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Promoters for pregenomic RNA of banana streak badnavirus are active for transgene expression in monocot and dicot plants

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Abstract

Two putative promoters from Australian banana streak badnavirus (BSV) isolates were analysed for activity in different plant species. In transient expression systems the My (2105 bp) and Cv (1322 bp) fragments were both shown to have promoter activity in a wide range of plant species including monocots (maize, barley, banana, millet, wheat, sorghum), dicots (tobacco, canola, sunflower, *Nicotiana benthamiana*, tipu tree), gymnosperm (*Pinus radiata*) and fern (*Nephrolepis cordifolia*). Evaluation of the My and Cv promoters in transgenic sugarcane, banana and tobacco plants demonstrated that these promoters could drive high-level expression of either the green fluorescent protein (GFP) or the β-glucuronidase (GUS) reporter gene (*uidA*) in vegetative plant cells. In transgenic sugarcane plants harbouring the Cv promoter, GFP expression levels were comparable or higher (up to 1.06% of total soluble leaf protein as GFP) than those of plants containing the maize ubiquitin promoter (up to 0.34% of total soluble leaf protein). GUS activities in transgenic *in vitro*-grown banana plants containing the My promoter were up to seven-fold stronger in leaf tissue and up to four-fold stronger in root and corm tissue than in plants harbouring the maize ubiquitin promoter. The Cv promoter showed activities that were similar to the maize ubiquitin promoter in *in vitro*-grown banana plants, but was significantly reduced in larger glasshouse-grown plants. In transgenic *in vitro*-grown tobacco plants, the My promoter reached activities close to those of the 35S promoter of cauliflower mosaic virus (CaMV), while the Cv promoter was about half as active as the CaMV 35S promoter. The BSV promoters for pregenomic RNA represent useful tools for the high-level expression of foreign genes in transgenic monocots.

Abbreviations: BSV, banana streak virus; CaMV, cauliflower mosaic virus; CFDV, coconut foliar decay virus; CoYMV, *Commelina* yellow mottle virus; GFP, green fluorescent protein; GUS, β-glucuronidase; MMV, mirabilis mosaic virus; RTBV, rice tungro bacilliform virus; ScBV, sugarcane bacilliform virus; sORF, small open reading frame.

Introduction

Banana streak virus (BSV) is a species of the genus *Badnavirus* (family Caulimoviridae). Isolates of BSV infect banana causing chlorotic and necrotic streak symptoms on leaves and pseudostem (Lockhart, 1986). The BSV genome is a circular non-covalently closed double-stranded DNA molecule of ca. 7.4 kb (Harper and Hull, 1998). The DNA sequence from the Nigerian BSV-Onne isolate has recently been sequenced and also has been found to be integrated into chromosomal DNA of some *Musa* genotypes

(Ndowora *et al.*, 1999; Harper *et al.*, 1999). The widespread occurrence of BSV isolates as integrated or non-integrated forms in most banana cultivars has recently raised questions in regard to safe germplasm exchange and breeding programmes. Four other isolates of BSV have been described recently and these sequences displayed a high degree of sequence variability (Geering *et al.*, 2000). The badnavirus genome comprises three long open reading frames and one large intergenic region (Pooggin *et al*., 1999). A single transcriptional start site and a poly(A) signal is positioned such that it allows the production of a terminally redundant pregenomic RNA which serves as both, a template for replication *via* reverse transcription and as a polycistronic mRNA (Rothnie *et al*., 1994). The DNA promoter sequence driving transcription of the pregenomic RNA is located close to the left border of the large intergenic region and overlaps with the coding region at its 5′ end. An unusually long leader sequence is present downstream of the transcriptional start site. It contains several short open reading frames and a stable hairpin structure which requires a complex ribosome shunting mechanism for efficient translation of the first long open reading frame (Pooggin *et al*., 1999). The DNA sequences that act as promoter elements for transcription of the pregenomic RNA may also be useful to drive the expression of transgenes in genetically modified plants.

Several promoters have been isolated that direct strong near-constitutive expression of transgenes in monocot plants. These include the maize ubiquitin promoter (Christensen and Quail, 1996), the rice actin1 promoter (McElroy *et al.*, 1990), various enhanced cauliflower mosaic virus (CaMV) 35S promoters (Kay *et al.*, 1987; Vain *et al.*, 1996; Mitsuhara *et al.*, 1996) and a promoter from sugarcane bacilliform virus (ScBV) (Tzafrir *et al*., 1998; Schenk *et al*., 1999). Tissue-specific viral promoters were isolated from *Commelina* yellow mottle virus (CoYMV) and rice tungro bacilliform virus (RTBV) (Medberry *et al.*, 1992; Bhattacharyya-Prakasi *et al.*, 1993). The use of different constitutive promoters for the expression of several transgenes in the same plant may avoid gene silencing that has been frequently observed in plants transformed with several genes that are each regulated by the same promoter (Flavell 1994; Matzke *et al.*, 1994; Park *et al.*, 1996).

We report an evaluation of two novel promoter sequences isolated from BSV, for high-level expression of recombinant genes in stably transformed sugarcane, banana and tobacco plants. A 2105 bp and a 1322 bp fragment upstream of the first open reading frame of the BSV isolates Mys and Cav, respectively, were chosen for assessment of promoter activity. These sequences include many putative *cis*-acting elements that could be essential for high-level expression of the pregenomic RNA. The results indicate that BSV-derived promoters are suitable for high-level expression of transgenes in vegetative tissues of monocot and dicot plants.

Materials and methods

Plant material

Maize (*Zea mays* cv. Iochief Improved), barley (*Hordeum vulgare* cv. Gilbert), millet (*Eleusine coracana*), wheat (*Triticum aestivum*), sorghum (*Sorghum vulgare*), tobacco (*Nicotiana tabacum* cv. W38), canola (*Brassica napus* cv. Westar), sunflower (*Helianthus annuus*) and *Nicotiana benthamiana* plants were grown from seeds in a growth cabinet or glasshouse. Fishbone fern (*Nephrolepis cordifolia*) leaves, tipu tree leaves (*Tipuana tipu*) and male pine tree inflorescences (*Pinus radiata*) were collected from ornamental gardens at the University of Queensland, St. Lucia, Australia. Banana leaves (*Musa* spp., group AAA, cv. Williams) for transient assays were provided by Dr A. Geering, QDPI, Plant Protection Unit, Indooroopilly, Australia. Sugarcane cv. Q117 embryogenic callus was established as described by Taylor *et al.* (1992). Banana cell cultures (group AAB, cv. Three Hand Planty) were established as described previously (Sagi *et al.*, 1995) from the *Musa* germplasm collection of the International Network for Improvement of Banana and Plantain (INIBAP) maintained at the Catholic University Leuven, Belgium.

