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)

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Abstract

Activity of three constitutive promoters and enhanced derivatives in transgenic grape (Vitis vinifera L. cv. Thompson Seedless) was characterized using a bifunctional fusion marker containing the enhanced green fluorescent protein (EGFP) and neomycin phosphotransferase (NPTII) genes. Relative differences in transient GFP expression and stable transformation efficiencies were used to compare promoter activity. Expression patterns in transformed somatic embryos revealed that the ACT2 promoter from Arabidopsis thaliana, previously shown to be a strong constitutive promoter in A. thaliana and other species, failed to promote strong expression in grape. In contrast, a promoter isolated from cassava vein mosaic virus (CsVMV) supported high levels of transgene expression equivalent to those achieved using an enhanced double cauliflower mosaic virus (CaMV) 35S promoter. Duplication of the 5'-upstream enhancer region of the CsVMV promoter further enhanced its ability to increase transgene expression. However, the pattern of transgene expression driven by these two viral promoters was significantly different at the whole plant level. The enhanced double CaMV 35S promoter was highly active in most tissues and organs including roots, mature leaves, shoot apices and lateral buds. In contrast, the CsVMV promoter and its double enhancer derivative induced relatively weak expression in these tissues. Our results suggest that activity of the CsVMV promoter, in contrast to the CaMV 35S promoter, was under developmental regulation in transgenic grape plants as compared with the CaMV 35S promoter. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Promoter activity; Gene expression; Fusion marker; Transgenic plants; Grape; Vitis vinifera

1. Introduction

Promoters effect gene transcription both quantitatively and qualitatively. The successful application of gene transfer technologies to crop improvement depends on the use of efficacious promoters. Over the years, numerous promoters have been isolated from a wide variety of organisms. There have been intensive efforts to characterize the effect of the molecular structure of promoters on mechanisms regulating transcriptional control of gene expression. As a result, several well-characterized promoters have been successfully adopted in the genetic engineering of plants. These promoters control transgene expression in transgenic plants to improve agronomic performance or to incorporate value-added features.

Engineering of crop plants for disease resistance requires the use of promoters capable of conferring strong and constitutive gene expression. Also, strong promoters are needed to promote the expression of marker genes in order to identify trans-
formed cells and transgenic plants during the transformation process. However, repeated use of identical promoters within the transferred DNA often induces transgene silencing [1]. Thus, in spite of the ready availability of promoters, there is currently a shortage of efficacious promoters for use in plant genetic transformation research. To date, the cauliflower mosaic virus (CaMV) 35S promoter and its derivatives are among the most commonly used [2,3]. Most promoters characterized thus far possess diverse structural and functional characteristics, and often display different levels of activity and altered patterns of expression. This is believed to be due to variation in the genetic background of the host and the interaction of promoter elements with the unknown factors present in different species [4–6]. Additional knowledge on the functionality of alternative promoters in target host plants is essential in our efforts to improve the efficacy of transgene expression.

Grape (Vitis vinifera L.) is of worldwide economic importance. It has long been recognized that effective approaches are needed to introduce and express genes encoding antimicrobial characteristics and disease resistance traits in elite grape genotypes. The availability of such techniques would facilitate efforts to enhance agronomic performance through improved local adaptation. Previous reports have described the production of transgenic grape plants and the expression of useful transgenes in these plants [7]. Several promoters including the CaMV 35S and its enhanced derivatives, and the nopaline synthase (NOS) promoter of Agrobacterium tumefaciens, have been used in transformation research with grape. Thus far, no studies have been conducted to characterize the activity and expression patterns of various promoters in grape.

Recently, we developed an improved protocol for A. tumefaciens-mediated transformation and regeneration of transgenic grape plants derived from various genotypes [8] (unpublished data). We subsequently initiated studies to analyze the performance of several constitutive promoters in grape. These promoters included an enhanced double CaMV 35S promoter [9], the ACT2 promoter of Arabidopsis thaliana [10], the cassava vein mosaic virus (CsVMV) promoter [11], and an enhanced version of the CsVMV promoter. To facilitate the monitoring of expression provided by these promoters, we developed a bifunctional fusion marker containing the enhanced Aequorea victoria green fluorescent protein (EGFP) gene and the kanamycin-resistance neomycin phosphotransferase (NPTII) gene. Unlike some of the previously developed fusion markers that utilized destructive reporter systems [12–14], the EGFP-containing fusion marker offered several advantages including the ability to continuously monitor gene expression in a non-destructive manner. The use of fusion marker genes enhanced not only the functionality and versatility of the reporter systems, but also reduced the number of promoters required for expression of multiple genes. This minimized unwanted interactions between adjacent promoters.

