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Full Length Research Paper

Expression analysis of the 35S CaMV promoter and its derivatives in transgenic hairy root cultures of cucumber (*Cucumis sativus*) generated by *Agrobacterium rhizogenes* infection

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The cauliflower mosaic virus (CaMV) 35S promoter is the most commonly used viral-based promoter to drive transgene expression in plants. Although, many studies have demonstrated the constitutive nature of this promoter, some reports have suggested varied expression levels in different parts of the plant. Therefore, our aim was to study the activity of the CaMV 35S promoter in the hairy root system. The CaMV 35S promoter, the duplicate CaMV 35S promoter (designated CaMV 35ST) and the duplicate CaMV 35S promoter containing a 5'- untranslated leader sequence from the alfalfa mosaic virus RNA4 promoter (designated CaMV 35ST/AMV) were compared to evaluate their effects on the expression of the *gus* reporter gene in transgenic hairy roots, which was mediated using the *Agrobacterium rhizogenes* A4 transformation system. The integration of T-DNA containing a *gus* reporter gene in hairy root lines was confirmed at low copy numbers ranging from 1 to 4 copies using quantitative real-time PCR. Histochemical staining of cucumber hairy roots showed over-expression of the *gus* gene when driven with the CaMV 35S promoter. The expression of the *gus* gene when driven with the CaMV 35ST promoter showed a lower expression than that driven by the CaMV 35S promoter. However, the expression of the *gus* gene driven by the CaMV 35ST/AMV promoter was slightly higher than that driven by the CaMV 35ST promoter. In this study, the reduced activity of the CaMV 35ST promoter was observed for the first time. Further investigation is required to elucidate the factors that mediate the decline in promoter activity.

Key words: *Cucumis* sativus L, hairy root, *Agrobacterium rhizogenes,* promoter 35S cauliflower mosaic virus (CaMV), β-glucuronidase (GUS), 5'UTR AMV.

INTRODUCTION

The development of genetic improvements in cucumbers has been used to produce desired traits and to study plant biological systems. The genetic transformation of cucumbers using *Agrobacterium rhizogenes* is not the optimal method to produce transgenic plants because the effects of this type of genetic transformation have not been well studied. In addition, it is difficult to regenerate transgenic plants from hairy root explants (Trulson et al., 1986; Yin et al., 2005). However, there are advantages to this transformation system when studying metabolic pathways of secondary metabolites and transgene expression in the hairy root system. The phenotype of the cucumber hairy root is plagiotropic and is characterized

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Abbreviation: CaMV, Cauliflower mosaic virus; **GUS,** β-glucuronidase.

by numerous branches with rapid growth even in the absence of plant growth regulators (PGR) (Amselem and Tepfer, 1992). Transgenic hairy roots are genetically and biochemically stable, have a rapid growth rate and synthesize natural compounds at levels that are comparable to those in intact plants (Christey and Braun, 2005; Georgiev et al., 2007; Srivastava and Srivastava, 2007). These characteristics make the *A. rhizogenes*-mediated transformation system ideal for studying plant gene expression. Previous studies have reported on the use of the hairy root system (Peebles et al., 2007; Banarjee et al., 2002, 2008; Lee Flem-Bonhomme et al., 2004). Plant hairy roots display secondary metabolic 'manufacturing' capabilities that are suitable for the genetic engineering of metabolic pathways. Additionally, the hairy root system can produce or synthesize more than one type of secondary metabolite (Lee Flem-Bonhomme et al., 2004).

Genetically, engineered root cultures have been used as a model system to study various aspects of the metabolic and molecular regulation of several secondary metabolite pathways. For example, the over-expression of a cDNA encoding *Panax ginseng* farnesyl diphosphate synthase (FPS) in the hairy roots of *Centella asiatica* caused an increase in the contents of phytosterol and triterpene (Kim et al., 2010). Additionally, Lee et al. (2010) reported an increase in the rosmarinic acid level in the hairy roots of *Nepeta cataria*. The foreign genes that were introduced into the plant genomes were driven by a promoter that had been designed for expression in plants (Vengadesan et al., 2004).

