CHAPTER III

RESULTS AND DISCUSSION

3.1 Preparation of *gus* fragment

The forward and reverse primers designed for amplification of a *gus* fragment based on the *gus* coding sequence in pCAMBIA1301 plasmid which was obtained from NCBI database (Accession No. AF234297). The sequence of the forward primer, designated as GUS-F (*Xba*I/*Asc*I) was 5′- TG**TCTAGA**GGCGCGCCAGACT GTAACCACGCGTC -3′ which contains *Xba*I and *Asc*I restriction sites at its 5′ end. The *Xba*I site is in bold letters and the *Asc*I site is underlined. The sequence of the reverse primer, designated as GUS-R (*Bam*HI/*Swa*I) was 5′- CA**GGATCC**ATTTA AATAATCACCACGATGCCATG -3′ which contains *Bam*HI and *Swa*I restriction sites at its 5′ end. The *BamH*I site is in bold letters and the *Swa*I site is underlined. In PCR reactions using pCAMBIA1301 plasmid as the template, these primers generated a product of approximately 490 bp which was the expected size of the *gus* fragment. The amplified product was separated by 1% agarose gel electrophoresis as shown in Figure 3.1. The *gus* DNA fragment was pulified from agarose gel using the QIAquick gel extraction kit. The purified *gus* fragment was then ligated into pGEM®-T vector. After the competent *E. coli* strain Top 10 cells were transformed with the ligation mixture, the transformants were selected by blue/white colony screening on ampicillin agar plates containing X-gal and IPTG. White colonies were randomly selected and cultured in LB broth containing 100 μg/ml of ampicillin at 37 °C overnight and the cultures were subjected to plasmid extraction. To confirm the insertion of the PCR product into the vector by restriction analysis, the potential recombinant plasmids were digested with *Nco*I and *Spe*I at 37 °C overnight. These

Figure 3.1 Agarose gel electrophoresis of the amplified fragment of the *gus* gene The PCR product was separated on a 1.0 % agarose gel and visualized by ethidium bromide staining.

Lane 1 *gus* fragment

Lane M pBR322/*Msp*I standard marker

reactions were analyzed by 1.0% agarose gel electrophoresis. The result in Figure 3.2 showed that DNA fragment of approximately 500 bp in length was obtained as expected. The resulting plasmid is called pGEM-*gus*.

3.2 Construction of *gus* inverted repeat

The construction of an inverted repeat of the *gus* gene involved a two-step cloning procedure facilitated by two pairs of restriction endonuclease sites included in the sequences of the forward and reverse primers that constitute the ends of the PCR fragment which was cloned into pGEM®-T vector (3.1). In the first cloning step, the recombinant plasmid (3.1) was digested with *Asc*I and *Swa*I at the innermost restriction sites at each end of the PCR product. The digested DNA fragments of approximately 460 bp from the *gus* fragment and 3,000 bp from the pGEM®-T vector were obtained (Figure 3.3) and purified. The purify product was analyzed by 1.0% agarose gel electrophoresis (Figure 3.4). The resulting *gus* fragment was then ligated into the *Asc*I and *Swa*I sites of pFGC5941 digested with the same enzymes (Figure 3.4). The ligation products were introduced to *E. coli* strain XL1-Blue. The transformants were selected by kanamycin resistance and colonies were randomly picked for plasmid extraction and analyzed by *Asc*I and *Swa*I digestion. Restriction analysis of a recombinant plasmid released two fragments of approximately 460 and 11,000 bp (Figure 3.5). The DNA fragment of 474 bp was the expected size of the *gus* fragment. The resulting recombinant plasmid from the first cloning step which contains the *gus* sequence in the sense orientation is called pFGC5941-sGUS and would be served as the template for the second cloning step.

In the second cloning step, the recombinant plasmid pGEM-*gus* (3.1) was digested with *Bam*HI and *Xba*I which their sites are distal to the *Asc*I and *Swa*I sites

