TECHNICAL ADVANCE

Evaluation of Constitutive Viral Promoters in Transgenic Soybean Roots and Nodules

Manjula Govindarajulu, James M. Elmore, Thomas Fester, and Christopher G. Taylor

Donald Danforth Plant Science Center, 975 N. Warson Road, St. Louis 63132, U.S.A.

Submitted 12 December 2007. Accepted 4 April 2008.

The efficiency of β -glucuronidase (GUS) expression was evaluated with five viral promoters to identify the most suitable promoter or promoters for use in soybean hairy roots, including applications to study the symbiotic interaction with *Bradyrhizobium japonicum*. Levels of GUS activity were fluorimetrically and histochemically assayed when the GUS (*uidA*) gene was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter and enhanced 35S (E35S) promoter, the *Cassava vein mosaic virus* (CsVMV) promoter, the *Figwort mosaic virus* (FMV) promoter, and the *Strawberry vein banding virus* (SVBV2) promoter. We demonstrate that GUS activity was highest when driven by the FMV promoter and that the promoter activity of 35S and SVBV2 was significantly lower than that of the CsVMV and E35S promoters when tested in soybean hairy roots. In mature soybean root nodules, strong GUS activity was evident when the FMV, 35S, and CsVMV promoters were used. These results indicate that the FMV promoter facilitates the strong expression of target genes in soybean hairy roots and root nodules.

Additional keywords: *Agrobacterium rhizogenes*, constitutive viral promoter.

Soybean (*Glycine max*), an agronomically important member of the Fabaceae family, is used worldwide and highly valued for the high levels of protein and oil in its seeds. The rising economic importance of soybean has led to increased efforts over the past several years to apply biotechnology strategies for the improvement of soybean germplasm, including genetic transformation. The most commonly used method for soybean transformation is *Agrobacterium*-mediated transformation (Christou 1997; Droste et al. 2000; Trick and Finer 1997, 1998; Santarém et al. 1998). Although substantial improvements have been made in the efficiency of stable transformation (Olhoft et al. 2003; Somers et al. 2003), the generation of transgenic soybean plants is still laborious and time consuming. Due to this difficulty in developing stable transgenic soybean, transient or composite transformation methodologies are an attractive approach to screen potential genes useful for soybean improvement. Regardless of the transformation method one chooses, the availability of promoters with varied strengths and tissue specificities will enhance the success of transgenic approaches in soybean improvement and functional analysis of genes. In order to drive efficient transgene expression in soybean, it is essential to use a promoter that regulates

gene expression to sufficient levels in tissues of interest. To this end, we report here on the promoter::*gus* driven profile of five different virus-derived promoters in *Agrobacterium rhizogenes*-induced soybean hairy roots and, subsequently, in nodules induced by *Bradyrhizobium japonicum*.

Several reports described constitutive plant promoters that confer high levels of transgene expression in dicot plants (An et al. 1996; Holtorf et al. 1995; Kelemen et al. 2002; Khoudi et al. 1997; Malik et al. 2002). Viral promoters that confer high levels of constitutive gene expression in transgenic plants were also identified. For example, the 35S promoter isolated from *Cauliflower mosaic virus* (CaMV) is the most widely used constitutive and efficient promoter in transgenic plants (Benfey and Chua 1990; Holtorf et al. 1995; Odell et al. 1985; Wilmink et al. 1995). Other viral promoters such as the *Figwort mosaic virus* (FMV) promoter (Maiti et al. 1997; Sanger et al. 1990), the *Cassava mosaic virus* (CsVMV) promoter (Verdaguer et al. 1996), and the *Carnation etched ring virus* promoter (Holmberg et al. 2002) were reported to have activity similar to the 35S-CaMV promoter in transgenic dicot plants. Previous studies showed that the levels of activity and differential expression patterns of a “constitutive” promoter can vary substantially depending on the plant species and tissue type. For example, the activity of the 35S-CaMV promoter is higher in the leaves of transgenic tobacco than in the leaves of alfalfa or *Arabidopsis* plants (Malik et al. 2002). In transgenic alfalfa plants, the activity of the 35S-CaMV promoter is moderate in the leaves, roots, and stems while the promoter is inactive in the stem pith cells, root cortical cells, or symbiotic zones of the nodules (Samac et al. 2004). The CsVMV promoter, however, shows a high level of β -glucuronidase (GUS) expression in the leaves, roots, and nodules in transgenic alfalfa (Samac et al. 2004). Recent studies by Auriac and Timmers (2007) reported that the 35S promoter is inactive in both the meristem and bacteroid-containing cells of nodules in *Medicago truncatula*.

Although several studies examined promoter expression in dicots, there is clearly a dearth of knowledge regarding the evaluation of tissue-specific promoters for expression of transgenes in soybean plants. A detailed study on the expression of different viral promoters in soybean is not available.

