Promoter activity associated with the intergenic regions of banana bunchy top virus DNA-1 to -6 in transgenic tobacco and banana cells

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Promoter regions associated with each of the six ssDNA components of banana bunchy top virus (BBTV) have been characterized. DNA segments incorporating the intergenic regions of BBTV DNA-1 to -6 were isolated and fused to the uidA (β-glucuronidase) reporter gene to assess promoter activity. In tobacco cell suspensions, the BBTV DNA-2 and -6 promoters generated levels of GUS expression 2-fold greater and similar to the 800 bp CaMV 35S promoter, respectively. Deletion analysis of the BBTV DNA-6 promoter suggested all the necessary promoter elements required for strong expression were located within 239 nucleotides upstream of the translational start codon. In transgenic tobacco plants, the BBTV-derived promoters generally provided a weak, tissue-specific GUS expression pattern restricted to phloem-associated cells. However, in callus derived from tobacco leaf tissue, GUS expression directed by the BBTV DNA-6 promoter was strong and, in some lines, comparable to the CaMV 35S promoter. Detectable promoter activity associated with the BBTV promoters in banana embryogenic cells was only observed using a sensitive green fluorescent protein (GFP) reporter. Promoters derived from BBTV DNA-4 and -5 generated the highest levels of transient activity, which were greater than that of the maize ubi-1 promoter. In transgenic banana plants, the activity of the BBTV DNA-6 promoter was restricted to the phloem of leaves and roots, stomata and root meristems.

Introduction

Banana bunchy top virus (BBTV) is a single-stranded DNA virus which infects members of the genus Musa. BBTV has small isometric virions of 18–20 nm, and is persistently transmitted by the aphid Pentalonia nigronervosa (Harding et al., 1991). Based on cytopathology and transmission characteristics, BBTV is considered phloem-limited (Magee, 1939). The BBTV genome consists of at least six components of circular ssDNA each about 1 kb in size (Harding et al., 1993; Burns et al., 1995). BBTV is most likely a member of an unclassified group of plant viruses that potentially includes subterranean clover stunt virus (SCSV) (Boevink et al., 1995), faba bean necrotic yellows virus (FBNYV) (Katul et al., 1997), milk vetch dwarf virus (MDV) (Sano et al., 1993) and coconut foliar decay virus (CFDV) (Rohde et al., 1990).

Each of the six DNA components associated with BBTV encodes at least one gene (Beetham, 1997; Beetham et al., 1997). BBTV DNA component 1 (BBTV DNA-1) contains two transcribed ORFs in the virion sense. The major DNA-1 gene encodes a replication initiation protein (Rep) (Harding et al., 1993, Hafner et al., 1997a), while Wanitchakorn et al. (1997) demonstrated that BBTV DNA-3 encodes the viral coat protein. The functions of the genes encoded by BBTV DNA-1 internal ORF, -2, -4, -5 and -6 are unknown.

The intergenic or non-coding regions of the BBTV DNA components have three regions of homology. The major common region (CR-M) incorporates a 66–92 nucleotide (nt) region (Burns et al., 1995) and is the binding site for an endogenous ssDNA primer which is capable of priming full-length complementary strand synthesis in vitro (Hafner et al., 1997a). The second region of homology, the stem–loop common region (CR-SL), incorporates a 69 nt region (Burns et al., 1995) and contains a stem–loop sequence similar to that found in the geminiviruses (Lazarowitz et al., 1992). Recently, in vitro translation studies with the BBTV DNA-1 Rep gene have demonstrated that the BBTV Rep is involved...
in the specific nicking and joining of the loop region (Hafner et al., 1997b). Thus, the CR-M and CR-SL appear to be intrinsic sequences to the rolling circle mechanism by which BBTV is proposed to replicate. The third region common to all BBTV DNA components is a potential TATA box, with consensus nonanucleotide sequence CTATA/ta/tAt/taA (Burns et al., 1995), and is one of a number of transcription elements identified within the intergenic region, suggesting the potential promoter function of these BBTV sequences.

