Plant Cell Rep (2001) 20:642–646
DOI 10.1007/s002990100348

GENETICS AND GENOMICS

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Promoters derived from banana bunchy top virus-associated components S1 and S2 drive transgene expression in both tobacco and banana

Received: 20 July 2000 / Revision received: 22 December 2000 / Accepted: 2 February 2001 / Published online: 23 August 2001

Abstract Potential promoter regions of the *Banana bunchy top virus* (BBTV)-associated DNA components S1 and S2 were fused to the β-glucuronidase reporter gene and assessed for activity in both tobacco (*Nicotiana tabacum* cv. Xanthi) and banana (*Musa* spp. cv. Bluggoe). Transient assays indicated that all the S1- and S2-derived promoters were active and had greater expression in tobacco than banana. In stably transformed tobacco and banana, the S1- and S2-derived promoters directed expression in root meristems and trichomes. The S1 promoter was also expressed in the vascular tissue of leaves and roots, while both the S1 and S2 promoters were active in tobacco leaf trichomes and pollen. In banana, expression was significantly enhanced by the inclusion of the maize polyubiquitin intron 3′ to the promoter. Interestingly, there was some evidence to indicate that S1 promoter fragments containing part of the open reading frame at the 5′ end of the promoter had enhanced activity, suggesting that promoter elements may not be confined to the non-coding region.

Keywords Banana bunchy top virus · Promoter · Vascular expression · Intron-mediated enhancement

Abbreviations *BBTV:* Banana bunchy top virus · *GUS:* β-Glucuronidase · *ORF:* Open reading frame · *uidA: Escherichia coli* gene encoding GUS

Introduction

A number of monocot-active, transcriptional promoters have been isolated from viruses infecting both dicotyledonous (dicot) and monocotyledonous (monocot) plants. Several of these promoters, such as those derived from the cauliflower mosaic virus (CaMV) (Terada and Shimamoto 1990), cassava vein mosaic virus (CVMV) (Verdaguer et al. 1996) and sugarcane bacilliform virus (ScBV) (Tzafrir et al. 1998; Schenk et al. 1999), show strong, near-constitutive expression in rice (CVMV), banana (ScBV) and oat (ScBV). In contrast, promoters isolated from rice tungro bacilliform virus (RTBV) (Bhattacharyya-Pakrasi et al. 1993) and *Commelina* yellow mottle virus (CoYMV) (Medberry et al. 1992) show vascular tissue-specific activity in rice and oat, respectively. With respect to the genetic engineering of plants, both classes of promoters may be valuable for different temporal and spatial expression of transgenes. However, a greater range of monocot-active promoters is required to expand the diversity of transgene expression patterns.

Banana bunchy top virus (BBTV), which infects members of the monocot genus *Musa*, has a genome consisting of at least six segments of circular, single-stranded DNA (cssDNA) (BBTV DNA-1 to -6), each about 1 kb (Harding et al. 1993; Burns et al. 1995). The intergenic regions of these components have been previously shown to possess promoter activity in vascular tissue and in undifferentiated and/or actively dividing cell types (Dugdale et al. 1998). This activity was increased 100- to 300-fold by the inclusion of the maize polyubiquitin or rice actin introns, respectively, 5′ to the reporter gene (Dugdale et al. 2001). Recently, several additional cssDNA components, BBTV-S1, -Y1 and -S2, have been isolated from different Taiwanese isolates of BBTV. These components are also approximately 1 kb (Wu et al. 1994; Yeh et al. 1994; Horser et al. 2000) and, like BBTV DNA-1, potentially encode replication initiation (Rep) proteins. However, based on their limited geographical distribution, different genome organisation and relatively low homology to the ‘master’ Rep protein encoded by BBTV DNA-1 (<40%), these additional cssDNA components do not appear to be integral components of the BBTV genome (Horser et al. 2000). Here, we report the identification of the promoter regions of

Communicated by R. Gilbertson

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BBTV-S1 and -S2, and their ability to direct gene expression in transgenic plants.

