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Deletion Analysis of the Sugarcane bacilliform virus Promoter Activity in Monocot and Dicot Plants

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Abstract: The Sugarcane bacilliform virus (SCBV) promoter confers strong constitutive expression in both monocot and dicot plants. To further characterize the SCBV promoter, we conducted a deletion analysis to identify cis-acting promoter elements and determine the minimal promoter sequence required for full promoter activity. Sequential deletions of the 5' end of a 1.4 kb SCBV promoter fragment fused to the *Escherichia coli gusA* reporter gene were used to transiently transform Black Mexican Sweet (BMS) corn (*Zea mays* L.) suspension culture cells and for the production of transgenic plants of *Arabidopsis thaliana* and *Avena sativa*. The results from the BMS cells and the stably transformed Arabidopsis seedlings indicate that the SCBV promoter contains cis-elements affecting promoter strength. Although database searches identified several putative cis-elements that may regulate SCBV promoter specificity, no regions that conferred tissue-specific expression within species were observed except for construct pSCBV-385d which showed constitutive expression in the petals of *A. thaliana*. Differences were observed in embryo expression between *A. sativa* and *A. thaliana*. The promoter activity of a fragment beginning at -254 relative to the TATA box was similar to that of the full-length promoter and therefore should be useful for use in transgene expression.

Key words: Banana streak virus (BSV), cauliflower mosaic virus (CaMV), commelina yellow mottle virus (CoYMV), cassava vein mosaic virus (CsVMV), figwort mosaic virus (FMV), Luciferase (LUC), 4-methyl-umbelliferyl-β-D-glucuronide (MUG), sugarcane bacilliform virus (SCBV), black mexican sweet corn (BSM)

INTRODUCTION

The rate of transcription is an important control point for the regulation of gene expression. Regulatory elements controlling transcription initiation and the rate of transcription reside within the promoter region of a gene (Doelling and Pikaard, 1995). Much of the regulation of expression is mediated by the binding of *trans-acting* proteins (transcription factors) to cis-acting promoter elements. Some transcription factors are present in all cell types while others are unique to specific cell types (Benfey *et al*., 1989). Therefore, the array and types of cis-elements in a promoter and the expression patterns of the transcription factors that interact with them determine the expression specificity of the promoter.

The PLACE database (Higo *et al*., 1999) for plant cis-acting regulatory DNA elements is a good resource for identifying putative cis-elements in a promoter sequence. The PLACE is a database of motifs found in plant cis-acting regulatory DNA elements, based on previously published reports of transcription factor binding sites. However, putative cis-elements predicted by this program must be tested for function in transient or stable transgenic cells or plants.

One of the most extensively characterized plant promoters in terms of its regulatory elements is the 35S promoter. The 35S promoter is the major promoter of the cauliflower mosaic virus (CaMV) and is highly active in most plant organs and during most developmental stages (Odell *et al*., 1985; Jefferson *et al*., 1987). The regulatory region of the 35S promoter is composed of multiple cis-elements that are involved in organ-specific expression, as well as providing for strong constitutive expression (Odell *et al*., 1985; Benfey *et al*., 1990). In transformed

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tobacco plants having 35S promoter deletion constructs, the level of expression similar to those of the full size promoter were retained in a promoter containing the region between -343 and +9 relative to the transcription start site (Odell *et al*., 1985). It has been suggested that the strength of the constitutive properties of the 35S promoter derive from additive and synergistic interactions between factors that bind to the cis-elements within these regions (Benfey *et al*., 1989).

The sugarcane bacilliform virus (SCBV) is a *Badnavirus* that like CaMV encapsidates a dsDNA genome produced by reverse transcription. A 1.4 kb fragment of the SCBV genome has constitutive promoter activity in both the monocot *Avena sativa* and the dicot *Arabidopsis thaliana* (Tzafrir *et al*., 1998). In *A. sativa,* constitutive promoter activity was detected in all organs with the exception of the anther (Tzafrir *et al*., 1998) and embryo (Al-Saady *et al*., 2004). In *A. thaliana*, constitutive promoter activity was detected in the rosette leaf, stem, stamen and root (Tzafrir *et al*., 1998). Constitutive expression patterns of the SCBV promoter were found in most organs of both banana and tobacco (Schenk *et al*., 1999). The constitutive pattern of expression of the SCBV promoter rather than the vascular-specific pattern of expression common to the promoters of the monocot-infecting Badnavirus, commelina yellow mottle virus (ComYMV) and the related *Tungrovirus* Rice tungro bacilliform virus (RTBV), suggests that it might have different regulatory elements.

The goal of this research was to determine the functional organization and minimal promoter of the SCBV full-length constitutive promoter. Deletion analyses of the 5' end of the SCBV promoter were conducted to determine the regions essential for promoter activity in *Arabidopsis thaliana, Avena sativa* and *Zea mays*. We have identified the region -254 relative to the TATA box (305 bp promoter fragment) that has activity similar to the parental 1.4 kb promoter.

MATERIALS AND METHODS

Construction of 5' end deletions of the SCBV promoter: A set of nested deletions of the 5' end of the 1.4 kb SCBV promoter (which will be referred to as the full length promoter) were generated and introduced into pMON755i and pOCA101 (Fig. 1a, b). Standard molecular biology procedures were used throughout (Ausubel *et al*., 1997: Sambrook *et al*., 1989). All coordinates on the SCBV genome used as described previously (Bouhida *et al*., 1993).