Promoter activity associated with the intergenic regions of two other BBTV-like viruses, SCSV and CFDV, has been characterized. In SCSV, promoter activity varies between the seven components and appears primarily vascular-associated (Surin et al., 1996). To date, one ssDNA component, potentially encoding a Rep, has been associated with CFDV (Rohde et al., 1990). A promoter region from this component has weak phloem-associated activity in tobacco, which reflects the tissue-limited accumulation of the virus within its natural monocot host (Rohde et al., 1995).

In order to better understand the replication and control of gene function in BBTV, we have assessed the activity of potential promoter sequences derived from BBTV DNA-1 to -6. Using *uidA* (β-glucuronidase) and green fluorescent protein (GFP) reporter genes, we report that the BBTV promoters are functional in both a dicot, tobacco, and a monocot, banana, the natural host of BBTV.

**Methods**

- **Isolation of BBTV-derived promoter sequences.** Molecular techniques were essentially as described by Sambrook et al. (1989). The generalized organization of BBTV DNA-1 to -6 is illustrated in Fig. 1(a). BBTV genomic nucleic acid was purified from BBTV-infected banana plants essentially as described by Harding et al. (1991). BBTV-derived intergenic containing fragments were isolated by either PCR or restriction digestion of previously cloned BBTV sequences (Fig. 1b). PCR reactions contained 20 pmol of each primer, with 50 mM KCl, 1 M MgCl\(_2\), 10 mM Tris–HCl pH 8.0, 200 μM dNTPs, 0.2 U AmpliTaq polymerase (Perkin Elmer), and 1 μl of a 1/1000 dilution (~0.1 ng) of nucleic acid extract. The reaction mix was subjected to an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 45 °C for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Nucleotide sequences were confirmed using an Applied Biosystems 373A DNA sequencer.

**BBTV DNA-1.** A 435 bp fragment of BBTV DNA-1 was amplified by PCR using primers, BT1RI.17 (5’ GAAACAAGTAATGACTTT 3’); BBTV DNA-1 sequence from nt 761–977) and BT1F4.30 (5’ GGAAAGGCTCTCTTGCCTGGGCACGACG 3’); BBTV DNA-1 sequence complementary to nt 250–285. The amplified product was subsequently digested with *TaqI* and blunt-ended. The resulting 225 bp intergenic fragment (promoter BT1.1) was cloned into *Smal*-digested pBl101.3 vector (Clontech). This construction was designated pBIN-BT1.1.

**BBTV DNA-2.** An 862 bp intergenic fragment of BBTV DNA-2 was isolated by *XbaI* (nt 362) and *AclI* (nt 566) digestion of a cloned full-length PCR product. The *AclI* site was blunt-ended using DNA polymerase I large (Klenow) fragment, and cloned into similarly prepared pGEM-3zf+ vector (Promega). The intergenic sequence (promoter BT2.1) was subsequently removed as a *HindIII/BamHI* fragment and cloned into *HindIII/BamHI*-digested pBl101.3. This construction was designated pBIN-BT2.1. The BT2.1 promoter fragment was designed to include the consensus TATA box described by Burns et al. (1995). Since that study, RNA transcripts were mapped to an ORF in DNA-2 and the consensus TATA box identified by Burns et al. (1995) was within this ORF (Beetham, 1997). Further analysis identified an alternative appropriately located TATA-like box that is most likely associated with this ORF. Thus, the BT2.1 promoter contains a 225 bp region of this ORF at its 3’ end. The translational start of this ORF, however, is not in frame with the *uidA* translational start.

**BBTV DNA-3.** A 526 bp intergenic fragment of BBTV DNA-3 was amplified by PCR using primers BT3FV (5’ GGCACTGACCCTTGATGTA 3’); BBTV DNA-3 sequence from nt 760–775 with *PstI* restriction site) and BT3C (5’ CCGGATCCCTATCTAGACACTGG 3’); BBTV DNA-3 sequence complementary to nt 198–212 with *BamHI* restriction site). The resulting fragment was cloned as a *PstI/BamHI* fragment into similarly digested pGEM-3zf+ vector. Subsequently, the intergenic sequence (promoter BT3.1) was inserted into *HindIII/BamHI*-digested pBl101.3 as a *HindIII/BamHI* fragment. This construction was designated pBIN-BT3.1.