A promoter is necessary to express genes newly introduced into plant DNA. In this study, three strategies were use to manipulate the expression of the reporter gene as follows: the 35S CaMV promoter was used to drive the expression of the *gus* reporter gene in the plant; the duplicate 35S CaMV promoter was used to over-express the reporter gene and the introduction of the 5'UTR of the alfalfa mosaic virus (AMV) sequence, an enhancer element, was used to enhance the translation process. The 35S CaMV promoter is a well-known viral promoter that is constitutively active in several different species (Odell et al., 1985). Previous studies have indicated that the introduction of tandem repeats of the 35S CaMV promoter increases the expression levels by 100 fold compared with the *nos* promoter (Govindarajulu et al., 2008) in certain tissue types, but these expression levels were not studied in hairy root tissue. The tandem repeat of the 35S CaMV promoter acts as an enhancer element for the core promoter to increase promoter activities. Lam and Chua (1989) identified activation sequence 2 (*as-2*), which is homologous to the SV40 enhancer core A element and the Box II element of pea *rbc*S. The *as*-2 motif contributes to the expression in plant non-root parts and the *as*-1 motif contributes to the expression in the root and the protoplast (Lam et al., 1990). Interestingly, 35S CaMV elements can uniquely enhance or activate the activity of an adjacent tissue- and organ-specific gene promoter (Zheng et al., 2007).

The introduction of the AMV 5'UTR as a leader sequence was previously shown to enhance the translation efficiency of foreign genes (Gallie et al., 1987; Gallie and Walbot, 1992). A leader sequence functions to prevent the formation of secondary mRNA structures and provide a smoother translation process to increase the levels of the final gene product. Although, a previous study (Jobling and Gehrke, 1987) did not evaluate the effect of the leader sequence on the expression of a foreign gene in the hairy root system, our findings will establish a better understanding of the effects of the 5'UTR leader sequence in the expression of foreign genes in the hairy root. This approach should be useful for obtaining high expression levels of foreign genes in transgenic plants.

MATERIALS AND METHODS

The pCAMBIA 1301 binary vector was donated by Dr. Richard A. Jefferson at CAMBIA in Australia. The modified pCAMBIA 1301 vectors were constructed with the promoter modifications that are illustrated in Figure 1. The three promoters were transformed into *Escherichia coli* for propagation and confirmation purposes and were later transferred into the *A. rhizogenes* strain A4 before infecting the cotyledons of cucumbers. Figure 1 shows the preexisting promoters in pCAMBIA 1301 vector, which contains the 35S CaMV promoter in its T-DNA region and its modification to incorporate the 35ST CaMV and 35ST CaMV/AMV promoters. Cucumber seeds of the 'Simpang Pulai' variety were acquired from the Malaysian Agriculture, Research and Development Institute (MARDI), Malaysia.

Explant preparation

Cucumber transformation using each promoter was performed according to the transformation procedure that was previously described by Amselem and Tepfer (1992). Cucumber seeds were sterilized by shaking in 20% Clorox (Clorox, Kuala Lumpur, Malaysia) in sterile water for 20 min. Sterilized seeds were rinsed with sterile water, blotted dry and placed into Murashige and Skoog (MS) medium without PGRs. Seedlings were grown at 25°C for one week under a 16 h photoperiod until germination. Cotyledons of the sterile cucumber seedlings were excised and cultured in MS medium for 2 days under similar conditions.

Plant transformation

A. rhizogenes strain A4 cells harboring the binary vector pCAMBIA 1301 or one of its derivatives and carrying the *gus* reporter gene were grown to 0.5 to 0.6 OD_{600} before co-cultivation. The cucumber cotyledons were transferred into a bacterial suspension for 20 min. Then, the cotyledon explants were placed into co-culture medium containing MS with 200 µM acetosyringone for 2 days at 25°C in the dark. Later, the cotyledons were transferred to MS medium containing 250 mg/l of cefotaxime (Duchefa, Haarlem, Netherland) to inhibit bacterial growth and they were cultured at 25°C with a 16 h photoperiod for two weeks. Two-centimeter-long hairy root initiates from the cotyledons were excised and placed onto MS medium containing 250 mg/l of cefotaxime to eliminate any residual bacterial under similar conditions.