The objective of this study was to compare the performance of various constitutive viral promoters using the *uidA* (*gus*) marker gene in soybean roots and in nodules induced by *B. japonicum*. We aimed to identify promoters suitable for driving high levels of expression of genes in soybean roots and *B. japonicum*-induced nodules and to evaluate their tissue-specific expression patterns. Because stable transformation in soybean is not quick or easily scalable for screening large numbers of

gene constructs, we adopted the composite plant system and used the *A. rhizogenes* strain K599 (Taylor et al. 2006) to produce transgenic hairy roots expressing our viral-derived promoter::*gus* fusion constructs on wild-type soybean shoots. We tested several constitutive viral promoters derived from plant pararetroviruses, including CaMV 35S and enhanced 35S (E35S), *Strawberry vein banding virus* (SVBV2), CsVMV, and FMV. For our analysis, we fluorimetrically measured GUS activity in hairy roots and root nodules of soybean and examined tissue-specific expression using histochemical staining. Here, we report that the highest level of GUS activity was obtained using the FMV promoter in soybean hairy roots. In soybean root nodules, constructs utilizing the FMV, 35S-CaMV, and CsVMV promoters showed high levels of GUS activity in different cell types of the nodule.

RESULTS

FMV promoter is a strong constitutive promoter in soybean hairy roots.

The activity of different constitutive viral promoters was tested in hairy roots of soybean plants by assaying GUS enzymatic activity. Transformed roots containing the viral promoter fused with the *gus* gene (Fig. 1A) were obtained by *A. rhizogenes*-mediated gene transfer. All roots were assessed as being transformed using a scorable green fluorescent protein (GFP) marker that was present on the same T-DNA as the GUS marker (Fig. 1A and B). Transformation efficiency in soybean plants, defined as the percentage of the plant shoots tested that produced at least one GFP-expressing root, ranged from 10 to 50% (comparable with that observed by Collier and associates [2005]). In 3-week-old hairy roots, GUS activity was analyzed both fluorimetrically and histochemically. No GUS staining was detected in roots transgenic for the promoterless::*gus* construct that was used as a negative control (Fig. 2A). The highest intensity of GUS staining was observed in roots transgenic

for the pFMV::*gus* construct (Fig. 2D) and was uniformly visible along the entire root. The pCsVMV::*gus* and pE35S::*gus* constructs produced roots with high levels of GUS staining, with the highest intensity in the root tip and central vascular bundle of the root (Fig. 2C and E, arrowheads). Roots transgenic for the p35S::*gus* and pSVBV2::*gus* constructs exhibited very weak GUS staining in the entire root except at the root tips (Fig. 2B and F). Cross-sections were made of GUS-stained transgenic roots to study cell-specific expression patterns (Fig. 2G to L). The pFMV::*gus* construct showed strong GUS staining in all root cells (Fig. 2J). The expression of pCsVMV::*gus* construct produced GUS staining throughout the root, with the strongest staining localized to the rhizodermis (R) and cells of the central cylinder (CC) (Fig. 2K). The pE35S::*gus* and p35S::*gus* constructs had reduced levels of GUS staining in the rhizodermis and cortical cells, with stronger staining localized to the endodermis and central cylinder regions (Fig. 2H and I, respectively). Transgenic root sections expressing the pSVBV2::*gus* construct showed limited GUS staining throughout the root, with the exception of those found in the root tip (Fig. 2L). The promoterless::*gus* construct produced no observable GUS staining in the root (Fig. 2G).

To verify whether the observations from the histochemical GUS staining were indicative of differential GUS protein levels due to promoter activity and not an artifact of the staining procedure, fluorimetric enzymatic assays were performed on hairy root GUS protein extracts. GUS enzyme activity was measured in 25 independent hairy roots from 25 individual soybean plants for each promoter::*gus* fusion construct. The pFMV::*gus* construct exhibited approximately 280-fold higher relative GUS enzyme activity compared with roots transgenic for the promoterless::*gus* control construct. The pE35S::*gus* and pCsVMV::*gus* constructs showed relatively lower levels of GUS enzyme activity (approximately 180- and 220-fold, respectively) in the hairy roots. Roots expressing the p35S::*gus* and pSVBV2::*gus* constructs showed the lowest levels of GUS

Fig. 1. Schematic representation of the promoter constructs that control β -glucuronidase (*gus*, *uidA*) expression in transgenic soybean roots and root nodules. **A**, Shuttle vector for promoter::*gus* assays contained the *uidA* gene driven by various viral promoters. Each shuttle (promoter::intron::*uidA*::terminator) was flanked by *Ascl* restriction sites. **B**, Binary vector used for testing promoter::*gus* constructs has a unique *Ascl* restriction site for cloning the promoter::*gus* shuttle, a kanamycin resistance gene (*NPT-II*), and a scorable marker (green fluorescent protein [*GFP*]) (Collier et al. 2005). Shuttle vector: Promoter = viral promoters p35S-CaMV, pE35S-CaMV, pSVBV2, pCsVMV, or pFMV; Intron = super-ubiquitin intron; GUS = β -glucuronidase gene (*uidA*); tNOS = nopaline synthase terminator. Binary vector: LB = T-DNA left-border sequence; RB = T-DNA right-border sequence; GFP (pSU::intron::GFP::tNOS) and NPT-II (pNOS::NPT-II::tNOS) from Collier and associates (2005).

