhibitory effect on promoter function. Similar results were reported for other *Caulimovirus* promoters such as the FMV Sgt promoter [8] in which the presence of extended leader sequences was shown to reduce promoter activity. The promoter activity was reduced probably through the effect of a longer untranslated leader sequence on transcription and subsequent translation [8]. This long untranslated leader sequence may have some effect on the formation of secondary structure of transcripts. More work will be needed to evaluate the regulatory role, if any, of untranslated leader sequence in promoter function.

#### 3.4. Finer deletion analysis of a 90 bp putative regulatory region (coordinate -392 to -302 from TSS)

Comparison of promoter activity of constructs 9 and 11 with 10 in the 5'-end deletion analysis of the SVBV FLt promoter (see Fig. 2B) indicated the presence of putative negative and positive regulatory elements in between regions (coordinates -392 to -353 from TSS) and (coordinates -352 to -302 from TSS), respectively. In order to dissect further the negative and positive regulatory cis-elements, a finer deletion analysis of these two regions was conducted. A schematic deletion map and results are shown in Fig. 3A. Three constructs (9a–c) were generated by successive deletion of 10, 20 and 30 bp from the 5'-end of construct 9 (coordinates -392 to +19 from TSS) to map the negative ele-

ments present in this region. Constructs 9a, 9b and 9c with coordinates -382 to +19, -372 to +19, and -362 to +19 from TSS, respectively, showed a little or no enhancement in GUS activity compared to construct 9 (coordinates -392 to +19 from TSS) that gave about 36% of maximal promoter activity (Fig. 3A). Construct 9c (coordinates -362 to +19 from TSS) with 10 additional nts at the 5'-end of construct 10, (representing 100% promoter activity) showed about 35% of maximal promoter activity. Taking these data together, in this context, it indicates that this 10-nt region (coordinates -362 to -353 from TSS) has significant inhibitory effect on promoter activity.

To map the putative positive regulatory elements present in region (coordinates -352 to -302 from TSS), four constructs (10a–d) were generated by successive deletion of 10, 20, 30 and 40-nts from the 5'-end of construct 10 (pSV10GUS, coordinates -352 to +19 from TSS). In construct 10a, deletion of 10-nt from the 5'-end of construct 10 resulted in 60% reduction of promoter activity (Fig. 3A). However, further deletion of 20, 30 or 40 nt in constructs 10b–d, respectively, from the 5'-end as in construct 10 resulted in a little or no increase in promoter activity compared to construct either 9a, 10a or 11; each of these three construct gave about 40% of maximal promoter activity. These results clearly indicate that this 10-nt cis-sequence (coordinates -352 to -343 from TSS) is essential for maximal promoter activity in this context. This positive cis-sequence may have some role in the formation of secondary structure needed for promoter function. The boundaries of these positive and negative regulatory elements may not be necessarily located in the deletion end points—these may be smaller or longer than 10-nt. More work will be needed to fully elucidate their regulatory role in promoter function. The existence of positive and negative cis-regulatory elements localized in different regions has been reported for a number of plant promoters [41–43]. The results described above showed that the SVBV FLt promoter has a negative and a positive cis-regulatory element, which affects the promoter activity quantitatively in a protoplast transient expression assay. Analysis of these cis-regulatory elements using the PLACE database [44] revealed limited homology with the sequences of other reported cis-regulatory elements. The putative AT-rich negative and GC-rich positive regulatory elements showed some homology with AT-rich CGF-1 [45] and GC-rich PRE1/PRE2 elements [41], respectively. More work will be needed to evaluate the significance, if any, of these sequence homology in promoter function.

#### 3.5. Gain-of-function experiment using putative regulatory domains

In order to evaluate whether these putative regulatory domains can function independently in promoter activity, four upstream regions of 20, 50, 100, and 150-nt (coordinates -352 to -333, -352 to -302, -352 to -252, and -352 to -202 from TSS, respectively) were fused with the minimal