Cloning and sequence analysis of putative promoter fragments from banana-infecting badnaviruses

Badnavirus isolates BSV-Mys and BSV-Cav were obtained from infected leaf material from banana cultivars Mysore (*Musa* group AAB) and Williams (*Musa* group AAA), respectively, from the Centre for Wet Tropics Agriculture, South Johnstone, North Queensland, Australia. Virus purification, extraction of genomic DNA and cloning of viral PCR fragments for both isolates were carried out as described previously (Geering *et al.*, 2000). Using the degenerate primers Badna3 and BadnaT (Lockhart and Olszewski, 1993)

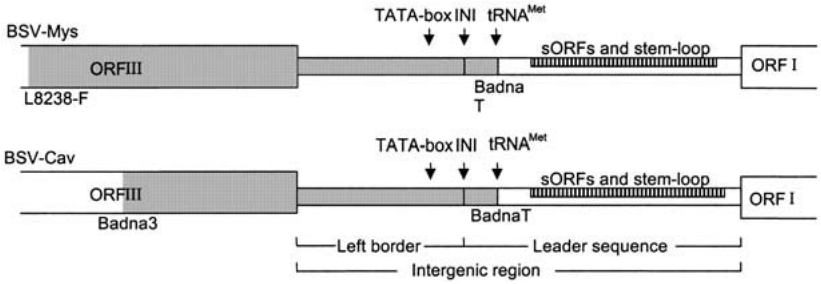

Figure 1. Genetic organisation and cloning strategy of putative promoter fragments My and Cv from BSV isolates Mys and Cav. Sequences were amplified to contain parts of the coding regions as well as the intergenic regions with primers L8238-F, Badna3 and BadnaT (shaded areas). Indicated are the left border of the intergenic region with the transcription initiation site (INI) at its 3′ end and the leader sequence containing the $tRNA^{Met}$ binding site and the assumed positions of small open reading frames (sORFs) and the RNA stem-loop structures.

a PCR fragment of 1322 bp was amplified from BSV-Cav DNA, cloned into pCR2.1 (Invitrogen) and sequenced (Figure 1). Several *Xho*II-digested genomic DNA fragments were cloned and sequenced from the BSV-Mys isolate and a continuous 2105 bp fragment was amplified (cloned into pCR-Script SK(+), Stratagene) using a virus-specific primer (L8238-F, Geering *et al.*, 2000) and the BadnaT primer (Figure 1).

Multiple sequence alignments were carried out with ClustalX 1.64 (gap open penalty 12, gap extension penalty 4). Putative *cis*-acting elements were identified in comparison with other promoter sequences (e.g. sequences from other pararetroviruses) or by using the PLACE database which describes elements from vascular plants (Prestridge, 1991; Higo *et al.*, 1999). Putative elements were checked for relevance (e.g. necessity of a repeat, distance between repeats, flanking sequences, distance from TATA-box) by comparing with the literature that describes the elements. Direct repeats were identified with the EREPEAT program provided by WebANGIS (Australian National Genomic Information Service).

5′ RACE

The start of transcription for promoter sequences was identified using 5′ RACE. Total RNA was extracted from a young leaf of a pCvGFP and a pMyGFP transformed mature glasshouse sugarcane plant by the method of Chirgwin *et al.* (1979). Reverse transcription was directed by a reverse primer gfp-B1 (5′-CAGCTTGTGCCCCAGGATGTTG-3′) specific to the *sgfp*(S65T) gene and by the capswitch oligonucleotide from the CapFinder PCR cDNA Library Construction Kit (Clontech) to bind to the 5′ CAP of the mRNA using the SuperScript II RNase H^- Reverse Transcriptase (GibcoBRL) according to the manufacturer's instructions. The cDNA was amplified in a 100 μl PCR reaction (conditions as described in SuperScript II protocol) by using a nested s*gfp*(S65T) gene specific reverse primer gfp-B2 (5′-GGTGTCGCCCTCGAACTTCAC-3′) and the 5′ PCR primer from the CapFinder kit. A second nested PCR using s*gfp*(S65T) gene-specific reverse primer gfp-B3 (5′-AGATGGTGCGCTCCTGGACG-3′) and the 5′ PCR primer from the CapFinder kit was necessary to yield a product of around 500 bp for both promoters. The PCR product was cloned into pCR2.1 (Invitrogen) and sequenced. The resulting RNA leader sequence was aligned with the promoter sequences to determine the transcription start site of the My and Cv promoters.

Preparation of reporter gene constructs containing BSV promoter sequences

Several constructs including direct gene transfer and binary vectors were made using the above PCR products as putative promoters in fusion with reporter genes coding for either GUS or GFP following standard molecular biology procedures (Sambrook *et al.*, 1989). The vector pUbiGUS was used as the basis for the construction of the promoter-reporter gene cassettes pMyGUS, pCvGUS and the promoterless GUS construct. The vector pUbiGUS contains the *Hind*III/*Bam*HI fragment with the maize ubiquitin promoter from pAHC18 (Christensen and Quail, 1996), the *uidA* reporter gene (Jefferson, 1987) and the nopaline synthase (nos) terminator sequence from *Agrobacterium tumefaciens* in pUC118. The plasmid pMyGUS contains the badnavirus PCR fragment from BSV-Mys (BadnaT primer at the 3′ end) instead of the maize ubiquitin promoter. It was constructed by ligating a blunt-ended *Bam*HI/*Not*I-cut 2.1 kb My fragment into the blunt-ended dephospho-