Fig. 2. Histochemical localization of the expression of β -glucuronidase (GUS) (*uidA*) constructs in soybean hairy roots. The *uidA* gene was driven by various viral promoters. **A and G**, Promoterless::*gus* control; **B and H**, p35S-*Cauliflower mosaic virus* (CaMV)::*gus*; **C and I**, pE35S-CaMV::*gus*; **D and J**, *Figwort mosaic virus* (pFMV)::*gus*; **E and K**, *Cassava vein mosaic virus* (pCsVMV)::*gus*; and **F and L**, *Strawberry vein banding virus* (pSVBV2)::*gus*. **A through E**, Arrows indicate location of strongest GUS activity in the central vascular bundle of the root and in the root tip. Dotted lines indicate the region of cross sections. **G through L**, Corresponding 12- μ m paraffin cross-sections. GUS staining (dark-blue color) can be seen in the rhizodermis (R), root cortex (RC), and central cylinder (CC). Pictures represent typical expression pattern seen in at least 25 roots. G, H, J, K, and L, bars = 65 μ m; I, bar = 35 μ m.

enzyme activity (approximately 30- and 60-fold, respectively) (Fig. 3) and were in accordance with the observed histochemical analysis.

Differential expression of GUS in soybean root nodules.

Soybean root nodules carrying different viral promoter::*gus* fusions were formed on transgenic hairy roots by inoculating transformed roots with *B. japonicum*. All nodules were assessed as transformed by using GFP as a scorable marker. The average number of nodules per GFP-expressing root ranged from four to six and was not significantly different from that of non-GFP-expressing (nontransgenic) roots (data not shown).

Mature 4-week-old nodules were histochemically examined for GUS activity. Promoter-driven expression patterns of GUS activity in nodules appear to be different from those observed in non-nodulated hairy roots. Paraffin sections of GUS-stained nodules clearly revealed that, within the central region of the nodule, GUS staining was observed with all viral promoters (Fig. 4B to F). However, in nodules transgenic for the pSVBV2::*gus* construct, there was no staining in the outer cortical cells of the nodule, indicative of limited promoter activity in these tissues (Fig. 4F). GUS staining was seen at the outer cortical cells of the nodule using the other four promoters tested (Fig. 4B to E), albeit at varying levels. As expected, the promoterless::*gus* construct failed to produce GUS activity in the nodule (Fig. 4A).

Cell-specific patterns of GUS staining using 12- μ m-thick longitudinal sections of paraffin-embedded nodules revealed differential staining with five viral promoters (using transmitted light from a dissecting microscope). The expression of the pFMV::*gus* construct was the strongest among the five promoters tested, and was found to be expressed in all cells of the nodule (Fig. 4J). The pCsVMV::*gus* construct was expressed strongly in the outer cortex (OC), vascular bundle (VB), and sclerenchyma cell layer (SC) of the nodule (Fig. 4K). The p35S::*gus* and pE35S::*gus* constructs (Fig. 4H and I, respectively) showed weaker staining in the outer cortex, sclerenchyma cell layer, and vascular tissues of the nodule compared with pFMV::*gus* and pCsVMV::*gus* constructs (Fig. 4J and K, respectively). However, the expression of the pSVBV2::*gus*

construct was the weakest and primarily localized to the vascular bundles of the nodule whereas cells of the outer cortex and sclerenchyma cell layer remained unstained (Fig. 4L).

In order to show differential expression within the inner cortex of the nodule (containing the uninfected and infected cells of the nodule), we verified the expression pattern using reflected light of a dissecting microscope. All promoters tested produced intense staining in the infected cells of the inner cortex (Fig. 4H to L, insets). Infected cells from nodules of the promoterless::*gus* construct showed no GUS staining, as expected (Fig. 4G, inset). Staining patterns of uninfected cells of the inner cortex for the five promoters tested produced variable results. The pFMV::*gus* construct showed intense staining while p35S::*gus*, pCsVMV::*gus*, and pSVBV2::*gus* constructs exhibited lower levels of GUS staining in uninfected cells of the inner cortex (Fig. 4J, H, K, and L insets, respectively). The uninfected cells of the inner cortex of pE35S::*gus* construct nodules showed little to no staining, similar to that observed in the promoterless::*gus* construct nodules (Fig. 4I and 4G insets, respectively).