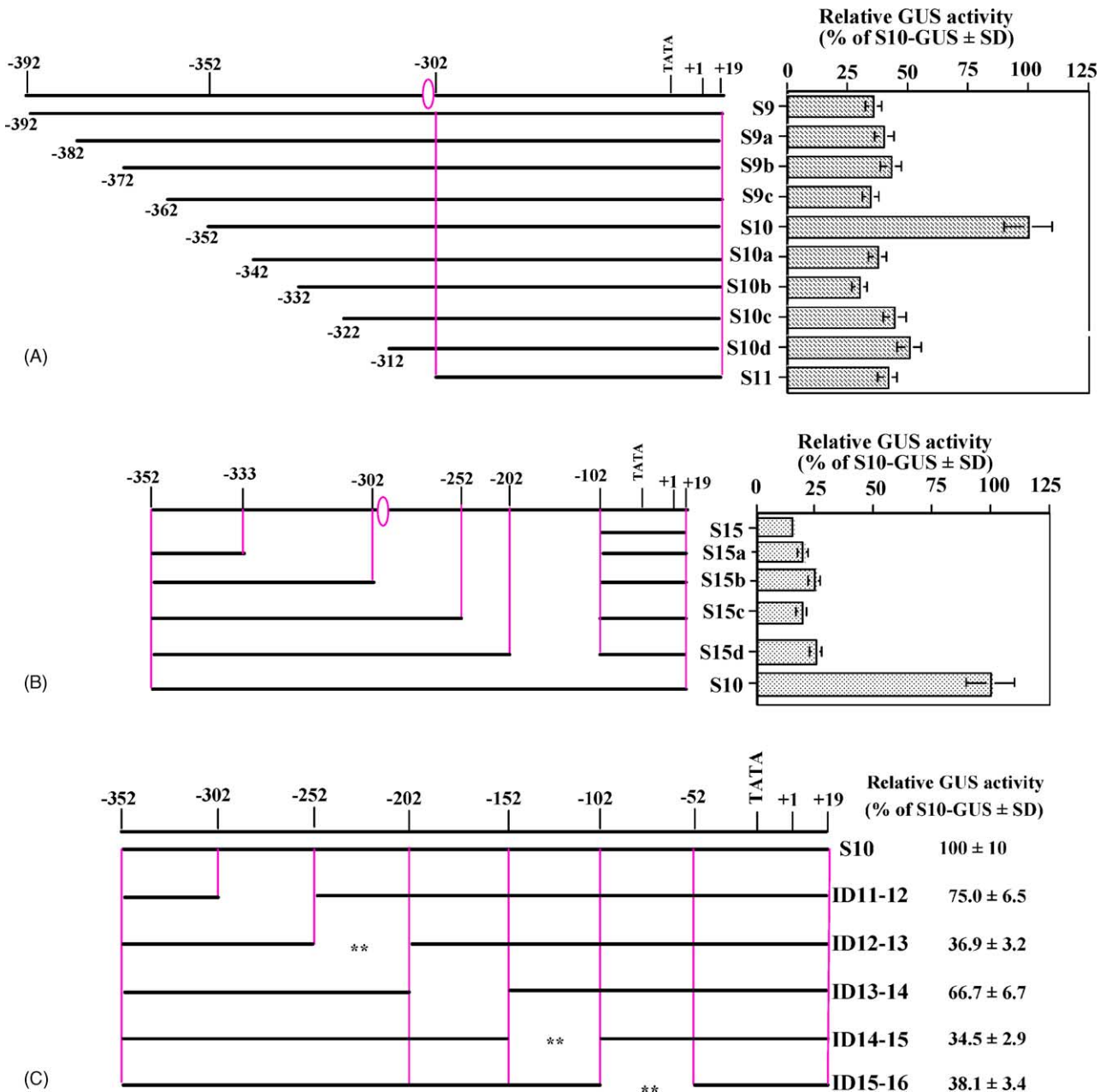


Fig. 3. (A) A schematic map of the GUS constructs developed for the finer deletion analysis of SVBV Flt promoter and expression analysis in tobacco protoplast transient expression assay. At the top, the relative position of the TATA box, transcription start site (TSS, +1) and the SVBV Flt promoter coordinates are shown. The 5'- and 3'-end coordinates of the relative deletion fragments are shown. The relative GUS activity of each construct with standard deviation is presented. (B) A schematic map of the GUS constructs developed for gain-of-function experiment of SVBV minimal promoter and expression analysis in tobacco protoplast transient expression assay. The relative GUS activity with standard deviation is presented. (C) A schematic map of the GUS constructs developed for internal deletion analysis of SVBV Flt promoter and expression analysis in tobacco protoplast transient expression assay. The relative GUS activity with standard deviation is presented. Each of these constructs was assayed at least three times in four independent experiments. 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  $<0.001$ .