Materials and methods

Transformation constructs

Putative promoter fragments (Fig. 1) were polymerase chain reaction (PCR)-amplified from cloned BBTV-S1 or -S2 (Horser et al. 2000). Fragment S1.1 was amplified using primers S1.1 (5′-GCAAGCTTGGTCTATGGTCCG-3′) and S1R (5′-GCGGATCCTGATGACGTAGGG-3′); fragment S1.2, using primers S1.2 (5′-GCAAGCTTCCGAAGATAGAATAAAG-3′) and S1R; and fragment S1.3, using primers S1.3 (5′-GCAAGCTTAGCCACGAAGGAAC-3′) and S1R. Fragment S2.1 was amplified using primers S2.1 (5′-GCGTCGACGGCTCACTGGGAGA-3′) and S2R (5′-GCTCTAGATGATGACGTCAGGG-3′); fragment S2.2, using primers S2.2 (5′-GCGTCGACAGAAGATAGAATA-3′) and S2R; and fragment S2.3, using primers S2.3 (5′-GCGTCGACGGCCCTTAATGGGCC-3′) and S2R. The PCR reactions contained 20 pmol of each primer, with 50 m*M* KCl, 15 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 8.0, 200 µ*M* dNTPs, 0.2 U Amplitaq polymerase (Perkin Elmer) and approximately 0.1 ng of BBTV template. The reaction mix was subjected to an initial denaturation step of 94°C for 5 min, followed by 35 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. The amplified products were cloned into pGEM-T (Promega) and subcloned into vectors for either *Agrobacterium*-mediated transformation or microprojectile bombardment. For *Agrobacterium*-mediated transformation, the promoter fragments were cloned upstream of the *Escherichia coli* gene encoding β-glucuronidase (*uid*A) and the nopaline synthase (*nos*) terminator in pBI101.3 (CLONTECH), creating pBINS1.1 to pBINS2.3. For microprojectile bombardment, the promoter fragments were cloned into pGEM-GN, which contained the *Hin*dIII/*Eco*RI *uid*A cassette from pBI101.3 in *Hin*dIII/*Eco*RI-digested pGEM3zf+ (Promega), creating pS1.1-GN to pS2.3-GN. Promoter constructs containing introns were prepared by excising the maize polyubiquitin 1 (*ubi*1) intron-*uid*A cassette (Christensen and Quail 1996) from the vector pUGR73 (*Bgl*II/*Sac*I digest) and cloning into *Bam*HI/*Sac*I-digested S1/2.2-GN, creating S1/2.2-*ubi*1-GN.

To compare promoter expression, we used two control plasmids: p35S-GN, which contained the 800-bp CaMV 35S promoter upstream of the *uid*A cassette from pBI121, and pUGR73, which consisted of the maize *ubi*1 promoter and intron (Christensen and Quail 1996) located upstream of a *uid*A reporter gene and the tobacco ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene 3′ untranslated region. For co-transformation experiments, equal amounts (1 µg) of the promoter constructs and pDHKan, which contained the CaMV 35S promoter driving the selectable marker gene neomycin phosphotransferase II (*npt*II), were used. Plasmid DNA was prepared using a Bresapure Plasmid Maxi Kit according to the manufacturer's instructions (Geneworks).

Agrobacterium-mediated transformation and regeneration

Tobacco (*Nicotiana. tabacum* cv. Xanthi) plants were grown on MS media (Murashige and Skoog 1962) and subcultured monthly. Purified binary vectors (pBINS1.1 to pBINS2.3, except pBINS2.1) were used to transform *Agrobacterium tumefaciens* LBA4404 by electroporation (Singh et al. 1993). The *Agrobacterium* transformants were then inoculated into LB medium containing kanamycin (100 mg/l) and grown overnight at 28°C. This culture was used to transform tobacco leaf discs as described by Horsch et al. (1988). Stably transformed tobacco plants were selected by the inclusion of kanamycin (100 mg/l) in the media. Timentin (200 mg/l) was also included to kill any residual *Agrobacterium*. Plantlets were regenerated as described by Horsch et al. (1988).