rylated *Bam*HI/*Hin*dIII-cut 4.8 kb vector fragment of pUbiGUS. The plasmid pCvGUS contains the badnavirus PCR product from BSV-Cav (BadnaT primer at the 3′ end) instead of the maize ubiquitin promoter. It was constructed by ligating the blunt-ended *Bam*HI/*Not*I-cut 1.3 kb Cv fragment into the blunt-ended dephosphorylated *Bam*HI/*Hind*III-cut 4.8 kb vector fragment of pUbiGUS. The vector pUbiGFP was used as the basis for the construction of promoter-reporter cassettes pMyGFP and pCvGFP. The plasmid pUbiGFP (Elliott *et al*., 1999) contains the maize ubiquitin promoter, a synthetic jellyfish green fluorescent protein reporter gene (s*gfp*(S65T); Chiu *et al.*, 1996) and a nos terminator sequence. The plasmid pMyGFP contains the badnavirus PCR fragment from BSV-Mys (BadnaT primer at the 3′ end) instead of the maize ubiquitin promoter. It was constructed by ligating the blunt-ended *Bam*HI-cut 2 kb My fragment into the blunt-ended dephosphorylated *Xba*I-cut 4.2 kb vector fragment of pUbiGFP. The plasmid pCvGFP contains the badnavirus PCR product from BSV-Cav (BadnaT primer at the 3′ end) instead of the maize ubiquitin promoter. It was constructed by ligating the *Xba*I/*Bam*HI-cut 1.3 kb Cv fragment into the dephosphorylated *Xba*I/*Bam*HI-cut 4.8 kb vector fragment of pUbiGFP. Furthermore, pBIN-mgfp5-ER was used as the basis for the construction of promoter-reporter cassettes for *Agrobacterium*-mediated plant transformation. It contains the CaMV 35S promoter, the ER-targeted mutated version of the GFP reporter gene m*gfp*5-ER and the nos terminator. The plasmid pMymGFP5-ER contains the badnavirus PCR fragment from BSV-Mys (BadnaT primer at 3′ end) instead of the 35S promoter. It was constructed by ligating the blunt-ended *Bam*HI 2.1 kb My fragment into the blunt-ended *Hin*dIII/*Bam*HI 13 kb fragment of pBIN-mgfp5-ER. The plasmid pCvmGFP5-ER contains the badnavirus PCR fragment from BSV-Cav (BadnaT primer at the 3′ end) instead of the 35S promoter. It was constructed by ligating the *Eco*RV/*Bam*HI-cut 1.3 kb Cv fragment from pCvGFP into the *Hind*III(blunt-ended)/*Bam*HI-cut 13 kb fragment of pBIN-mgfp5-ER.

Particle bombardment of leaves for transient expression

Leaves (male inflorescences for pine tree) were freshly cut from plants and placed adaxial side-up in petri dishes containing moist filter paper. Particle bombardments were carried out as described previously (Schenk *et al*., 1998) using gold particles with a diameter of 1.6 μm (BioRad) and freshly prepared plasmid DNA as promoter-GUS constructs (50–500 ng per bombardment; Qiaprep Mini spin kit). Plant material was then kept under high humidity for 48 h before transferring the leaves to X-Gluc staining solution (Jefferson *et al*., 1987) and incubation at 37 °C for 12 h. Banana leaves were stained with modified X-Gluc solution (Schenk *et al*., 1999). No background GUS activity was observed using this staining solution. GUS activity indicated by the number and sizes of blue spots was used to monitor promoter activity. The experiments were carried out and repeated on 4–10 leaves of each species.

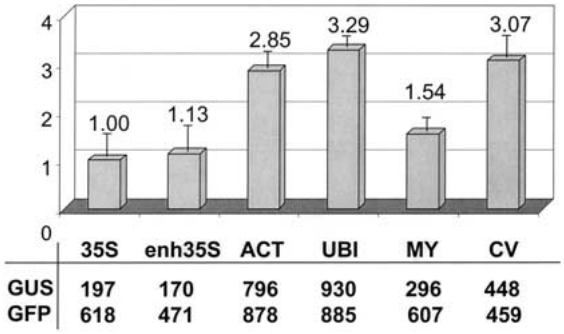

Figure 2. Normalised transient expression of different promoter-GUS constructs in particle-bombarded maize leaves in comparison to the CaMV 35S promoter (35S) using an internal s*gfp*(S65T) standard. For each bombarded leaf, numbers of GUS-producing blue foci were divided by the number of GFP-producing cells (standard deviations shown in bars) and average ratios for each construct were normalised to the average ratio obtained for the CaMV 35S promoter (set to 1.00) (enh35S, enhanced CaMV 35S promoter; ACT, rice actin-1 promoter; UBI, maize ubiquitin promoter).

To take into account variability during particle bombardments, a standardised transient assay (Schenk *et al.*, 1998) was used to evaluate the My and Cv promoter-GUS constructs (40 ng per bombardment) on eight maize leaves per construct. The vector pUbiGFP (described above) was used as an internal standard (30 ng per bombardment). Other GUS constructs included pBI221 (CaMV 35S promoter; Mitsuhara *et al.*, 1996), pMG221 (enhanced 35S promoter with a 3′ exon/intron insertion from the maize *shrunken-1* gene; Maas *et al*., 1991), pAct1-D (rice actin1 gene promoter; McElroy *et al.*, 1990) and pUbiGUS. Relative average GUS activity was calculated for each assay by dividing the total number of blue spots of eight leaves by the number of fluorescing cells for the same leaves. GUS activities were calculated for each assay rather than each leaf to reduce the effects of vari-

ability. Standard deviations were calculated from data on each leaf containing at least 20 spots. The relative GUS activity value of the pBI221 construct was set at 1.00.

Production of transgenic sugarcane, banana and tobacco plants

Embryogenic sugarcane calluses (cv. Q117) were bombarded using gold particles coated with pMyGFP and pCvGFP following a procedure described by Bower *et al.* (1996). Sugarcane plants transformed with pUbiGFP were described previously (Elliott *et al.*, 1999). After bombardment, sugarcane calluses were placed on selective callus-inducing medium (MSC3) containing 45 mg/l geneticin (Sigma). Plantlets (15 independent lines for each construct) were regenerated from the first 15 calluses producing GFP (unbiased for GFP expression level) and transferred to soil. GFP activity was monitored at 2, 7, 12 and 19 months after bombardment by fluorescence microscopy. At 22 months, sugarcane stems were cut down to soil level and the ratoon crop was analysed again after 8 months by fluorescence microscopy.

Embryogenic banana cell suspensions were established and transgenic banana plants (cv. Three Hand Planty) were produced as described previously (Sagi *et al.*, 1995). After co-transformation with pAct1F-neo and pMyGUS or pCvGUS by particle bombardment, transgenic cell cultures were selected in the presence of 50 mg/l geneticin for 2 months prior to regeneration. Banana plants transformed with pWRG1515 (enhanced CaMV 35S promoter fused to the alfalfa mosaic virus leader region and the *uidA* reporter gene), pAHC27 (maize ubiquitin promoter fused to *uidA* reporter gene) and pAct1-D (rice actin promoter fused to *uidA* reporter gene) were produced previously (Sagi *et al.*, 1995).

Tobacco plants (cv. W38) were transformed using *Agrobacterium tumefaciens* strain LBA4404 containing the binary vectors pBIN-mgfp5-ER, pMYmGFP5-ER or pCVmGFP5-ER according to Ellis *et al.* (1987). Fluorescence microscopy was used to monitor transgenic shoot-forming calluses and roots.