The plasmid pSCBV-3 m promoter was used as the backbone for constructs for transient expression assays and stable transformation of *A. sativa* (Tzafrir *et al*., 1998). pSCBV-3m includes the full-length SCBV promoter fused to *gusA* gene and the maize alcohol dehydrogenase-1 (*Adh*I) intron for enhanced expression in monocots as designated by the letter m. From here on the pSCBV-3m full length promoter used in monocots will be referred to as pSCBVm.

The following deletions were made within the plasmid containing the 1.4 kb SCBV promoter by digesting with the restriction enzyme *Hpa*I plus the following restriction enzymes: *Sph*I, *Age*I, *Bgl*II and *Stu*I, respectively. The resulting pSCBVm fragments were polished using T4 DNA polymerase to produce blunt ends and self ligated. The resulting deletions were designated SCBV-555m, SCBV-385m and SCBV-254m and promoter-less relative to the putative TATA box, respectively. Further deletion constructs were made for pSCBVm. For deletion constructs pSCBV-104m and pSCBV-42m, forward primers GCACCTGTGCCACTTTATTCC and GCAGTGCGTGTGTGACACC and reverse primer GGATAGCATACAAGAATCAGCG were designed to amplify the regions 7262-7420 and 7329-7420 (relative to the numbering), respectively (Bouhida *et al*., 1993). A *Pst*I site was engineered into the amplifying primers at positions 7262 and 7329. The resulting fragments were digested with *Pst*I and *Stu*I and constructs were prepared as mentioned above.

For transformation of *A. thaliana,* the binary vector pOCA101 (Medberry and Olszewski, 1993) was used. The plasmid pOCA101 is a derivative of pOCA28 (Medberry *et al*., 1990) in which the polylinker has been replaced by the promoter-less GUS gene contained on a *Hin*dIII-*Eco*RI fragment from pBl101. The promoter region of pSCBVm was digested with *Stu*I and *Pst*I and ligated to pBluescript SK- (Stratagene, La Jolla, CA) that was digested with restriction enzymes *Sma*I and *Pst*I. The above mentioned deletions were made in pBluescript SK- and then the *Sal*I-*Xba*I fragments were isolated from the resulting recombinant constructs and ligated into pOCA101 vector that was digested with *Sal*I-*Xba*I. The resulting plasmids were designated pSCBVd (full length promoter), pSCBV-555d, pSCBV-385d and pSCBV-254d using d to signify deletions for dicot transformation. All promoter deletion plasmids were confirmed by restriction mapping and sequencing.

Transient assay: To determine the strength of the SCBV promoter deletions by transient expression, the plant expression vector pSCBV m, pSCBV-555 m, pSCBV-385 m, pSCBV-254 m, pSCBV-104 m pSCBV-42 m and the promoter-less construct were used in maize BMS suspension cells. The Biolistic PDS 1000-Helium gun

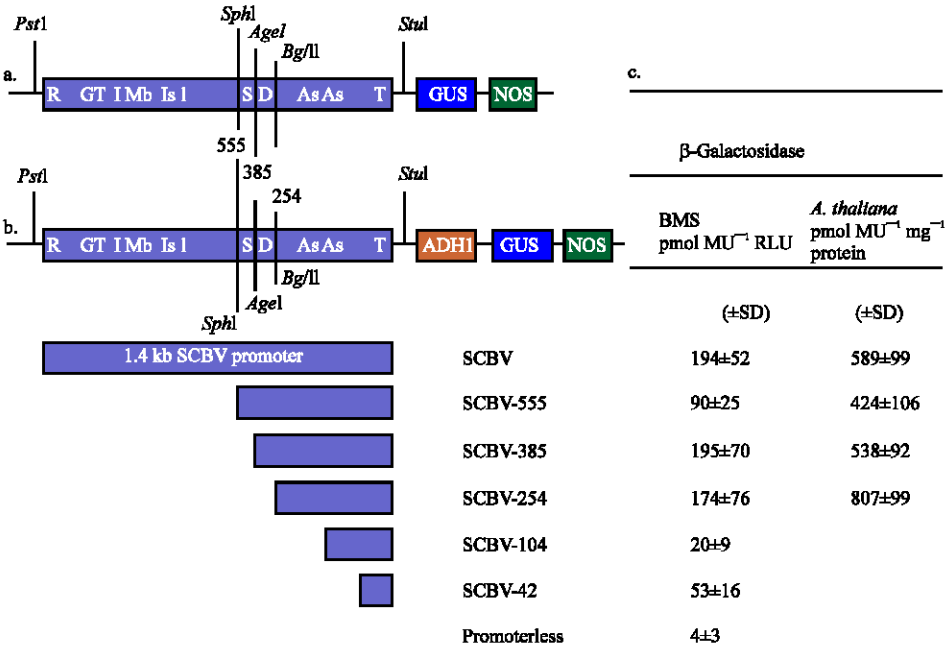

Fig. 1: A schematic map of the SCBV full-length promoter and 5' end deletion constructs in (a) pMON755i monocot expression construct (b) pOCA101 dicot expression construct. Deletions are indicated by the bars below the maps, with numbers designated relative to the 5'end of the TATA box T. The maps show the different putative elements identified in the SCBV promoter using the database. The promoter elements are labeled as follows: R is the RAVIAAT motif, GT is the GT1 motif, I is the Ibox motif, Mb is the MYBIAT motif, Is is the INTRNSPADB motif, S is the SEF3 motif, D is DOFCOREZM motif, Is is the ASF1 motif and T is the TATA box. (c) β-glucuronidase activity which included MUG fluorometric BMS assay activities of BMS cells and MUG assays on protein extracts from transgenic Arabidopsis. For the GUS assay of BMS cells each construct was independently transformed 5 times. The data were analyzed as an average of the 5 independent transformations for each construct. Expression data were determined as a ratio of β-glucuronidase (GUS) to luciferase (LUC) in pmol MU/min/RLU. For the MUG assays on protein extracts from transgenic Arabidopsis, soluble proteins were prepared from 3 week old shoots of homozygous plants and assayed for GUS activity as described in the Materials and Methods. Each extract was prepared from >10 shoots and extracts from two or more sets of plants were assayed for each line. The data indicate the mean activity of the extracts of an independent transgenic line. SD indicates the standard deviation errors