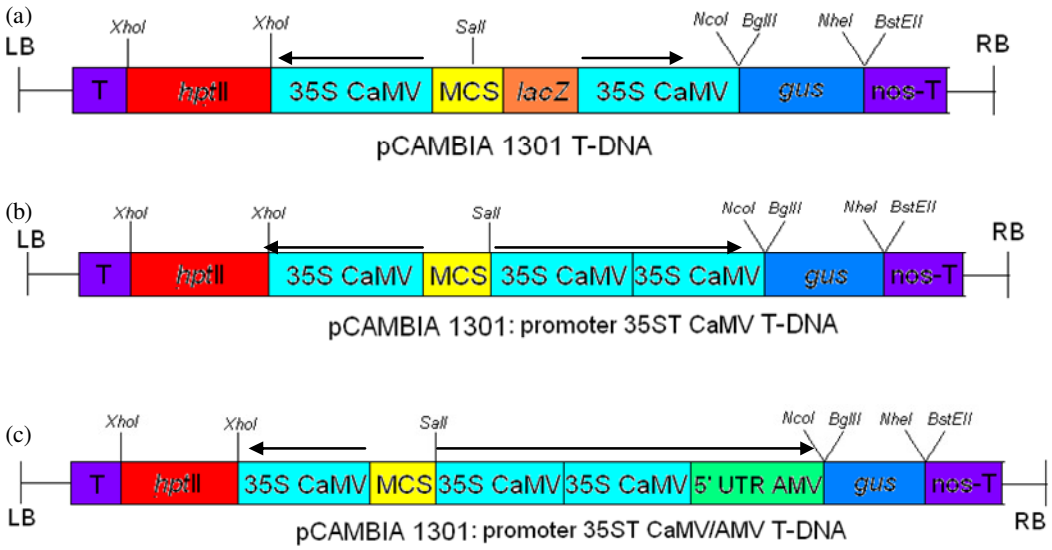

Figure 1. Linear map of pCAMBIA 1301 T-DNA region and modification of its 35S CaMV promoter. (a) T-DNA region for pCAMBIA 1301 with 35S CaMV promoter, (b) 35ST CaMV promoter replacing 35S CaMV promoter and (c) 35ST CaMV promoter with 5' UTR as leader sequence for *gus* gen. *RB* right border, *LB* left border, *hpt hygromicin phosphotransferase* II, MCS multiple cloning sites, *gus- β-Glucuronidase gen , nos-T* - nopaline terminator.

Histochemical analysis of GUS expression

The hairy root lines were tested using a histochemical GUS assay as previously described (Jefferson, 1987) to localize the distribution of GUS activity in transformed roots. One-centimeter-long hairy roots from each individual line were excised and transferred into histochemical assay buffer containing 0.1 M phosphate buffer, 0.5 mM ferricyanide (Sigma, St. Louis, USA), 0.5 mM ferrocyanide (Sigma, St. Louis, USA), 0.1% Triton X-100 (Sigma, St. Louis, USA), 10.0 mM EDTA (Sigma, St. Louis, USA), 20% methanol (Sigma, St. Louis, USA) and 1.0 mM 5-bromo-3-indolyl-glucuronide (X-gluc) (Duchefa, Haarlem, Netherlands). The samples were incubated for 16 h at 37°C in the dark. After staining, the sample solutions were removed and replaced with FAA solution [45% absolute ethanol, (Hmbg, Hamburg, Germany), 5% glacial acetic acid (Hmbg, Hamburg, Germany), 5% formaldehyde (Hmbg, Hamburg, Germany)] for fixation. The samples were later examined under a light microscope.

Fluorometric assay

A fluorometric assay of *gus* gene expression was performed following the protocol that was provided with the FluorAce β-glucuronidase reporter assay kit (BioRad, Hercules, USA) to determine the activity of the *gus* gene by calculating the amount of methylumbelliferone (MU) present in the hairy root extract. The total protein content of the hairy root extract was determined using the Bradford assay (Bradford, 1976). Three replicates for each sample line were measured using a VersaFluor fluorometer (BioRad, Hercules, USA). For the negative control references, non-transgenic roots and hairy roots without the *gus* gene were subjected to analysis.

PCR confirmation of transformants

Ten putative positive lines for each promoter were subjected to PCR using the *gus* and *rol*B primers. The primer set for the *gus* gene was: F: 5'-CGCCGATGCAGATATTCGTA-3'; R: 5'-ATTAAT GCGTGGTCGTGCAC-3'. The primer set for the *rol*B gene was: F: 5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAAGC-3'; R: 5'-ATGGA TCCCAAATTGCTATTCCTTCCACGA-3'.

