



Bi-directional duplex promoters with duplicated enhancers significantly increase transgene expression in grape and tobacco

Zhijian T. Li¹, Subramamnian Jayasankar² & D.J. Gray^{1,*}

¹Mid-Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 2725 Binion Road, Apopka, FL 32703-8504, USA

²Department of Plant Agriculture, Ontario Agricultural College, University of Guelph, Vineland Campus, 4890 Victoria Ave. N., P.O. Box 7000, Vineland Station, Ontario, Canada L0R 2E0

Received 16 May 2003; revised 11 September 2003; accepted 3 December 2003

Key words: bi-directional duplex promoter, gene expression, grape (*Vitis vinifera*), promoter activity, tobacco (*Nicotiana tabacum*), transgenic plants

Abstract

Novel bi-directional duplex promoters (BDDP) were constructed by placing two identical core promoters divergently on both upstream and downstream sides of their duplicated enhancer elements. Estimates of promoter function were obtained by creating versions of CaMV 35S and CsVMV BDDPs that contained reporter marker genes encoding β -glucuronidase (GUS) and enhanced green fluorescent protein (EGFP) interchangeably linked either to the upstream or downstream core promoters. GUS was used for quantitative analysis of promoter function, whereas, EGFP allowed visual qualitative evaluation. In addition, the GUS and EGFP genes placed in downstream positions were modified by translational fusion with neomycin phosphotransferase (NPTII) to allow simultaneous monitoring of promoter activity and selection of stable transformants. These versions of BDDP were compared with each other and with equivalent unidirectional constructs by evaluating their expression in grape and tobacco. For 35S promoter constructs tested in grape somatic embryos (SE), BDDP exhibited transient GUS expression 206- and 300-fold greater in downstream and upstream configurations, respectively, compared to a unidirectional 35S core promoter. Compared with a unidirectional double enhanced 35S promoter, BDDPs exhibited 0.5- and 3-fold increased GUS expression from downstream and upstream core promoters, respectively. The same differences in expression levels determined quantitatively with GUS were distinguished qualitatively with EGFP. Constructs using CsVMV core promoters yielded results relative to those obtained with 35S promoter. For example, the upstream BDDP CsVMV core promoter provided a 200-fold increase in GUS expression compared to a unidirectional core promoter. However, CsVMV promoter was found to have higher promoter activity than 35S promoter in both BDDP and unidirectional constructs. Incorporation of an additional duplicated enhancer element to BDDPs resulted in increased expression. For example, a 35S BDDP with two divergently arranged duplicated enhancer elements resulted in over a 6-fold increase in GUS expression in stably transformed tobacco plants compared to a BDDP with one duplicated enhancer element. Data demonstrate that BDDP composed of divergently-arranged core promoters separated by duplicated enhancers, all derived from a single promoter sequence, can be used to significantly enhance transgene expression and to direct synchronized expression of multiple transgenes.

Abbreviations: CaMV 35S (35S for short) – Cauliflower mosaic virus 35S; CsVMV – Cassava vein mosaic virus; EGFP – Enhanced green fluorescent protein; GUS – β -Glucuronidase; NOS – Nopaline synthase; NPTII – Neomycin phosphotransferase; PCR – Polymerase chain reaction.

Introduction

Transcriptional promoters that regulate gene expression in eukaryotes encompass a core promoter and

associated upstream regulatory sequences. An average core promoter is approximately 100 bp in length and contains a TATA box along with a transcription start site; these are critical elements for transcription initiation. Upstream regulatory sequences include enhancers, silencers, insulators and DNA motifs that achieve spatial and temporal gene expression by mod-

* Author for correspondence
E-mail: djg@mail.ifas.ufl.edu

ulating transcriptional activity (Lee & Young, 2000). This unique functional organization facilitates manipulation of promoter elements for improved transgene expression in plants (Poulsen & Chua, 1988; Fang et al., 1989).

Enhancers in eukaryotic promoters often function in an orientation- and position-independent fashion to up-regulate transcriptional activity (Dyner, 1989). The multifaceted functionality of enhancers permits development of bi-directional duplex promoters (BDDP) using homologous promoter sequences. BDDP reported thus far are constructed by placing a homologous core promoter in a divergent orientation upstream of an existing enhancer-containing promoter such that the shared enhancer functions to regulate bi-directional transcriptional activity of both upstream and downstream core promoters. Although natural bi-directional promoters were tested in transgenic plants previously (Velten & Schell, 1985), Barfield and Pua (1991) first reported the use of synthetic BDDP in plants. In their study, a CaMV 35S core promoter was placed in a divergent orientation upstream of the native 35S promoter containing a single enhancer. The resultant BDDP was used to simultaneously direct expression of the *nptII* and the *gusA* genes; this led to recovery of kanamycin-resistant transgenic plants that also had GUS expression. Xie et al. (2001) demonstrated that similar BDDP could be developed from other natural promoters. These studies did not delineate relative strengths of upstream and downstream orientation nor did they demonstrate superiority in expression level compared to conventional unidirectional promoter complexes.

There are several advantages to the use of BDDP if their expression levels can be improved, including the following: they allow the expression of multiple transgenes synchronized by a single transcriptional regulatory element, thus consolidating and reducing the use of promoter sequences. They facilitate efforts to evaluate promoter modifications to improve transcriptional activity. Potentially, they allow much higher levels of gene expression than other types of promoter architectures (such as those that are multiply-enhanced and/or truncated).

Preliminary research to study multiple transgene expression in grape (*Vitis vinifera* L.) led to development of transformation vectors containing a 35S core promoter fused to the upstream side of its double enhanced version. This BDDP was similar to that previously reported (Barfield & Pua, 1991;

Xie et al., 2001), but differed by incorporation of a doubled enhancer element. We found that separation of divergently-arranged core promoters by doubled enhancer elements resulted in dramatic increase of promoter activity. In the present study, we constructed a series of novel BDDP composed of 35S and CsVMV core promoters with duplicated enhancers to compare with their unmodified core promoters and tandem-arranged double enhanced versions. We compared BDDP and unidirectional promoter function by linking *gusA* and *egfp* marker genes in all combinations to upstream and downstream promoters and by measuring their expression both quantitatively (GUS) and qualitatively (EGFP). In addition, *gusA/nptII* and *egfp/nptII* translational fusion genes were used to facilitate recovery of stable transformants by antibiotic selection. *Agrobacterium*-mediated transformation was utilized to study expression of the vectors in grape and tobacco (*Nicotiana tabacum* L.).