(Bio-Rad, Hercules CA) was used to deliver the various DNA constructs into maize BMS cell lines (Torbert *et al*., 1995). Cells at mid-log growth phase were collected by filtration onto 5.5 cm filter paper to form a thin layer of cells. The filters were then transferred to petri dishes containing MS2D medium (Fromm *et al*., 1986). Co-bombardments were done using the plasmid pMON755i, which uses the 35S as the promoter and derivatives of pMON755i using the SCBV promoter. All transformations were co-transformed with the plasmid pMON722i, which has the 35S promoter and the reporter gene luciferase (LUC). pMON772i was used as an internal control to normalize for any variations in plasmid introduction efficiency as well as extract preparation. Each construct was independently transformed 5 times. The data were analyzed as an average of the five independent transformations for each construct.

LUC and MUG assays: BMS cells were ground in GUS extraction buffer (0.1 M KPO_4, pH 7.8, 1 mM EDTA, 10 mM DTT, 0.8 mM PMSF, 5 % glycerol). Extracts were clarified by centrifugation (5000x g, 5 min) at 4°C in a micro centrifuge. The supernatant was recovered and used for MUG and LUC assays. GUS activity was determined using, 4-methyl-umbelliferyl-β-D-glucuronide (MUG Sigma) by the method of Jefferson *et al.* (1987) and quantified for 50 μL of extract as pmol 4-methylumbelliferone (MU) produced per minute.

Luciferase assays were performed according to the method of Nguyen *et al.* (1988). Luciferase (LUC)

quantification was carried out by injecting 0.3 mL luciferase assay buffer (25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 0.5 mg mL^{-1} BSA) and 0.1 mL of 0.65 mM luciferin into 50 μL of the same protein extract in a Berthold LB9501 luminometer. Activity was recorded as Relative Light Units (RLU) produced in the first 10 sec after injection. Expression data was determined as a ratio of β-glucuronidase (GUS) to luciferase (LUC) in pmol MU/min/RLU. The promoter activities were compared statistically using the pooled Student's t-test (Montgomery and Runger, 1994).

For Arabidopsis MUG assays were conducted on protein extracts from the transgenic plants. Soluble proteins were prepared from 3 week old shoots of homozygous plants and assayed for GUS activity as described above. Each extract was prepared from >10 shoots and extracts from two or more sets of plants were assayed for each line.

Transformation and regeneration of *A. sativa*: Callus tissue derived from the GAF-30/Park genotype of oat (*Avena sativa* L.) was subjected to particle bombardment as mentioned in the transient assay. The deletion constructs along with pH24 plasmid were co-transformed into embryogenic callus derived from mature embryos (Torbert *et al*., 1998). The pH24 plasmid contains the 35S-*npt II* cassette which confers resistance to paramomycin. Regenerated plants were placed in the growth chambers set at 20°C day, 15°C night with a 12 h day-length of light intensity 300 to 400 μE m^{-2} sec^{-1} at canopy top from an adjustable height fixture of incandescent and cool white fluorescent lamps until the plants were about 20 to 25 cm tall. Plants regenerated from transgenic tissue culture were designated T_0 plants. T_0 plants were selfed to produce the T_1 generation. T_1 seeds were stained in a section of the endosperm to identify GUS-positive seeds. Ten GUS-positive T_1 seeds from the GUS-positive T_1 lines were selected and used for further analysis. The T_1 generation plant tissues were stained for GUS activity.

Plants were sampled and sections stained for GUS at the following stages: callus; germination, a section of the seminal root was cut and stained; 3 leaf stage, the third leaf section was stained; tillering stage, a tiller was removed and leaf and stem sections were stained; developing panicles still enclosed by the leaf sheath (booting stage), leaf, stem, roots and spikes were stained; and mature T_2 seeds were cut into two half grain portions containing half embryos and stained. Three sections of each leaf were stained: the leaf tip, middle portion of the leaf (anywhere between the top section and the bottom section) and the bottom of the leaf including the collar. Stem sections were also taken from three locations: top (section beneath the second youngest leaf), middle (any section between the top and bottom sample which includes a node) and the bottom of the stem. Random root sections were stained. The entire panicle at booting stage was removed, cut into pieces and stained.

Transformation and regeneration of *A. thaliana*: To determine the expression patterns of the above mentioned deletions in *A. thaliana* the deletion constructs in pOCA101 were introduced into the *Agrobacterium tumefaciens* strain C58CI (pMP90) (Koncz and Schell, 1986) by electroporation (Mersereau *et al*., 1990). The resulting recombinant bacteria were used to transform eight *A. thaliana* Colombia plants by the vacuum infiltration method of Van Hoof and Green (1996). Seeds from the transformed T_0 plants were sown on Kanamycin-containing plates as described by Feldmann (1992). Individual kanamycin-resistant plants designated T_1 plants were transferred to soil. Plants were sampled for the leaf, stem and root at 4, 6 and 10-days and at bolting. At bolting the inflorescence was also sampled and stained for GUS activity. GUS staining was also determined in mature *A. thaliana* seeds.