Genomic DNA was isolated from each individual hairy root line using a previously described method (Doyle and Doyle, 1987). All of the PCR reactions were performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany). Samples containing 200 ng of genomic DNA were first heated at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 60°C (for the *gus* gene) or 57°C (for the *rol*B gene) for 1 min and 72°C for 1 min followed by a 7-min final extension at 72°C. In total, 100 ng of plasmid DNA was used as a positive control. The PCR reactions contained 200 µM of each primer, 10 mM dNTP mix, 15 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% (v/v) Triton X-100, 2 U *Taq* DNA polymerase and 200 ng of template DNA in 2X reaction buffer. The amplified DNA was analyzed using 1.5% agarose gel electrophoresis.

Quantitative real-time PCR

Quantitative real-time PCR was performed according to the manufacturer's instructions using an IQ SYBR Green Supermix real-time PCR kit (BioRad, Hercules, USA). Quantitative real-time PCR was used to determine the copy number of the *gus* gene per genome in the transgenic cucumber hairy roots. The method of quantification used a standard curve for a series of copies of the *gus* gene. Plasmid pCAMBIA 1301 containing a single copy of *gus* gene per plasmid was prepared and the copy number of the *gus* gene was calculated per molecular weight. A primer set for real-time PCR was designed according to the manufacturer's instructions. The primer set for the *gus* gene was F: 5'-CAAC GGGGAAACTCAGCAAG-3'; R: 5'-AGCGTCGCAGAACATTACAT-3'. Quantitative and relative real-time PCR were conducted using an IQ5 real-time PCR detection thermal cycler (BioRad, Hercules, USA).

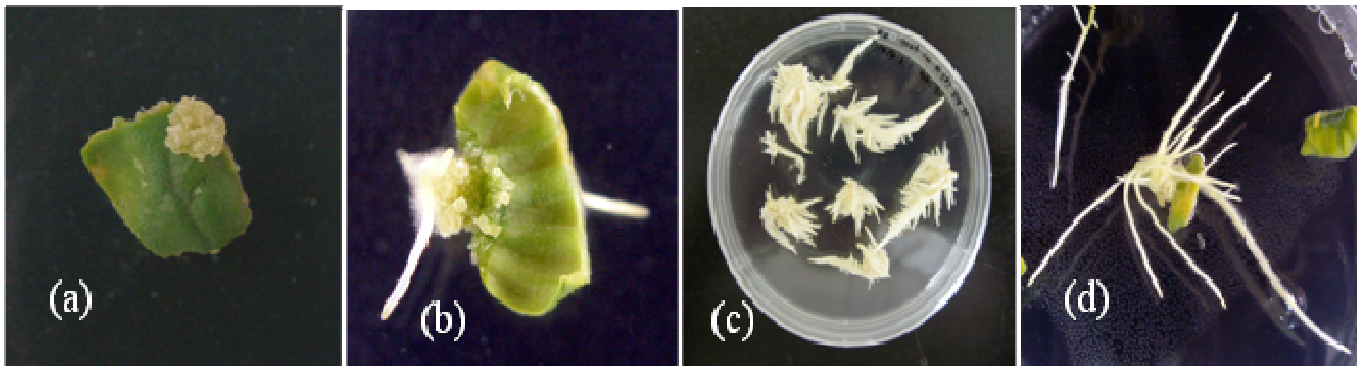

Figure 2. Picture of hairy root formation from wounded cucumber cotyledon after infection with *A. rhizogenes.* (a) Callus formation on wounding and infected site, (b) first hairy root initiation from callus, (c) elongation of hairy roots and (d) hairy root survived and growth without original cotyledon on MS medium without PGRs.