**BBTV DNA-4.** A 659 bp intergenic fragment of BBTV DNA-4 was amplified by PCR using primers BT4FV (5’ GCTCTAGAATGTTGATGTA 3’); BBTV DNA-4 sequence from nt 662–676 with *Xhol* restriction site) and BT4C (5’ CCGGATCCCTATCTAGACACTGG 3’); BBTV DNA-4 sequence complementary to nt 262–278 with *BamHI* restriction site). The resulting fragment (promoter BT4.1) was cloned as an *Xhol/BamHI* fragment into a similarly digested pBl101.3 vector. This construction was designated pBIN-BT4.1.

**BBTV DNA-5.** A 613 bp fragment of BBTV DNA-5 was amplified by PCR using primers BT129F3.17 (5’ GTTATCATGCAGATCCGACG 3’); BBTV DNA-5 sequence from nt 639–655) and BT5C (5’ GCGGATCCCTATCTAGACACTGG 3’); BBTV DNA-5 sequence complementary to nt 216–233 with *BamHI* restriction site). The amplified product was digested with *AclI* (nt 795), blunt-ended and the resulting 456 bp sequence was digested with *BamHI* and cloned into a similarly prepared pGEM-3zf+ vector. The intergenic fragment (promoter BT5.1) was then cloned as a *HindIII/BamHI* fragment into a similarly digested pBl101.3 vector. This construction was designated pBIN-BT5.1.

**BBTV DNA-6.** A 623 bp fragment of BBTV DNA-6 was amplified by PCR using primers BTeV1 (5’ CTGGCAGATGTTGTCGTAATT 3’); BBTV DNA-6 sequence from nt 747–763 with *PstI* restriction site) and primer BT6C1 (5’ GATCCCTATCTAGACACTGG 3’); BBTV DNA-6 sequence complementary to nt 264–280 with *BamHI* restriction site). The amplified product was cloned into *PstI/BamHI*-digested pUC19 as a *PstI/BamHI* fragment. Subsequently, the intergenic sequence (promoter BT6.1) was cloned as a *HindIII/BamHI* fragment into a *HindIII/BamHI*-digested pBl101.3. This construction was designated pBIN-BT6.1.

For micro-particle bombardment studies, the BT-*uidA*-nos cassette from each construction was cloned as a *HindIII/EcoRI* fragment into *HindIII/EcoRI*-digested pGEM-3zf+ (except the BT4.1-*uidA*-nos cassette which was cloned as an *Xhol/EcoRI* fragment). These constructions were designated pBT1.1-GN to pBT6.1-GN. A generalized cloning scheme is depicted in Fig. 1(c). The CaMV 35S-*uidA*-nos cassette from pBl121 (Clontech) was similarly cloned as a positive control for NT-1 transient assays. This plasmid was designated p35S-GN. For promoter comparisons in banana embryogenic cells using the *uidA* reporter gene, the vectors pUGR73 and pGUS-2 were used. Plasmid pUGR73 consists of...
BBTV promoter activity

Fig. 1. Schematic representations of (a) general BBTV cssDNA genome organization, (b) BBTV DNA-1 to -6 promoter fragments and (c) cloning strategy. Promoter fragments incorporating the intergenic regions of BBTV DNA-1 to -6 were isolated by PCR or restriction digestion from cloned components, and inserted upstream of the uidA reporter gene in pBI101.3 for Agrobacterium-mediated transformation of tobacco. The BT-uidA-nos cassettes from each construction were subsequently cloned into pGEM3zf+ for micro-particle bombardment transient assays.