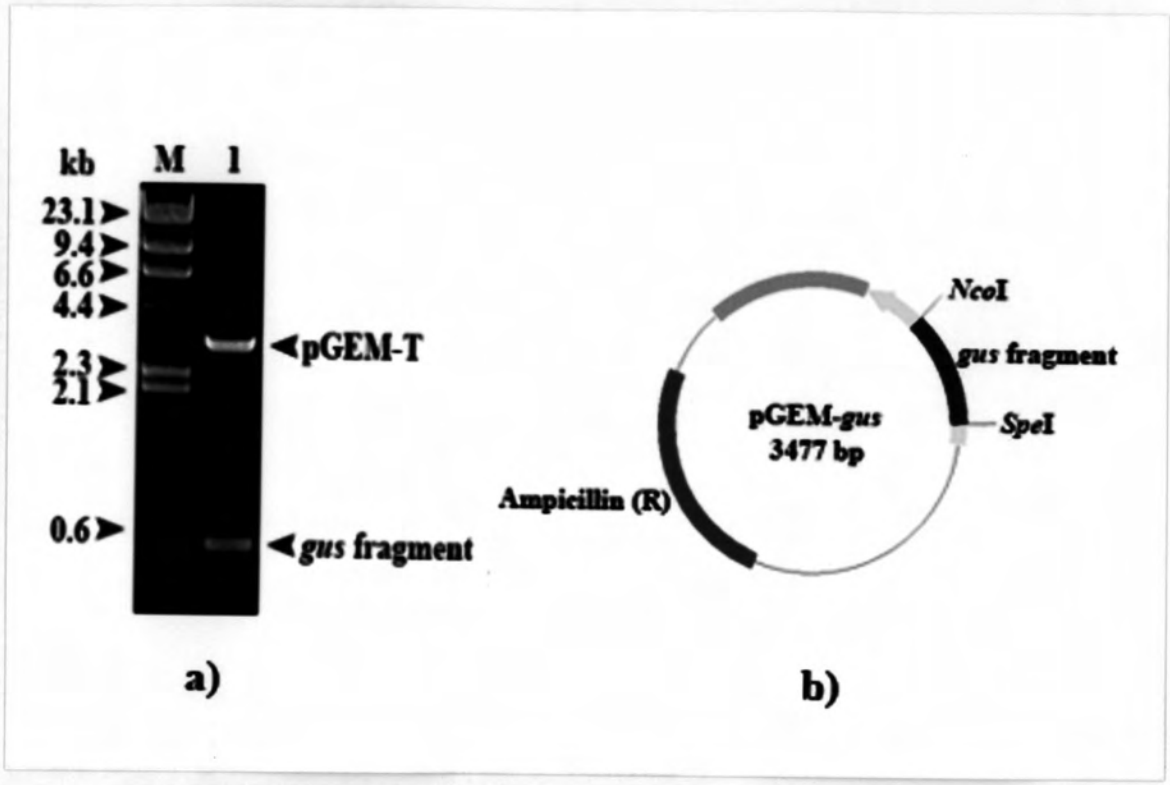


Figure 3.2 Agarose gel electrophoresis of recombinant plasmid pGEM-*gus* on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM-*gus* digested with *Nco*I and *Spe*I

b) Schematic diagram of the recombinant pGEM-*gus* containing the *gus* fragment

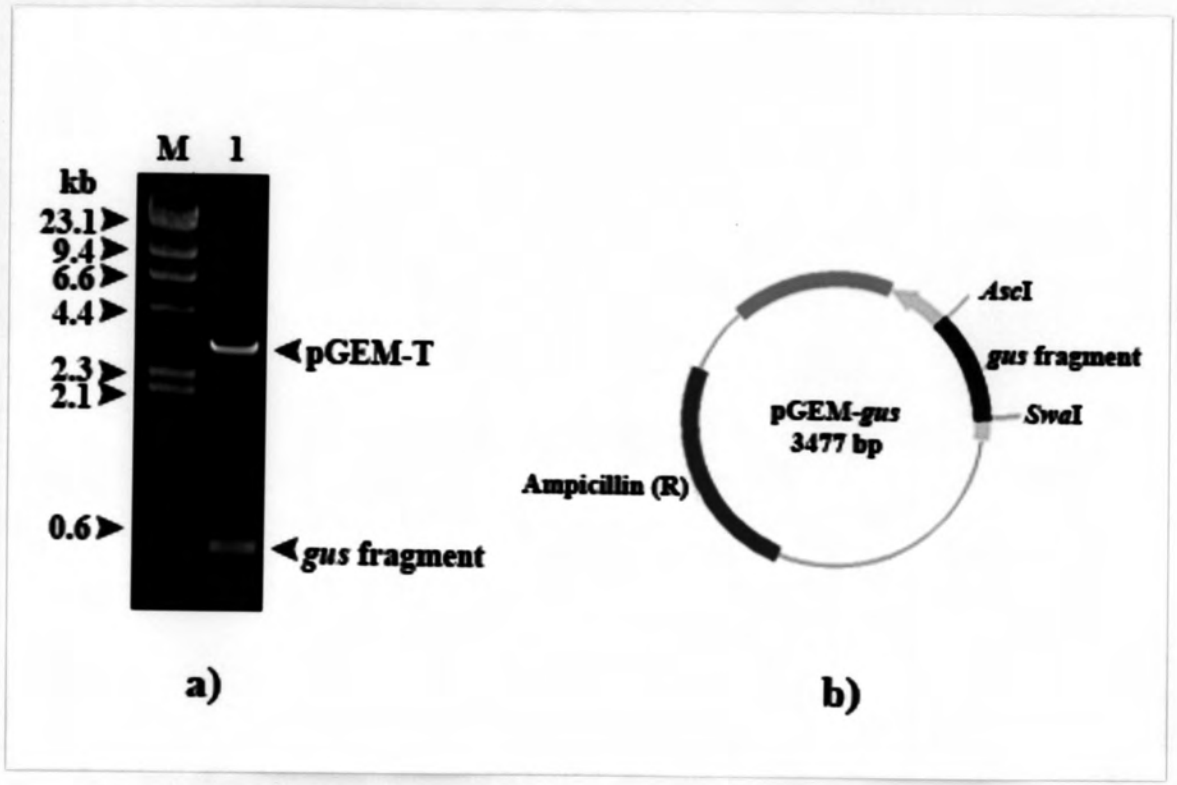


Figure 3.3 Agarose gel electrophoresis of recombinant plasmid pGEM-*gus* on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM-*gus* digested with *Asc*I and *Swa*I