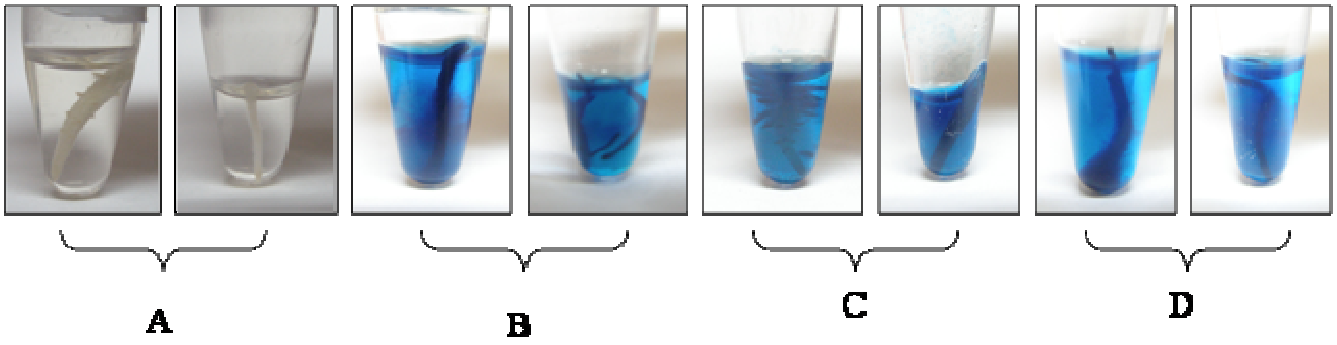

Figure 3. Early detection of *gus* gene expression via histochemical GUS assay after overnight incubation of hairy root lines. (A) Negative control, (B) hairy root lines with 35S CaMV promoter, (C) hairy root lines with 35ST CaMV promoter and (D) hairy root lines with 35ST CaMV/AMV promoter.

Relative real-time PCR

Relative real-time PCR was used to determine the expression level of the GUS gene in each transgenic hairy root line, which was normalized to the level of an endogenous gene in cucumbers, the *nadph-po* (NADPH-protochlorophyllide oxidoreductase) gene. The cDNA from hairy roots that were transformed using *A. rhizogenes* A4 without any promoter was used to calibrate PCR products using a quantification method based on the Pfaffl method (Pfaffl, 2001). This method was chosen because the efficiency of the PCR reaction was in the acceptable range of 95 to 105% and the PCR process exponentially (2^n) amplified cDNA templates. The primer set for the endogenous gene used was: F: 5'-AAACG GCTCATCATTGTCG-3'; R: 5'-ATCGCTTGTGGAACTCTTGC-3'.

The total RNA was extracted from hairy root lines using an RNase plant mini kit (Qiagen, Germantown, USA) and was treated with RNase-free DNase-I to remove residual genomic DNA. The first-strand cDNA was synthesized using the RevertAid first strand cDNA synthesis kit (Fermentas, Burlington, Canada).

RESULTS AND DISCUSSION

The A4 strain of *A. rhizogenes* containing the binary vector was infectious to cucumbers. After 2 weeks, roots formed from explants and spread throughout the medium. These roots exhibited the same characteristics that have been previously reported (McInnes et al., 1991; Nader et al., 2006) and included abundant roots, auxiliary root growth and a plagiotropic phenotype. Figure 2 shows the formation of the hairy root phenotype from cucumber cotyledons after the transformation using *A. rhizogenes*.

Figure 3 shows histochemical GUS staining in transgenic hairy root lines. The presence of GUS activity and its localization were observed in all of the tissues of the root, especially in transgenic cucumber hairy root lines with the CaMV 35S and CaMV 35ST/AMV promoters. The observation of GUS activity was concentrated in the meristematic tissue and the vascular tissues of the root and the auxiliary roots. Figure 4 shows the specific localization of GUS activity in different tissue types. The transgenic cucumber hairy roots lines with the CaMV 35S promoter or the CaMV 35ST promoter showed localized GUS activity only in the vascular bundles.

Benfey and Chua (1990) reported that 2 domains in the 35S promoter confer gene expression in different tissues; domain A confers gene expression in meristematic tissue