Materials and methods

Construction of transformation vectors

A series of transformation vectors were constructed to compare the relative activity between BDDP, individual and tandem-arranged promoters. Marker genes used to test promoters were *egfp*, *gusA*, and in frame translational fusions *egfp/nptII* and *gusA/nptII* (Datla et al., 1991; Li et al., 2001). Individual expression units containing promoter, target gene and terminator were first constructed by using cloning plasmid pUC18 and verified by DNA sequencing. Final transformation vectors were assembled on a pBIN19-derived binary backbone and introduced into *A. tumefaciens* strain EHA105 as described by Burrow et al. (1990).

T-DNA regions of the nine transformation vectors used in this study are presented in Figure 1. Transformation vector pd35G contained a single GUS expression unit consisting of a *gusA/nptII* fusion gene (Datla et al., 1991) controlled by a double enhanced 35S promoter with a tandem duplicated enhancer repeat (−343 to −89 linked to −343 to +9 relative to transcription start site) (Kay et al., 1987) and the terminator and polyadenylation signal of the *nos* gene of *Agrobacterium* (Figure 1). This vector was utilized as the basic platform for construction of additional transformation vectors by inserting alternative expression units into the T-DNA region.

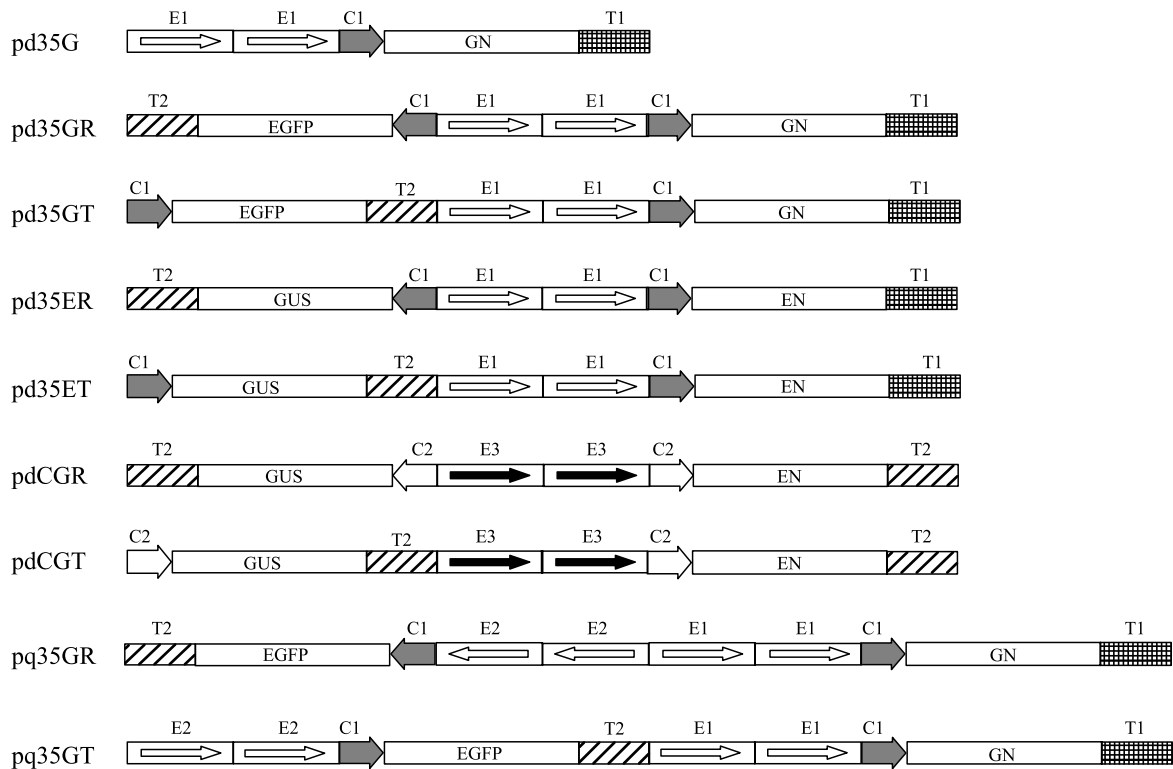


Figure 1. Schematic illustration of T-DNA regions of transformation vectors. *EGFP*, enhanced green fluorescent protein gene; *GUS*, β -glucuronidase; *GN*, *GUS-NPTII* fusion gene (Datla et al., 1991); *EN*, *EGFP-NPTII* fusion gene (Li et al., 2001); C1, core promoter derived from the CaMV 35S promoter (–90 to +1); C2, core promoter derived from the CsVMV promoter (–123 to +1); E1, enhancer fragment isolated from the 35S promoter (–343 to –90); E2, enhancer fragment isolated from the 35S promoter (–419 to –90); E3, enhancer fragment derived from the CsVMV promoter (–443 to –123); T1, terminator and polyadenylation signal sequences from the *NOS* gene of *Agrobacterium*; T2, terminator and polyadenylation signal sequences from the 35S transcript. All vectors were developed based on pBIN19-derived binary backbone.

To construct transformation vectors pd35GR and pd35GT, an intermediate plasmid pME containing an EGFP expression unit was first prepared. This expression unit was composed of an *egfp* gene (Clontech Lab. Inc., Palo Alto, CA, USA) under the control of a 35S core promoter (–90 to +1) (Kay et al., 1987) and the terminator and polyadenylation signal of the 35S transcript. The entire EGFP expression unit was isolated as a *Hind*III fragment from pME and then inserted into the unique *Hind*III site 5' upstream of the *GUS* expression unit in pd35G, resulting in transformation vectors pd35GR and pd35GT, depending on the orientation of inserts. The EGFP expression unit in pd35GR was in a divergent or bi-directional orientation relative to the transcription direction of the downstream *GUS* expression unit. This placement resulted in a unique BDDP that contained two identical, divergently oriented, 35S core promoters separated by an internal

tandem duplicated enhancer. Within this BDDP the sense oriented (downstream) core promoter/duplicated enhancer drives the *gusA/nptII* fusion gene, whereas the upstream core promoter directs the *egfp* gene. In pd35GT, the *egfp* gene functioned as an independent expression unit because it was in a tandem (or sense) orientation relative to the transcription direction of the downstream *GUS* expression unit (Figure 1).

To construct transformation vectors pd35ER and pd35ET, an intermediate plasmid pMG was prepared to contain a *GUS* expression unit consisting of a 35S core promoter, *gusA* gene, and the terminator and polyadenylation signal of the 35S transcript, sequentially. In addition, an intermediate binary vector pd35E was constructed by substituting the *gusA/nptII* fusion gene in pd35G with an *egfp/nptII* fusion gene (Li et al., 2001). The *GUS* expression unit was excised from pMG as a *Hind*III fragment and inserted into the

unique *Hind*III site 5' upstream of the EGFP/NPTII expression unit in pd35E, resulting in transformation vectors pd35ER (bi-directional) and pd35ET (tandem), each with a different insertion orientation relative to the transcription direction of downstream the EGFP/NPTII expression unit (Figure 1). Vector pd35ER contained a BDDP structurally identical to that of pd35GR except that positions of the reporter genes were switched so that a *gusA* gene was attached to the upstream core promoter and a *egfp/nptII* fusion gene was attached to the downstream core promoter (Figure 1).