b) Schematic diagram of the recombinant pGEM-*gus* containing the *gus* fragment

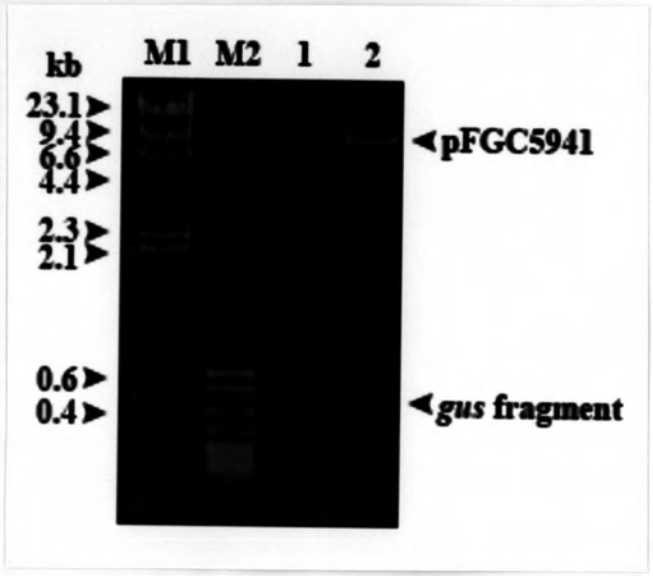


Figure 3.4 Purified products of the *gus* fragment and the pFGC5941 analyzed on 1.0 % agarose gel.

Lane M1 λ/*Hin*dIII standard marker

Lane M2 pBR322/*Msp*I standard marker

Lane 1 purified *Asc*I/*Swa*I-digested *gus* fragment

Lane 2 purified *Asc*I/*Swa*I-digested pFGC5941

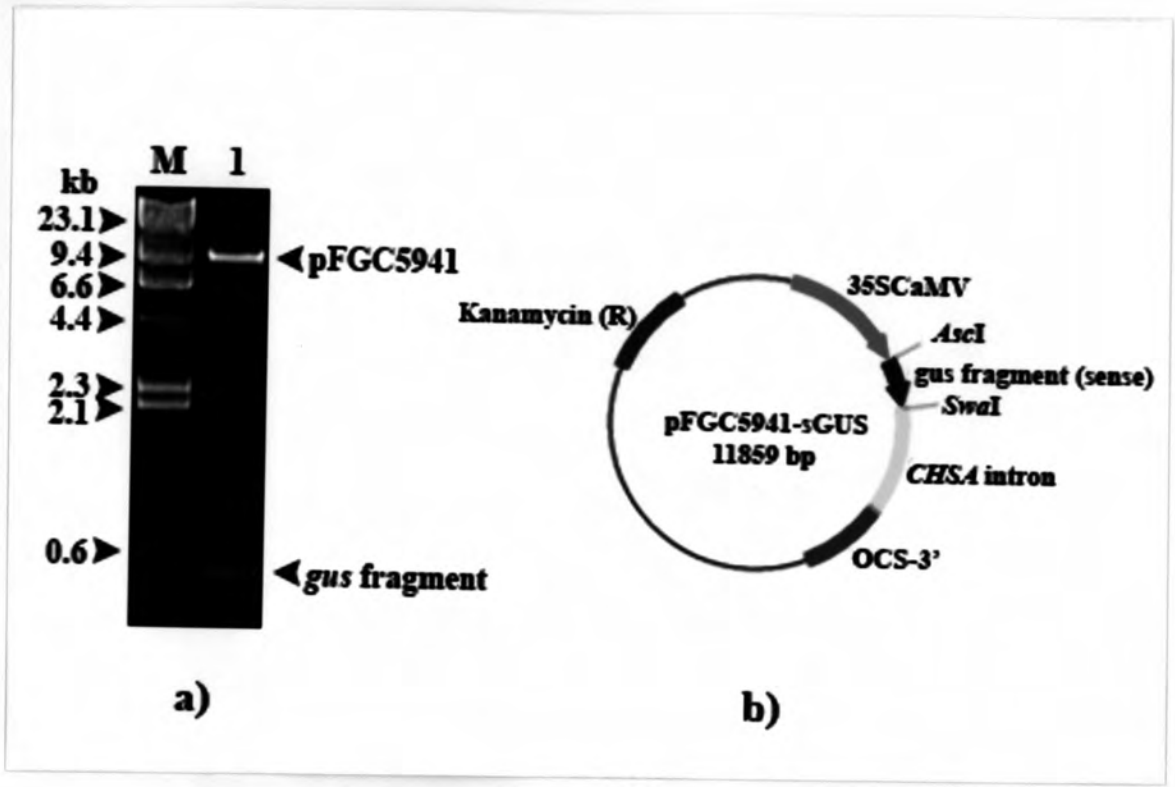


Figure 3.5 Agarose gel electrophoresis of recombinant plasmid pFGC5941-sGUS on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pFGC5941-sGUS digested with *Asc*I and *Swa*I

b) Schematic diagram of the recombinant pFGC5941-sGUS containing *gus* sequence in sense orientation

at the termini of the *gus* fragment. The digested DNA fragment of approximately 475 bp from the *gus* fragment and 2990 bp from the pGEM®-T vector were obtained (Figure 3.6) and purified. The purified product was analyzed by 1.0% agarose gel electrophoresis (Figure 3.7). The resulting *gus* fragment was ligated into *Bam*HI and *Xba*I sites of pFGC5941-sGUS digested the same enzymes (Figure 3.7). The ligation products were transformed to *E. coli* strain XL1-Blue. The transformants were selected by kanamycin resistance and colonies were randomly picked for plasmid extraction and analyzed by *Bam*HI and *Xba*I digestion. Restriction analysis of a recombinant plasmid released two fragments of approximately 480 and 11,600 bp (Figure 3.8). The DNA fragment of 486 bp was the expected size of the *gus* fragment. Because of the orientation of the restriction sites in the pFGC5941 plasmid, the second ligation inserted the second *gus* fragment in the opposite orientation to the first one creating an inverted repeat separated by the chalcone synthase A (*CHSA*) gene intron (hpGUS). The resulting recombinant plasmid from the second cloning step was called pFGC5941-hpGUS.