In this report, we compared promoter activity by examining both transient GFP expression and stable transformation derived from the expression of the bifunctional fusion marker under the control of various promoters. Patterns of transgene expression in various tissues and at various stages of plant development were also determined using transgenic grape plants containing these promoters.

2. Materials and methods

2.1. Plant material and growth conditions

All chemicals for tissue culture were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). Somatic embryos (SE) of grape (V. vinifera L. cv. Thompson Seedless) were initiated from immature leaves of in vitro-grown shoots as described by Scorza et al. [7] and were maintained on X6 medium consisting of a modified MS medium [15] lacking glycine and supplemented with 3.033 g l⁻¹ KNO₃ and 0.364 g l⁻¹ NH₄Cl (as the sole nitrogen source), 60.0 g l⁻¹ sucrose, 1.0 g l⁻¹ myo-inositol, 7.0 g l⁻¹ TC agar (Phytotechnology laboratories, LLC, Shawnee Mission, KS, USA), 0.5 g l⁻¹ activated charcoal and a pH value adjusted to 6.0 prior to autoclaving. All SE cultures were kept in the dark at 26°C and subcultures were made at 6-week intervals. Prior to transformation, SE at the midcotyledonary stage of development were selected and preconditioned by culturing them on DM medium for 1 week. The DM medium contained
DKW basal salts [16] supplemented with 0.3 g l\(^{-1}\) KNO\(_3\), 1.0 g l\(^{-1}\) myo-inositol, 2.0 mg l\(^{-1}\) each thiamine-HCl and glycine, 1.0 mg l\(^{-1}\) nicotinic acid, 30.0 g l\(^{-1}\) sucrose, 5.0 µM benzyladenine (BA), 2.5 µM each β-naphthoxyacetic acid (NOA) and 2,4-dichlorophenoxyacetic acid (2,4-D), and 7.0 g l\(^{-1}\) TC agar at pH 5.7.

2.2. Construction of a bifunctional fusion gene and binary vectors

Procedures for DNA manipulations were as described by Maniatis et al. [17]. The NPTII gene was amplified by polymerase chain reaction (PCR) from plasmid pBI121 (Clontech Lab. Inc., Palo Alto, CA, USA) using primers GK-1 (5\(^{-}\%\)-gga-cgccgctcatctaccatcatgtgtagtacctgaagcaggt-3\(^{-}\%\)) and GK-2 (5\(^{-}\%\)-gtagcggccttatcagaagaactcgtcatcgcaaggagggcttcatctaccatcatgtgtagtacctgaagcaggt-3\(^{-}\%\)) to produce a DNA fragment containing a 6× His tag followed by an NPTII gene sequence lacking a start codon. The EGFP gene was amplified from plasmid pEGFP (Clontech) using primers GK-3 (5\(^{-}\%\)-agaggatccccgggtaccggtcgc-3\(^{-}\%\)) and GK-4 (5\(^{-}\%\)-gccctgcccttgtacagctcgtc-3\(^{-}\%\)), resulting in a DNA fragment containing the EGFP gene lacking a stop codon. The two amplified fragments were joined together at their common PstI site to give rise to a 5′-EGFP–6× His–NPTII-3′ in-frame translational fusion gene. The fusion gene was verified by DNA sequencing and subsequently cloned into a bacterial expression vector for confirmation of gene functionality using Escherichia coli strain DH5α.