BBTV DNA-6 intergenic deletions. The first 5' deletion of BT6.1 was generated using a unique Accl restriction site (nt 1019). Clone pUC-BT6.1 was digested with Accl, which was present in both the pUC19 multiple cloning site and BT6.1, and subsequently blunt-digested using DNA polymerase I large (Klenow) fragment. The resulting digestion was ligated to produce BT6.2. Promoter BT6.3 was isolated by PCR amplification using primer BT6C1 and primer BT6V2 (5'-CTGCAGCATGACGTCAGCAAGG; BBTV DNA-6 sequence from nt 42–57 with PstI restriction site). BT6.3 was amplified and cloned as previously described for promoter BT6.1. The final 5' deletion was generated using a HaeIII restriction site (nt 98). Clone pUC-BT6.1 was digested with HaeIII and BamHI. The resulting fragment was cloned into HindIII/BamHI-digested pUC19. A 3' deletion of BT6.1 was produced by PCR amplification using oligonucleotides: primer BT6V1 and primer BT6C2 (5'-GGATCCTGGACCGGGCTT; BBTV DNA-6 sequence complementary to nt 119–134 with BamHI restriction site). BT6.5 was amplified and cloned as previously described for BT6.1. All BBTV DNA-6 intergenic fragments were cloned as HindIII/BamHI fragments upstream of the uidA reporter gene and nos 3' UTR in pGEM3zf+. These plasmid constructions were designated pBT6.2-GN to pBT6.5-GN, respectively. Each of the BT6 deletions is schematically represented in Fig. 2(a).

GFP reporter constructions. The vector, pblue-SGFP-TYG-nos (SK), contains a codon altered gene encoding the green fluorescent protein (GFP) (Chiu et al., 1996) cloned upstream of a nos 3' UTR in pBluescript. The GFP-nos cassette from pblue-SGFP-TYG-nos was initially subcloned as a BamHI/KpnI fragment into BamHI/KpnI-digested pGEM-3zf+. This vector was designated pGEM-GFP. The GFP-nos cassette was subsequently excised from pGEM-GFP as a BamHI/SacI fragment and inserted into the BamHI/SacI restriction sites of pBT1.1-GN to pBT6.1-GN to replace the original uidA reporter gene. These constructions were designated pBT1.1-GFP to pBT6.1-GFP. A CaMV 35S–GFP fusion was similarly generated by excision of the GFP-nos cassette from pGEM-GFP as a BamHI/SacI fragment and insertion into the BamHI/SacI restriction sites of pGUS-2, to replace the original uidA-35S 3' UTR cassette. This construction was designated p35S-GFP. A maize ubi-1–GFP fusion was generated by insertion of the maize ubi-1 promoter and intron into the HindIII/BamHI restriction sites located upstream of the GFP gene in pGEM-GFP. This construction was designated pUbi-GFP.

Agrobacterium-mediated transformation. Constructs pBIN-BT1.1 to pBIN-BT6.1 were used for Agrobacterium-mediated transformation of tobacco. The plasmid pBI121 was included as a positive control. All BT promoter fusions and the positive control were introduced into Agrobacterium tumefaciens strain LBA4404 by electroporation (Singh...
et al., 1993). Agrobacterium was then used to infect Nicotiana tabacum cv. Xanthi leaf discs essentially as described by Horsch et al. (1988). Leaves from plants transformed with the BT6.1-uidA fusions (1 month old) were transferred to basic MS media containing 1 mg/l BAP and 0-1 mg/l NAA to induce callus formation.

**Preparation of tissues for bombardment**

**Tobacco cells.** Ten days prior to bombardment an N. tabacum cell line (NT-1) was subcultured at high density in NT media as described by An (1985). Three days prior to bombardment, the NT-1 cells were harvested by low speed centrifugation (130 g). Aliquots of 400 µl of NT cell suspension, containing packed cells/liquid NT medium ratio of 1:1 (v/v), were dispensed onto NT media solidified with 0-7% agar (Sigma) and air-dried for 2–3 h.

**Banana cells.** Embryogenic suspension cultures of banana cv. ‘Bluggoe’ (Musa spp. ABB group) were initiated and maintained essentially as described by Dhed’a et al. (1991). Cells were subcultured fortnightly in maintenance medium containing 7.5 µM 2,4-D and 1 µM zeatin (BH media). Suspension cells were collected 5 days after subculture and passed through a 450 µm filter. The filtrate was then centrifuged for 10 min (130 g) and sufficient supernatant was removed to leave a packed cell volume/liquid medium ratio of 1:5. Cells were resuspended and dispensed in 200 µl aliquots onto filter paper discs (Whatman no. 1) to form a thin layer of evenly dispersed cells. Discs were placed in 90 mm Petri dishes containing BH media solidified with 0-7% agar. Cells were bombarded 5 days after plating.