To construct transformation vectors pdCGR and pdCGT using sequences of the CsVMV promoter, an intermediate binary vector pdCG first was developed in which an enhanced CsVMV promoter with a tandem duplicated enhancer (−443 to −123 linked to −443 to +1) was linked to an *egfp/nptII* fusion gene followed by the terminator and polyadenylation signal of the 35S transcript within the T-DNA region. Additionally, a new GUS expression unit was developed in an intermediate plasmid pMCG by replacing the 35S core promoter in the above-mentioned pMG with a CsVMV core promoter (−123 to +1). This GUS expression unit was then excised from pMCG as a *Hind*III fragment and introduced into the unique *Hind*III site at the 5' terminus of the pdCG T-DNA region. The insertion of this fragment in both bi-directional and tandem orientations relative to the downstream EGFP/NPTII expression unit resulted in transformation vectors pdCGR and pdCGT, respectively (Figure 1). Vector pdCGR was used to compare a CsVMV promoter-derived BDDP with that of 35S promoter used in pd35ER (Figure 1).

For preparation of transformation vectors pq35GR and pq35GT, an EGFP expression unit first was constructed in an intermediate plasmid pD2E by placing an enhanced 35S promoter modified to contain the following: a tandem enhancer repeat with extended enhancer sequences (−419 to −89 linked to −419 to +1) as described by Mitsuhashi et al. (1996), the *egfp* gene, and the terminator and polyadenylation signal of the 35S transcript together, sequentially. The resulting EGFP expression unit was then excised from pD2E as a *Hind*III fragment and inserted into the unique *Hind*III site 5' upstream of the GUS expression unit in pd35G, leading to the formation of pq35GR (bi-directional) and pq35GT (tandem), as shown in Figure 1. Divergent placement of two different expression units in vector pq35GR resulted in a BDDP with two divergently arranged enhancer repeats.

Plant material and culture maintenance

Somatic embryos (SE) of grape (*V. vinifera* cv. Thompson Seedless) were initiated from young leaves of *in vitro* propagated shoot-tip cultures as previously described (Gray, 1992, 1995). All SE cultures were maintained on X6 medium in the dark at 26°C and subcultured onto fresh medium at 6-week intervals (Li et al., 2001). SE at the mid-cotyledonary stage of development were used in transformation experiments. *In vitro* plants of tobacco (*N. tabacum* cv. Samsun) were initiated from sterilized seeds and maintained in Magenta GA-7™ culture vessels containing 1/2 strength Murashige and Skoog (1962) medium at 26°C and a 16 h light/8 h dark photoperiod.

Agrobacterium-mediated transformation of grape SE

Agrobacterium-mediated transformation of grape SE was performed as described by Li et al. (2001), with minor modifications. Overnight cultures of *A. tumefaciens* strain EHA105 harboring transformation vectors were prepared in liquid Luria broth (LB) medium containing appropriate antibiotics at 26°C on an orbital shaker at 185 rpm. When culture density reached an OD₆₀₀ value of 0.8–1.0, the bacterial culture was mixed with an equal volume of liquid DM medium composed of DKW basal salts (Driver & Kuniyuki, 1984) supplemented with 0.3 g L^{−1} KNO₃, 1.0 g L^{−1} *myo*-inositol, 2.0 mg L^{−1} each of thiamine-HCl and glycine, 1.0 mg L^{−1} nicotinic acid, 30.0 g L^{−1} sucrose, 5.0 μM BA, 2.5 μM each of NOA and 2,4-D, at pH 5.7 prior to autoclaving. The mixture was then maintained under the same culture conditions for another 4 h before use in the inoculation of tissue explants. For transformation, SE explants were submerged in bacterial solution for 10 min and then transferred onto DM medium supplemented with 7.0 g L^{−1} TC agar (semi-solid DM medium). The cultures were maintained in the dark for 48 h. After being washed three times in sterile water and once in liquid DMcc medium (DM medium containing 200 mg L^{−1} each of carbenicillin and cefotaxime), SE were placed on semi-solid DMcc medium and cultured at 26°C in darkness for 10 days prior to subsequent transgene expression analysis.

Agrobacterium-mediated transformation of tobacco

Transgenic tobacco plants were obtained after *Agrobacterium*-mediated transformation of leaf disks

according to Burrow et al. (1990). Transgenic plants were identified by kanamycin-resistance and homo-genous expression of reporter genes in cells and tis-sues of roots, stems and leaves as determined by histochemical GUS staining (Jefferson, 1987) and mi-croscopic observation of GFP-specific fluorescence, as described below. Transgenic tobacco plants from independent transformation events were obtained by isolation from different leaf disks, transferred to pot-ting soil and maintained in a growth chamber at 25°C under a 16 h light/8 h dark photoperiod.

Monitoring GFP expression

GFP expression in transformed grape SE was monitored microscopically to evaluate activity of EGFP-carrying expression units. A fluorescence ste-reomicroscope (Leica MZFLIII) equipped with an illuminator powered by an HBO 100 W mercury lamp and a GFP filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (495 nm) and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland) was used to visual-ize GFP-dependent fluorescence as early as 4 days after *Agrobacterium*-mediated transformation in grape SE and routinely over time in tobacco. Fluorescence images were recorded with a FinePix S-1 Pro color di-gital camera (Fuji Photo Film Co. Ltd., Tokyo, Japan) and manufacturer-provided image-recording software. GFP expression in transformations with individual transformation vectors was analyzed using images of at least 30 SE. Representative images are presented.

GUS assay and data treatment

Grape SE explants were collected 10 days after trans-formation. Excessive moisture was removed by a brief exposure of SE to sterile paper towels. After recording fresh weight, samples were immediately ground into a slurry in GUS extraction buffer (Jefferson, 1987) and briefly centrifuged. An aliquot of supernatant was taken from each sample and then used in a fluoro-genic assay to determine the relative GUS activity defined as pmol MU/mg tissue/min with the substrate 4-methylumbelliferyl glucuronide (MUG) following the procedure described by Jefferson (1987). In these experiments, each sample treatment contained 30–40 SE with three replicates per vector treatment. Exper-iments were repeated three times. For comparative analysis of GUS expression, average relative GUS activities from all treatments were converted into per-

centages of mean activity derived from the indicator vector pq35G.

Relative GUS activity in young leaves of trans-genic tobacco plants was determined using a similar fluorogenic assay procedure. Fully expanded young leaves were collected from independent transgenic plants and assayed for relative GUS activity. Assays were repeated three times within a time interval of 1 week. Activity data obtained were averaged for subsequent expression analysis.