As illustrated in Fig. 1, four transformation vectors with a pBIN19-derived binary backbone [13] were constructed for the expression of the fusion gene under different promoters. The promoter in vector pD35S contained a double CaMV 35S promoter (−419 to −90 linked to −419 to +1) and the Ω leader sequence of tobacco mosaic virus (TMV), all of which were derived from plasmid pE2113-GUS [9]. A single CsVMV promoter (−443 to +72) was used in vector pCsVM [11]. A double CsVMV promoter (−443 to −123 joined by −443 to +72) was employed in vector pDCsVM. The ACT2 promoter fragment (−611 to +619) in vector pACT included the first intron (442 bp) of the ACT2 gene of A. thaliana [10]. These transformation vectors were introduced into A. tumefaciens strain EHA105 by the freeze–thaw method as described by Burrow et al. [18].

2.3. Transformation and plant regeneration

Preconditioned SE were transformed by incubation in Agrobacterium suspension (OD\(_{600}\) = 0.8–1.0) for 10 min and co-cultivation on DM medium for 2 days. The SE were then rinsed three times in sterile water containing 200 mg l\(^{-1}\) each carbenicillin and cefotaxime, and cultured on DM medium containing the same concentration of antibiotics (DMcc) for 4 days. Selection for kanamycin-resistant callus was carried out by culturing SE on DMcc medium supplemented with 70 mg l\(^{-1}\) kanamycin (DMcck70) for 20 days followed by subcultures on X6 medium containing 200 mg l\(^{-1}\) each carbenicillin and cefotaxime (X6cc) for 40 days with a 20-day subculture interval. Transgenic SE derived from a single transformation event in a particular location of the original SE explant were identified visually and designated as an independent transgenic line. Plant regeneration from transgenic SE was accomplished by culturing SE on MS1B medium consisting of MS medium supplemented with 1 µM BA under light (165 µmol s\(^{-1}\) m\(^{-2}\)) with a 16-h photoperiod.

Fig. 1. Physical maps of T-DNA region of transformation vectors containing the EGFP–NPTII fusion gene under control of various promoters. CaMV 35S-E, Enhancer sequence (−419 to −90) of CaMV 35S promoter; CaMV 35S-P, promoter sequence (−419 to +1) of CaMV 35S transcript; Ω, leader sequence of TMV; CsVMV-P, promoter sequence (−443 to +72) of CsVMV; CsVMV-E, enhancer sequence (−443 to −123) of CsVMV; ACT2, promoter sequence (−611 to +619) of actin 2 gene of A. thaliana; CaMV 35S-3′, the termination site and polyadenination signal of the CaMV 35S transcript; RB, right border; LB, left border.
2.4. Analysis of transgene expression

GFP expression in transformed SE was monitored using a stereomicroscope equipped with a fluorescence illuminator and a GFP filter system. Transformation frequency based on transient GFP expression and kanamycin-resistant callus formation was determined by continuously monitoring the percentage of GFP-expressing SE versus the total number of inoculated SE over a 2-month period following transformation. In these experiments, 30 SE were used in each culture dish, with three replicate dishes per treatment. Experiments were repeated two or three times depending on the availability of SE.

3. Results

3.1. Construction and expression of a bifunctional fusion gene

GFP has a cylindrical structure in which both peptide termini are exposed on the surface of the mature protein [2]. This stable protein structure provides a significant degree of tolerance to terminal fusion with other polypeptides. We fused the NPTII protein to the C-terminal of GFP. A 6×His peptide was incorporated into the fusion protein to serve as a bridge between these two fusion partners in an attempt to minimize protein folding interactions between the fusion partners and to maximize the functionality of each fused protein.

The dual function of the EGFP–NPTII fusion protein was first examined in E. coli. Bacterial cells expressing the fusion gene were capable of colonization on medium containing high concentrations of kanamycin. Transformed E. coli using a bacterial expression plasmid containing either the EGFP–NPTII fusion gene or an NPTII gene produced near identical frequencies of colony formation on kanamycin-containing selective medium (data not shown). These data indicated that the fusion protein retained its NPTII activity. All kanamycin-resistant colonies also produced high levels of green fluorescence comparable with those of bacteria expressing the EGFP gene, while none of the colonies with the NPTII-containing plasmid produced any green fluorescence (data not shown). Following this confirmation of dual functionality, the fusion gene was utilized in the construction of a series of plant transformation vectors directed by various promoters.