GUS enzyme activity was fluorimetrically measured 4 weeks post inoculation with *B. japonicum* in 25 nodules each from an independent hairy root from 25 individual soybean plants. Results from our fluorimetric enzymatic assays confirmed the observation that nodules expressing the pFMV::*gus* construct had the highest relative GUS enzyme activity (approximately 330-fold) followed by the p35S::*gus* (approximately 300-fold) compared with the promoterless::*gus* control root nodules (Fig. 5). The pE35S::*gus* and pCsVMV::*gus* constructs exhibited less GUS activity (approximately 150- and 250-fold) in the transgenic soybean root nodules relative to the promoterless::*gus* control. The lowest level of GUS activity was observed for the pSVBV2::*gus* construct (approximately 11-fold). No GUS activity was detected in nodules transgenic for the promoterless::*gus* control (Fig. 5).

DISCUSSION

The main objective of this study was to assess the activity of various viral promoters that could be used to drive high levels

Fig. 3. Relative β -glucuronidase (GUS) activity of different viral promoter::*gus* constructs in soybean hairy roots. GUS activity was measured using a fluorimetric assay. GUS activities were compared with that of the promoterless::*gus* control (assigned a value of 1). Each bar represents the standard errors of three experimental repeats each evaluating 25 independent roots. According to Student's *t* test, measurements for promoter::*gus* constructs were significantly different from the control promoterless::*gus* construct ($P < 0.05$) in all cases.

of gene expression in soybean roots and nodules. All promoters identified and analyzed could be used to drive expression of genes, each with their own different expression levels and tissue specificities. Our results indicated that the 35S-CaMV

promoter was a relatively weak promoter in soybean roots, active only in the root tip region and to a much lesser extent in the central vascular bundle of soybean roots. Auriac and Timmers (2007) reported similar results where the 35S-CaMV

Fig. 4. Histochemical localization of β -glucuronidase (GUS) activity in soybean nodules. **A through F**, Longitudinal paraffin sections (12 μ m) of mature soybean nodules showing GUS activity driven by various viral promoters. **A and G**, Promoterless::gus control; **B and H**, p35S-*Cauliflower mosaic virus* (CaMV)::gus; **C and I**, pE35S-CaMV::gus; **D and J**, *Figwort mosaic virus* (pFMV)::gus; **E and K**, *Cassava vein mosaic virus* (pCsVMV)::gus; and **F and L**, *Strawberry vein banding virus* (pSVBV2)::gus. **G through L**, Higher magnification of the nodule section as observed in **A through F**. Dissecting microscopy (using transmitted light) shows GUS staining (dark-blue color) in the outer cortex (OC), sclerenchyma (SC), nodule vascular bundle (VB), infected inner cortex (IC), and uninfected inner cortex (UC). Inset: Dissecting microscopy (using reflected light) shows a detailed image of GUS staining in the infected inner cortex (IC) and uninfected inner cortex (UC). Pictures represent typical expression patterns seen in at least 25 nodules. **A through F**, bars = 400 μ m and **G through L**, bars = 65 μ m.

promoter was active only in the root tip region and the central vascular bundle in the roots of *M. truncatula*. Their confocal microscopic analysis of GFP-expressing roots showed that the 35S-CaMV promoter gives strong expression in the root cap but is significantly weaker in the meristem. A similar pattern of expression was observed in cotton (Sunilkumar et al. 2002), tobacco (Mitsuhara et al. 1996), and alfalfa (Samac et al. 2004). Moreover, Yang and Christou (1990) showed relatively low expression of the 35S-CaMV promoter specifically in the procambium, phloem, and root cortical cells of soybean.

The FMV promoter exhibited strong constitutive gene expression that was uniformly expressed through the entire soybean root. The CsVMV promoter was weaker than the FMV promoter but still drove expression throughout most of the root similar to that observed by Samac and associates (2004). Their results showed high levels of GUS activity in *M. sativa* roots transformed using a similar pCsVMV::gus construct. In transgenic tobacco plants, the FMV subgenomic transcript (Sgt) promoter expression level in roots was higher than the 35S-CaMV promoter (Bhattacharyya et al. 2002). A full-length transcript (FLt) of the FMV promoter that is equivalent to the 35S transcript of CaMV drives high levels of expression in the vascular tissues of tobacco roots compared with the 35S-CaMV promoter (Maiti et al. 1997).

Soybean hairy roots transgenic for pE35S::gus showed strong GUS activity in the root tip and central vascular cylinder. GUS activity was lower in the cortical region of hairy roots. In transgenic tobacco plants, where the E35-CaMV promoter was used to drive gus expression, GUS activity was observed in the root elongation zone and vascular tissues and, to a lesser extent, in the root apex (Elmayan and Tepfer 1995). However, in *Allocasuarina* spp., the E35S-CaMV promoter showed activity throughout the root system (Obertello et al. 2005). Certainly, based on our results, the E35S-CaMV promoter was stronger than the 35S-CaMV promoter in soybean roots and would be the better choice if a 35S-CaMV-based promoter is to be used in soybean.

From our analysis, the SvBV2 promoter appeared to be the weakest promoter tested in soybean hairy roots. This was in contrast to transgenic tobacco plants where Pattanaik and associates (2004) showed that an FLt SvBV2 promoter was a strong

constitutive promoter capable of driving expression greater than that observed for the 35S-CaMV promoter. Of the five viral promoters tested, our data indicate that the FMV promoter is the best choice when a strong, constitutive promoter is required for transgene expression in soybean hairy roots.