Results

Construction of transformation vectors

The transformation vectors constructed in this study incorporated the marker genes *egfp* and *gusA* to measure relative promoter strengths. Both genes were interchangeably placed in all positions in the con-structs in order to utilize both the convenient qual-itative visualization of EGFP along with the highly sensitive quantification afforded by MUG assay of GUS activity. In this way, upstream and downstream core promoters of BDDP and unidirectional promoters were compared both with EGFP and GUS. When used in downstream positions, these markers were modi-fied into the in-frame translational fusions *egfp/nptII* and *gusA/nptII* (Datla et al., 1991; Li et al., 2001) so that antibiotic selection could be employed to ob-tain stable transformants without the use of additional promoters. In addition to positional effects, relative performance of the 35S promoter was compared with the CsVMV promoter and the effect of additional enhancer elements was determined (Figure 1).

Vector pqd35GR was used to test the effect of two enhancer repeats in a BDDP (Figure 1). How-ever, in preliminary experiments, we were unable to obtain such a BDDP using expression units contain-ing identical double enhanced 35S promoters possibly due to unfavorable intrinsic DNA sequence inter-action. All ligation products recovered contained a tandem arrangement of two expression units similar to that of pq35GT (Figure 1). Subsequently, it was found that formation of BDDP with two divergently arranged enhancer repeats required the use of expres-sion units containing asymmetrical enhancer repeats, each with slightly different sequence length (at least 30 bp). As illustrated in Figure 1, the 5' enhancer re-peat [$2 \times (-419 \text{ to } -90)$] is 76 bp longer in length than the 3' enhancer repeat [$2 \times (-343 \text{ to } -90)$] in pq35GR.

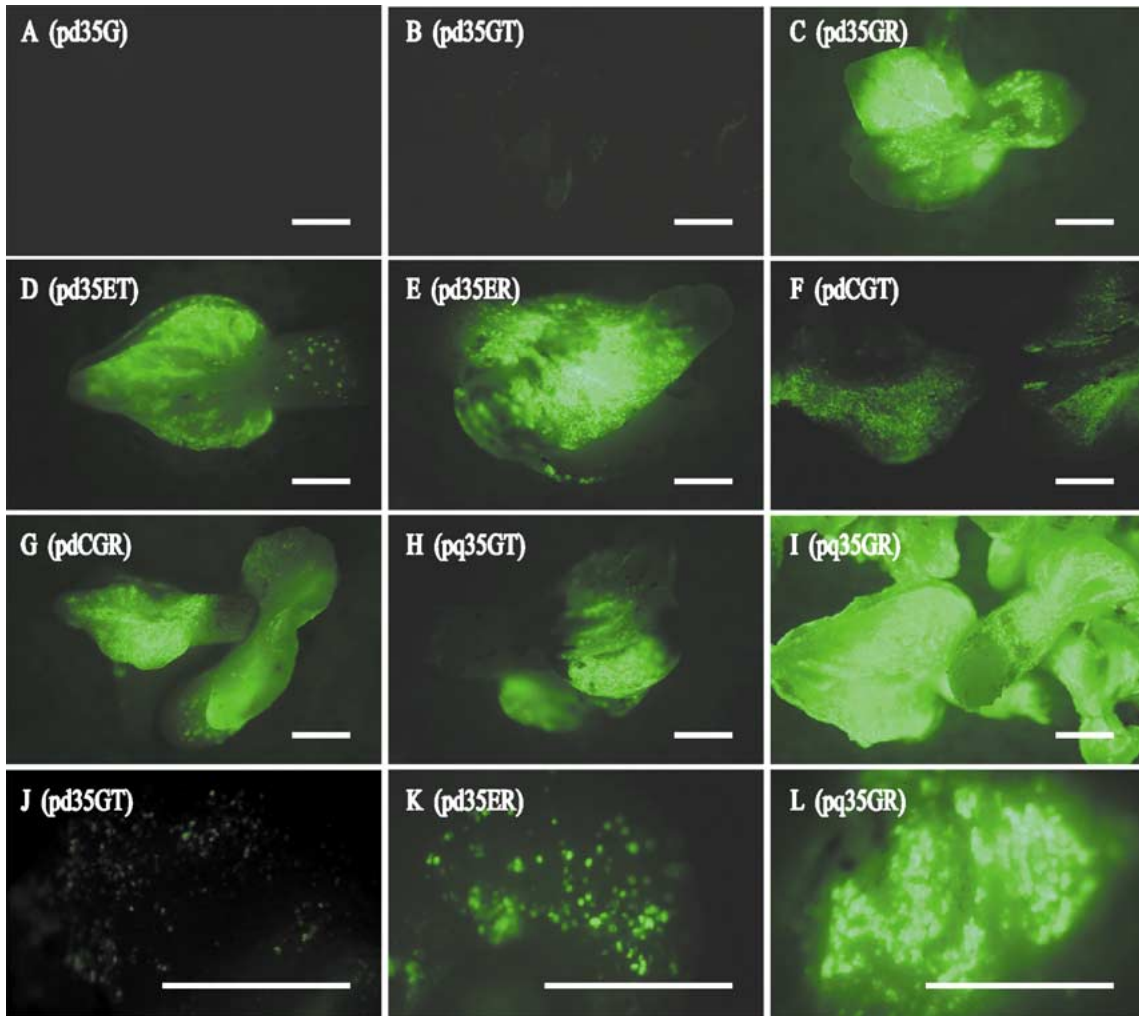


Figure 2. Transient GFP expression in grape SE transformed with *Agrobacterium* harboring various transformation vectors. Fluorescent images of grape SE were taken 4 days after *Agrobacterium*-mediated transformation. Vectors used in each transformation are indicated in parenthesis. Bars placed on the lower right corner of each panel represent 1 mm.

Analysis of transient GFP expression in transformed grape SE

SE transformed with pd35G containing only a *gusA/nptII* fusion gene did not show GFP-dependent fluorescence (Figure 2(A)). Transformation with pd35GT resulted in a barely visible level of GFP fluorescence (Figure 2(B)). This weak transcription activity suggested that the 35S core promoter functioned independently without the influence of downstream enhancers when placed in tandem upstream of another expression unit. GFP fluorescence levels in SE transformed with pd35GR were significantly greater than in those SE transformed with pd35GT (Figure 2(C) and

B)) indicating that the activity of the same core promoter divergently placed upstream of the duplicated enhancer in a BDDP configuration in vector pd35GR was dramatically increased.