At least 6 independent T_2 lines homozygous for each transgene construct were identified and T_3 seeds were collected from these lines. Individual homozygous plants were selected based on kanamycin resistance and GUS staining. For each line >10 plants were grown on MS medium and the shoots were harvested when the plants were three weeks old. The shoots were frozen in liquid nitrogen and stored at -80°C until they were used to prepare protein extracts for MUG assays. The protein extracts were prepared and the MUG assay was performed as described above.

Analysis of GUS activity: GUS analysis was done according to the histochemical staining method described earlier (Jefferson, 1987; Kosugi *et al*., 1990). *Avena sativa* tissues were hand sectioned from different organs during different developmental stages and placed in GUS staining buffer pH 8.0 containing the indigogenic substrate X-Gluc (5-bromo-4-chloro-3indolyl glucuronide) with 20% v/v methanol. The GUS staining solution was vacuum-infiltrated into tissue by subjecting it to a vacuum for 10 min to ensure that the stain is transferred throughout the tissues. After staining, chlorophyll was removed from green tissues by soaking them in 70% ethanol. *A. sativa* florets were collected from mature plants (those in which leaves have senesced). *Avena sativa* and *A. thaliana* progeny (T_1) plants were subjected to GUS analysis.

Scoring of GUS-staining: The GUS staining intensity was scored according to the following scale: high (+++), medium (++) low (+) or none (-) staining intensities. Each plant within a line was scored according to the above scale and tallied based on the staining intensities. A line was scored as high, medium, low or no staining intensity based on the staining intensities observed in the majority of plants within a line (Table 1 and 2). GUS expression pattern was scored as positive in a line if the same pattern was observed in more than one plant per line.

Table 1: GUS staining of oat lines transformed with the full-length and deletions fragments of the ScBV promoter fused to *gusA* in different organs during different developmental stages

	pSCBVm					pSCBV-555m					pSCBV-385m					pSCBV-254m				
	GUS expression					GUS expression					GUS expression					GUS expression				
Stages	+++	++	+	-	Expressing	+++	++	+	-	Expressing	+++	++	+	-	Expressing	+++	++	+	-	Expressing
Mature seed																				
Endosperm	9	-	-	1	9	6	-	-	-	6	8	-	-	-	8	7	4	2	-	13
Embryo	-	-	-	10	0	-	-	-	6	0	-	-	-	8	0	-	-	-	13	0
Seminal Root	-	-	4	6	4	-	-	2	4	2	-	-	2	6	2	4	-	-	9	4
3 Leaf	1	4	2	3	7	-	1	1	4	2	1	1	6	-	8	1	2	6	4	9
Tillering stage																				
Leaf	-	2	4	4	6	-	-	1	5	1	-	4	4	-	8	-	1	9	3	10
Stem	1	4	4	1	9	-	3	1	2	4	1	3	4	-	8	-	3	7	3	10
Booting stage																				
Leaf	4	2	2	2	8	3	-	3	-	6	3	3	1	1	7	3	2	2	6	7
Stem	3	2	1	4	6	4	-	2	-	6	4	2	-	2	6	4	-	1	8	5
Root	-	5	-	5	5	-	2	2	2	4	-	-	2	6	2	4	1	2	6	7
Spike	4	3	1	2	8	6	-	-	-	6	4	4	-	-	8	-	4	1	8	5
Anther	-	-	2	8	2	-	-	-	6	0	-	-	-	8	0	-	2	-	11	2
Filament	-	4	2	4	6	-	-	-	6	0	-	-	2	6	2	-	-	1	12	1
Pollen	-	-	-	10	0	-	-	-	6	0	-	-	-	8	0	-	-	1	12	1
Ovary	-	-	2	8	2	-	-	-	6	0	-	-	-	8	0	-	-	2	11	2
Stigma	-	-	2	8	2	-	-	-	6	0	-	-	-	8	0	-	-	1	12	1
Style	-	-	2	8	2	-	-	-	6	0	-	-	-	8	0	-	-	-	13	0

Transgenic lines were scored into four groups; high (+++), medium (++), low (+) and none (-) based on staining intensity. Ten to fifteen T_1 plants per line were analyzed. Each plant within a line was scored according to the above scale and tallied based on the staining intensities. GUS expression pattern was interpreted as representing promoter specificity in a line if the same pattern was observed in more than one plant per line

Table 2: GUS staining of *A. thaliana* lines transformed with the full-length and deletion fragments of the ScBV promoter fused to gusA in different organs during different developmental stages