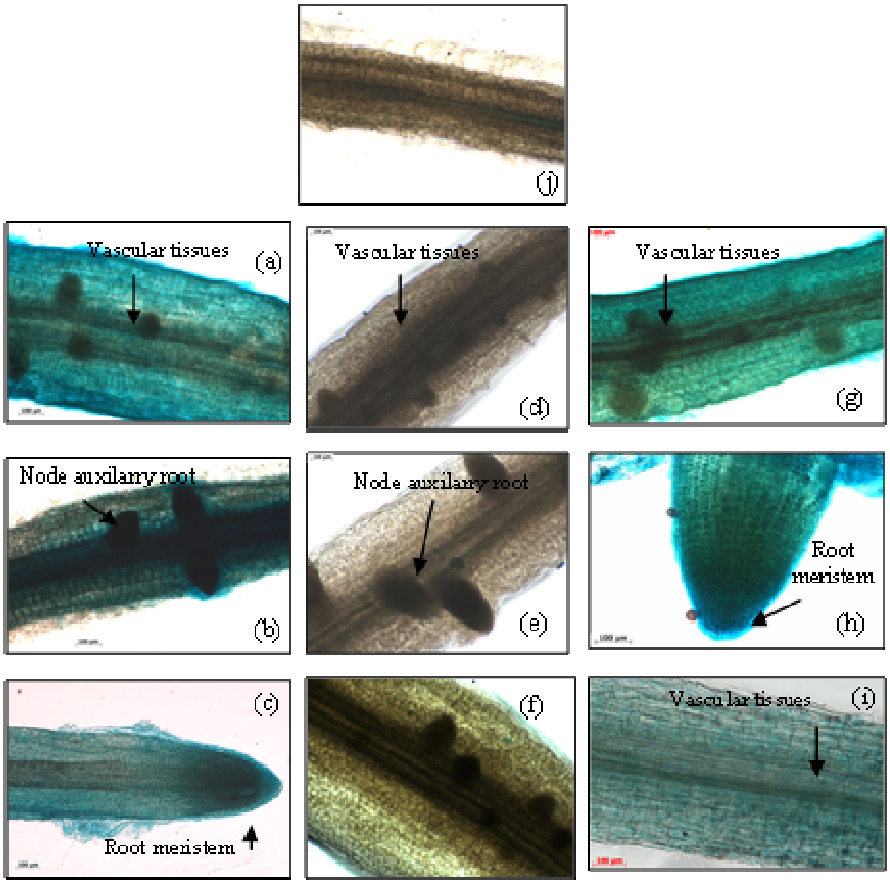

Figure 4. Picture from above, (a), (b) and (c); GUS distribution in hairy root lines transformed with 35S CaMV promoter, (d), (e) and (f); hairy root lines with 35ST promoter, (g), (h) and (i); hairy root lines with 35ST/AMV promoter. (j); negative control of hairy root transform with *A. rhizogenes* wild type.

and domain B confers gene expression in vascular bundles. Their report suggests that using the full-length 35S CaMV promoter may confer gene expression in these tissue types, while using the duplicate repeat of the 35S CaMV promoter was expected to increase the gene expression in these tissues. In this study, we demonstrated that using the tandem repeat promoter sequence reduced the promoter activity and conferred gene expression only in the vascular bundles and not in the root meristematic tissue.

A fluorometric GUS assay was performed to determine the expression level in transgenic hairy root lines that were previously analyzed using a histochemical GUS assay. Cucumber roots and cucumber hairy roots that were transformed using *A. rhizogenes* without a binary vector were used as negative controls. The GUS activity for each transgenic hairy root line with each promoter is shown in Figure 5. Based on the fluorometric results, we showed that the highest GUS expression in the hairy root line was in the plants transformed using the 35S CaMV promoter (3288 pmol/µg/min), whereas the hairy root line that was transformed using the 35ST CaMV promoter showed the lowest GUS expression (353 pmol/µg/min). Very low endogenous GUS activity was detected in the control samples. These results show that cucumber hairy root tissue exhibits very low or negligible endogenous GUS expression and is suitable for the *gus* reporter gene studies. Jefferson and Wilson (1991) have suggested that higher plants show low or no GUS activity all endogenously.

PCR confirmation of the *gus* and *rol*B gene expression profiles (Table 2) revealed the presence of both genes in all of the putative transgenic hairy root lines. This result shows that the T-DNA region of the binary vector and pRi was successfully transferred into plant cells. The integration of the *gus* gene was confirmed using quanti-tative RT-PCR.

Quantification of the copy number of the *gus* gene using absolute quantification in real-time PCR revealed a low copy number of the GUS gene per genome. The results of this quantification are shown in Table 1. Figure 6 shows the copy number of the *gus* gene in each transgenic hairy root line. The determination of the gene copy number was required to gauge the level of gene