Vectors pd35ET and pd35ER were used to evaluate GFP expression derived from the BDDP-associated core promoter downstream of the duplicated enhancer. Transient GFP expression indicated that both vectors were capable of inducing a high level of GFP fluorescence (Figure 2(D and E)). However, based on visual comparison, the intensity level of GFP fluorescence derived from pd35ET was lower than that from pd35ER (Figure 2(D and E)). These results indicated that the BDDP configuration in pd35ER was

also capable of further enhancing the activity of a core promoter located downstream of the duplicated enhancer.

To determine whether similar BDDP derived from other promoters also provide transcriptional enhancement, transformation vectors pdCGR and pdCGT constructed using CsVMV promoter sequences were tested. In both vectors, an *egfp/nptII* fusion gene was driven by the CsVMV core promoter downstream of the duplicated enhancer (Figure 1). Transformation using both vectors resulted in intense GFP fluorescence. However, the fluorescence intensity level in pdCGT-transformed SE was noticeably less than that found in pdCGR-treated SE (Figure 2(F and G)). These results were in accord with observations of 35S promoter-derived BDDP constructs but demonstrated that the CsVMV promoter version was stronger.

Transformation vectors pq35GT and pq35GR were utilized to investigate effects of BDDP containing divergently positioned multiple enhancer repeats (Figure 1). Transformation with pq35GT resulted in significantly lower GFP fluorescence intensity when compared to that from the BDDP-containing pq35GR (Figure 2(H and I)). The level of fluorescence intensity produced by pq35GR was the highest ever recorded in this laboratory. Thus, a BDDP containing two enhancer repeats exhibited better transcriptional efficiency than with one.

The number of GFP-expressing cells and the amount of GFP molecules accumulated in individual cells together contribute to the observed level of transient GFP expression. To further determine cellular accumulation of GFP molecules associated with BDDP configurations, SE transformed with vectors pd35GT, pd35ER or pq35GR were examined under higher magnification. Results indicated that the number of GFP-expressing cells per unit surface area remained near constant, whereas fluorescence intensity within individual cells varied greatly (Figure 2(J–L)). Among three tested vectors, the intensity of cellular fluorescence ranged from low [pd35GT, Figure 2(J)], to intermediate [pd35ER, Figure 2(K)], to high [pq35GR, Figure 2(L)], well in accord with the relative activity strength of *gfp* gene-associated core promoters observed above (Figure 1). Thus, increased promoter activity derived from the BDDP configuration led to production and accumulation of more GFP molecules in transformed cells.

Stably transformed grape calli were obtained using these vectors with levels of GFP fluorescence similar

to those noted in transient expression analysis (data not shown).

Analysis of transient GUS expression in transformed grape SE

Promoter activity was quantified fluorimetrically through transient analysis of the *gusA* gene and its fusion with the *nptII* gene within the same vectors that harbored the *gfp* gene (Figure 1). In all experiments, no GUS activity was detected in non-transformed SE (Figure 3, CK). Diverse levels of relative GUS activity ranging from 0.2 to up to 638.2 pmol MU/mg/min were obtained after transformation using *gusA* gene-containing vectors (data not shown). For comparisons, GUS activity of each vector was converted into the percentage of the mean activity from transformation with base vector pd35G (Figure 3).

When compared to vector pd35G, tandem vector pd35GT exhibited approximately 80% GUS activity, even though both vectors harbored a *gusA/nptII* fusion gene driven by the same 35S core promoter downstream of the duplicated enhancer. Apparently, 5' insertion of an EGFP expression unit in a tandem orientation reduced the expression level of the downstream GUS/NPTII expression unit in pd35GT. In contrast, BDDP-containing vector pd35GR exhibited almost twice (175%) as much GUS activity when compared to pd35G (Figure 3). This demonstrated a significant increase in activity of a downstream 35S core promoter/duplicated enhancer complex when placed in a BDDP configuration.

Transformation with pd35ET resulted in only 1.5% of the GUS activity level obtained with pd35G (Figure 3). The relatively low GUS expression level of pd35ET correlates with the observed low GFP expression level of pd35GT (Figure 2(B)); both genes were driven by an independent 35S core promoter similarly configured in the two vectors. Transformation using BDDP vector pd35ER containing an upstream *gusA* gene resulted in a 300% activity level of that derived from vector pd35G (Figure 3). In other words, the same 35S core promoter exhibited 206 times more GUS expression activity in a BDDP (pd35ER) versus a tandem (pd35ET) configuration (1.5% v.s. 303.3%, Figure 3). In BDDP-containing vectors, GUS expression was higher in upstream (pd35ER) versus downstream configurations (pd35GR) (Figure 3).

Use of a CsVMV promoter to compare BDDP and tandem vectors yielded results similar to that of 35S

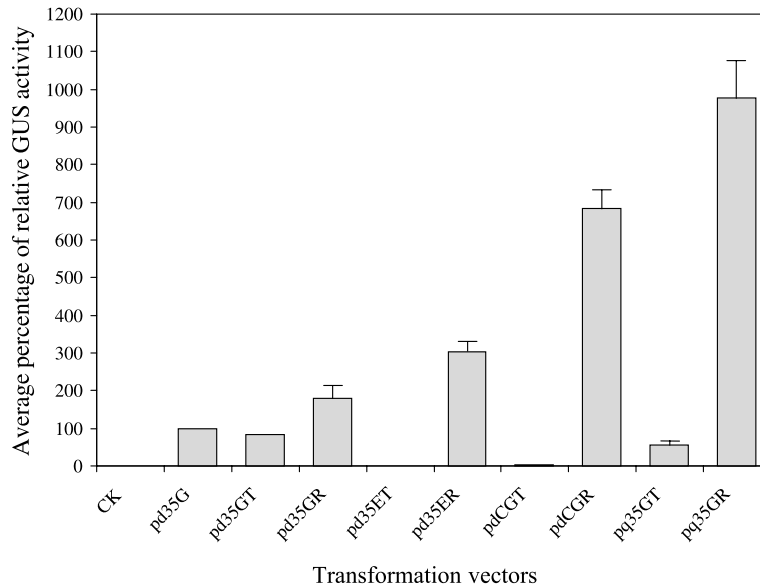


Figure 3. Transient GUS expression in grape SE after *Agrobacterium*-mediated transformation with various transformation vectors. Data were derived from three repeated independent experiments. GUS activity from individual transformation vectors is expressed as a percentage of mean relative GUS activity from vector pd35G.

promoter. However, GUS expression levels were uniformly higher (Figure 3). Comparing tandem vectors, SE transformed with pdCGT yielded about twice the GUS activity of pd35ET, which was about 3.5% of that obtained from pd35G-transformed SE (Figure 3). Thus, GUS expression analysis, which correlated with visible differences in GFP expression (pd35GT, Figure 2(B)), indicated that the independent CsVMV core promoter used in this study provided a basal level of promoter activity slightly higher than that of the 35S core promoter. However, when the same CsVMV core promoter was placed in the upstream position of a BDDP (pdCGR), a large increase in GUS activity (680%) was observed in transformed SE, when compared to that found in pd35G-transformed SE (Figure 3).