3.2. Influence of promoter activity on transient GFP expression and stable transformation

In order to evaluate their ability to direct transgene expression in grape cells, different promoters were placed individually upstream of the bifunctional EGFP–NPTII fusion gene and the resulting expression units were used in A. tumefaciens-mediated transformation of grape SE. Transient GFP expression was evident 2 days after transformation. Significant differences in the intensity of GFP fluorescence were observed among the SE transformed with the various vectors. As illustrated in Fig. 2a–d, SE transformed with pD35S (Fig. 2a) and pCsVM (Fig. 2b) produced similar levels of fluorescence. A much higher level of GFP fluorescence was achieved when pDCsVM was used (Fig. 2c). In contrast, SE transformed with pACT yielded a very low level of GFP fluorescence (Fig. 2d). Since all the vectors were, with the exception of the promoter fragments, identical, we attributed the differences in GFP expression to the capacity of the different promoters to direct transgene expression in grape SE cells. The enhanced double CsVMV promoter was the most efficacious in driving transgene expression. Both the single CsVMV promoters and the Ω-containing enhanced double CaMV 35S promoter provided similar, but moderate levels of transgene expression. The ACT2 promoter supported only a low level of transgene expression.

Efficacy of transgene expression among the transformation vectors was further evaluated quantitatively by examining transformation frequencies via transient GFP expression. Transformation of grape SE using pD35S, pCsVM and pDCsVM generally produced high frequencies of transient GFP expression (Fig. 3). In contrast, transformation of grape SE using pACT resulted in a transformation frequency less than one-half of the transformation frequencies achieved with other vectors 2 weeks after transformation.

Kanamycin-resistant and GFP-expressing transgenic calli were recovered from SE transformed with all vectors 2 months after transformation (Fig. 3). Transformation using pD35S or pCsVM yielded stable transformation frequencies of about
Fig. 2. Transient and stable GFP expression in various tissues of grape (*V. vinifera* cv. Thompson Seedless) after transformation using the EGFP–NPTII fusion gene under control of various promoters. (a)–(d) Transient GFP expression in representative grape SE transformed with four different binary vectors (showed on upper corner). Pictures were taken 5 days after transformation initiation under a stereomicroscope equipped with a fluorescence illuminator and GFP filter system. (e), (f) Fluorescent images of somatic embryos derived from transgenic embryos lines T-D35S-1 and pDCsVM-3, respectively. Pictures were taken microscopically using identical magnification and exposure time. (g), (h) Light and fluorescent images of germinating somatic embryos of transgenic embryo line pDCsVM-1. (i), (j) Light and fluorescent images of developing roots derived from transgenic plant lines T-D35S-1 (upper) and T-DCsVM-1 (lower). (k), (l) Light and fluorescent images of a root tip from transgenic plant line T-DCsVM-2. (m), (n) Light and fluorescent images of a root portion of transgenic plant line T-DCsVM-2. (o), (p) Light and fluorescent images of GFP-expressing callus produced from root tissue of transgenic plant line T-CsVM-2. (q)–(t) Light (q and s) and fluorescent (r and t) images of a developing shoot apex from transgenic lines T-D35S-3 and T-DCsVM-2, respectively. (u), (v) Fluorescent images of a lateral bud-containing stem node derived from transgenic plant lines T-D35S-1 and T-DCsVM-2, respectively. (w), (x) Fluorescent images of mature leaf lamina from transgenic plant lines T-D35S-1 and T-DCsVM-1, respectively. Bars placed on the lower left corner of the image represent 1 mm; bars on the lower right corner represent 1 cm.
5%. Higher frequencies of up to 11% were obtained using pDCsVM. A stable transformation frequency of 2.4% was obtained with pACT. In addition, the density of transgenic calli (the number of transgenic calli per responsive SE explant) was highest in SE transformed with pDCsVM and lowest in SE transformed with pACT (data not shown). Both callus growth and visual level of green fluorescence of the kanamycin resistant calli recovered within a similar culture period were noticeably affected by the vector type. Transgenic calli transformed with pDCsVM were larger in size and produced the highest level of GFP fluorescence when compared with transgenic calli with either pD35S or pCsVM. The lowest level of GFP fluorescence intensity, similar to that of transient GFP expression (Fig. 2d), was observed in transgenic calli with pACT. In most instances, pACT-derived transgenic calli were chimeric being composed of both transformed and non-transformed cells.