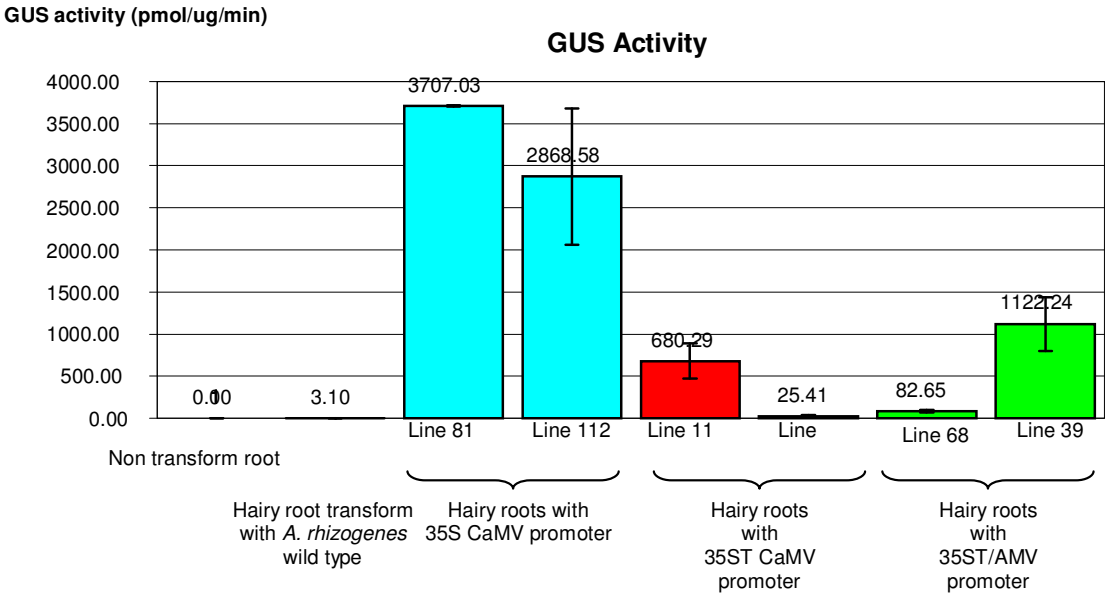

Figure 5. A fluorometric GUS assay shows the pattern of expression for each tested hairy root line with different promoters

Table 1. Quantitative real-time PCR result for *gus* gene quantification per genome.

Cucumber hairy root line	Copy/genome
81 with CaMV 35S promoter	4
112 with CaMV 35S promoter	1
14 with CaMV 35ST promoter	1
11 with CaMV 35ST promoter	1
68 with CaMV 35ST/AMV promoter	1
39 with CaMV 35S/AMV promoter	1

Table 2. PCR confirmation for putative positive lines of hairy root harbouring particular promoters.

Promoter	Number of positive line from Histochemical GUS/ actual line tested	Number of line performing PCR		Number of line successfully confirmed through PCR	
		GUS	RolB	GUS	RolB
35S CaMV	18/300	11	11	10	11
35ST CaMV	20/300	10	10	10	10
35ST CaMV/ AMV	75/300	9	10	9	9

expression. Kohli et al. (1999) reported that a high rate of foreign gene insertion may contribute to the instability or a reduced level of gene expression. Fortunately, *Agrobacterium*-based transformation induces a low rate of foreign gene insertion as suggested by Gelvin (2003). Kohli et al. (1999), Dong et al. (2001) and Khristaleve and Kik, (2001) reported that the integration of foreign genes is random and that foreign genes tend to integrate into distal chromosomal regions of the genome.

Similar to our observations in the fluorometric assay, relative real-time PCR also showed a similar pattern of gene expression for each of the transgenic hairy root lines. This result shows that the transcription and post-translation of the *gus* gene are consistent with our findings. The results of the relative real-time PCR are shown in Figure 6. The highest recorded difference in the gene expression was seen in transgenic plants with the 35S CaMV promoter (14-fold difference compared with that of wild-type plants). The lowest recorded difference in the gene expression was seen in transgenic plants with

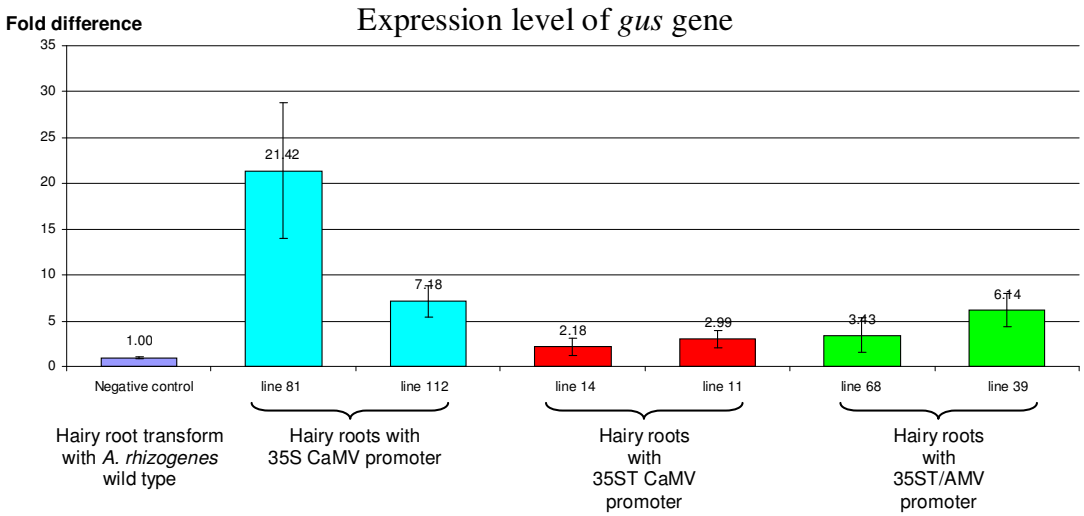

Figure 6. Relative real-time PCR results for each corresponding promoter constructed in cucumber hairy root lines.