	pSCBVd					pSCBV-555d					pSCBV-385d					pSCBV-254d				
	GUS expression					GUS expression					GUS expression					GUS expression				
Stages	+++	++	+	-	Expressing	+++	++	+	-	Expressing	+++	++	+	-	Expressing	+++	++	+	-	Expressing
Mature seed	10	-	-	-	10	10	-	-	-	10	15	-	-	-	15	15	-	-	-	15
Seedlings 4 days																				
Leaf	10	-	-	-	10	6	4	-	-	10	15	-	-	-	15	15	-	-	-	15
Stem	10	-	-	-	10	6	4	-	-	10	15	-	-	-	15	15	-	-	-	15
Root	10	-	-	-	10	10	-	-	-	10	15	-	-	-	15	15	-	-	-	15
6 days																				
Leaf	8	2	-	-	10	5	3	-	2	8	15	-	-	-	15	15	-	-	-	15
Stem	8	2	-	-	10	8	-	-	2	8	15	-	-	-	15	15	-	-	-	15
Root	8	-	2	-	10	9	-	-	1	9	15	-	-	-	15	15	-	-	-	15
10 days																				
Leaf	6	2	1	1	9	6	2	-	2	8	15	-	-	-	15	15	-	-	-	15
Stem	6	2	-	2	8	3	3	2	2	8	15	-	-	-	15	15	-	-	-	15
Root	6	2	-	2	8	6	4	-	-	10	15	-	-	-	15	15	-	-	-	15
Bolting																				
Leaf	8	-	2	-	10	2	6	-	2	8	11	4	-	-	15	11	2	2	-	15
Stem	8	-	2	-	10	2	6	-	2	8	12	3	-	-	15	10	5	-	-	15
Root	8	-	-	4	6	3	6	-	1	9	12	3	-	-	15	9	4	2	-	15
Petals	-	-	2	8	2	-	6	-	4	6	8	3	-	4	11	7	7	-	1	14
Anther	-	4	2	4	6	-	-	-	10	0	-	-	2	13	2	-	2	-	13	2
Pollen	-	-	-	10	0	-	-	-	10	0	-	-	-	15	0	-	-	-	15	0
Ovary	-	-	2	8	2	-	4	-	6	4	-	11	-	4	11	-	8	2	5	10
Style	-	2	-	8	2	-	4	-	6	4	11	-	-	4	11	8	-	2	5	10
Stigma	-	-	2	8	2	-	4	-	6	4	-	11	-	4	11	-	8	2	5	10

Transgenic lines were scored into four groups; high (+++), medium (++), low (+) and none (-) based on staining intensity. Ten to fifteen T_1 plants per line were analyzed. Each plant within a line was scored according to the above scale and tallied based on the staining intensities. GUS expression pattern was interpreted as representing promoter specificity in a line if the same pattern was observed in more than one plant per line

RESULTS

To directly test the function of the multiple cis-elements present in the SCBV full-length promoter, a set of nested deletions were produced using convenient restriction enzyme sites. Figure 1a and b show the full length promoter, deletion constructs as well as the putative cis-elements predicted within the SCBV promoter by the PLACE database analysis. The deletion constructs were used to produce transgenic oat and Arabidopsis plants. Tissue-specificity and development regulation of the SCBV deletion constructs were determined in T_1 progeny of multiple independent events in oats and Arabidopsis using histochemical GUS staining (Table 1, 2, Fig. 2).

Mature dried T_2 seeds were collected and sectioned both in cross-section and longitudinally and stained for GUS. All four constructs produced strong GUS activity in the endosperm of mature oat seeds as indicated in Table 1 and Fig. 2a. Consistent with our previous observations (Al-Saady *et al*., 2004), no embryo staining was produced by any of the constructs. The four constructs pSCBV m, pSCBV-555 m, pSCBV-385 m and pSCBV-254 m exhibited strong GUS staining in transgenic oat callus as shown in Fig. 2b.

Seminal root sections from seedlings growing on filter paper in petri dishes were stained for GUS activity (Table 1). For the full length construct pSCBVm, expression in the seminal root was mainly observed in the root tip, for constructs pSCBV-555m, pSCBV-385m and pSCBV-254m expression was localized to the vascular tissue.

At the 3 leaf stage, sections from the third leaf were stained for GUS activity. All 4 constructs produced similar staining patterns but the intensity of the staining was variable (Table 1). All leaf sections showed a constitutive pattern of expression with stronger staining in the vascular tissue (Fig. 2c).

During the tillering stage, a tiller was removed and stem and leaf sections were stained for GUS activity. All four constructs showed GUS activity in both the leaf and stem. In the leaves GUS staining was more intense in the vascular tissue.

During the booting stage, leaf, stem and root sections as well as panicles were analyzed for GUS activity (Table 1, Fig. 2c and d). All four constructs caused GUS staining in the leaves, stem and roots. The glumes, lemma and palea of some of the lines from each construct had detectable GUS staining. pSCBV m had 2 lines with GUS staining in the ovary, stigma and style. No GUS activity was observed in the reproductive tissues of pSCBV-555 m (Table 1). pSCBV-385 m had 2 lines with expression in the filament. Limited GUS activity was observed in the ovary, stigma and style of pSCBV-254 m lines. Overall there were no major changes in promoter specificity that could be related to the promoter deletions.

Arabidopsis thaliana seeds from plants transformed with all four constructs exhibited GUS staining. Similarly, 4, 6 and 10-day old seedlings transformed with all four constructs had similar GUS staining in the leaf, stem and root (Fig. 2e, g and h). All transgenic lines transformed with pSCBV-385d and pSCBV-254d had high GUS staining in all organs tested during the three different seedling stages. Lines transformed with pSCBVd and pSCBV-555d had more variable staining intensities (Table 2). At 4 days, GUS activity was constitutive throughout the seedling except at the root tip (Fig. 2f). At 6 days, GUS staining was constitutive in the root and more vascular in the hypocotyl and cotyledons. At 10 days, the staining pattern was mainly constitutive. pSCBVd had weaker staining intensity at 10 days compared to SCBV-385d and SCBV-254d plants (Table 2).

During the bolting stage, GUS staining was observed in the leaf, stem and root for all four constructs (Table 2). Fewer pSCBV-555d lines with strong staining intensities were observed compared to pSCBVd, pSCBV-385d and pSCBV-254d plants. One difference was observed in the petals of pSCBV-385d, which showed constitutive staining patterns compared to the other three constructs, which had a vascular pattern of expression (Fig. 2i-*l*). All four constructs caused GUS staining in all the reproductive tissues except for the pollen grains. As shown in Table 2, a few lines for pSCBVd, pSCBV-385d and pSCBV-254d had some GUS staining in the anthers, whereas lines for pSCBV-385d had no GUS staining in the anthers (Table 2). Two, four, eleven and ten lines for pSCBVd, pSCBV-555d, pSCBV-385d and pSCBV-254d had GUS activity in the ovaries and stigma, respectively. All lines that had expression in the style had a constitutive pattern of expression. As of oat, promoter specificity was generally similar for the three deletion constructs and the full-length control.