The insertion of both *gus* fragments cloned in the sense and antisense orientations in pFGC5941 vector was confirmed by restriction analysis. The pFGC5941-hpGUS recombinant plasmid was digested with *Pst*I and *Eco*RI and analyzed by 1.0% agarose gel electrophoresis. Two DNA fragments of approximately 4500 and 7750 bp were obtained (Figure 3.9). The DNA fragment of approximately 4500 bp, which was the expected size of the hpGUS cassette includes the 35SCaMV, the hpGUS and the OCS3' sequence. In addition, the nucleotide sequence of hpGUS construct was confirmed by sequence analysis. The result showed 100% nucleotide sequence identity with the published sequence of the *gus* coding sequence in pCAMBIA1301 vector which was obtained from the NCBI database (Accession No. AF234297)

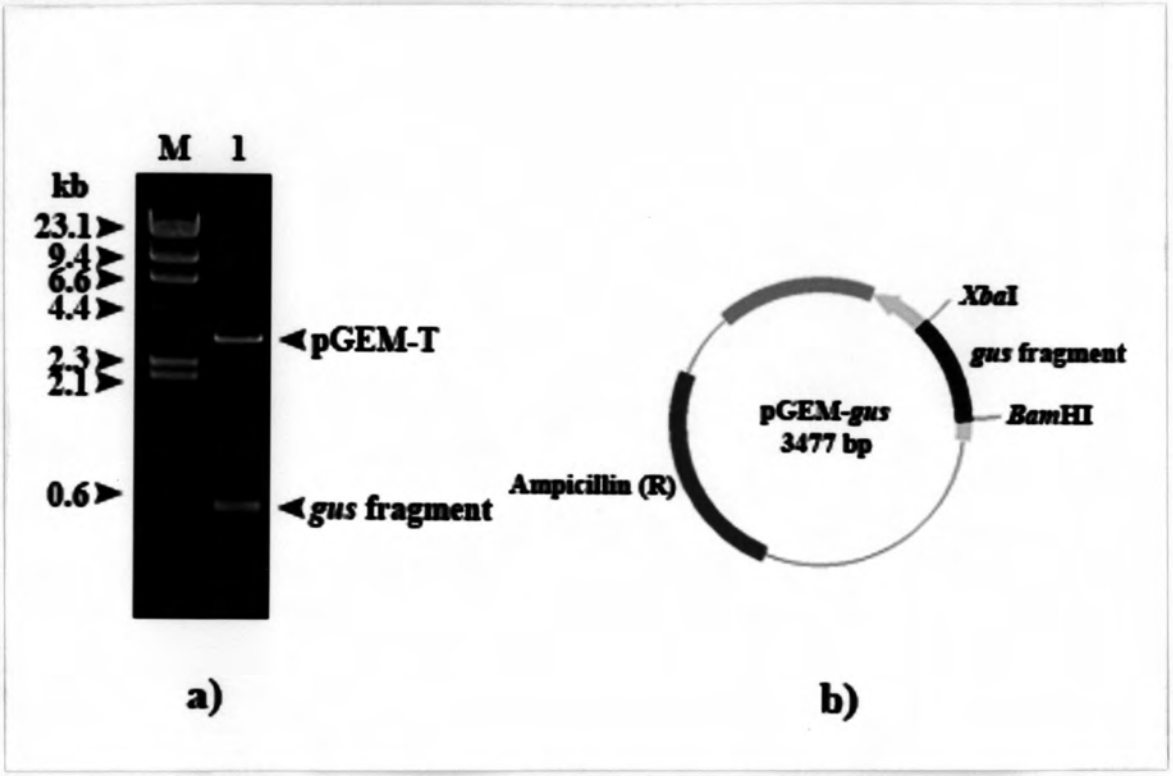


Figure 3.6 Agarose gel electrophoresis of recombinant plasmid pGEM-*gus* on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pGEM-*gus* digested with *Bam*HI and *Xba*I

b) Schematic diagram of the recombinant pGEM-*gus* containing *gus* fragment

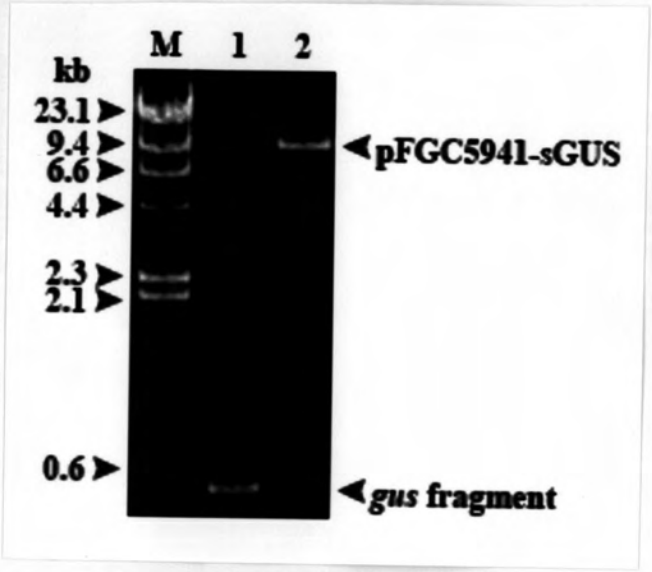


Figure 3.7 Purified products of the *gus* fragment and the pFGC5941-sGUS analyzed on 1.0 % agarose gel.