Microprojectile bombardment and regeneration

The pGEM-GN vectors were introduced into tobacco callus and banana embyrogenic cell suspensions using a particle inflow gun. Target tissue was prepared essentially as described by Dugdale et al. (1998). The preparation of microcarrier gold particles and coating of plasmid DNA were essentially as described by Becker et al. (2000). For transient expression studies, tissues were assayed 48 h post-bombardment. Transformed banana plants were selected and regenerated essentially as described by Becker et al. (2000). All plants were PCR-tested for incorporation of the S1 or S2 promoters using primers and the PCR conditions described previously.

Analysis of promoter strength

β-glucuronidase (GUS) activity was measured both histochemically and fluorometrically (Jefferson et al. 1987). For histochemical GUS assays, tissue was incubated in buffer containing 100 m*M* sodium phosphate (pH 7.0), 50 m*M* ascorbate and 1 m*M* 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). Samples were incubated for up to 12 h and cleared with acetic acid:ethanol (3:1). For the fluorometric GUS assays, total protein was extracted from leaf tissue and GUS activity assayed spectrophotometrically using 4-methylumbelliferone as substrate.

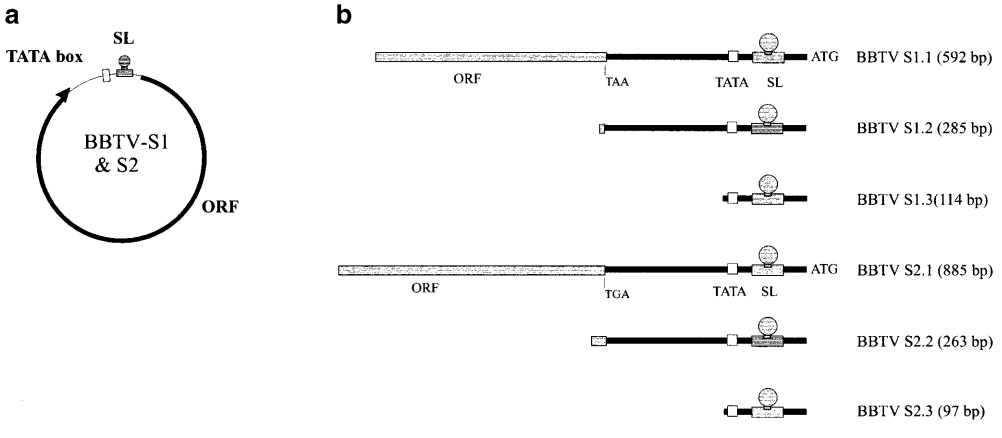

Fig. 1a, b Schematic representations of: **a** cssDNA genome organisation of BBTV-S1 and-S2, **b** BBTV-S1.1 to -S2.3 promoter fragments. The putative TATA box, stem-loop sequence (*SL*) and open-reading frame (*ORF*) are shown. Promoter fragments incorporating the intergenic regions and upstream regions of BBTV-S1 and -S2 were amplified by PCR from cloned components and inserted upstream of the *uid*A reporter gene in pGEM3zf+ for microprojectile bombardment and in pBI101.3 for *Agrobacterium*-mediated transformation