About 10 weeks after transformation, homogeneous transgenic embryos were recovered only from SE transformed with pD35S, pCsVM and pDCsVM. Despite repeated efforts, including the use of other genotypes, transgenic calli with pACT failed to produce transgenic embryos. Thus, transgenic embryo lines transformed with pD35S, pCsVM and pDCsVM (lines T-D35S, T-CsVM and T-DCsVM, respectively) were used in subsequent efforts to study the patterns of transgene expression at different stages of SE and plant development.

3.3. Transgene expression in embryonic tissues of transgenic embryos

Four independent T-D35S lines, two independent T-CsVM lines, and nine independent T-DCsVM lines were used to examine GFP expression and transgene expression patterns at various developmental stages. Transgenic SE from these independent lines were cultured on X6 medium to promote embryo growth and propagation. GFP expression in SE was monitored periodically. Transgenic SE from all transgenic T-D35S lines examined showed a homogeneous pattern of GFP expression in all stages of embryo development (Fig. 2e), suggesting that the transgenic embryos were derived from single transformed cells and were not chimeric. GFP expression in these T-D35S SE, at a late cotyledonary stage of development, was present predominantly in the hypocotyl and radicle regions, whereas expression...
in cotyledons was somewhat reduced (Fig. 2e). The majority of transgenic SE derived from T-CsVM and T-DCsVM lines also exhibited homogeneous GFP expression in all embryonic tissues at all stages of embryo development. This is exemplified in SE of transgenic line T-DCsVM-3 (Fig. 2f). However, in contrast to those SE of T-D35S lines, no reduction of GFP expression in SE of T-DCsVM lines was observed in cotyledons of SE during cotyledonary development. Visual comparison indicated that the GFP fluorescence level among transgenic SE of T-CsVM and T-DCsVM lines were relatively higher than that of transgenic SE of T-D35S lines (Fig. 2e versus Fig. 2f). No visually detectable difference in the GFP fluorescence intensity of SE was found within T-CsVM and T-DCsVM lines (data not shown).

During germination when SE were cultured on MS1B medium, GFP expression in cotyledons of SE derived from T-D35S lines was reduced, and GFP expression in hypocotyls and roots remained high (data not shown). GFP expression in root tissues of the majority of T-CsVM and T-DCsVM lines showed a gradually changing pattern with a high level in the radicles of mature SE (Fig. 2f), and an almost non-detectable level in the developing roots of germinated SE (Fig. 2g,h). In most instances, when roots of these germinated SE reached about 3–4 cm in length, GFP expression was not detected in the majority of cell types within the lower two-thirds portion of the root.

3.4. Transgene expression in root and vegetative tissues of transgenic plants

Observed differences in GFP expression in the roots of germinated SE of various transgenic lines prompted us to further examine expression patterns in roots of regenerated plants. Roots of in vitro-grown T-D35S plants continuously produced high levels of GFP expression (Fig. 2i,j, upper specimen). In contrast, the level of GFP expression in the majority of the roots of T-CsVM and T-DCsVM plant lines was, at best, barely visible (Fig. 2i,j, lower specimen). GFP expression was, however, observed occasionally in cells located outside the root tips (Fig. 2k,l) and on the surface of mature roots (Fig. 2m,n). Calli developed from root epidermal cells also retained a high level of GFP expression among T-DCsVM plant lines (Fig. 2o,p).