Variation in the intensity and frequency of plants exhibiting GUS staining in both species suggested that there may be changes in promoter strength associated with specific deletions. To test this possibility, promoter strength was determined using the MUG assay in transient transformation of BMS cells and transgenic *A. thaliana* plants. As shown in Fig. 1C, in BMS cells the full-length construct pSCBVm produced about 194 pmol MU/min/RLU. pSCBV-555m promoter activity was reduced to almost half (90 pmol MU/min/RLU) the activity of pSCBVm ($p<0.05$). pSCBV-385m and pSCBV-254 m had promoter activity similar to pSCBVm ($p>0.05$) (Fig. 1c). Of

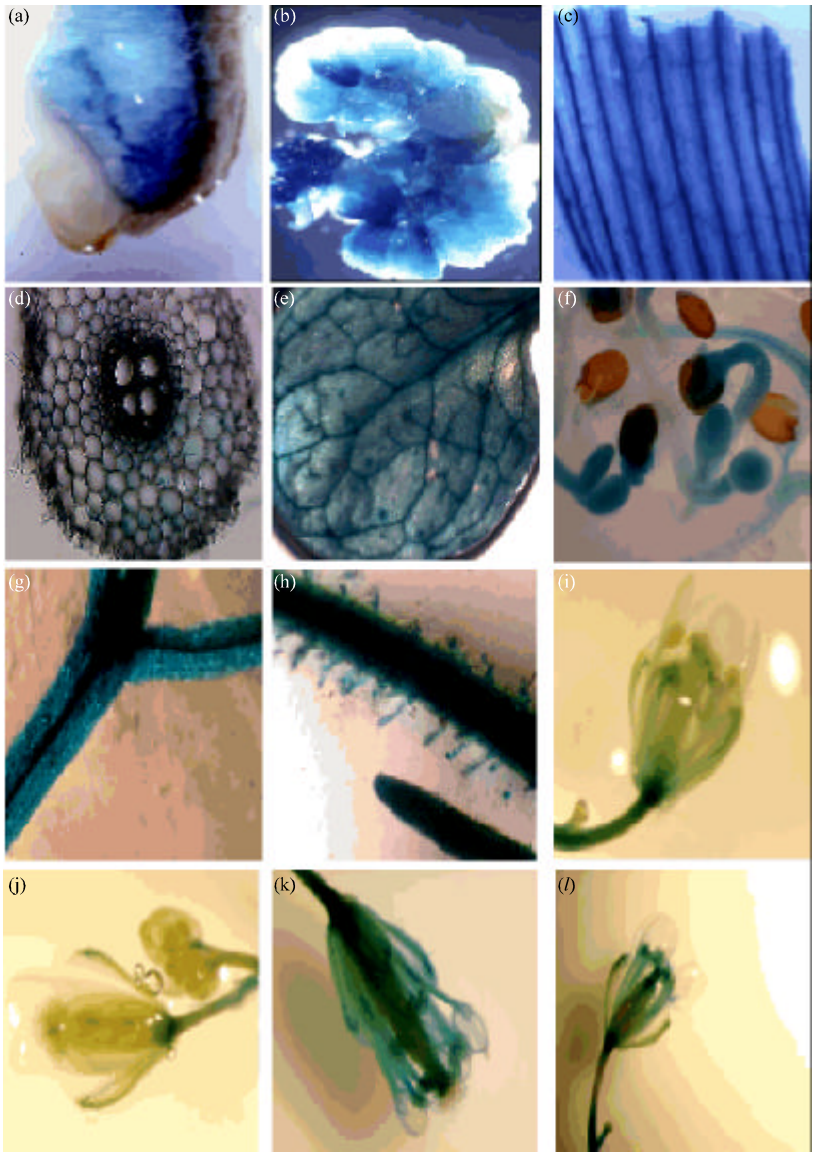

Fig. 2: Histochemical analysis of GUS expression in transgenic oat and Arabidopsis transformed with the deletion series. Pictures are representative of the lines analyzed. (a) Oat embryo, (b) Oat callus, (c) Oat leaf, (d) Oat root, (e) Arabidopsis leaf, (f) Arabidopsis seedings, (g) Arabidopsis stem, (h) Arabidopsis root, (i-*l*) Flowers of pSCBVd, SCBV-555d, SCBV-385d and SCBV-254d lines, respectively

the other two additional constructs used in the transient assay of BMS maize cells, SCBV-104m had low levels of promoter activity. Deletion of the next 62 bp (SCBV-42 m) slightly increases promoter activity. The promoter-less construct showed minimal promoter activity (4 pmol MU/min/RLU).

The effect of the different deletions on the SCBV promoter activity in stably transformed 3 week old Arabidopsis shoots was determined (Fig. 1b, c and Table 2). For each promoter, the β-glucuronidase activity in protein extract prepared from shoots of at least 6 independent homozygous T_3 lines was determined using a MUG assay. For each promoter construct, the different T_3 lines had considerable variation in the MUG activity and there was no significant difference in the activity conferred by the promoters. However, the general trend in activity paralleled the transient expression results obtained with BMS cells in that the SCBV-555 promoter had the lower activity and SCBV-254 promoters had greater activity then the full length promoter.