Results and discussion

To determine whether the BBTV-S1 and -S2 sequences contained promoter activity, various fragments derived from these components were fused to the *uid*A reporter gene (Fig. 1) and tested for transient activity via microprojectile bombardment in both tobacco callus (NT-1) and banana embryogenic cell suspensions. In NT-1 cells, the S1- and S2-derived promoter fragments showed varying levels of activity (Fig. 2). The S2.1 promoter provided the highest level of transient GUS expression, about 1.5- to 2-fold higher than that of the CaMV 35S promoter (p35S-GN), whereas the S2.3 promoter showed the weakest activity, just above that of the untransformed control. The S1.1 and S1.2 promoters both showed similar levels of GUS expression, and these were equivalent to the CaMV 35S promoter. The S1.3 and S2.2 promoters provided lower levels of expression than CaMV 35S. In banana embryogenic cells, GUS activity from the six promoter-*uid*A fusions did not differ significantly from that of untransformed tissue, whereas the CaMV 35S and *ubi*1 promoters showed 20- to 100-fold greater expression levels than untransformed tissue, respectively (results not shown). Dugdale et al. (1998) reported similar results with the BBTV DNA-1 to -6 promoters, which were active in NT-1 cells but showed very little expression in banana embryogenic cells.

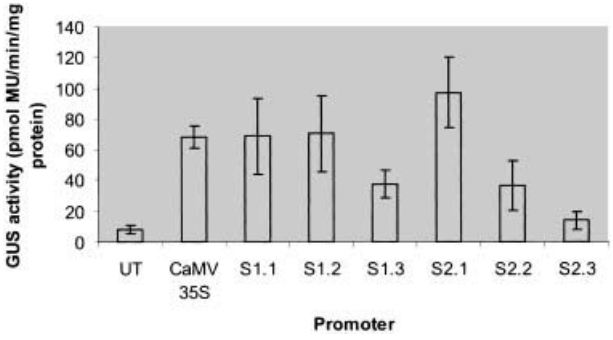

Fig. 2 Transient activity of the S1 and S2 promoter truncations. Six fragments from BBTV-S1 and -S2 were fused to the *uid*A reporter gene and introduced into tobacco NT-1 cells via micro-particle bombardment. GUS expression was assessed fluorimetrically 48 h post-bombardment. The CaMV 35S promoter was included as a promoter comparison, and untransformed (*UT*) tobacco tissue was included as a background control. Values are shown as mean GUS activity (pmol MU/min per mg protein) from four replicates ± the standard error

To analyse the activity and expression patterns of the BBTV-S1 and -S2 promoters in transgenic plants, we generated between five and ten stably transformed tobacco and banana lines for most constructs. All lines were tested by PCR to confirm the presence of the promoter-GUS cassette (results not shown). The S1.1 promoter showed strong activity in the vascular tissue of both leaves and roots of tobacco (Fig. 3a, b) and banana (Fig. 3j). Expression was also found in the stigma, pollen (Fig. 3g, h) and leaf trichomes of tobacco, in the vascular tissue of the petals and sepals of tobacco flowers (Fig. 3i) and in root meristems and trichomes of both tobacco (Fig. 3b) and banana. The smaller S1 promoters, S1.2 and S1.3, showed relatively little or no expression in tobacco leaf and root vascular tissue and meristems (Fig. 3c, d, e) and were undetectable in banana. The S2.1 promoter was not tested in tobacco, and no visible expression was detected in transgenic banana, although certain non-regenerable tissues were GUS-positive (re-