A second aim of this study was to test the function of these viral promoters in nodules formed during the symbiotic interaction between soybean roots and *B. japonicum*. Recent studies showed that 35S-CaMV promoter activity is restricted to the noninvaded cells of *M. truncatula* nodules (Auriac and Timmers 2007). Similar results were also reported for nodules of *Vicia hirsuta* (Quandt et al. 1993) and *M. sativa* (Samac et al. 2004). Moreover, Samac and associates (2004) showed high activity in nodule tissues of *M. sativa* using the CsVMV promoter. In our study, GUS activity could be detected in the infected cells of the inner cortex within the mature nodules in soybean regardless of the viral promoter used. However, differences in GUS activity between the promoters were observed in the outer cortex, sclerenchyma cell layer, and uninfected cells of the inner cortex. In the inner cortex, GUS staining for the pE35S::gus construct was primarily localized to the infected cells of the nodule, with little staining observed in uninfected cells. The p35S::gus construct produced GUS activity in the infected cells but weak staining was observed in the uninfected cells of the inner cortex. However, in the outer cortex, sclerenchyma cell layer, and vascular bundles of the nodule, few differences were observed between the two promoters. These results are intriguing, suggesting that a possible duplication of *cis*-acting elements of the 35S-CaMV promoter in the E35S promoter or a loss of a cryptic *cis*-acting site during the construction of the E35S promoter may lead to a decrease in expression of uninfected cells of the inner cortex.

The pFMV::gus and pCsVMV::gus constructs produced GUS activity throughout the entire nodule, although the GUS activity in the CsVMV::gus nodules appeared to be lower in the uninfected cells of the inner cortex compared with the pFMV::gus construct. The reason for this is unknown but may reflect different *cis*-acting elements within the CsVMV promoter that are not active in this region. The fluorimetric data obtained from nodules further reflected these findings, show-

Fig. 5. Relative β -glucuronidase (GUS) activity of different viral promoter::gus constructs in nodules of soybean hairy roots. The GUS activity was measured by a fluorimetric assay of 25 different nodules for each construct. GUS activities were compared with that of the promoterless::gus control (assigned a value of 1). Each bar represents the standard errors of three experimental repeats. According to Student's *t* test, measurements for promoter::gus constructs were significantly different from the promoterless::gus construct ($P < 0.001$) in all cases.

ing that overall GUS activity on a per-nodule basis was not statistically different among the 35S-CaMV, E35S-CaMV, FMV, and CsVMV promoters. However, fluorimetric data from the SVBV2 promoter was significantly lower in the nodule than the other four promoters tested.

This study highlights the importance of choosing an appropriate promoter when attempting to express transgene constructs in soybean roots. If transgenes are required to be expressed in soybean hairy root tissues, the 35S, E35S, or SVBV2 are probably not the best promoter to use due to their low expression in these tissues. However, these promoters may be useful for targeting gene expression to the infected cortical cells of the nodule or to the root tip. If high levels of gene expression are required, the FMV promoter is one of the best viral promoters currently available due to its strong expression patterns in the root and nodule. From our studies, we concluded that the constitutive FMV promoter, followed by the CsVMV promoter, were the promoters of choice for use in developing sense or antisense strategies, RNAi-based approaches, overexpression, or localization studies aimed at understanding the function of genes in soybean hairy roots or nodule tissues. Other promoters might be more suitable if targeting specific tissues or when weaker gene expression is required.

In summary, we used the scorable marker gene (*uidA*) as a marker to study the expression pattern of several constitutive viral promoters in soybean root and nodule tissues. Unique activity and expression patterns by these promoters were observed in soybean hairy roots and nodules. Our data provide useful information relevant to the application of these promoters to direct transgene expression in specific target tissues to maximize the efficacy of genetic engineering efforts in this economically important plant.

MATERIALS AND METHODS

Plasmid construction for plant transformation.

The viral promoters for 35S-CaMV, E35S-CaMV, FMV, CsVMV, and SvBV2 were polymerase chain reaction (PCR) amplified and inserted into the shuttle plasmid pAKK 1431 (containing a promoterless *uidA* gene with an intron in the 5' untranslated region and terminated with the NOS terminator) to create a recombinant promoter::*gus* transcription module. The promoter::intron::*gus*::terminator (referred to as promoter::*gus*) recombinant construct was moved into the binary vector, AKK 1448, using two flanking *AscI* sites. The binary plasmid, AKK 1448, is based on a modified binary construct system (Collier et al. 2005) with six unique 8-bp restriction sites located between the right and left border sequences of a modified pBIN19 plasmid (Bevan 1984). AKK 1448 contains a *gfp* expression module (described by Collier and associates [2005]) driven by the superubiquitin promoter from *Pinus radiata* and a kanamycin (*NPT-II*) plant-selectable module driven by the nopaline synthase promoter from *A. tumefaciens*.