GUS activity levels of pq35GT were about 55% of that of pd35G, similar to the relatively reduced GUS expression observed with vector pd35GT containing a downstream GUS expression unit (Figure 3). GUS activity levels from pq35GR were more than 1000% greater than that of pd35G (Figure 3). This large increase in GUS expression also coincided with high levels of transient GFP expression observed with the same vector (Figure 2(I and L)). These results demonstrated that two divergently positioned enhancer repeats in a BDDP configuration are more effective in enhancing the transcrip-

tional activity of both up and downstream core promoters.

Analysis of stable GUS expression in transgenic tobacco plants

Transgenic tobacco plants were generated for analysis of stable transgene expression. A total of 34 independent transgenic tobacco plants were utilized: four for pCsVM (a control vector that did not contain a *gusA* gene; Li et al., 2001), seven each for pd35G and pq35GT, and 16 for pq35GR. Results of the quantitative analysis of relative GUS activity are shown in Figure 4. GUS-specific activity was not detected in plants transformed with pCsVM that contained an *egfp/nptII* fusion gene driven by a native CsVMV promoter, although the plants did express GFP fluorescence (Li et al., 2001). GUS activity levels in pd35G-transformed plants were approximately 40 pmol MU/mg/min. GUS activity levels in plants transgenic for pq35GT were more than twice that of pd35G-containing plants (94 v.s. 40 pmol MU/mg/min) (Figure 4). The relative GUS activity in pq35GR-transformed plants averaged 242 pmol MU/mg/min – 6-fold greater than the value derived from pd35G-transformed plants (Figure 4). This measurement of stable GUS expression in

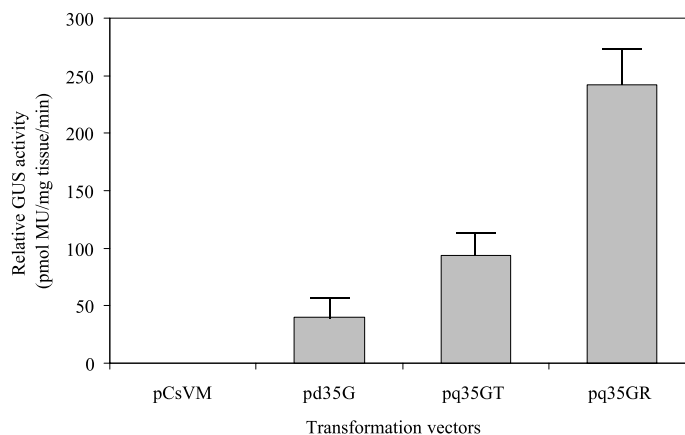


Figure 4. Quantitative analysis of GUS expression in transgenic tobacco plants. Leaf samples were collected from independent transgenic plants and assayed three times at a time interval of 1 week. Bar values represent the averaged relative GUS activity obtained from these repeated analyses.

transgenic tobacco plants paralleled the values obtained from transient analyses of grape SE (Figure 3).

Discussion

Transcriptional enhancement associated with BDDP

Potential advantages of bi-directional promoters for concurrent expression of multiple transgenes in plants have been discussed previously. Velten and Schell (1985) described the use of a small natural bi-directional complex composed of two heterologous core promoters isolated from the T-DNA of *A. tumefaciens* to simultaneously drive expression of *nptII*, *cat* and chloramphenicol acetyltransferase (CAT) genes. However, quantitative expression analysis of CAT and GUS revealed that such natural closely-linked, heterologous promoters displayed significantly different, independently regulated gene expression patterns and thus failed to confer concurrent expression of multiple transgenes (Peach & Velten, 1991). To study the incorporation of enhancers into bi-directional promoters, Barfield and Pua (1991) first positioned a 35S core promoter in a divergent orientation upstream of a native 35S promoter containing a single enhancer element and demonstrated simultaneous expression of two transgenes from the resultant BDDP. Xie et al. (2001) extended this concept by testing similarly configured BDDP using other source promoters. However, these artificially constructed BDDP containing a single enhancer did not confer

the transcriptional enhancement shown in the present study; instead, they led to reduced transgene expression and transgene silencing (Barfield & Pua, 1991; Pua, 2000; Xie et al., 2001).

In this study, a series of transformation vectors containing BDDP with duplicated enhancers were developed and characterized for their expression activity in grape SE and transgenic tobacco plants by comparison with equivalent tandem arranged vectors and single gene expression units. Based on GFP and GUS expression analyses, BDDP with duplicated enhancers placed between identical divergently-oriented core promoters exhibited increased transcriptional activity.

Results of this study showed that a double enhanced unidirectional 35S promoter (pd35G) produced 58-fold more GUS activity than an independent 35S core promoter (pd35ET); these results were similar to those obtained by Kay et al. (1987). However, the same 35S core promoter placed divergently upstream of a duplicated enhancer in a BDDP configuration (pd35ER) was capable of significantly increasing transient GUS expression levels up to 206-fold when compared to an independent 35S core promoter (pd35ER v.s. pd35ET). In addition, the same BDDP configuration approximately doubled transcriptional activity of the core promoter downstream of the duplicated enhancer, when compared to an independent double enhanced 35S promoter with identical duplicated enhancers (pd35ER v.s. pd35G). Similar enhancement of transcriptional activity was observed with the use of CsVMV promoter-derived BDDP containing duplicated enhancers. Noticeably, in a BDDP configuration, the upstream CsVMV core promoter

(pdCGR) resulted in twice the GUS activity of the equivalent 35S promoter version (pd35ER). This was in agreement with our previous findings that, when compared to 35S promoter, the CsVMV promoter and its enhanced derivatives were more active in grape SE cells (Li et al., 2001). Furthermore, additional increased activity resulted when a second set of duplicated enhancers was added in a divergent orientation to the first in a BDDP. Contrary to BDDP, all tandem arranged expression units did not show transcriptional enhancement from both promoters. Thus, the BDDP architecture allowed concurrently increased transgene expression without altering basic expression capabilities and characteristics of the source promoters. The expression enhancement associated with various BDDP was observed in both transient and stable expression analyses involving grape and tobacco hosts, suggesting that such enhancement is achieved at epi-chromosomal DNA level as well as at integrated genomic DNA level.

Previous studies extensively analyzed several composite 35S promoters containing different tandem enhancer repeats (Mitsuhara et al., 1996). These studies suggested that multiple enhancer elements significantly improved the transcriptional activity of a downstream core promoter. However, the extent of transcriptional enhancement was not always positively correlated with an increased number of enhancer repeats that were arranged in a tandem orientation in composite promoters (Mitsuhara et al., 1996). In this report, up to 10-fold increases in expression level were achieved by using BDDP containing two divergently positioned enhancer repeats when compared to expression level from a double enhanced 35S promoter (pq35GR v.s. pd35G). This indicated that the BDDP configuration allowed the incorporation of an increased number of enhancers to achieve further enhanced promoter activity.