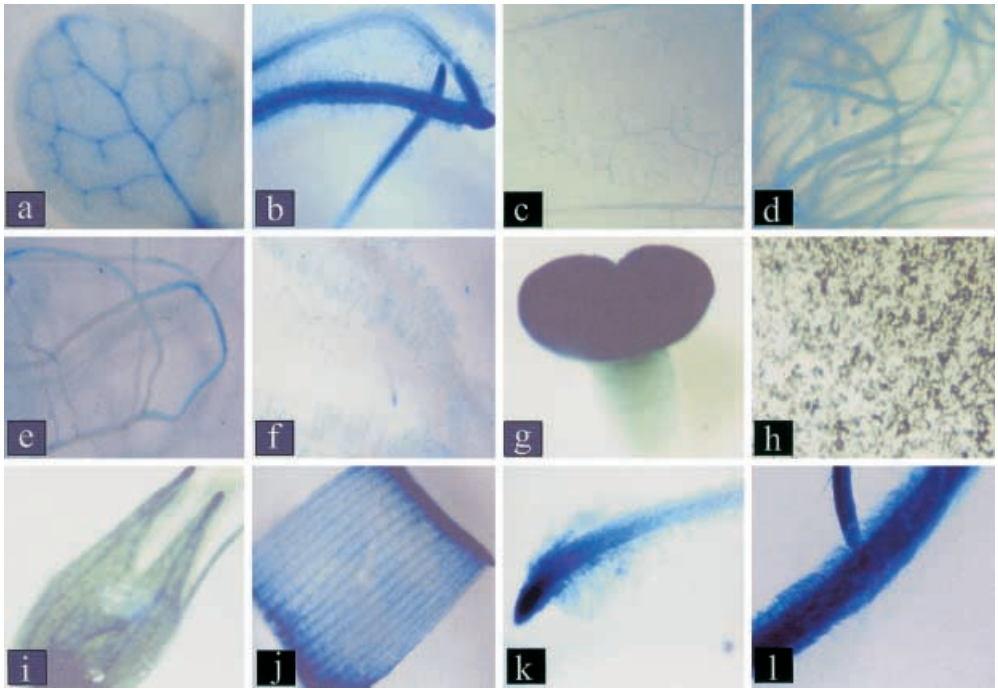

Fig. 3a–l Histochemical localisation of GUS expression in transgenic tobacco and banana containing the BBTV-S1/S2-derived promoter-*uid*A gene fusions. GUS activity is indicated by an indigo dye precipitate after addition of the X-gluc substrate. Pictures presented are from S1.1, S1.2, S1.3, S2.2 and S2.2-*ubi*1 transgenic plants. **a** S1.1 tobacco leaf section, **b** S1.1 tobacco roots, **c** S1.2 tobacco leaf section, **d** S1.2 tobacco roots, **e** S1.3 tobacco roots, **f** S2.2 tobacco roots, **g** S1.1 tobacco stigma, **h** S1.1 tobacco pollen, **i** S1.1 tobacco sepals, **j** S1.1 banana leaf, **k** S2.2 banana root, **l** S2.2-*ubi*1 banana root

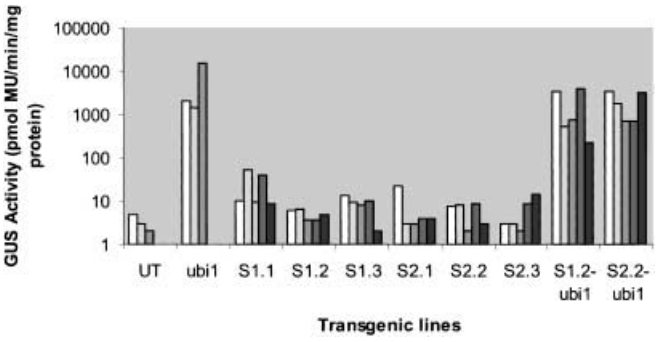

Fig. 4 Comparison of promoter activity in stably transformed transgenic banana. Fluorometric GUS assays of leaves from five lines of each of the S1, S2 and S1/2.2-*ubi*1 promoters. The *ubi*1 promoter and intron (*ubi1*) and untransformed (*UT*) banana were included as controls. GUS activity is represented as pmol MU/min per mg protein