In T-D35S plants, high levels of GFP expression were evident in most vegetative tissues, including developing shoot apices, lateral buds, and fully expanded leaves (Fig. 2q,r,u,w). In contrast, much lower levels of GFP expression were observed in corresponding tissues in most T-CsVM and T-DCsVM plants (Fig. 2s,t,v,x). Most noticeably, the GFP expression in T-CsVM and T-DCsVM plants was relatively higher in the leaflet tips of immature leaves (Fig. 2t) and in the laminar portion between leaflets of mature leaves (Fig. 2x). Green fluorescence level in the vascular tissues of leaf, petiole and stem of all plants examined was relatively low, possibly due in part to a lower cell cytoplasmic density and storage capability of the vascular tissues when compared with other types of tissues. Variations in the level of GFP expression among different plants within a line or among different lines transformed with the same transformation vector were observed; nonetheless, visual examination of shoots and roots derived from various transgenic plant lines indicated that GFP expression levels in all T-D35S lines was significantly higher than the level observed in T-CsVM and T-DCsVM lines, where GFP expression level in T-CsVM and T-DCsVM lines was essentially identical (Fig. 4).

4. Discussion

We report the construction of a bifunctional marker gene encoding the reporter marker EGFP and a selectable marker NPTII. The dual functionality of this fusion marker was confirmed in both E. coli and grape cells. In contrast to most other fusion marker systems that use destructive and substrate-requiring reporter markers, including LUC [12], GUS [13], or LacZ [14], this EGFP-based fusion reporter system permitted assays for transgene expression at the cellular level in a non-destructive manner. It also facilitated continuous monitoring of temporal and spatial control of transgene expression during various stages of plant development. We successfully used this fusion marker to track GFP expression in transformed grape cells from transformation through plant regeneration. In addition, the use of this bifunctional fusion marker reduced the requirements for multiple promoters and the potential for interactions between adjacent promoters that typi-
cally complicate interpretation of promoter functionality data in promoter characterization studies. This fusion marker containing the NPTII protein and a 6×His polypeptide tag could also allow the quantitative determination of transgene expression based on an NPTII activity assay, as previously demonstrated [14,19], or based on quantitative analysis of the fusion proteins using available 6×His-related assay products. We are currently evaluating different methods for the quantitative analysis of this fusion protein using cell extracts from transgenic grapes.

Three promoters derived from different origins, and their enhanced derivatives examined in the present study are reported to be constitutive and capable of conferring high levels of transgene expression in various cell types in plants of diverse taxonomic origins [9–11]. However, data from this study indicated that the ACT2 promoter from A. thaliana failed to support high levels of transgene expression in cells of grape SE (c.v. Thompson Seedless). Although transgenic calli were recovered after transformation using the bifunctional fusion marker gene under the control of the ACT2 promoter, these calli were mostly chimeric and expressed a low level of GFP fluorescence and kanamycin resistance. Such low levels of transgene expression provided by the ACT2 promoter precluded the recovery of any transgenic grape embryos under selection conditions. These findings were somewhat in agreement with previous reports indicating that little or no transgene expression could be detected in seed coats or hypocotyls of A. thaliana when an ACT2/GUS fusion construct was used [10].

The cause for lack of expression activity with the ACT2 promoter in grape SE cells remains unknown. The ACT2 promoter may be subjected to developmental and tissue-specific regulatory control in grape SE cells. This has been shown to be the case in tobacco, in which high levels of promoter activity were mainly associated with vegetative tissues [10]. That is, regulatory cis-elements within the A. thaliana ACT2 promoter may not be recognizable by trans-acting factors present in certain types of cells, including the cells of grape SE and callus. Actins in eukaryotic organisms are encoded by a multigene family. The number of gene members in the actin multigene family may vary among different species [20,21]. Although actin genes are relatively conserved at the DNA level, distinct patterns of expression regulation and protein functions occur among different actin gene members within a species [20]. Additional charac-

![Fig. 4. GFP expression in shoot and root tissues of in vitro-grown transgenic plantlets of grape (cv. Thompson Seedless) as affected by different promoters. Shoot tips of similar size were excised from various transgenic lines and cultured on a hormone-free MS medium with four shoot tips per culture dish. One month after culture initiation, GFP expression in newly produced shoots and roots was estimated using a subjective scale from 0 to 5, based on the relative level of green fluorescence observed under a stereomicroscope equipped with a fluorescence illuminator and GFP filter unit. Values for vector pD35S were averaged from four independent lines with a total of 28 plantlets; values for vector pCsVM were averaged from one line with a total of eight plantlets; values for vector pDCsVM represent average data from three independent lines with a total of 29 plantlets. Vertical lines represent standard errors.](image-url)
terization of the transcriptional activity of the A. thaliana ACT2 promoter in different types of tissues and organs in the non-host species V. vinifera is needed before attempts are made to utilize this promoter to confer transgene expression in transgenic grapes. Nonetheless, we suggest that caution be exercised in the use of the ACT2 promoter to provide expression of marker genes for the purpose of transformant recovery in genetic transformation processes that utilize SE.