All recombinant promoter::*gus* fusions binary constructs were electroporated into *A. rhizogenes* strain K599, selected on kanamycin, and used for composite plant production (Collier et al. 2005; Taylor et al. 2006).

Transformation of soybean hairy roots.

Soybean composite plants were generated according to Taylor and associates (2006) with some modifications. In short, soybean seed (cv. Williams 82) were sterilized using chlorine gas. Sterilized seed were sown in soil (4 M; Hummert International, Earth City, MO, U.S.A.) and grown in the greenhouse for 2 weeks. Hairy root transformation was carried out with *A. rhizogenes* strain K599 transformed with the GUS binary constructs carrying different promoters, along

with the promoterless GUS control. *A. rhizogenes* cultures were grown in Luria-Bertani (LB) broth supplemented with kanamycin (50 µg/ml) in a flask at 225 rpm at 28°C overnight. Bacterial cells were spun down at 3,000 × *g* for 10 min at 23°C and resuspended in sterile water to an optical density at 600 nm (OD₆₀₀) of 0.3. Sterilized FibrGro cubes (1 cm³; Hummert International) were inoculated with *A. rhizogenes* bacterial suspension. Apical stem sections (meristem with one fully expanded leaf) were excised from 2-week-old greenhouse-grown soybean plants and inserted into the inoculated cubes. Shoots were incubated in the growth chamber (temperature, 22°C; light, 15 µmol/m²/s; humidity, 30%; 16-h day and 8-h night photoperiod) for 1 week. Shoots were removed from FibrGro cubes and each shoot was placed in individual 4-in. pots containing sterilized vermiculite/perlite (3:1) wetted with nitrogen-free plant nutrient solution (Lullien et al. 1987). Shoot-containing pots were incubated in the growth chamber (temperature, 26°C; light, 200 µmol/m²/s; humidity, 60%; 16-h day and 8-h night photoperiod) for two additional weeks. Plants were watered every other day, alternating water with nitrogen-free plant nutrient solution. Production of transgenic hairy roots on soybean stems was monitored by observing the formation of GFP-expressing roots. Hairy roots used in this analysis were verified as transgenic by GFP epifluorescence using a Zeiss (Thornwood, NY, U.S.A.) Stemi SV11 microscope outfitted with a 480-nm excitation/515 emission fluorescein isothiocyanate (FITC) filter. Independent hairy roots were used for histochemical and fluorimetric enzyme activity analysis. Composite plants were also inoculated with *B. japonicum* (described below) and nodules analyzed for GUS activity.

Soybean nodulation using *B. japonicum*.

Soybean composite plants were inoculated with *B. japonicum* USDA110 strain for nodulation. Cultures were grown in HEPES-MES (HM) liquid medium (Cole and Elkan 1973) and chloramphenicol at 20 µg/ml and 30°C for 2 days with agitation to an OD₆₀₀ of 0.5. *B. japonicum* cells were centrifuged at 7,000 × *g* for 15 min at 10°C, and the pellet washed with sterile water and then resuspended in nitrogen-free plant nutrient solution to a final OD₆₀₀ of 0.08 (approximately 10⁸ cells ml⁻¹). After 2 weeks of shoot growth under high-light conditions (200 µmol/m²/s) in vermiculite/perlite, 10 ml of the *B. japonicum* bacterial suspension was inoculated onto each plant. Control plants (noninoculated) were mock inoculated with 10 ml of nitrogen-free plant nutrient solution. Four weeks after *B. japonicum* inoculation, independent mature nodules were used for histochemical and fluorimetric enzyme analysis. Nodules used in this analysis were verified as transformed by their GFP epifluorescence using a Zeiss Stemi SV11 microscope outfitted with a 480-nm excitation/515 emission FITC filter.

Histochemical localization of GUS activity and sectioning.

For histochemical analysis, GUS expression was evaluated by staining hairy roots and nodules from composite soybean in 1× GUS solution (Jefferson et al. 1987). Root or nodule material was placed in 12-well microtiter plates and 2 ml of 1× GUS solution (0.1 M NaPO₄ at pH 7.0, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], 10 mM EDTA, 0.1% Triton X-100, and 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-glucuronide dissolved in dimethyl sulfoxide) was added. The tissue was placed under vacuum infiltration for 15 min, then incubated at 37°C overnight. GUS expression was examined using a Zeiss Stemi SV11 dissecting microscope equipped with a digital camera. In all, 25 independent roots or nodules collected from 25 GFP-expressing roots were examined. Each construct was tested in roots and nodules in duplicate.

For sectioning soybean roots and nodules, tissues were first GUS stained overnight and then dehydrated through a graded water/ethanol series: 10, 25, 50, 75, and 95% for 1 h each with constant agitation, refreshing solutions after 30 min. Fixed dehydrated tissues were then infiltrated in a graded series of Histoclear in ethanol: 50, 75, and 95% Histoclear for 2 h each, and 100% Histoclear overnight. Tissues were infiltrated in 1:1 Histoclear/Paraplast tissue embedding medium for 8 h at 56°C and then infiltrated in Paraplast tissue embedding medium for 96 h (Hawes and Satiat-Jeunemaitre 2001). Nodule and root sections (12 µm thick) were generated with a wax microtome and examined using a Nikon SMZ 1500 dissecting microscope and a Nikon Eclipse E800 microscope with bright field optics.