Lane M λ/*Hin*dIII standard marker

Lane 1 purified *Bam*HI/*Xba*I-digested *gus* fragment

Lane 2 purified *Bam*HI/*Xba*I-digested pFGC5941-sGUS

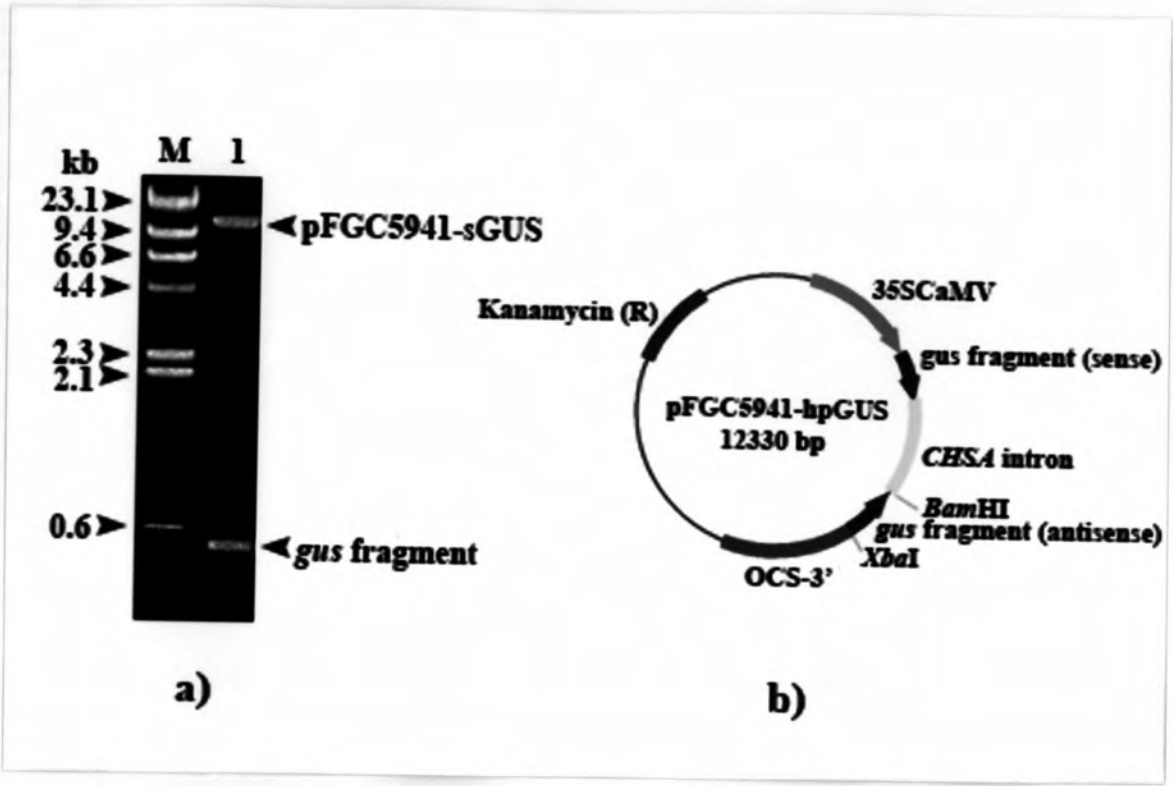


Figure 3.8 Agarose gel electrophoresis of recombinant plasmid pFGC5941-hpGUS on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pFGC5941-hpGUS digested with *Bam*HI and *Xba*I

b) Schematic diagram of the recombinant pFGC5941-hpGUS containing *gus* sequence in antisense orientation