promoter, construct 15 (coordinates  $-102$  to  $+19$  from TSS, Fig. 2) to generate constructs: 15a–d. A schematic deletion map and results are shown in Fig. 3B. Constructs 15a–d are devoid of regions (coordinates  $-332$  to  $-103$ ,  $-301$  to  $-103$ ,  $-251$  to  $-103$ , and  $-201$  to  $-103$  from TSS, respectively) as compared to construct 10 (coordinates  $-352$  to  $+19$  from TSS). Quantitative GUS assay for promoter ac-

tivity in constructs 15a–c showed about 40–70% increment over the minimal promoter, construct 15 (coordinates  $-102$  to  $+19$  from TSS). However, these constructs 15a–d gave about 20–25% of maximal promoter activity, compared to construct 10 that gave maximal promoter activity (Fig. 3B). Taking together these data, it indicates that a 100-bp region (coordinates  $-201$  to  $-103$  from TSS) may be essential for

maximum promoter activity (compare promoter activity in construct 15d with construct 10, Fig. 3B). Thus, a 371-bp promoter fragment in construct 10 (coordinates  $-352$  to  $+19$  from TSS) was found sufficient for maximal promoter activity and was chosen for further internal deletion analysis as shown below.

### 3.6. Internal deletion analysis of SVBV FLt promoter

In order to map the position of essential cis-elements and to gain further insight about the essential role of various cis-sequence domains present in the SVBV FLt promoter, internal deletion analysis was carried out with the construct 10 (a 371 bp fragment, coordinates  $-352$  to  $+19$  from TSS) that gave maximum promoter activity. A general scheme of the internal deletion and results are shown in Fig. 3C. Five internal deletion constructs: ID11-12, ID12-13, ID13-14, ID14-15 and ID15-16 with an internal successive deletion of 50-bp (coordinates  $-301$  to  $-253$ ,  $-251$  to  $-203$ ,  $-201$  to  $-153$ ,  $-151$  to  $-103$  and  $-101$  to  $-53$  from the TSS, respectively) showed about 75, 37, 67, 35 and 38% of maximal promoter activity, respectively. Deletion of defined 50-bp in respective construct ID12-13, ID14-15 and ID15-16 resulted in a considerable decrease in promoter activity (Fig. 3C). This observation indicates that these three 50-nt domains (coordinates  $-251$  to  $-203$ ,  $-151$  to  $-103$ , and  $-101$  to  $-53$  from TSS) are essential for promoter activity in this context. Sequence analysis revealed that the deleted regions contain several transcription factor core recognition sequences: DOF (AAAG) [39], as-1/OCS-like sequence [38], and E-box (CANNTG) [40], which may be essential for the SVBV FLt promoter activity. Taken together, the data of gain-of-function and internal deletion analysis experiments suggest that the function of SVBV FLt promoter is the result of combinatorial and synergistic interaction of cis-elements in a position dependent manner as reported for other promoters [8,14].

### 3.7. Transient expression analysis of the SVBV FLt promoter in tobacco and maize protoplasts and comparison with the CaMV 35S promoter

A 371-bp SVBV FLt promoter fragment (pSV10GUS, coordinates  $-352$  to  $+19$  relative to the TSS), that showed the highest GUS activity in tobacco protoplast transient expression assays, was chosen for further analysis. A comparative expression analysis of the SVBV FLt promoter with the CaMV 35S promoter was performed both in tobacco and maize protoplasts using GUS as a reporter gene. Protoplast isolation from suspension cell cultures of tobacco cv. Xanthi and maize cv. BMS, and electroporation with plasmid DNA were done as described in Section 2. Results are shown in Fig. 4A–D. The activity of the CaMV 35S promoter was about seven- and nine-fold higher in tobacco and maize protoplasts, respectively, compared to that of the SVBV FLt promoter (Fig. 4A, B). Real-time qRT-PCR anal-