Fluorimetric assays for GUS activity.

GUS enzyme activity was quantified using the fluorimetric assay described by Jefferson and associates (1987) with the following modifications. Individual whole root pieces (approximately 15 to 25 mg) and whole nodules (approximately 5 to 7 µg) were placed in a 1.5-ml microfuge tube, frozen in liquid nitrogen, pulverized, and then ground in GUS extraction buffer (10 µl/mg tissue) (Jefferson et al. 1987) using a motorized pestle. Insoluble material was pelleted by centrifugation at 14,000 × g for 10 min twice. Samples of 10 µg of protein per 50 µl were assayed in triplicate in extraction buffer with 150 µl of 2 mM 4-methylumbelliferyl β-D-glucuronide trihydrate (GoldBio Tech, St. Louis) in 200-µl reactions in 96-well black-sided assay plates. Plates were incubated at 37°C for 10 min prior to plate reading. Fluorescence of the 4-methylumbelliferone produced was measured over 1 h at 37°C using a Gemini fluorescence spectrophotometer (Molecular Devices, Toronto, Canada) equipped with a 360/40 nm excitation filter and 450/50 nm detection filter. Protein concentration was determined using the BioRad Protein assay (BioRad Laboratories, Hercules, CA, U.S.A.) with bovine serum albumin as the standard. Each tissue sample was assayed in triplicate and each experiment was carried out twice.

ACKNOWLEDGMENTS

We thank G. Stacey, S. Clough, H. Marella, and C. Ehret for critically reviewing the manuscript. We gratefully acknowledge the generous gift of the FMV promoter from The Monsanto Company; the CsVMV promoter from The Scripps Research Institute; and the 35S-CaMV, E35S-CaMV, and SVBV2 promoters from the Akkadix Company. This work was funded in part by the National Science Foundation (grant number 0421620).

LITERATURE CITED

An, Y.-Q., McDowell, J. M., Huang, S., McKinney, E. C., Chambliss, S., and Meagher, R. B. 1996. Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. *Plant J.* 10:107-121.

Auriac, M.-C., and Timmers, A. C. J. 2007. Nodulation studies in the model legume *Medicago truncatula*: Advantages of using the constitutive *EF1α* promoter and limitations in detecting fluorescent reporter proteins in nodule tissues. *Mol. Plant-Microbe Interact.* 20:1040-1047.

Benfey, P. N., and Chua, N.-H. 1990. The *Cauliflower mosaic virus* 35S promoter: Combinatorial regulation of transcription in plants. *Science* 250:959-966.

Bevan, M. W. 1984. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* 12:8711-8721.

Bhattacharyya, S., Dey, N., and Maiti, I. B. 2002. Analysis of *cis*-sequence of subgenomic transcript promoter from the *Figwort mosaic virus* and comparison of promoter activity with the *Cauliflower mosaic virus* promoters in monocot and dicot cells. *Virus Res.* 90:47-62.

Christou, P. 1997. Biotechnology applied to grain legumes. *Field Crop Res.* 53:83-97.

Cole, M. A., and Elkan, G. H. 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Anti-*

microb. Agents Chemother. 4:248-253.

Collier, R., Fuchs, B., Walter, N., Lutke, W. K., and Taylor, C. G. 2005. Ex vitro composite plants: An inexpensive, rapid method for root biology. *Plant J.* 43:449-457.

Droste, A., Pasquali, G., and Bodanese-Zanettini, M. H. 2000. Integrated bombardment and *Agrobacterium* transformation system: An alternative method for soybean transformation. *Plant Mol. Biol. Rep.* 18:51-59.

Elmayan, T., and Tepfer, M. 1995. Evaluation in tobacco of the organ specificity and strength of the *rolD* promoter, domain A of the 35S promoter and the 35S2 promoter. *Transgenic Res.* 4:388-396.

Hawes, C., and Satiat-Jeunemaitre, B. 2001. *Plant Cell biology* (The Practical Approach Series), 2nd ed. Oxford University Press, Oxford.

Holmberg, N., Harker, M., Gibbard, C. L., Wallace, A. D., Clayton, J. C., Rawlins, S., Hellyer, A., and Safford, R. 2002. Sterol C-24 methyltransferase type 1 controls the flux of carbon into sterol biosynthesis in tobacco seed. *Plant Physiol.* 130:303-311.

Holtorf, S. N., Apel, K. and Bohlmann, H. 1995. Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*. *Plant Mol. Biol.* 29:637-646.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3901-3907.