Thus far, a large number of transgenic grape plants containing the described 35S promoter-derived BDDP have been obtained and maintained in the greenhouse for up to 4 years without loss of their high-level transgene expression activity. This demonstrates that the BDDP promoter architecture is capable of providing high genetic stability, most likely due to the incorporation of duplicated enhancer elements in a unique configuration. It is important to note that BDDP constructs described here were developed using DNA sequences from a single source promoter for concurrent expression of multiple transgenes. This sequence requirement was set forth based on the well-documented

functional compatibility of core promoters with activating enhancers and context-dependent transcriptional regulation for gene-specific promoter activity (Kay et al., 1987; Wefald et al., 1990; Gatz et al., 1991; Fry & Farnham, 1999; Smale, 2001).

Tandem arrangement of repeat promoters reduced promoter activity

In this study, we observed that GUS expression from pd35GT and pq35GT was lower than that from pd35G in transformed grape SE. In these vectors, the same 35S core promoter downstream of the duplicated enhancer directed the expression of *gusA/nptII* fusion gene, and the only difference between them was that in pd35GT and pq35GT a second expression unit was positioned in a tandem orientation 5' upstream of the *gusA/nptII* expression unit (Figure 1). Thus, tandem arrangement of two expression units tends to reduce expression level of the downstream-positioned expression unit. Such reduced gene expression is probably due to the presence of a sense terminator sequence immediately upstream of the affected promoter. Upstream terminator sequences frequently negatively affected transcription initiation of downstream promoters (West et al., 2002). In addition, transcription of two tandem arranged genes was found to be interfered with by promoter occlusion induced by differences in the strength of two gene promoters (Valerius et al., 2002). Eszterhas et al. (2002) reported that convergent and tandem arrangements of two transgene expression units integrated in the chromosome caused high, but often unpredictable, levels of transcription suppression, whereas a divergent arrangement resulted in higher levels of transcription. Thus, the common use of a tandem arrangement of expression units in transformation vectors containing multiple transgenes should be reconsidered, since it may have heretofore unrecognized negative consequences (Goderis et al., 2002).

Molecular interplays within duplex promoters for enhanced promoter activity

The BDDP described here differed from previously reported versions in that they were configured with divergently positioned tandem enhancer repeats, which separated the core promoters. Such unique configuration may provide an advantageous physical and molecular environment for enhanced promoter activity. Repetitive DNA sequences, such as tandem array,

inverted repeats and palindromic sequences, are conducive to dynamic DNA conformational changes due to intrinsic bonding interactions between homologous nucleic acid residues contained in a unique DNA sequence context (Pérez-Martín & De Lorenzo, 1997). DNA conformational changes can also be induced and/or augmented by molecular interplay with DNA-binding proteins (Goosen & Van de Putte, 1995; Seong et al., 2002). Such DNA conformational changes within enhancer and promoter regions in both prokaryotes and eukaryotes play an important role in facilitating effective recruitment of transcription activators and providing critical molecular environments for the formation of productive transcriptional machinery (Pérez-Martín & De Lorenzo, 1997; Ptashne & Gann, 1997; Fry & Farnham, 1999; Shlyakhtenko et al., 2000). We speculate that the unique repeat configurations of core promoters and enhancers within BDDP may promote intrinsic interactions between homologous DNA sequences and ultimately trigger dynamic conformational changes leading to formation of stable secondary DNA structures (Pérez-Martín & De Lorenzo, 1997). Such secondary structures, once formed within BDDP, may extrude critical regulatory sequence motifs in the enhancer region, provide a greater accessibility to associated transcription factors, and give rise to the subsequent formation of transcriptional machinery that functions more efficiently than the unidirectional native promoter (Lemon & Tjian, 2000).

In addition, an alternative model for gene transcription involving formation of immobilized transcription factories, as previously discussed by Cook (1999), may provide explanation for transcription enhancement by the herein-described BDDP. According to Cook (1999), multiple polymerases are attached (as opposed to being free in the conventional model) to large structures called transcription factories where the immobilized polymerases are capable of reeling in templates and extruding newly synthesized nucleic acids, simultaneously. With this model, accessibility of promoter to immobilized polymerases determines the rate of transcription initiation. Repeated enhancers and closely linked core promoters used in BDDP may increase the association of transcriptional regulatory proteins with enhancer and promoter sequences, improve promoter attachment to transcription factories, and thus increase transcriptional activity. In accordance with this model, two divergently oriented promoters may reduce rate-limiting steps in transcription by allowing simultaneous reeling in, and extruding

out, of templates by immobilized polymerases from both directions. Such bi-directional clearing of templates and nascent transcripts during transcription is believed to be dynamically more efficient as compared to unidirectional operation (Cook, 1999). The inability to achieve expression enhancement by using tandem arranged expression units in this study supports such an interpretation.

In summary, we have demonstrated that various BDDP with duplicated enhancer repeats increase transgene expression in grape SE and transgenic tobacco plants. Bi-directional promoters composed of heterologous regulatory elements are commonly found in nature; many have been isolated from organisms ranging from viruses to plants to mammals (Beck & Warren, 1988; Keddie et al., 1994; Bell et al., 1995; Shigekane et al., 1999; Dresser et al., 2001). Possibly, bi-directional promoters offer certain structural and functional advantages, including providing compact DNA sequence organization; enhancing communication and interplay between enhancer and promoter sequences and transcriptional factors; and increasing efficacy of transcription regulation and gene expression. Functionality of BDDP as described in this study not only support these ideas but also offer synchronized and enhanced expression activity using limited promoter resources. Such synchronized control of transcription by BDDP will facilitate efforts in genetic engineering to improve the complex traits and other value-added characteristics that require high-level expression and/or control of multiple transgenes as discussed by François et al. (2002).

Acknowledgements

We thank NJ Barnett, MM Van Aman, K Kelley and A Ravindrar for excellent technical assistance. Gratefully acknowledgement is made to Drs RL Jarret and N Murai for their critical review of this manuscript and constructive comments. This work was supported in part by grants from Florida Viticultural Advisory Committee. Florida Agricultural Experiment Station Journal Series Number R-09956.

References

- Barfield DG and Pua EC (1991) Gene transfer in plants of *Brassica juncea* using *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Rep* **10**: 308–314.
- Beck CF and Warren RA (1988) Divergent promoters, a common form of gene organization. *Microbiol Rev* **52**: 318–326.