Extraction of DNA from plant tissue and PCR amplification for analysis of transgenic plants

Total plant DNA was isolated from leaf tissue according to a modified method of Dellaporta *et al.* (1983) described by Schenk *et al.* (1999). Alternatively, a procedure described by Graham *et al.* (1994) was used. The presence of the promoter and GFP reporter gene in transgenic sugarcane plants was confirmed by PCR using promoter-specific forward primers Ubi-A (5′-GATGTGGTGTGGTTGGGCGG-3′) for the ubiquitin promoter, My-A (5′-AGAGGCGCCCCTGGTATTGG-3′) for the My promoter and Cv-A (5′-CCTAACGATG CGGGAAGCCG-3′) for the Cv promoter together with the s*gfp*(S65T)-specific primer GFP-B (5′-AGATGGTGCGCTCCTGGACG-3′). The presence of the BSV promoters and the reporter gene *uidA* in transgenic banana plants was confirmed by PCR with primers My-A and Cv-A in combination with GUS1R (CTTGTAACGCGCTTTCC-CACC) which were used to amplify a fragment of 450 bp and 350 bp, respectively. In addition, primers neoA (5′-GAGGCTATTCGGCTATGACTG-3′) and neoB (5′-ATCGGGAGCGGCGATACCGTA-3′) were used to amplify a 700 bp fragment of the *nptII* gene. The presence of the promoter and reporter gene in transgenic tobacco was shown by PCR with promoter-specific primers 35S-A (5′-CACTATGTCGACCAAGACCCTTCCTCTATATAA G-3′ for the CaMV 35S promoter; a courtesy of Rebecca Brown), Cv-A and My-A together with the m*gfp*5-ER-specific primer mgfp-B (5′-GGCGCTCTTGAAGAAGTCGTG-3′). These primer combinations amplified fragments of ca. 400, 670 and 560 bp from pBIN-mgfp5-ER-, pMYmGFP5-ER- and pCVmGFP5-ER-transformed tobacco plants, respectively.

Southern blot analyses

Genomic DNA was isolated from young leaves of mature transgenic glasshouse-grown sugarcane plants following the procedure described by Graham et al. (1994). Genomic DNA (10 μg) was digested with XbaI for plants transformed with pUbiGFP and pCvGFP, and with XhoI for plants transformed with pMyGFP. These enzymes have a single restriction site in the transformation vector. Using promoter-specific forward primers Ubi-A, Cv-A and My-A together with the GFP-B primer, DIG-labelled probes were made specific to the promoter-reporter gene region in the pUbiGFP, pCvGFP and pMyGFP transformation vectors, respectively. Construction of DIG-labelled probes and Southern blot analyses were performed using a DIG DNA labelling and detection kit (Roche) according to the manufacturer's instructions.

Western blot analyses of transgenic plants

Comparative western blot analyses were performed on protein extracts of the youngest leaf from transgenic sugarcane plants by grinding 150 mg tissue in 1.5 ml ice-cold extraction buffer (Grof *et al.*, 1998) and by loading 20 μl (the equivalent of 2 mg tissue) per lane (Grof *et al*., 1996). A commercial antibody preparation consisting of a mixture of two mouse monoclonal antibodies directed against GFP was used (Roche).

Histological and fluorometric assays of reporter gene products

Histological examinations of maize, sugarcane and tobacco tissue expressing GFP was carried out with a Leica MZ6 stereomicroscope with fluorescence GFP Plus filter module as described by Elliott *et al.* (1999). Confocal microscopy studies of individual cell layers were performed using a confocal microscope (Zeiss) with a BioRad MRC600 light source and appropriate filters for excitation at 488 nm with emission measured at 509 nm. Digital images of sugarcane leaves expressing GFP (adaxial side up) were produced using a superimposed Z series of three or nine serial scans at 9 μm distance.

The GFP content of transgenic sugarcane and tobacco plants was determined with a Fluoroskan Ascent FL fluorometer (Labsystems) with narrow band emission filters adapted for sGFP(S65T) or mGFP5-ER, respectively, as described previously (Remans *et al.*, 1999). The sGFP(S65T) content was quantified in transgenic sugarcane plants by taking a sample from the second youngest leaf of each plant, and each protein extract was measured three times. The level above which measurements were significant was set at 50 ng sGFP(S65T) per mg soluble protein which equals the average background plus 1.5 times the standard deviation. To determine the GFP concentration in transgenic tobacco leaves the method of Remans *et al.* (1999) was modified by the addition of saturated $(NH_4)_2SO_4$ at a pH of 8.0 to the protein samples to a final saturation of 45%. After centrifugation for 20 min at 13 000 rpm and 4 °C, the supernatant was used for analysis of GFP concentration. Three samples were taken from each transgenic tissue-cultured tobacco plant and the supernatant of each salt-precipitated protein extract was measured as duplicates. The addition of a salt precipitation step decreased the background variation and increased the sensitivity of the assay about two-fold. As a result mGFP5-ER amounts as little as 0.34 μg mGFP5-ER per mg protein could be considered significant.

Histochemical analyses and fluorometric enzymatic assays of GUS in transgenic banana tissues were carried out as described previously (Schenk *et al.*, 1999).

Statistical data analyses and pooled t-tests were carried according to Montgomery and Runger (1994).

Results

Identification and sequence analysis of putative promoter sequences from two Australian BSV isolates

Based on sequence analysis of other badnaviruses, two putative promoter fragments, My and Cv, were chosen and amplified from the Australian isolates BSV-Mys and BSV-Cav, respectively. These contain parts of the 3′ end of the coding region (1537 bp and 846 bp for the My and Cv fragments, respectively) as well as 568 bp and 476 bp, respectively, of the left border of the long intergenic region, including 166 bp and 154 bp, respectively, of the long leader sequence of BSV and ending at the $tRNA^{Met}$ binding site (Figure 1). Sequences of the viral Cv and My fragments were analysed for putative *cis*-acting elements typical of plant and viral promoters and for homology to sequences of other BSV isolates to determine which areas may be of importance for promoter activity. The transcription initiation sites for the Cv and My promoter fragments were determined by 5′ RACE using RNA from transgenic sugarcane plants expressing s*gfp*(S65T) driven by the Cv and My fragments (see below). The 5′ ends of transcripts were located at position 1151 (GenBank accession number AF215815) starting with ACGCAACACAACGCGAGCTTACTTC... and at position 2035 (AF214005) beginning with AGAGTCGGAAATACCAGACTGCTTA... for the Cv and My promoter fragments, respectively. Sequences of ca. 600 bp were aligned and compared to the isolates BSV-Onne and BSV-GF. These included ca. 450 bp upstream and 150 bp downstream of the transcription initiation sites and bordered to the sequence for the putative $tRNA^{Met}$ binding site at the 3′ end. Small areas of sequence similarity between the four BSV pregenomic promoter regions were found but the sequences were most similar across the putative TATA boxes and within the leader sequences downstream of the transcriptional start sites.