Kelemen, Z., Mai, A., Kapros, T., Feher, A., Gyorgyey, J., Waterborg, J. H., and Dudits, D. 2002. Transformation vector based on promoter and intron sequences of a replacement histone H3 gene. A tool for high, constitutive gene expression in plants. *Transgenic Res.* 11:69-72.

Khoudi, H., Vézina, L.-P., Mercier, J., Castonguay, Y., Allard, G., and Laberge, S. 1997. An alfalfa rubisco small subunit homologue shares *cis*-acting elements with the regulatory sequences of the RbcS-3A gene from pea. *Gene* 197:343-351.

Lullien, V., Barker, D. G., de Lajudie, P., and Huguet, T. 1987. Plant gene expression in effective and ineffective root nodules of alfalfa (*Medicago sativa*). *Plant Mol. Biol.* 9:469-478.

Maiti, I. B., Gowda, S., Kiernan, J., Ghosh, S. K., and Shepherd, R. J. 1997. Promoter/leader deletion analysis and plant expression vectors with the *Figwort mosaic virus* (FMV) full length transcript (FLt) promoter containing single or double enhancer domains. *Transgenic Res.* 6:143-156.

Malik, K., Wu, K., Li, X.-Q., Martin-Heller, T., Hu, M., Foster, E., Tian, L., Wang, C., Ward, K., Jordan, M., Brown, D., Gledde, S., Simmonds, D., Zheng, S., Simmonds, J., Miki, B., Tian, L., Wang, C., Ward, K., Jordan, M., Brown, D., Gledde, S., Simmonds, D., Zheng, S., Simmonds, J., and Miki, B. 2002. A constitutive gene expression system derived from the tCUP cryptic promoter elements. *Theor. Appl. Genet.* 105:505-514.

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y., and Ohashi, Y. 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* 37:49-59.

Obertello, M., Santi, C., Sy, M.-O., Laplace, L., Auguy, F., Bogusz, D., and Franche, C. 2005. Comparison of four constitutive promoters for the expression of transgenes in the tropical nitrogen-fixing tree *Albizia verticillata*. *Plant Cell Rep.* 24:540-548.

Odeh, J. T., Nagy, F., and Chua, N.-H. 1985. Identification of DNA sequences required for activity of the *Cauliflower mosaic virus* 35S promoter. *Nature* 313:810-812.

Olhoft, P. M., Flagel, L. E., Donovan, C. M., and Somers, D. A. 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta* 216:723-735.

Pattanaik, S., Dey, N., Bhattacharyya, S., and Maiti, I. B. 2004. Isolation of full-length transcript promoter from the *Strawberry vein banding virus* (SVBV) and expression analysis by protoplast transient assays and in transgenic plants. *Plant Sci.* 167:427-438.

Quandt, H.-J., Pühler, A., and Broer, I. 1993. Transgenic root nodules of *Vicia hirsuta*: A fast and efficient system for the study of gene expression in indeterminate-type nodules. *Mol. Plant-Microbe Interact.* 6:699-706.

Samac, D. A., Tesfaye, M., Dornbusch, M., Saruul, P., and Temple, S. J. 2004. A comparison of constitutive promoters for expression of transgenes in alfalfa (*Medicago sativa*). *Transgenic Res.* 13:349-361.

Sanger, M., Daubert, S., and Goodman, R. M. 1990. Characteristics of a strong promoter from figwort mosaic virus: Comparison with the analogous 35S promoter from *Cauliflower mosaic virus* and the regulated mannopine synthase promoter. *Plant Mol. Biol.* 14:433-443.

Santarém, E. R., Trick, H. N., Essig, J. S., and Finer, J. J. 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: Optimization of transient expression. *Plant Cell Rep.* 17:752-759.

- Somers, D. A., Samac, D. A., and Olhoft, P. M. 2003. Recent advances in legume transformation. *Plant Physiol.* 131:892-899.
- Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C., and Rathore, K. S. 2002. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant Mol. Biol.* 50:463-479.
- Taylor, C. G., Fuchs, B., Collier, R., and Lutke, W. K. 2006. Generation of composite plants using *Agrobacterium rhizogenes*. Pages 155-167 in: *Agrobacterium Protocols. Methods in Molecular Biology*. K. Wang, ed. Humana, Totowa, NJ, U.S.A.
- Trick, H. N., and Finer, J. J. 1997. SAAT: Sonication assisted *Agrobacterium*-mediated transformation. *Transgenic Res.* 6:329-336.
- Trick, H. N., and Finer, J. J. 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean (*Glycine max* (L.) Merrill) embryogenic suspension culture tissue. *Plant Cell Rep.* 17:482-488.
- Verdaguer, B., deKochko, A., Beachy, R. N., and 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.
- Wilmink, A., Ven, B. C. E., and Dons, J. J. M. 1995. Activity of constitutive promoters in various species from the Liliaceae. *Plant Mol. Biol.* 28:949-955.
- Yang, N.-S., and Christou, P. 1990. Cell type specific expression of a CaMV 35S-GUS gene in transgenic soybean plants. *Dev. Genet.* 11:289-293.