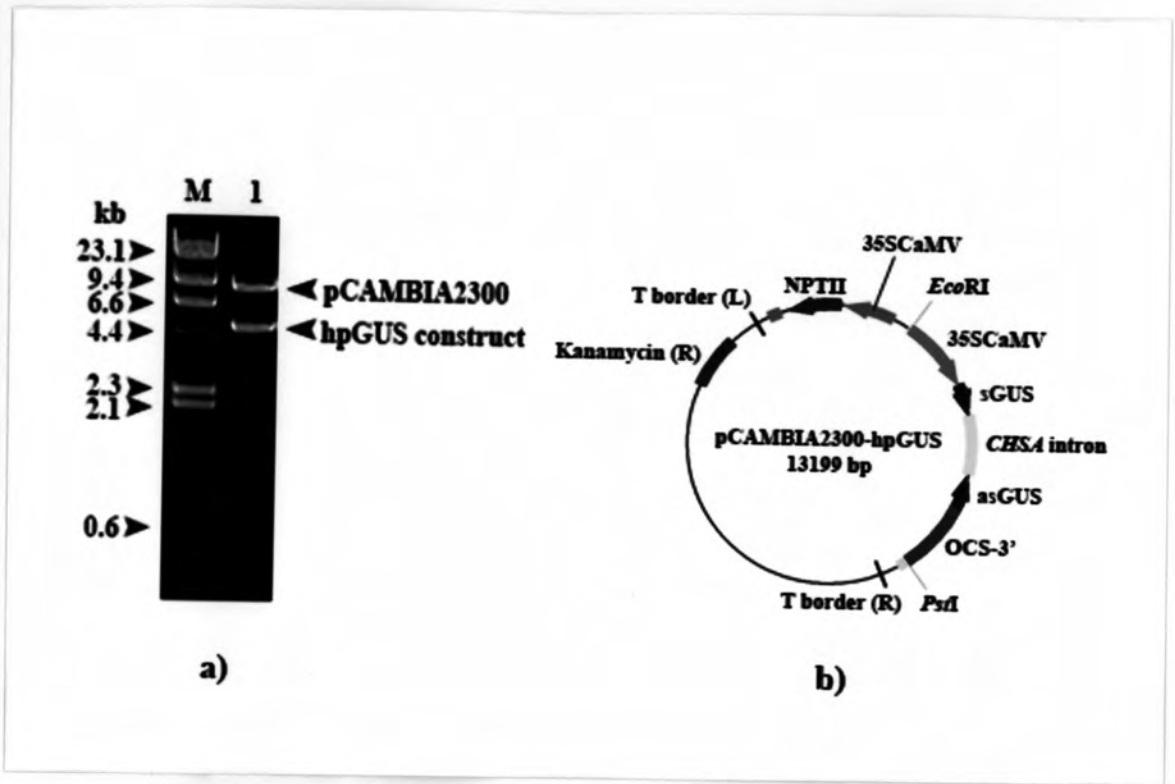


Figure 3.9 Agarose gel electrophoresis of recombinant plasmid pFGC5941-hpGUS on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pFGC5941-hpGUS digested with *Eco*RI and *Pst*I

b) Schematic diagram of the recombinant pFGC5941-hpGUS containing hpGUS construct

as shown in Figure 3.10 and a schematic diagram of the hpGUS construct depicting the confirmed orientation of the *gus* sequences inserted into pFGC5941 is shown in Figure 3.11.

The result supports the notion that this pFGC5941 vector is the appropriate RNAi vector for gene silencing in plants because of the core structure of the vector is a chalcone synthase A intron that was obtained from petunia (dicotyledous plant) flanked by 2 multiple cloning sites (MCSs), which supply multiple enzymes with compatible sites. The multiplicity of its MCSs facilitates the insertion of a gene of interest in both sense and antisense directions with use of only one pair of primers. The simplicity of pFGC5941 makes it possible to construct an RNAi structure in a much quicker and easier way. Therefore, it is a practical vector to study gene function and has been used for inhibiting homologous gene expression.

3.3 Construction of plant expression vector for silencing a *gus* gene

The pCAMBIA2300 was employed for plant transformation. The pCAMBIA2300 contains a aminoglycoside phosphotransferase (kanamycin resistant: aadA) gene as a bacterial selectable marker, a neomycin phosphotransferase (kanamycin resistant: *npt*II) gene as a plant selectable marker and a LacZ alpha within the T-DNA region. It was used as a binary vector to transfer the hpGUS sequence into plants. The pFGC5941-hpGUS was digested with *Pst*I and *Eco*RI and the desired hpGUS fragment of approximately 4,500 bp was separated by 1% agarose gel electrophoresis (Figure 3.9). This fragment was purified and ligated into *Pst*I and *Eco*RI sites of pCAMBIA2300 digested with the same enzymes (Figure 3.12). *E. coli* strain XL1-Blue cells were transformed with the ligation mixture. The transformants were then selected by blue/white colony screening on kanamycin agar plates containing X-gal and IPTG.