DISCUSSION

In recent years, studies of several badnavirus promoters have provided insight into their expression patterns and potential for driving the expression of transgenes in plants (Medberry *et al*., 1992; Bhattacharya-Pakrasi *et al*., 1993; Medberry and Olszewski, 1993; Yin and Beachy, 1995). In this study the activity of the promoter was assessed by determining the pattern of GUS activity in transgenic *A. sativa* and *A. thaliana* containing deletion series of the SCBV:GUS reporter gene. Studies of eukaryotic promoters have shown that promoters are composed of multiple cis-elements, which are required for promoter function. Several of these promoter elements are common to most promoters, which function as the basal elements of the promoter, such as the TATA box and the CAAT box, while other promoter elements are unique to specific promoters (Ow *et al*., 1987).

Based on transient assays of the deletion series of the SCBV promoter in maize BMS cells, a 50% reduction in promoter activity was observed upon deletion of about 800bp (pSCBV-555 m) from the 5' end of the full-length SCBV promoter (pSCBVm). This indicates the presence of a positive regulatory element in pSCBV-555 m sequences. According to the PLACE database search many putative cis-elements in the SCBV promoter fragment were identified (Fig. 1a, b).

Several CAAT box, an upstream promoter element which usually resides near position -80 and important for the transcription (Ow *et al*., 1987), were identified upstream of the region -555 relative to the TATA box. Another element identified in the region upstream of -555 is the GATA motif (as-2), which has been shown to be important for leaf tissue expression in the 35S promoter (Lam *et al*., 1989). A putative GT1 consensus (GRWAAW) was identified upstream of the region -555. GT1 element has been identified in many light-regulated genes of plants such as pea, oat, rice, tobacco and *A. thaliana* and has been reported to stabilize the TFIIA-TBP-DNA complex, a transcription factor complex that binds to the TATA box to initiate transcription (Villain *et al*., 1996; Le Gourrierec *et al*., 1999). In the SCBV promoter a putative GT1 consensus was identified in three positions, two within the region upstream of -555 and one within the region -555 and -385. Some elements are required in multiple copies to be functional (Meshi and Iwabuchi, 1995) and some transcription factors need to form homodimers.

Many elements unique to the region upstream of the deletion -555 such as the IBox consensus sequence GATAAG, INTRONLOWER sequence TGCAGG, INTRNTPSADB sequence YTCANTYY, RAV1AAT, several transcription factor MYB recognition sites and sugar responsive elements and other transcriptional activators were also identified (Fig. 1a). The IBox is a binding site for the transcription factor leMYB1, which has been shown to be a transcriptional activator in *Arabidopsis thaliana* (Donald and Cashmore, 1990). Mutations in the IBox sequences profoundly affect expression from the *Arabidopsis* rcbS-1A promoter. Deletion of this putative element in the SCBV promoter could have contributed to the reduced promoter activity seen in the case of SCBV-555m.

Upon further deletion of the next 170 bp (pSCBV-385 m), the activity level went back up to full-length promoter activity level. Some promoter elements act as negative regulators resulting in reduced promoter activity, which when deleted could result in increased activity levels. From the database search, no negative regulatory element was identified within the region -555 and -385. Some promoter elements are known to function as binding sites for both activators and repressors (Meshi and Iwabuchi, 1995). A putative element such as GT1, which is important for transcription could also bind transcription factors that are capable of interacting with other transcription factors that down regulate transcription, therefore upon deletion of such elements as in the case of pSCBV-385 m and pSCBV-254 m, the activity level of the SCBV promoter was restored.

Deletion of about 1.3 kb from the 5' end of the promoter resulted in very low levels of promoter activity indicating that most of the elements necessary for the strength of the SCBV promoter reside upstream of the region -104 relative to the TATA box (Fig. 1b).

A few differences were observed for the deletion constructs in *A. thaliana* transgenic plants. No GUS staining was observed in the reproductive tissues of the constructs pSCBV-555 m and pSCBV-385 m, whereas the other two constructs had a few lines that showed some staining in some of the reproductive tissues of oat. Tissue and developmental specific expression patterns are determined by the combination of spatial orientation of 'cis-elements and the presence of transcription factors that interact with these elements (Dynan, 1989). Even though several late pollen-specific element GTGANTG10 (GTGA) were identified upstream of -555, all promoter fragments showed no expression in the pollen grains of both monocots and dicots. Therefore, these pollen-specific elements might not be functional in the SCBV promoter.

Strong GUS expression was observed in the endosperm of transgenic oat and Arabidopsis lines. Several endosperm-specific elements and storage protein

elements were identified throughout the SCBV promoter. Eleven copies of the endosperm specific putative element DOFCOREZM consensus sequence AAAG (Yanagisawa, 2000) were identified upstream of -555 and five copies of the storage protein element E-box of the *napA* storage protein of *Brassica napus* were identified, three within the region -555 and -385 and two within the region -42 (Stalberg *et al*., 1996).

Subtle differences in the deletion constructs were observed when tested in one species such as those in the reproductive tissues of oat and in the seminal root of lines transformed with pSCBVm, which showed expression primarily in the root tips compared to vascular expression in the deletion constructs. These differences could be due to low number of oat lines analyzed and the low number of lines expressing GUS possibly due to silencing.