sults not shown). The S2.2 promoter expressed in root meristems and trichomes in both tobacco (Fig. 3f) and banana (Fig. 3k) and in leaf trichomes and pollen of tobacco; expression in other vegetative tissues was either weak or undetectable. The S2.3 promoter showed very little expression, with the exception of one tobacco line in which weak GUS expression was detected in root trichomes. Inclusion of the *ubi*1 intron 5′ of the GUS reporter gene in S1.2 and S2.2 enhanced expression in both banana leaves and roots without appearing to alter the spatial expression patterns (Fig. 3l). In leaves, expression remained strongest in the vascular tissue; however, tissue specificity in roots was difficult to determine due to the high level of GUS expression. Fluorometric GUS assays on banana leaf tissue taken from five lines transformed with each of the BBTV-S1 and -S2 promoter fragments showed that the samples generally had weak expression that was not significantly different from untransformed tissue levels (Fig. 4). However, two lines of S1.1 did have two- to three-fold greater expression than untransformed tissue. The highest level of GUS expression was found for constructs enhanced with the maize polyubiquitin intron, S1.2-*ubi*1-GN and S2.2-*ubi*1-GN, both of which showed at least a 100-fold increase in expression over untransformed tissue and were equivalent to the *ubi*1 promoter (Fig. 4).

The BBTV-S1 and -S2-derived promoters showed strong expression in root meristems and trichomes of tobacco and banana and, in the stigma (S1.1 only) and pollen of tobacco flowers (Fig. 3b, d, f–i, k). Interestingly, promoters from BBTV DNA-6 (Dugdale et al. 1998), CVMV (Verdaguer et al. 1996), RTBV (Yin and Beachy 1995) and CoYMV (Medberry et al. 1992; Torbert et al. 1998) also show high levels of expression in root meristems (BBTV DNA-6, RTBV, CVMV), trichomes (CVMV), stigma (RTBV) and pollen (RTBV, CoYMV, CVMV). The reasons why these promoters are active in such dissimilar tissues remain unknown.

The activity of the S1.1 promoter in the vascular tissue of both tobacco and banana is consistent with the expression patterns observed for the other BBTV-derived promoters (Dugdale et al. 1998) as well as those of other nanoviruses such as subterranean clover stunt virus (Surin et al. 1996) and coconut foliar decay virus (Rohde et al. 1995). Interestingly, S1.1 promoter expression was detected in a greater range of tobacco and banana tissues than any of the other smaller S1 derived-promoters, suggesting that it may have enhanced activity. This is supported by the high expression of two S1.1 transgenic banana lines, leading to the hypothesis that the BBTV-S1 ORF region present in the S1.1 promoter may contain *cis*-regulatory elements that contribute to activity. The presence of such elements outside the intergenic region has previously been reported in a number of plant DNA viruses, including both CaMV (Benfey et al. 1989) and tomato golden mosaic virus (Sunter and Bisaro 1997). However, transient assays in tobacco callus has failed to support this, suggesting that any enhanced S1.1 activity may be limited to certain cell types or tissues such as vascular tissue.

In conclusion, the activity of the BBTV-S1 and -S2-derived promoters described in this report may make them useful for tissue-specific transgene expression in both monocots and dicots. Furthermore, in monocots the level of expression can be altered by the addition of heterologous introns.

Acknowledgements The authors are grateful to the Boyce Thompson Institute for Plant Research (Cornell University, Ithaca), for providing the NT-1 cell line, and the Division of Plant Industry (CSIRO, Canberra), for providing the constructs pDHKAN and pUGR73. We are also grateful to Cathy Horser and Mirko Karan for providing the full-length BBTV-S1 and -S2 clones, and Ben Dugdale for assistance in experimental design and analysis. This work was funded by an Australian Research Council grant. SRH was supported by an Australian Postgraduate Research Award.