the 35ST CaMV promoter (2.6-fold difference compared with that of wild-type plants).

Based on the previous studies of 35S CaMV promoter, we expected a high level of expression of the *gus* gene (Kay et al., 1987; Omirulleh et al., 1993; Govindarajulu et al., 2008). Previous studies have shown that the duplicate 35S CaMV promoter induces a greater increase in the gene expression when compared with the 35S CaMV promoter alone. Interestingly, we found a reduced expression of the reporter gene when using the duplicate 35S CaMV promoter. This report is the first to identify the reduced activity of the duplicate 35S CaMV promoter in the hairy root system.

Histochemical staining of putative positive cucumber hairy roots showed the over-expression of the *gus* gene when the expression was driven by the 35S CaMV promoter. These findings were confirmed using a fluorometric GUS assay and relative real-time PCR. The expression of the *gus* gene driven by the CaMV 35ST promoter was lower than that driven by the CAMV 35S promoter. GUS expression driven by the CaMV 35ST promoter displayed a decrease of 9.3 fold using the fluorometric GUS assay (Figure 5) and a decrease of 5.5 fold using relative real-time PCR (Figure 6). The expression of the *gus* gene driven by the CaMV 35ST/AMV promoter was slightly increased compared with that driven by the CaMV 35ST promoter; the expression level was found to be increased by 1.7 fold using the fluorometric GUS assay (Figure 5) and by 1.9 fold using relative real-time PCR (Figure 6). Differences in the fold change between the fluorometric GUS assay and relative real-time PCR may be attributed to differences in the stages of gene expression at the time of the analysis. The fluorometric analysis detected the expression at the translation level, whereas the relative real-time PCR compared the expression at the transcription level.

Figures 5 and 6 show that even though the hairy root lines harbored similar promoters, individual transgenic hairy root lines produced varied expression levels that may be regulated by internal and external factors. Therefore, the mean expression activity was used to represent lines containing the promoter of interest. The variation in foreign gene expression in transgenic hairy roots may have contributed to differences in the localization of gene integration, the silencing effects during transcription or post-translation or the effects of endogenous protein factors (Jorgensen et al., 1996; Wakimoto, 1998; Wagner and Garcia-Blanco, 2001; Schubert et al., 2004).

The introduction of the tandem 35S CaMV promoter resulted in reduced expression levels of the *gus* gene and potentially contributed to the absence of motifs or sequences that were necessary for elevated gene expression in cucumber hairy roots. The 35ST promoter has a double -343 to -46 sequences, which is the optimal sequence that is required for the elevated expression *in planta* (Odell et al., 1985). However, the full-length 35S CaMV promoter contains a downstream sequence from -343 that may contain unknown elements that may increase the expression of genes, especially in the cucumber hairy root system. Further studies are required to test the hypothesis that unknown elements downstream of the -343 sequence of the 35S CaMV promoter may contribute to the regulation of gene expression.

The introduction of the 5'UTR of AMV increased *gus* gene activity. The results from the fluorometric assay and relative RT-PCR indicate that the AMV sequence enhanced gene expression at the transcriptional and translational levels. The AMV sequence, similar to the TMV Ω leader sequence, exhibits a translational enhancer capability such that this sequence improves the stability and the translation efficiency of mRNA (Kozak,

1987; Gallie, 1991; Gallie and Walbot, 1992). The results show that the 5'UTR sequence can be used to enhance foreign gene expression in the hairy root system, especially in cucumbers.

Conclusion

In this study, the expression of the *gus* gene was successfully elevated and showed increased expression in cucumber hairy roots. Although, our strategy to modify the CaMV 35S promoter was not successful, we showed that the 35S CaMV and AMV enhancer elements strongly affected the expression of the *gus* gene in the cucumber hairy root. This approach is beneficial for understanding the root system and is an excellent model for *A. rhizogenes*-mediated transformation.

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