**Micro-particle bombardment.** All plasmids used for micro-particle bombardment were purified using Qiagen Maxi Plasmid Purification columns according to the manufacturer’s specifications. Tissue was bombarded using a particle inflow gun. Preparation of 1-0 µm microcarrier gold particles (Bio-Rad) and coating of plasmid DNA were essentially as described by Mahon et al. (1996), except that 2 µg of plasmid DNA was used to coat gold particles and 5 µl of the DNA–gold suspension was used for each bombardment. Target tissues were placed on a platform 7-5 cm from point of particle discharge and covered with...
a protective baffle of 210 µm stainless steel mesh during bombardment. Tissue was bombarded in a chamber evacuated to 600 mm Hg and particles delivered with a helium pressure of 550 kPa. Transient reporter gene expression was assessed 3 days post-bombardment.

**Transformation and regeneration of banana.** Banana cv. ‘Bluggoe’ was transformed and regenerated using a modified method of Sagi et al. (1995). Test plasmids used for stable transformation of banana included pBT6.1-GN and pBT6.1-GFP, while control plasmids included pBIN-3SS-GN/BT6.3-NPT and p3SS-GFP/BT6.3-NPT. Plasmids pBT6.1-GN and pBT6.1-GFP were co-transformed with an equal amount (1 µg) of pDHKAN for selection of transformants with geneticin (10 mg/l). The plasmid pDHKAN consists of a CaMV 3SS 5’ UTR- NPTII-CaMV 3SS 3’ UTR cassette in pUC18.

**Reporter gene assays and detection.** β-Glucuronidase (GUS) activity was assayed histochemically and fluorometrically essentially as described by Jefferson et al. (1987). Fluorescence was measured using a Perkin Elmer LS50B luminescence spectrophotometer. GFP expression was visualized using a Leica MZ12 stereo microscope with GFP-Plus fluorescence module and green barrier filter (BGG2, Chroma Technology). The number of green fluorescent foci was counted within an eyepiece graticule region, equivalent to 40 mm² on the Petri dish.

**Analysis of transgenic plants**

PCR. Genomic DNA was isolated from leaves of 2–3-month-old tissue culture plants essentially as described by Stewart & Via (1993). Full-length uidA or GFP genes were amplified using, respectively, primer pairs GUS1 (5’ ATGTTAGCTCCTGTAGAAAACC 3’), GUS2 (5’ TCATTGTTCGCCCTCCCTGCTGC 3’), GFP1 (5’ ATGGTGACCCAGGGCGAGGAG 3’), GFP2 (5’ TTACTTGTACAGCTCGTCAG 3’). The PCR reaction contained 20 pmol of each primer, 200 µM dNTPs, 0.1–1 µg genomic DNA, and 0.5 U Expand polymerase with manufacturer’s buffer system 3. The reaction mix was subjected to an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min, and a final extension step of 72 °C for 10 min.

**Southern hybridization.** Transgenic plants confirmed to contain the uidA or GFP genes by PCR were further analysed by genomic Southern hybridization. Total genomic DNA was isolated from leaf material of 2–3-month-old tissue culture plants essentially as described by Stewart & Via (1993). Genomic DNA (10 µg) was digested with BamHI, electrophoresed in a 1% agarose gel and transferred to Zetaprobe GT blotting membrane (Bio-Rad). RNA transcript probes were generated essentially as described by Hafner et al. (1995) using an Ambion MAXiScribe SP6/ T7 kit. NPTII specific probes were transcribed from linearized pGEM-3zf+ vector containing the NPTII gene with T7 polymerase, and hybridized at 55 °C. Hybridization signal was detected with X-OMAT AR scientific imaging film (Kodak) and scanned using a Hewlett Packard Scanjet II CX. The image was manipulated in Corel Photo-Paint V 7.0.

**Results**

### Transient activity of the BBTV-derived promoters in tobacco cell suspensions

The BT-uidA fusions were tested for transient activity in tobacco NT-1 cells via micro-particle bombardment. GUS activities in NT-1 cells from three independent experiments, in which there were four replicates for each promoter, were measured using the plasmid p3SS-GN as a positive control (Fig. 3). Of the six BBTV promoters, BT2.1 provided the highest level of transient GUS activity, about 2- to 3-fold higher than the 800 bp CaMV 35S promoter. The BT6.1 promoter consistently provided expression levels similar to that of the CaMV 35S promoter, while promoters BT3.1, BT4.1 and BT5.1 showed similar levels of GUS expression, about 2-fold lower than that of the CaMV 35S promoter. GUS activity from promoter BT1.1 was not significantly different from background levels, although between 1 to 10 transformation events (blue foci) were observed by histochemical assays.