Transient gene expression by the BSV promoters in monocots, dicots, gymnosperm and fern leaves

To directly assay promoter activity, constructs linking the Cv and My fragments to reporter genes were prepared. The *uidA* reporter gene was chosen for the construction of direct gene transfer vectors for both, transient assays and banana transformation. The putative promoter fragments My and Cv in pMyGUS and pCvGUS, respectively, were evaluated in different plant species by particle bombardment followed by histochemical GUS assays. Transient gene expression activity as indicated by blue spots was observed for both promoter fragments in all plants tested (data not shown). These included leaves of different monocots (maize, barley, wheat, millet, banana, sorghum) and dicots (tobacco, canola, sunflower, *Nicotiana benthamiana,* tipu tree) as well as fishbone fern leaves (*Nephrolepis cordifolia*) and leaves of male inflorescences of radiata pine trees (*Pinus radiata*). Because of the high variability in the number and intensity of GUS-stained spots per leaf observed after particle bombardments, the expression of the GFP gene from the co-transformed plasmid pUbiGFP was used as an internal standard to monitor the effectiveness for each bombardment in maize. In total, both BSV promoters and four additional well-characterised promoters; CaMV 35S, enhanced CaMV 35S, rice actin-1, and maize ubiquitin were assayed by co-bombardment with pUbiGFP into eight maize leaves for each of the six promoters (Figure 2). Using this transient assay, the Cv promoter showed roughly 3 times the activity of the 35S promoter, and it was comparable to the activities measured for both the actin and the ubiquitin promoter. The My promoter showed about 1.5 times the activity of the CaMV 35S promoter (99.95% significant difference) and about half the activity of the ubiquitin promoter.

*Sugarcane plants transformed with the BSV-Cv promoter fused to s*gfp*(S65T) show strong near-constitutive gene expression*

A synthetic gene encoding the green fluorescent protein sGFP(S65T) (Chui *et al.*, 1996) was chosen as the reporter for direct gene transfer vectors for sugarcane transformation. Transgenic sugarcane plantlets expressing pMyGFP, pCvGFP or pUbiGFP were regenerated from the first 15 calluses which formed on geneticin-containing medium for each construct and that showed GFP expression. PCR was carried out for some plantlets to confirm their transformation status. GFP expression in transgenic calluses after two months was generally very strong for all three constructs. Transgenic glasshouse-grown sugarcane plants were examined for GFP expression in leaves and roots at nine and twelve months after transformation under a fluorescence stereomicroscope. The intensity of GFP expression appeared to be strongest for the Cv and the ubiquitin promoter and significantly weaker for the My promoter. Expression of the Cv promoter construct appeared to be in a near-constitutive manner for both leaf and root tissue (data not shown). This was confirmed by more detailed analyses of the leaf tissue by confocal microscopy where sGFP(S65T) accumulation was visible in the nuclei of most leaf cells examined, including vascular tissue, bundle sheath cells, leaf parenchyma and epidermal cells (Figure 3). Expression was strongest in the parenchyma cells surrounding the vascular bundles. This was also observed for leaf tissue containing the My and ubiquitin promoter, where other leaf cells did not show a clear fluorescence and were probably below the detection limit (data not shown).

The intensity of GFP expression for each transgenic line was monitored by visual assessment under the fluorescent microscope, and the five strongest expressing lines for each construct were chosen for further analyses. Western blot analyses using antibodies directed against sGFP(S65T) were used for a semi-quantitative comparison of the selected lines. The intensity of bands was strongest for lines transformed with pCvGFP followed by lines transformed with pUbiGFP and pMyGFP (Figure 4). The band intensities for lines transformed with pCvGFP were estimated to be at least 3-fold stronger (measurement of band areas) than those obtained for pMyGFP. At 19 months after transformation, sugarcane plants reached maturity and were examined by Southern blot analyses and quantitative fluorometric GFP assays. Southern blot hybridisations showed the presence of the promoter and the reporter gene in five independent lines of pUbiGFP, pCvGFP and pMyGFP transformed sugarcane plants (Figure 4). Enzymes were used for digestion of the genomic DNA that cut once within the transformation vector. The presence of multiple hybridisation bands is consistent with integration of the transgene at multiple locations and/or a complex transgene array at a single locus. Quantitative fluorometric GFP assays of tissue from the second youngest unfolded leaves of mature transgenic sugarcane plants transformed with the Cv promoter (19 months af-

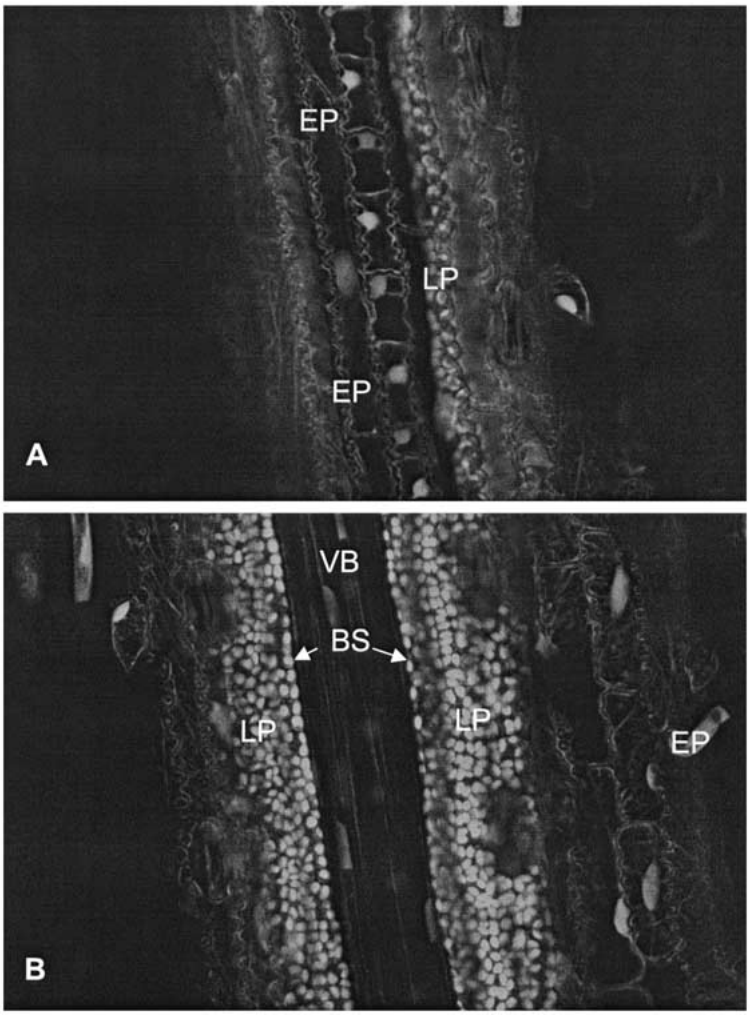

Figure 3. Two sets of three superimposed confocal microscopy images of upper (A) and middle (B) cell layers of a transgenic sugarcane leaf (longitudinal view, adaxial side up) expressing the s*gfp*(S65T) gene controlled by the Cv promoter from BSV (EP, epidermal cells; VB, vascular bundle; BS, bundle sheath cells; LP, leaf parenchyma surrounding the vascular tissue).