References

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL (2000) Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv 'Grand Nain' via microprojectile bombardment. Plant Cell Rep 19:229–234

Benfey PN, Ren L, Chua N-H (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J 8:2195–2202

Bhattacharyya-Pakrasi M, Peng J, Elmer JS, Lacco G, Shen P, Kaniewska MB, Kononowicz H, Wen F, Hodges TK, Beachy RN (1993) Specificity of a promoter from the rice tungro bacilliform virus for expression in phloem tissues. Plant J 4:71–79

Burns TM, Harding RM, Dale JL (1995) The genome organisation of banana bunchy top virus: analysis of six ssDNA components. J Gen Virol 76:1471–1482

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Dugdale B, Beetham PR, Becker DK, Harding RM, Dale JL (1998) Promoter activity associated with the intergenic regions of banana bunchy top virus DNA-1 to -6 in transgenic tobacco and banana cells. J Gen Virol 79:2301–2311

Dugdale B, Becker DK, Harding RM, Dale JL (2001) Intron-mediated enhancement of the banana bunchy top virus DNA-6

promoter in banana (*Musa* spp.) embryogenic cells and plants. Plant Cell Rep 20:220-226

Harding RM, Burns TM, Hafner GJ, Dietzgen R, Dale JL (1993) Nucleotide sequence of one segment of the banana bunchy top virus genome contains a putative replicase gene. J Gen Virol 74:323–328

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT (1988) Leaf disc transformation. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer, Dordecht, pp A5/1–A5/9

Horser CL, Karan M, Harding RM, Dale JL (2000) Additional Rep-encoding DNAs associated with banana bunchy top virus. Arch Virol 145:1–16

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Medberry SL, Lockhart BEL, Olszewski NE (1992) The *Commelina* yellow mottle virus promoter is a strong promoter in vascular and reproductive tissue. Plant Cell 4:185–192

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Rohde W, Becker D, Randles JW (1995) The promoter of coconut foliar decay-associated circular single-stranded DNA directs phloem-specific reporter gene expression in transgenic tobacco. Plant Mol Biol 27:623–28

Schenk PM, Sagi L, Remans T, Dietzgen RG, Bernard MJ, Graham MW, Manners JM (1999) A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. Plant Mol Biol 39: 1221–1230

Singh A, Kao TH, Lin JJ (1993) Transformation of *Agrobacterium tumefaciens* with T-DNA vector using high-voltage electroporation. Focus 15:84–87

Sunter G, Bisaro DM (1997) Regulation of a geminivirus coat protein promoter by AL2 protein (TrAP): evidence for activation and derepression mechanisms. Virology 232:269–280

Surin B, Boevink P, Keese P, Chu P, Larkin P, Waterhouse P (1996) Expression patterns of novel plant-active promoters isolated from the genome of subterranean clover stunt virus. In: Aust Soc Biochem Mol Biol Aust Soc Plant Physiol Comb Conf. SYM-21-03, Canberra, Australia

Terada R, Shimamoto K (1990) Expression of CaMV 35S-GUS gene in transgenic rice plants. Mol Gen Genet 220:389–392

Torbert KA, Gopalraj M, Medberry SL, Olszewski NE, Somers DA (1998) Expression of the *Commelina* yellow mottle virus promoter in transgenic oat. Plant Cell Rep 17:284–287

Tzafrir I, Torbert KA, Lockhart BEL, Somers DA, Olszewski NE (1998) The sugarcane bacilliform badnavirus promoter is active in both monocots and dicots. Plant Mol Biol 38:347–356

Verdaguer B, de Kochko A, Beachy RN, Fauquet C (1996) Isolation and expression in transgenic tobacco and rice plants of the cassava vein mosaic virus (CVMV) promoter. Plant Mol Biol 31:1129–1139

Wu RY, You LR, Soong TS (1994) Nucleotide sequences of two circular single-stranded DNAs associated with banana bunchy top virus. Phytopathology 84:952–958

Yeh HH, Su HJ, Chao YC (1994) Genome characterisation and identification of viral-associated dsDNA component of banana bunchy top virus. Virology 198:645–652

Yin Y, Beachy RN (1995) The regulatory regions of the rice tungro bacilliform virus promoter and interacting nuclear factors in rice (*Oryza sativa* L.). Plant J 7:969–980