When deletions were made to BT6.1 and the transient promoter activity in NT-1 cells examined, the BT6.2 promoter provided about a 2-fold higher level of GUS expression than the BT6.1 and CaMV 35S promoters (Fig. 2b). A further deletion of 112 bp, including the majority of the CR-SL
(promoter BT6.3), produced no significant change in GUS activity from BT6.2. GUS activity associated with the BT6.4 promoter was significantly lower than the BT6.3 promoter (about 2-fold), but not significantly different from that observed with the BT6.1 promoter. A 3’ deletion of 147 bp including the TATA box resulted in GUS activity equivalent to that detected of background.

Activity of the BBTV-derived promoters in tobacco plants

Tobacco was transformed with the plasmids pBIN-BT1.1 to pBIN-BT6.1 by Agrobacterium-mediated transformation using the plasmid pBI121 as a positive control. Seven to fourteen independently transformed lines were obtained for each promoter fusion. Integration of the uidA gene in these plants was confirmed by Southern hybridization using HindIII-digested genomic DNA, and each line was found to contain between one and six copies of the uidA gene (data not shown). When assayed histochemically, the majority of transformants had very weak GUS activity limited to vascular-associated cells of the leaves, stems and roots. In contrast, the CaMV 35S promoter directed strong constitutive GUS expression in all tissue types tested. Of the BBTV-derived promoters, BT6.1 appeared to give the highest level of GUS expression in phloem-associated cells (Fig. 4a). However, the low levels of
Fig. 5. Transient activity of BBTV-derived promoters in banana embryogenic cells. BBTV promoters fused to the (a) uidA and (b) GFP reporter genes were transiently introduced into banana embryogenic cells via micro-particle bombardment. Reporter gene expression was assessed 3 days post-bombardment. GUS activity is illustrated as pmol MU/min/mg protein. GFP expression is measured as the number of green fluorescent foci within a 40 mm² area of cells. Bars represent mean activities for ten independent bombardments. Standard error did not exceed 10% of independent means.

GUS expression associated with these plants suggested a significant difference in promoter activity between stable and transient transformation studies. Furthermore, there was a significant reduction in GUS expression in these transformants following extended tissue culturing with no visible GUS expression observed in leaf and root sections of the majority of plants tested after 1 year.

Leaves were taken from tobacco plants transformed with pBIN-BT6.1, including plants without visible GUS expression, and transferred to callus induction media. Upon de-differentiation, callus was excised and GUS activity assayed histochemically. Visible GUS expression was evident in all callus tested, although the level of expression varied between independent lines. In some cases, intensity of GUS expression in the induced callus was visibly comparable to that of callus derived from plants transformed with the CaMV 35S-uidA fusion (Fig. 4b). The increase in GUS activity between leaf and callus ranged from 2- to 100-fold using fluorometric GUS assays (data not shown).

Transient activity of the BBTV-derived promoters in banana cells

The BT-uidA fusions (pBT1.1-GN to pBT6.1-GN) were tested for transient activity in banana embryogenic cells by micro-particle bombardment (Fig. 5a). For promoter comparisons, the plasmids pUGR73 (maize ubi-1 promoter and intron) and pGUS-2 (530 bp CaMV 35S promoter) were included as controls. Using fluorometric assays, the BBTV-derived promoters provided low levels of GUS activity, about 2- to 3-fold greater than the non-bombarded negative control, in two independent experiments, consisting of five replicates for each promoter. Using histochemical assays, between 1 to 20 transformation events (blue foci) were observed for each BBTV promoter construction. Of the other promoters tested, the ubi-1 promoter and intron had the greatest activity, which was about 100-fold greater than the BT promoters. The CaMV 35S promoter was about 4-fold less active than the ubi-1 promoter and intron.