ter bombardment) revealed expression levels of up to 10.62 μg sGFP(S65T) per milligram soluble protein (Figure 5). The maize ubiquitin promoter showed expression of up to 3.4 μg/mg sGFP(S65T). One line transformed with pUbiGFP, which had previously (six months before this measurement) been selected by virtue of high-level expression, showed values below the detection limit. Similarly, all five selected sugarcane lines transformed with pMyGFP showed GFP expression that was below the detection limit of 50 ng sGFP(S56T) per milligram protein. Fluorescence microscopy of ratoon plants revealed that all lines containing the Cv promoter construct still showed strong GFP activity.

Banana plants transformed with BSV promoters fused to uid*A show near-constitutive gene expression*

Transgenic banana plants (cv. Three Hand Planty) were produced by co-bombardment of embryogenic cell suspensions with pMyGUS and pAct1F-neo or pCvGUS and pAct1F-neo vectors. A total of 65 lines and 61 lines were regenerated, maintained and micropropagated on geneticin-containing MS medium. Analyses of these putative transgenic plants by PCR and histochemical GUS staining on samples from corms, pseudostems and leaves revealed that ca. 90% of these lines contained the GUS transgene and the *nptII* gene. Longitudinal and cross sections of roots, corms, pseudostems and leaves revealed blue stain-

ing in all cell types analysed (data not shown). The strongest expression was observed for the corm and the vascular tissue. In roots, a high staining intensity was observed in vascular tissue and emerging side roots. Quantitative GUS enzymatic assays were performed on protein extracts of leaves and pseudostem/corm of randomly selected *in vitro* plants that were transformed with pMyGUS (3 lines) and pCvGUS (2 lines) and previously found to contain the *uidA* gene. The GUS activities of these plants were compared with those of an untransformed *in vitro*-grown plant and with one line (selected for high expression by histochemical GUS assays) that was previously transformed with pAHC27 containing the maize ubiquitin promoter (Figure 6A). In addition, leaves of glasshouse-grown plants (height 20–30 cm) were analysed: plants transformed with pMyGUS or pCvGUS were compared to plants (selected for high expression) that were previously transformed with pAct1-D (rice actin-1 promoter), pAHC27 (maize ubiquitin promoter) or pWRG1515 (enhanced CaMV 35S promoter). Three leaf samples were taken from each plant and all leaf samples were measured three times (Figure 6B). The results indicate that the My promoter is much more active in transgenic banana plants than any of the other promoters tested, with average expression levels reaching up to 6299 and 10650 nmol MU per hour per milligram protein for leaf and pseudostem/corm tissue, respectively. The expression levels of GUS in pCvGUS-transformed *in vitro*-grown plants were comparable to the transgenic *in vitro*-grown line containing the ubiquitin promoter construct (Figure 6A). Expression in pseudostem/corm tissue was generally higher than in leaf tissue, confirming the histochemical observations. The transition of *in vitro*-grown to glasshouse-grown plants appeared to result in a general reduction of transgene expression. This was indicated by measurements obtained for transgenic lines My1, My2 and Cv1. The latter line did not show any measurable GUS activity when grown under glasshouse conditions, despite the previous detectable expression (fluorometric or histochemical GUS assay) in *in vitro*-grown plants. Nevertheless, the results demonstrate that using the BSV-My promoter, it was possible to obtain glasshouse-grown banana plants with high-level transgene expression that showed four times the activity of the maize ubiquitin or enhanced CaMV 35S promoter.

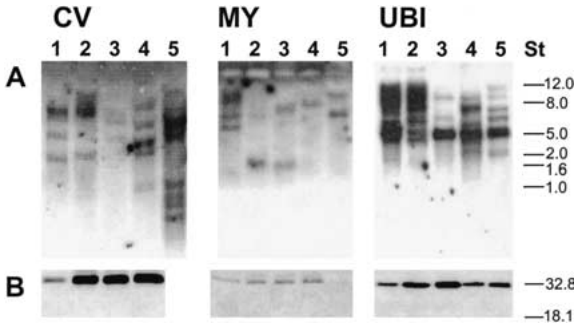

Figure 4. Southern blot analysis (A) and western blot analysis (B) of independent sugarcane plants each expressing s*gfp*(S65T) fused to either BSV promoters (MY or CV) or the maize ubiquitin promoter (UBI) with probes and antibodies directed against GFP. Size markers (St) are shown in kb for A and kDa for B.

In vitro-*grown tobacco plants transformed with the My promoter show transgene expression levels similar to those observed with the CaMV 35S promoter*

The ER-targeted mGFP5-ER was used as the reporter for binary vectors for *Agrobacterium*-mediated tobacco transformation. Tobacco plants (cv. W38) were transformed using *Agrobacterium tumefaciens* containing the binary vector pBIN-mgfp5-ER, pCVmGFP5-ER or pMYmGFP5-ER. A total number of 19, 23 and 26 T_0 lines were regenerated and maintained on kanamycin-containing basal MS medium for pBIN-mgfp5-ER, pCVmGFP5-ER and pMYmGFP5-ER transformed tobacco plants, respectively. The presence of the CaMV 35S, Cv and My promoters and of the m*gfp*5-ER gene was shown by PCR analysis in all lines.