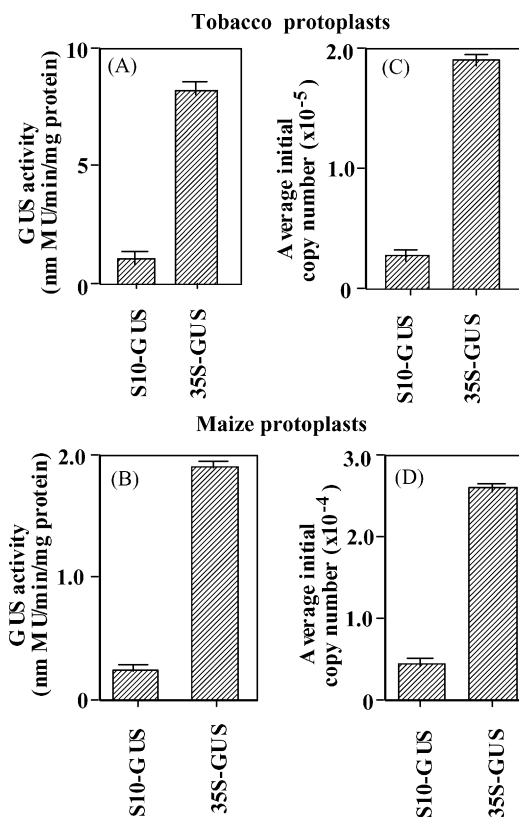


Fig. 4. Comparative expression analysis of the SVBV FLt promoter and CaMV 35S promoters in the protoplast transient expression assay. Average GUS activity in (A) tobacco and (B) maize protoplasts electroporated with constructs pSV10GUS and pCaMV35S GUS. Average initial copy number of GUS transcripts per 100 ng of total RNA in (C) tobacco and (D) maize protoplasts electroporated with constructs pSV10GUS and pCaMV35S GUS. Each construct (pSV10GUS and pCaMV35S GUS) was assayed at least three times in three independent experiments. The average GUS activity/GUS transcript copy number with standard deviation is presented in the histogram. Error bars show the 95% confidence intervals on the means. The statistical ANOVA analysis showed a  $P$  value  $<0.001$ .

ysis was performed to determine whether this difference in the GUS enzyme activity was related to the accumulation of more GUS-specific mRNA in protoplasts electroporated with the CaMV 35S promoter construct. The transcript analysis revealed that the accumulation of GUS-specific mRNA was considerably higher in protoplasts electroporated with the CaMV 35S promoter construct compared to the SVBV FLt promoter. The level was about six-fold higher in tobacco (Fig. 4C) and eight-fold higher in maize protoplasts (Fig. 4D). The expression level of the SVBV FLt promoter was about six-fold higher in tobacco compared to that in maize protoplasts.

### 3.8. Expression analysis of the SVBV FLt promoter in transgenic plants and comparison with the CaMV 35S promoter

Twelve independent tobacco transgenic lines were developed for the construct pKSV10GUS. The seedlings of the



Fig. 5. Histochemical localization of GUS activity in transgenic tobacco seedlings (R1 progeny, 2nd generation, 21 days old) generated for the construct pKSV10GUS and untransformed Samsun NN.

transgenic lines (R1 progeny, second generation) showing the expected segregation ratio (KanR:KanS::3:1) for the kanamycin marker gene were selected for further analysis. RT-PCR analysis was conducted using total RNA isolated from 4-week-old seedlings to amplify the full-length GUS transcript. All the transgenic lines gave the expected 1.8 kb GUS product (data not shown). Histochemical staining of whole seedlings expressing the construct pKSV10GUS showed intense GUS staining in root tissue compared to leaf and stem tissue (Fig. 5). Quantitative GUS activity assay indicated that the activity of the promoter was about three-fold higher in root tissue compared to leaf (Fig. 6A). Real-time qRT-PCR analysis showed that the difference in