In the dicot *A. thaliana* GUS staining data we see a trend in promoter activity similar to the results obtained in the transient assay. The full-length promoter construct had lines with variable staining intensities at 6 and 10 days but also most of the lines had strong GUS activity in the organs tested. pSCBV-555d had fewer *A. thaliana* lines with strong GUS activity compared to the other constructs. The strongest promoter activity was observed with pSCBV-385d and pSCBV-254d, where during the different seedling stages, all lines had strong expression in all organs tested (Table 2). This indicates that possibly the same elements were involved in the regulation of the SCBV promoter GUS activity in BMS cells as well as in *A. thaliana*. At bolting, only lines transformed with pSCBV-385d and pSCBV-254d had strong GUS activity. pSCBV-254d seemed to be a strong promoter in *A. thaliana*, as shown by the MUG analysis (Fig. 1c, Table 2).

In terms of staining patterns again similar patterns of expression were observed between the full-length promoter fragment and the deletion constructs as in *A. sativa*. The major difference observed was in the petals of the pSCBV-254d transformants, which showed increased staining in both the vascular and non vascular tissue (Fig. 2k). This could possibly be due to the presence of a cis-element involved in petal tissue expression in the region -385 and -254 that was down-regulated by other elements upstream or downstream of this region and upon deletion of the upstream regions the petal *cis*- element was expressed. It is not likely that this pattern of expression was due to position effect since the number of lines showing this pattern of expression was high, 14 out of 15 lines (Table 2).

The major difference observed between the expression patterns of the monocot and dicot with the SCBV promoter was in the embryo. No GUS staining was observed in the embryos of *A. sativa* (Fig. 2a), whereas GUS staining was observed in *A. thaliana* embryos. A soybean embryo factor binding site SEF3 (AACCCA) (Allen *et al*., 1989) was identified in the SCBV promoter but since the deletion constructs showed no change in embryo expression upon deletion of this putative element. Another embryo specific element the DPBFCOREDCDC3 (ACANNG) was identified within the region upstream of -555 and within the -254 (Kim *et al*., 1997). DPBFCOREDCDC3 is a binding core sequence for a novel class of bZIP transcription factors found in the Dc3 gene promoter of carrots. Despite the presence of several of these elements in the SCBV promoter no GUS expression was detected for all four constructs in oats. In Arabidopsis GUS expression was observed indicating the possibility that these elements could be functional in dicots or that the required transcription factor for binding to this element is present in dicots. Another difference between *A. sativa* and *A. thaliana* was in the roots. In *A. sativa* root GUS expression was very variable and only sections of the roots showed GUS staining, whereas in *A. thaliana* very strong and consistent GUS staining was observed throughout the root system. According to the database analysis of the SCBV promoter two putative as-1-like elements were identified at positions -233 and -196 relative to the TATA box (Fig. 1A, 1B, Table 3). The -233 as-1-like element of SCBV is identical to the as-1-like elements of cassava vein mosaic virus (CsVMV) and banana streak virus (BSV). The SCBV putative as-1-like elements share sequence homology with a 1 bp difference among each other and with the as-1 elements of CoYMV, CaMV and figwort mosaic virus (FMV) promoters as indicated in Table 3. The CaMV as-1 element has been shown to be important for root expression (Benfey *et al*., 1989).

Several putative environmental and biological stress inducible elements were also identified within the SCBV promoter. In this study we did not identify specific

Table 3: Comparison of the putative SCBV AS-1 like element with other viral promoter AS-1 like elements. The TGACG repeats are underlined and bases that differ from the consensus TGACG are shown in bold. Distances given are relative to the 5'end of the TATA box. SCBV is the Sugarcane bacilliform virus, CoYMV is the Commelina yellow mottle virus, CaMV is the cauliflower mosaic virus, FMV is the figwort mosaic virus and CsVMV is the cassava vein mosaic virus and BSV is the banana streak virus

Putative SCBV	Activator sequence-1 (AS-1) like	Distance from TATA box
SCBV	AGACGTAAGCAATGACG	233
SCBV	TGACGTCAGGGATGACC	196
CoYMV	TGATGATGTCATTGACG	176
CaMV	TGACGTAAGGGATGACG	34
FMV	TGACGAACGCAGTGACG	22
CsVMV	AGACGTAAGCAATGACG	174
BSV	AGACGTCAGCAATGACG	288

promoter regions among the regions deleted that conferred tissue specific expression. This may indicate that elements involved in tissue specificity reside downstream of -254. One putative element identified in the SCBV promoter from the database is the element NtBBF1 is the binding site in *Agrobacterium rhizogenes rolB* gene required for tissue specific-expression (Baumann *et al*., 1999). This putative element is located downstream of -254. Also the putative as-1 elements identified in the SCBV promoter which are important for root expression reside downstream of -254. These and more elements located downstream of the region -254 could be involved in tissue specificity. The SCBV promoter consists of several elements such as NtBBF1 element, Dof core transcription binding sites and as-1 like element which have been shown to confer tissue specific regulation of the BSV promoter (Remans *et al*., 2005). Thus the region downstream of -254 will have to be analyzed to determine regions that confer tissue specificity.

In conclusion, most of the cis-elements necessary for strong expression of the SCBV promoter appeared to reside within the 254 bp upstream of the TATA box. Promoter elements necessary for tissue specificity also appeared to reside within the region downstream of -254. Subtle differences do exist between the deletion constructs and the full-length promoter in both species, but larger number of lines will have to be analyzed to confirm these differences. In both monocots and dicots, the deletion constructs SCBV-555 had a much weaker expression pattern, which agrees with the transient data that showed reduced GUS activity upon deletion of the 800 bp of the 5'end of the full-length promoter. The results from this study indicate that the SCBV promoter is made up of multiple cis-elements that confer promoter strength. The transient data as well as the *A. thaliana* stable data show the possibility of negatively regulating elements being present since upon deletion of the region -555 to -385 strong promoter activity was observed. In this study we identified the boundaries of the minimal promoter.

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