Fluorometric GFP analyses showed that GFP expression in *in vitro*-grown tobacco plants transformed with the My promoter was close to the expression in plants transformed with the CaMV 35S promoter, while plants transformed with the Cv promoter showed GFP expression levels that were about half of those expressing the CaMV 35S promoter (Figure 7). This was shown by using average data of all lines or of the one quarter of the lines that expressed highest (Figure 7C and 7D, respectively). The proportions of transgenic lines above the detection threshold of this assay were 58%, 26% and 62% for plants containing the CaMV 35S, Cv and My promoter, respectively. Measurements for plants harbouring the Cv promoter construct were significantly different from data obtained from plants containing the other promoter constructs ($P < 0.0025$). Measure-

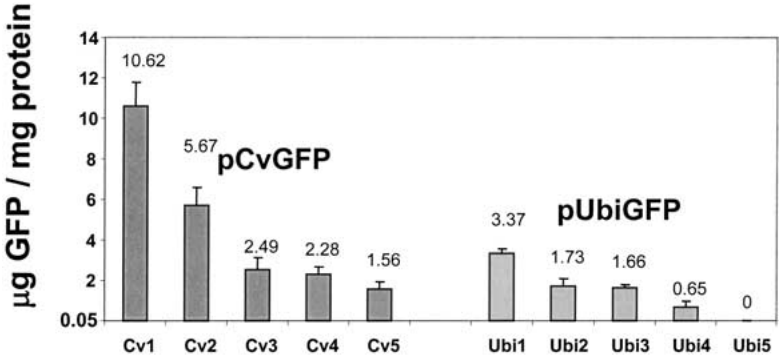

Figure 5. Fluorometrically measured GFP content of leaf extracts of mature transgenic sugarcane plants (the five highest-expressing lines) transformed with the Cv promoter (Cv1-Cv5) or the maize ubiquitin promoter (Ubi1-Ubi5) 19 months after gene transfer. Bars indicate standard deviations of three measurements for each line.

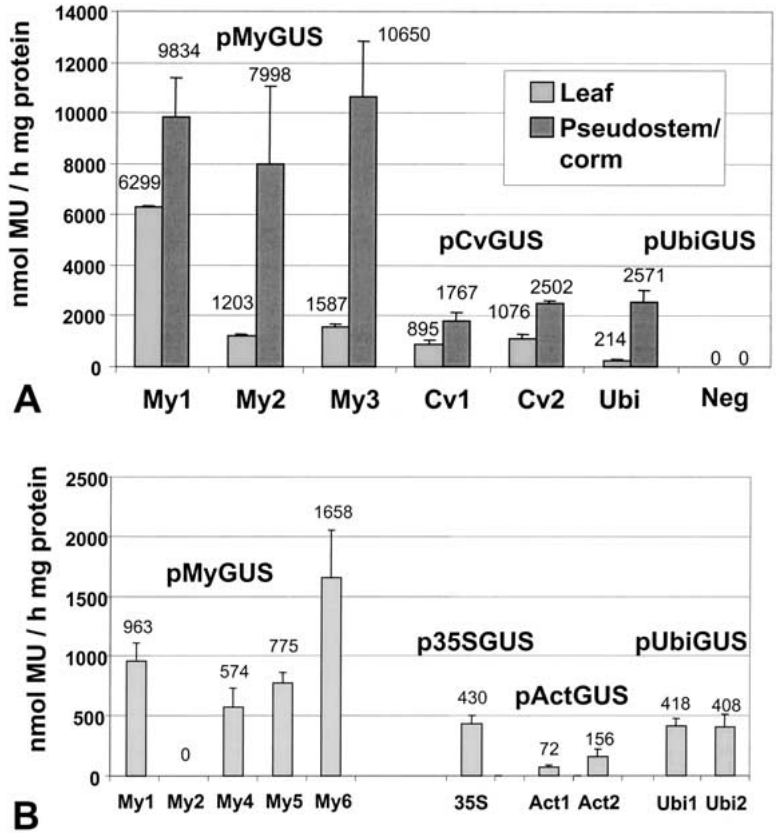

Figure 6. Fluorometrically measured GUS enzymatic activities of leaf and pseudostem/corm extracts of *in vitro* grown banana plants (A) and of leaf extracts of glasshouse-grown banana plants (B) transformed with BSV promoters (pMyGUS or pCvGUS), the maize ubiquitin promoter (pUbiGUS), the CaMV 35S promoter (p35SGUS), or the rice actin-1 promoter (pActGUS) averaged typically from three measurements with standard deviation error bars.