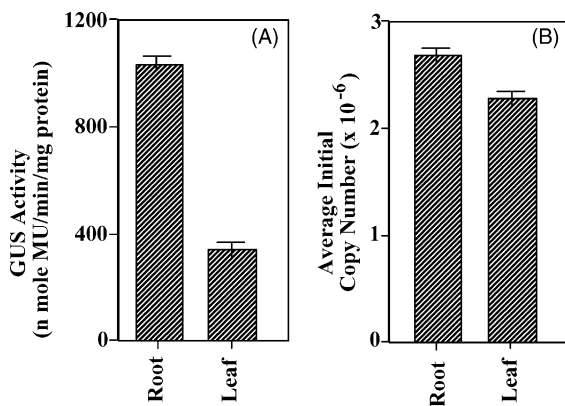


Fig. 6. Expression analysis of the SVBV FLt promoter in transgenic tobacco plants. (A) Average GUS activity in root and leaf tissue of pKSV10GUS- transformed tobacco seedlings. (B) Average initial copy number of GUS transcripts per 100 ng of total RNA in root and leaf tissue of pKSV10GUS- transformed tobacco seedlings. The average GUS activity/GUS transcript copy number with standard deviation is presented in the histogram.

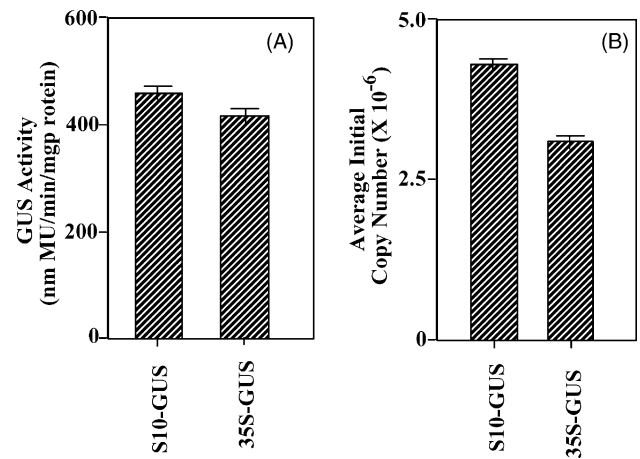


Fig. 7. Comparative expression analysis of the SVBV FLt promoter and CaMV35S promoter in transgenic tobacco plants. (A) Average GUS activity in the 4-week-old transgenic tobacco seedlings (R1 progeny) developed for the constructs pKSV10GUS and pKCaMV35S GUS. (B) Average copy number of GUS transcripts per 100 ng of total RNA isolated from transgenic tobacco seedlings harboring pKSV10GUS and pKCaMV35SGUS. The average GUS activity/GUS transcript copy number with standard deviation is presented in the histogram.

GUS activity was due to the accumulation of more GUS specific mRNA in root tissue (Fig. 6B). Melting temperature ( $T_m$ ) curves of the samples and the standards were analyzed to test the specificity of the amplified PCR products. The  $T_m$ -curve was seen as a single uniform peak at 82 °C for both sample and standard indicating the homogeneity of the amplified products (data not shown). The full-length or sub-genomic transcript promoters from other *Caulimoviruses* such as CaMV, FMV and PCISV have been shown to be more active (about two- to five-fold) in roots compared to leaves [5,6,8]. The strength of the SVBV FLt promoter was compared with the CaMV35S promoter in transgenic tobacco plants with respect to GUS enzyme activity and accumulation of GUS-specific transcripts. Quantitative GUS activity assay showed that the strength of the SVBV FLt promoter was greater than CaMV35S promoter in transgenic plants (Fig. 7A). Real-time qRT-PCR analysis revealed that plants harboring the SVBV FLt promoter fusion construct accumulated more GUS mRNA than did CaMV35S-transformed plants (Fig. 7B). This is in contrast to our results obtained with tobacco protoplast transient expression experiments. This could indicate that protoplasts may lack necessary transcription factors needed for full promoter activity. The results described in the foregoing sections demonstrate that the SVBV FLt promoter is a strong constitutive promoter and can direct expression of genes in transgenic plants. The strength of the SVBV FLt promoter is dependent on the types of cells used for analysis. The SVBV FLt promoter is less active in protoplasts, tobacco as well as maize, compared to the CaMV35S promoter. However, in transgenic tobacco plants the situation is reversed; the strength of the SVBV FLt promoter is greater than the CaMV 35S promoter. The SVBV FLt promoter

has great application potential in plant genetic engineering. For the metabolic engineering of plants and to express polygenic valuable traits in plants, the use of different promoters with non-homologous sequences will be useful, and will minimize the genetic instability due to recombination between identical promoter sequences. We are interested to analyze the molecular interaction between cis-elements and the cognate trans-elements involved in SVBV FLt promoter function and its regulation.

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