- Bell PJ, Bissinger PH, Evans RJ and Dawes IW (1995) A two-reporter gene system for the analysis of bi-directional transcription from the divergent MAL6T-MAL6S promoter in *Saccharomyces cerevisiae*. *Curr Genet* **28**: 441–446.
- Burrow MD, Chlan CA, Sen P and Murai N (1990) High frequency generation of transgenic tobacco plants after modified leaf disk cocultivation with *Agrobacterium tumefaciens*. *Plant Mol Biol Rep* **8**: 124–139.
- Cook PR (1999) The organization of replication and transcription. *Science* **284**: 1790–1795.
- Datla RS, Hammerlindl JK, Pelcher LE, Crosby WL and Selvaraj G (1991) A bifunctional fusion between β -glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. *Gene* **101**: 239–246.
- Dresser DW, Jamin SP, Atkins CJ and Guerrier D (2001) An expressed *GNRP*-like gene shares a bi-directional promoter with *SF3A2* (*SAP62*) immediately upstream of *AMH*. *Gene* **277**: 163–173.
- Driver JA and Kuniyuki AH (1984) *In vitro* propagation of Paradox walnut rootstock. *HortScience* **19**: 507–509.
- Dynan WS (1989) Modularity in promoters and enhancers. *Cell* **58**: 1–4.
- Eszterhas SK, Bouhassira EE, Martin DIK and Fiering S (2002) Transcriptional interference by independently regulated genes occurs in any relative arrangement of the genes and is influenced by chromosomal integration position. *Mol Cell Biol* **22**: 469–479.
- Fang RX, Nagy F, Sivasubramaniam S and Chua NH (1989) Multiple *cis* regulatory elements for maximum expression of the cauliflower mosaic virus 35S promoter in transgenic plants. *Plant Cell* **1**: 141–150.
- François IEJA, Broekaert WF and Cammue BPA (2002) Different approaches for multi-transgene-stacking in plants. *Plant Sci* **163**: 281–295.
- Fry CJ and Farnham PJ (1999) Context-dependent transcriptional regulation. *J Biol Chem* **274**: 29583–29586.
- Gatz C, Katzek J, Prat S and Heyer A (1991) Repression of the CaMV 35S promoter by the octopine synthase enhancer element. *FEBS Lett* **293**: 175–178.
- Goderis IJWM, De Bolle MFC, François IEJA, Wouters PFJ, Broekaert WF and Cammue BPA (2002) A set of modular plant transformation vectors allowing flexible insertion of up to six expression units. *Plant Mol Biol* **50**: 17–27.
- Goosen N and Van de Putte P (1995) The regulation of transcription initiation by integration host factor. *Mol Microbiol* **16**: 1–7.
- Gray DJ (1992) Somatic embryogenesis and plant regeneration from immature zygotic embryos of muscadine grape (*Vitis rotundifolia*) cultivars. *Am J Bot* **79**: 542–546.
- Gray DJ (1995) Somatic embryogenesis in grape. In: Jain SM, Gupta PK and Newton RJ (eds), *Somatic Embryogenesis in Woody Plants*. Vol. 2 (pp. 191–217) Kluwer Academic Publishers, Dordrecht.
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* **5**: 387–405.
- Kay R, Chan A, Daly M and McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **236**: 1299–1302.
- Keddie JS, Tsiantis M, Piffanelli P, Cella R, Hatzopoulos P and Murphy DJ (1994) A seed-specific *Brassica napus* oleosin promoter interacts with a G-box-specific protein and may be bi-directional. *Plant Mol Biol* **24**: 327–340.
- Lee TI and Young RA (2000) Transcription of eukaryotic protein-coding genes. *Annu Rev Genet* **34**: 77–137.
- Lemon B and Tjian R (2000) Orchestrated response: a symphony of transcription factors for gene control. *Genes Develop* **14**: 2551–2569.
- Li Z, Jayasankar S and Gray DJ (2001) Expression of a bifunctional green fluorescent protein (GFP) fusion marker under the control of three constitutive promoters and enhanced derivatives in transgenic grape (*Vitis vinifera*). *Plant Sci* **160**: 877–887.
- Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol* **37**: 49–59.
- Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473–497.
- Peach C and Velten J (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol Biol* **17**: 49–60.
- Pérez-Martín J and De Lorenzo V (1997) Clues and consequences of DNA bending in transcription. *Annu Rev Microbiol* **51**: 593–628.
- Poulsen C and Chua NH (1988) Dissection of 5' upstream sequences for selective expression of the *Nicotiana plumbaginifolia* rbcS-8B gene. *Mol Gen Genet* **214**: 16–23.
- Ptashne M and Gann A (1997) Transcriptional activation by recruitment. *Nature* **386**: 569–577.
- Pua EC (2000) Transgenic brown mustard (*Brassica juncea*). In: Bajaj YPS (ed), *Transgenic Crops I. Biotechnology in Agriculture and Forestry*. Vol. 46 (pp. 225–242) Springer, Berlin.
- Seong GH, Kobatake E, Miura K, Nakazawa A and Aizawa M (2002) Direct atomic force microscopy visualization of integration host factor-induced DNA bending structure of the promoter regulatory region on the *Pseudomonas* TOL plasmid. *Biochem Biophys Res Commun* **291**: 361–366.
- Shigekane H, Kawaguchi Y, Shirakata M, Sakaguchi M and Hirai K (1999) The bi-directional transcriptional promoters for the latency-relating transcripts of the pp38/pp24 mRNAs and the 1.8 kb-mRNA in the long inverted repeats of Marek's disease virus serotype 1 DNA are regulated by common promoter-specific enhancer. *Arch Virol* **144**: 1893–1907.
- Shlyakhtenko LS, Hsieh P, Grigoriev M, Potaman VN, Sinden RR and Lyubchenko YL (2000) A cruciform structural transition provides a molecular switch for chromosome structure and dynamics. *J Mol Biol* **296**: 1169–1173.
- Smale ST (2001) Core promoters: active contributors to combinatorial gene regulation. *Genes Develop* **15**: 2503–2508.
- Valerius O, Brendel C, Duvel K and Braus GH (2002) Multiple factors prevent transcriptional interference at the yeast ARO4-HIS7 locus. *J Biol Chem* **277**: 21440–21445.
- Velten J and Schell J (1985) Selection-expression plasmid vectors for use in genetic transformation of higher plants. *Nucleic Acids Res* **13**: 6981–6997.
- Wefald FL, Devlin BN and Williams RS (1990) Functional heterogeneity of mammalian TATA-box sequences revealed by interaction with a cell-specific enhancer. *Nature* **344**: 260–262.
- West AG, Gaszner M and Felsenfeld G (2002) Insulators: many functions, many mechanisms. *Genes Develop* **16**: 271–288.
- Xie M, He Y and Gan S (2001) Bidirectionalization of polar promoters in plants. *Nat Biotech* **19**: 677–679.