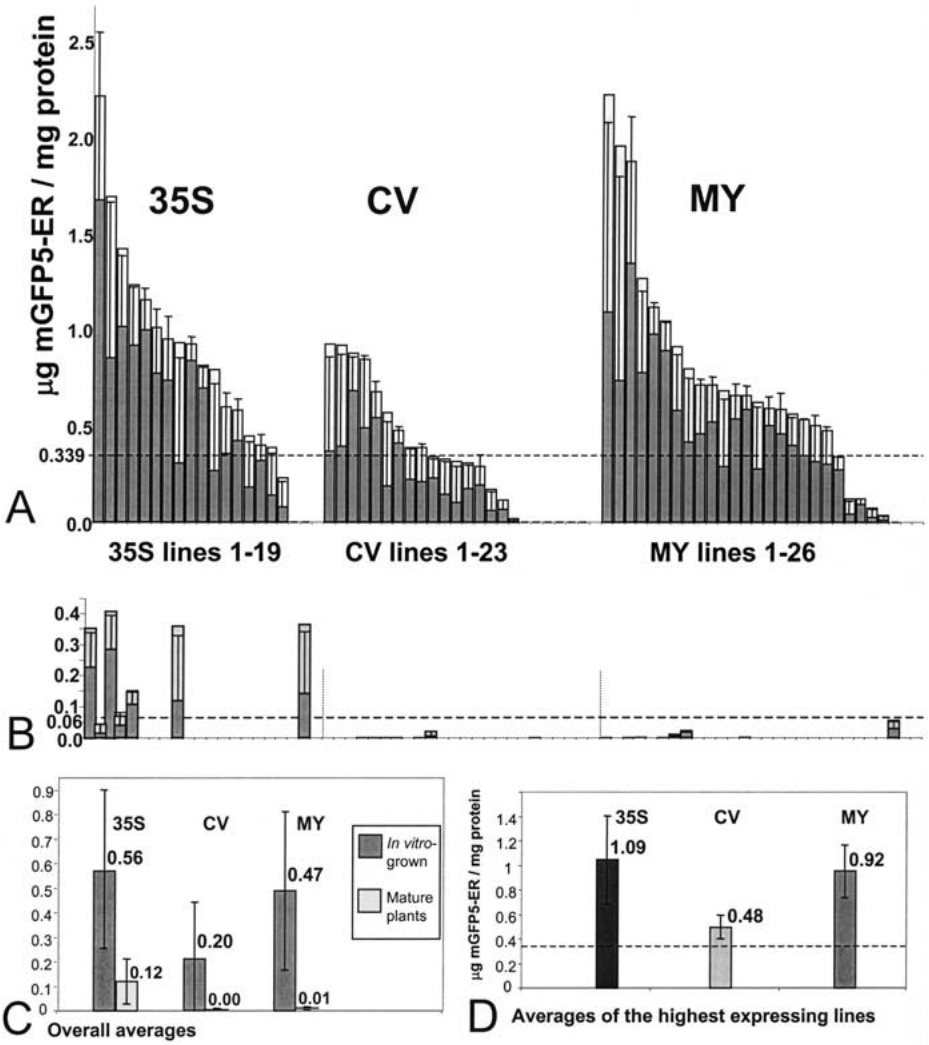

Figure 7. Fluorometric GFP assays of independent tobacco lines transformed with the BSV promoters (CV or MY) or the CaMV 35S promoter. Shown are the average activities of three independent measurements (dark bars) as well as the highest measurements (grey bars) and the standard deviations (error bars) for each line of *in vitro*-grown plants (A) and of seven selected lines of mature glasshouse-grown plants (B). The average background plus two times standard deviation is indicated by the dotted line. Overall averages and averages of the 25% of the highest expressing *in vitro*-grown lines are shown in C and D, respectively. Scales of the Y-axes are different for each histogram.

ments for glasshouse-grown tobacco plants containing either BSV promoter were below the detection threshold of the fluorometric assay (Figure 7B). Reduced expression was also measured for glasshouse-grown plants harbouring the CaMV 35S promoter in comparison with *in vitro*-grown plants.

Discussion

The data presented here demonstrate that the putative promoter regions from the BSV-Mys and BSV-Cav genomes show promoter activity in a range of monocot and dicot species. High levels of reporter gene expression were measured for monocots (e.g. up to 1% of total soluble protein in sugarcane). Interestingly, the Cv promoter showed the highest activity in transgenic sugarcane, while the My promoter led to highest transgene expression in banana. This reflects the diversity of different BSV isolate sequences. BSV-Mys and BSV-Cav display only 74.8% amino acid sequence identity within the conserved RNase H domain (Geering *et al*., 2000) and only 41.7% nucleotide

sequence identity within the promoter fragments used in this study.

Various promoters of viral origin, including promoters from several pararetroviruses, have been successfully used for gene expression in plants (for an overview, see Pooggin *et al*., 1999). Their expression pattern in transgenic plants seems to correlate with the virus distribution during infection. While promoters from the dicot-infecting caulimoviruses and the badnaviruses ScBV, BSV-Mys and BSV-Cav can be used for gene expression in both monocot and dicot plants with near-constitutive patterns, the promoters of CoYMV and RTBV mediate mostly expression in vascular tissues where they appear during infection. ScBV and BSV are distributed throughout the host plant (Lockhart, 1986; Bouhida *et al*., 1993). The observation that the BSV promoters were highly active in monocots is not unexpected considering that they replicate in banana and that many putative *cis*-acting promoter elements found were described previously in monocot plants, such as cereal glutenin box (Shirsat *et al.*, 1989), prolamin box (Vicente-Carbajosa *et al.*, 1997), low-temperature-responsive element (Dunn *et al.*, 1998), gibberellin-responsive element (Gubler *et al.*, 1995) and Dof protein-binding sites (Yanagisawa, 2000). Transgene expression from the BSV promoter was highest in and around vascular tissue. Phloem specificity of promoters from RTBV and CFDV in transgenic plants is thought to be regulated by repetitive CCA motifs ($(CCA(N)_n)_n$TGG) just upstream of the TATA box and a repeated AGAAG motif in RTBV (Yin *et al.*, 1997; Hehn and Rohde, 1998; Klöti *et al.*, 1999). A similar repeated CCAGAAG element and CCA repeats ending in CCANTGG are also present in the BSV-Cav pregenomic promoter which may contribute to the increased promoter activity in vascular tissue.

Based on a sequence analysis of badnaviruses, putative promoter fragments were chosen and amplified in this study. These fragments contain parts of the 3′ end of the coding region and of the left border of the long intergenic region, but only 166 bp and 154 bp, respectively, of the long leader sequence (Figure 1). The fragments therefore included putative enhancer elements downstream of the transcriptional start site and within the 5′ end of the leader. Untranslated plant viral sequences have been found to stimulate expression of a downstream reporter gene (Day Dowson *et al.*, 1993), for example, within the first 90 bp of the RTBV leader, between the first 11 bp and the first 64 bp of the figwort mosaic virus F1t leader and between the first 33 bp and 63 bp of the mirabilis mosaic virus (MMV) leader (Chen *et al.*, 1996; Maiti *et al.*, 1997; Dey and Maiti, 1999). Several putative *cis*-acting elements that could act as enhancers were also located within the first 150 bp of the BSV-Cav and BSV-Mys leader sequence including a 38 bp CT-rich region present in the BSV-Mys promoter in the region between +53 and +90. This corresponds in sequence and position to the shorter CT-rich enhancer region found at the 5′ end (+51/+70) of the RTBV pregenomic transcript (Chen *et al.*, 1996). A $(CT)_n$ sequence motif repeat at the beginning of the CaMV 35S transcribed region also increased expression, probably due to translational effects (Fütterer *et al.*, 1990). At the 5′ end of the BSV-Cav leader there is a direct repeat of 18 bp (containing a GT-1 consensus sequence) that are partially overlapped by three 7 bp repeats. Two 18 nucleotide direct repeats and three 10 nucleotide direct repeats were also located in the +2 to +63 region of the MMV leader that may have some regulatory function (Dey and Maiti, 1999). Further experimentation such as detailed deletion analyses will be necessary to confirm the functionality of the numerous putative *cis*-acting elements that were found in the BSV promoters.

On the other hand, the BSV promoter sequences used for plant transformation did not contain the small open reading frames and the stem-loop structure of the BSV leader sequence that may otherwise reduce translation efficiency. The first small open reading frame (sORF A) of the CaMV leader was found to be important for more efficient viral gene expression and a stem-loop structure was reported as a conserved feature among pararetroviruses (Pooggin *et al.*, 1998). Several small open reading frames and a stem-loop structure were also located in the leader of BSV-Onne (Pooggin *et al.*, 1999). Ribosome shunting was suggested to play a role in overcoming the otherwise adverse effects of short open reading frames for efficient translation. While short open reading frames, ribosome shunting and the particular secondary structure of the RNA leader may have stimulatory effects on viral gene expression and possible encapsidation signal recognition, they could have some adverse effects for the high level expression of heterologous genes. This notion has been supported by transient reporter gene assays using the pregenomic promoter from sugarcane bacilliform virus that was found to have 1.5 times stronger activity once this part of the leader had been removed (P. Schenk, unpublished results).

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