Transient activity of the BBTV promoters was also examined using the GFP reporter. In two independent experiments, each comprising five replicates for each promoter construction, all BBTV-derived promoters generated detectable levels of GFP expression (Fig. 5b). The CaMV 35S promoter was consistently the strongest of the promoters tested based on the intensity and number of green fluorescent foci. In comparison, BT4.1 and BT5.1 promoters were about 0.75-fold as active as the CaMV 35S promoter, while the maize ubi-1, BT6.1 and BT3.1 were about 2-fold less active. Promoters BT1.1 and BT2.1 were about 5-fold and 8-fold less active than the CaMV 35S promoter, respectively.

BBTV DNA-6 promoter activity in transgenic banana plants

Between 7 to 23 independently transformed banana plants were regenerated for each of the test plasmids, pBT6.1-GN and pBT6.1-GFP, and control plasmids, pBIN-35S-GN/BT6.3-NPT
Southern hybridization of transgenic banana plants. Genomic DNA was isolated from four independent lines of banana plants transformed with test constructs pBT6.1-GN and pBT6.1-GFP, and with control plasmids pBIN-35S-GN/BT6.3-NPT and p35S-GFP/BT6.3-NPT. Genomic DNA, digested with \textit{Bam}HI, was immobilized on a nylon membrane and hybridized with radiolabelled RNA probes specific for the NPTII gene. Controls included P = linear plasmid DNA (about 1 ng of pGEM-NPT) and U = untransformed banana genomic DNA.

Discussion

We have demonstrated using transient assays that (i) the intergenic regions of BBTV DNA-1 to -6 have promoter activity in both tobacco and banana cells, (ii) the activities of different intergenic regions vary considerably, and (iii) the relative activities of the different intergenic regions vary between tobacco and banana. In regenerated transgenic tobacco plants, expression from the BBTV-derived promoters was essentially limited to vascular tissue, which supports previous observations that BBTV is a phloem-associated virus. However, when callus was re-initiated from transformed tobacco leaf tissue, the expression of GUS driven by promoter BT6.1 was significantly enhanced. Interestingly, early studies by Magee (1939) reported disorganization of the phloem elements and surrounding parenchyma cells in banana at the point of initial BBTV infection, with subsequent hypertrophy and rapid cell division. The apparent high activities of the BT6.1 promoter in similar unspecialized, actively dividing cell types of tobacco suggests at least one of the BBTV promoters has adapted to high level expression in these cell types. The relative promoter activities of the six intergenic regions in tobacco probably has little relevance to BBTV replication as tobacco is a non-host, although it would seem that promoter activity is not the primary block to BBTV infecting tobacco.

The relative promoter activities from the different BBTV components in banana cell suspensions are, however, probably relevant to BBTV replication. Promoter activity associated with the BBTV intergenic regions in banana embryogenic cells was greatest using a sensitive codon modified GFP reporter gene, as opposed to the \textit{uidA} reporter, suggesting the BBTV promoters are generally weak promoters in their host species. Using GFP, highest expression was directed by the BBTV DNA-4 and -5 promoters, which were about 0.75-fold as active as the CaMV 35S promoter. The functions of the genes encoded by these two components have not been definitively determined. However, the major translational product of BBTV DNA-4 contains a \( \beta \)-sheet of 30 hydrophobic residues toward
the N terminus (Burns et al., 1995), which is similar to a transmembrane domain identified in the V1 proteins of the cereal-infecting geminiviruses (Boulton et al., 1993), suggesting this protein may be involved with cell-to-cell movement. BBTV DNA-5 is the most efficiently primed of all six BBTV components (Hafner et al., 1997a). Further, the predicted gene product encoded by this component contains an LXXE motif (R. Wanitchakorn, G. J. Hafner, R. M. Harding & J. L. Dale, unpublished) that is associated with retinoblastoma (Rb) binding-like proteins of animal DNA tumour viruses (Picksley & Lane, 1994). A functional Rb-binding motif has been identified in the RepA early gene product of members of the subgroup I geminiviruses (Xie et al., 1995; Collin et al., 1996; Ach et al., 1997) and evidence exists for a novel plant encoded Rb-like family of regulatory proteins (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997). Based on these findings, we hypothesize that following BBTV infection, the BBTV DNA-5 gene product is the first translated and may function in creating a cellular environment permissive for viral DNA replication and transcription. The strong activities associated with the BBTV DNA-4 and -5 promoters suggested the gene products encoded by these components are most likely intrinsic to the viral infection process. In contrast, low levels of promoter activity were observed with the BBTV DNA-1 intergenic region. This suggested that the Rep protein, although required at all stages of DNA replication, is expressed at relatively low levels as would be expected for a regulatory protein.