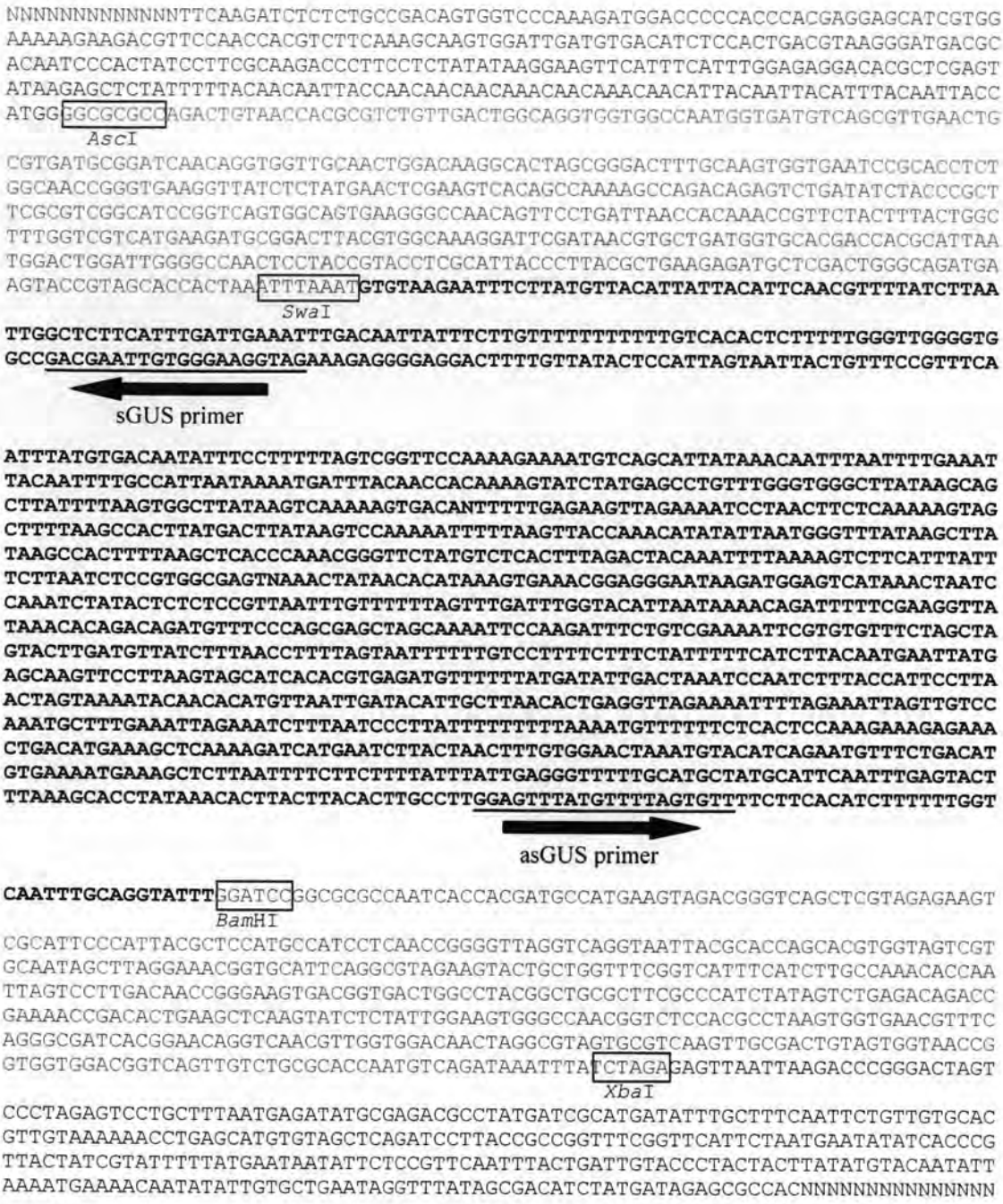


Figure 3.10 Nucleotide sequence of hpGUS in the pFGC5941-hpGUS plasmid. The underlined letters represent sequencing primer binding sites and the bold letters indicate the CHSA intron. The red letters show the nucleotide sequence of sGUS derived from the sGUS primer and the blue letters show the nucleotide sequence of the asGUS derived from the asGUS primer.

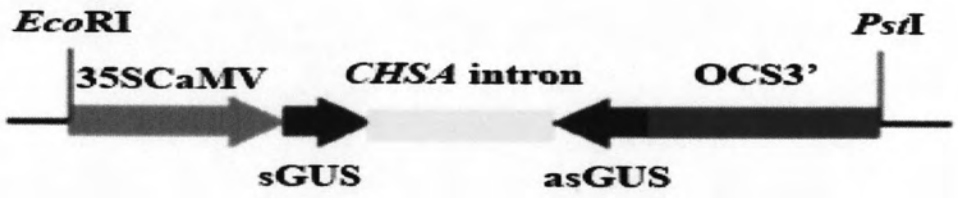


Figure 3.11 Schematic diagram of the hpGUS cassette of pFGC5941-hpGUS vector Abbreviations used: 35SCaMV - cauliflower mosaic virus promoter; sGUS - *gus* fragment in sense orientation; *CHSA* intron - intron obtained from petunia chalcone synthase A; asGUS - *gus* fragment in antisense orientation; OCS3′ - 3′ sequence of the octopine synthase.

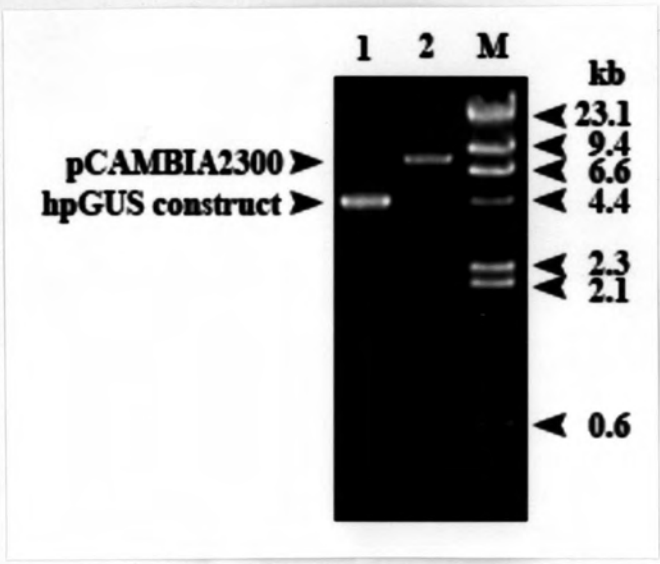


Figure 3.12 Purified products of the hpGUS cassette and the pCAMBIA2300-hpGUS analyzed on 1.0 % agarose gel.

Lane 1 purified *Eco*RI/*Pst*I-digested hpGUS construct

Lane 2 purified *Eco*RI/*Pst*I-digested pCAMBIA2300-hpGUS

Lane M λ/*Hin*dIII standard marker

white colonies were picked and their plasmids were extracted. The potential recombinant plasmids were digested with *Pst*I and *Eco*RI and the expected DNA fragment of approximately 4,500 bp was obtained from a resistant colony (Figure 3.13). The resulting plasmid is designated as pCAMBIA2300-hpGUS which contains the hpGUS construct for silencing a *gus* gene.

3.4 Plant transformation

A model system for this study is a *gus*-overexpressing *N. tabacum* cv. Virginia Coker transformant which was previously transformed by introducing a pCAMBIA1301 plasmid carrying a *gus* gene driven by the 35SCaMV promoter. In this study, we made double transformants by introducing the pCAMBIA2300-hpGUS (silencing construct) into the *gus*-overexpressing transformant. For the control, we made double transformants by introducing the pCAMBIA2300 alone. All were generated by *Agrobacterium*-mediated transformation.

Agrobacterium-mediated binary vector system is an excellent model of the natural exchange of genetic material from a prokaryote to a eukaryote. This method is widely used for genetic manipulation of more than 120 species of at least 35 families, including vegetables, fruits and trees (Birch, 1997) especially in dicotyledonous plants because it has many advantages. Integration of a single copy or low number of copies of transgenes is among the most important features of *Agrobacterium*-mediated transformation (Hiei *et al.*, 1997 and Koncz *et al.*, 1994), which leads to better and more reliable transgene expression.