The 5'-upstream region of the CaMV 35S promoter could be used in the form of tandem repeats to further enhance gene transcription without altering tissue-specific expression patterns in many plant species [9,22,23]. Results obtained from our study using promoter construct pD35S also confirmed the efficacy of this enhanced promoter to confer high levels of constitutive transgene expression in all types of tissues of grape.

The CsVMV promoter was highly active in grape SE tissues. A single CsVMV was capable of conferring transgene expression at levels equivalent to those conferred by the enhanced double CaMV 35S promoter. This finding is in agreement with the results of similar transformation studies using tobacco and cassava protoplasts [11]. We demonstrated that even higher frequencies of transient and stable transformation in grape SE tissues can be achieved using the enhanced double CsVMV promoter.

In spite of the strong expression activity conferred by the CsVMV promoter in transgenic SE tissues, this promoter produced relatively low levels of expression in roots of transgenic grape plants through all developmental stages. Based on the observations that transgene expression remained high in cells on the surface of the root, and in root-derived callus cells, we suggest that the lack of expression in transgenic grape roots may have been the result of transcriptional regulation, and not the result of gene silencing phenomenon as previously discussed [1]. The lack of expression in roots of transgenic grape plants containing the CsVMV promoter was in disagreement with previous reports indicating that high levels of transgene expression could be achieved in root tissues of other species, including tobacco and rice, using the CsVMV promoter [11].

The CsVMV promoter, in both its single and double enhancer versions, also generated a relatively low level of expression in mesophyll cells of transgenic grape plants at various developmental stages, when compared with those provided by the CaMV 35S promoter. In a recent study, Verdaguer et al. [24] demonstrated that an as1 motif located 168 bp upstream from the TATA box, and other unidentified elements, within the CsVMV promoter, were involved in regulating expression in root tips of transgenic tobacco. Promoter analysis using deletion mutations suggested that expression in tobacco mesophyll cells was associated with a GATA motif located downstream of the as1 motif and the synergistic interactions between these elements in the CsVMV promoter, as in the case of the CaMV 35S promoter [24,25]. However, the number and the relative position of both as1 and GATA elements are significantly different between the CsVMV and the CaMV 35S promoter [11,25]. In addition, as pointed out by Verdaguer et al. [24], the GATA motif identified in the CsVMV promoter was not identical to the GATA element found in the CaMV 35S promoter, but was more similar to a GATA motif identified in the rice tungro bacilliform badnavirus (RTBV) promoter [26,27]. We speculate that the low activity of the CsVMV promoter in various tissues of grape plants may have been influenced by unique structural characteristics of this promoter. For instance, variations in the pattern of transgene expression, as induced by different promoters isolated from various para-retroviruses, including CsVMV and RTBV, were reported previously [11,28–30].

In summary, we developed and utilized a bifunctional fusion EGFP–NPTII marker to characterize the functionality of several constitutive promoters in grape. Unique promoter activity and transgene expression patterns supported by these promoters were observed in transgenic grape plants. These data provide useful information relevant to the use of these promoters to direct transgene expression in specific target tissues in this economically important crop plant in order to maximize the efficacy of genetic engineering efforts. For example, if transgenes are required to be expressed in root tissues of transgenic grapes, the CsVMV is probably not the promoter of choice, due to its inability to support strong expression in grape root tissues. Alternatively, identification of cis-acting elements in the CaMV 35S promoter, which are responsible for strong expression in root and leaf tissues of transgenic grape, may help us
better understand transcriptional regulation requirements. Such information may also facilitate the development of improved CsVMV promoter constructs that are capable of conferring strong expression in these grape tissues.

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