In transgenic banana plants, the BT6.1 promoter displayed a tissue-specific pattern of expression limited to the phloem of leaves and roots, stomata and root meristems. This pattern of expression was analogous to that observed in transgenic tobacco, and may reflect the tissue-limited expression of the BBTV DNA-6 gene product within its natural host. However, it is likely that BBTV gene expression and replication is a complex mechanism. Studies with related ssDNA geminiviruses have revealed promoter activity is influenced via the interaction of virus-encoded gene products (Haley et al., 1992; Sunter & Bisaro, 1992; Zhan et al., 1993) and gene expression in certain cell types may be dependent on complex transcriptional transactivation mechanisms (Sunter & Bisaro, 1997). Therefore, the expression patterns associated with each BBTV promoter alone may differ from the expression patterns from each promoter in a natural infection.

Apart from their involvement in the regulation of BBTV gene expression, the intergenic regions of BBTV may also have applications for heterologous transgene expression. Therefore, we investigated whether any of the cis-elements identified in the BBTV DNA-6 intergenic region were involved in promoter regulation in tobacco. Removal of a 272 bp region from the 5′ end of the BT6.1 promoter increased promoter activity by about 2-fold. The significantly lower activity of the BT6.1 promoter in comparison to BT6.2 may have been due to inhibitory effects of conserved termination signals present at the 5′ end of the promoter (Beetham, 1997), or this region may potentially harbour a down-regulatory element. A significant reduction in promoter activity was observed between promoter BT6.3 and BT6.4, although this level of activity was comparable to the full BT6.1 promoter. Relatively strong expression associated with the minimal BT6.4 promoter suggested the sequences with homology to the G-box and I-box cis-elements may be involved in promoter activity. The G-box core has been shown to interact with several plant transcription factors (reviewed in Katagiri & Chu, 1992), and is responsive to plant hormones such as abscisic acid, jasmonate and ethylene. The location of the I-box consensus (GATAAG) with respect to the G-box, and in relation to the downstream TATA box, implies these motifs may be functionally equivalent to those identified in the rbcS gene promoters (Donald & Cashmore, 1990). The absence of promoter activity associated with the BT6.5 promoter implied that the 147 bp region at the 3′ end of the BT6.1 promoter was essential for promoter activity. Importantly, this region contained the TATA box. Deletion analysis therefore indicated that the CR-M and CR-SL are not essential for promoter activity, and all the transcription elements required for strong promoter activity from the BT6.1 promoter were located within 239 bp of the translation start codon. Furthermore, a 56 bp region, located 3′ of the common stem–loop structure, contributed to at least half of this promoter strength. This region contained a 10 bp sequence, CATGACGTCA, which has strong homology with the 3′ end of the 20 bp consensus incorporating the ocs-element of the Agrobacterium tumefaciens T-DNA promoters (Bouchez et al., 1989). Internal to this sequence is a TGACG motif (ASF-1) which exists as a tandem repeat in the as-1 element of the CaMV 35S and related promoters (Lam et al., 1989; Sanger et al., 1990; Verdaguer et al., 1996). The as-1 element is able to confer expression primarily in root tissues, but also interacts synergistically with other cis-elements (Lam et al., 1989) and has been shown to bind AS1 tobacco nuclear factor (Fromm et al., 1989). This region also contains a sequence, ACGTCA, with homology to the hexamer motif of plant histone promoters (Miki et al., 1987). The presence of this motif in the genomes of several other ssDNA plant viruses has been suggested to reflect an evolutionary conservation of transcription control mechanisms within this group (Morozov et al., 1994). The hexamer motif is most likely associated with strong promoter activity in undifferentiated, actively dividing cell types, as histone genes are expressed specifically in the S-phase of the cell cycle (Nakayama et al., 1992).

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