To generate double transformants, transgenic tobacco leaf discs were initially cocultured with *A. tumefaciens* strain EHA105, harboring the binary vector pCAMBIA2300 or pCAMBIA2300-hpGUS. Leaves from *gus*-overexpressing transgenic

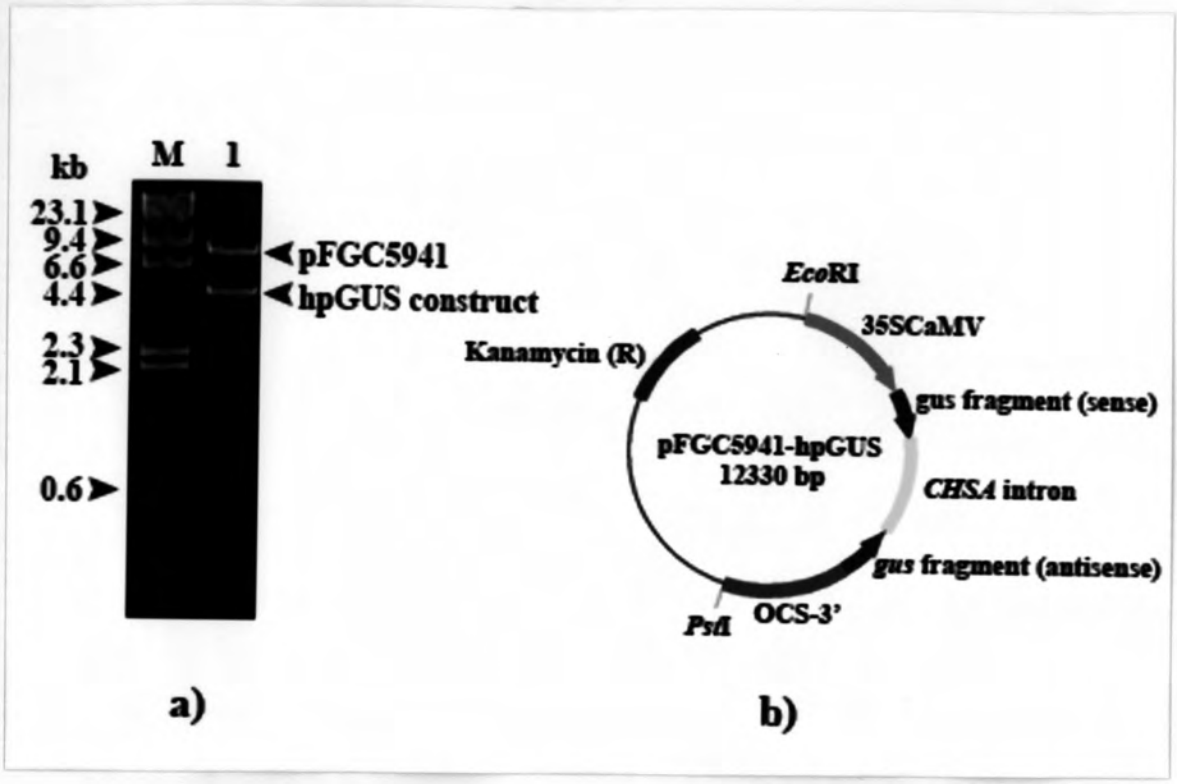


Figure 3.13 Agarose gel electrophoresis of recombinant plasmid pCAMBIA2300-hpGUS on 1.0 % agarose gel.

a) Lane M λ/*Hin*dIII standard marker

Lane 1 pCAMBIA2300-hpGUS digested with *Eco*RI and *Pst*I

b) Schematic diagram of the recombinant pCAMBIA2300-hpGUS containing hpGUS construct

tobacco grown on MS medium were used as the starting material for co-cultivation (Figure 3.14). The infected leaf explants after 2 days of co-cultivation were transferred to fresh selection medium supplemented with 50 mg/l kanamycin and 250 mg/l cefotaxime for 7 days to select transformed cells and to eliminate *Agrobacterium* overgrowth, respectively. Then, these leaves were transferred to the shoot induction medium. Two weeks later, some explants had grown larger and thicker and then a lot of shoots from kanamycin-resistant cells gradually appeared from the edges of these explants within one month. A lot of shoots were left to elongate on the shoot induction medium until they were 3.0 to 5.0 cm long (Figure 3.14) and then they were excised from the base of the explants and placed on MS medium to generate the root system (Figure 3.14). Several putative transgenic lines were obtained from regeneration of the kanamycin-resistant cells, indicating that the cointroduced *npt*II gene is expressed. The *npt*II gene is at the end of T-DNA near the left border therefore, it is likely that the T-DNA region of pCAMBIA2300-hpGUS or pCAMBIA2300 was completely transferred from *Agrobacterium* into the genome of the transgenic tobacco plants because *Agrobacterium* cells transfer T-DNA from the 5′ to 3′ direction, initiating at the right T-DNA border and terminating at the left T-DNA border (Citovsky *et al*., 1992, Howard *et al*., 1992 and Tinland *et al*., 1992.). Furthermore, there were no morphological differences between the control and the hpGUS-expressing double transformants.

3.5 PCR analyses of transgenic tobacco plants

Three putative independent lines from each of the control and hpGUS-expressing double transformants were selected for PCR analysis which was used to confirm integration of the *gus* gene and the hpGUS construct into their genomes by amplifying

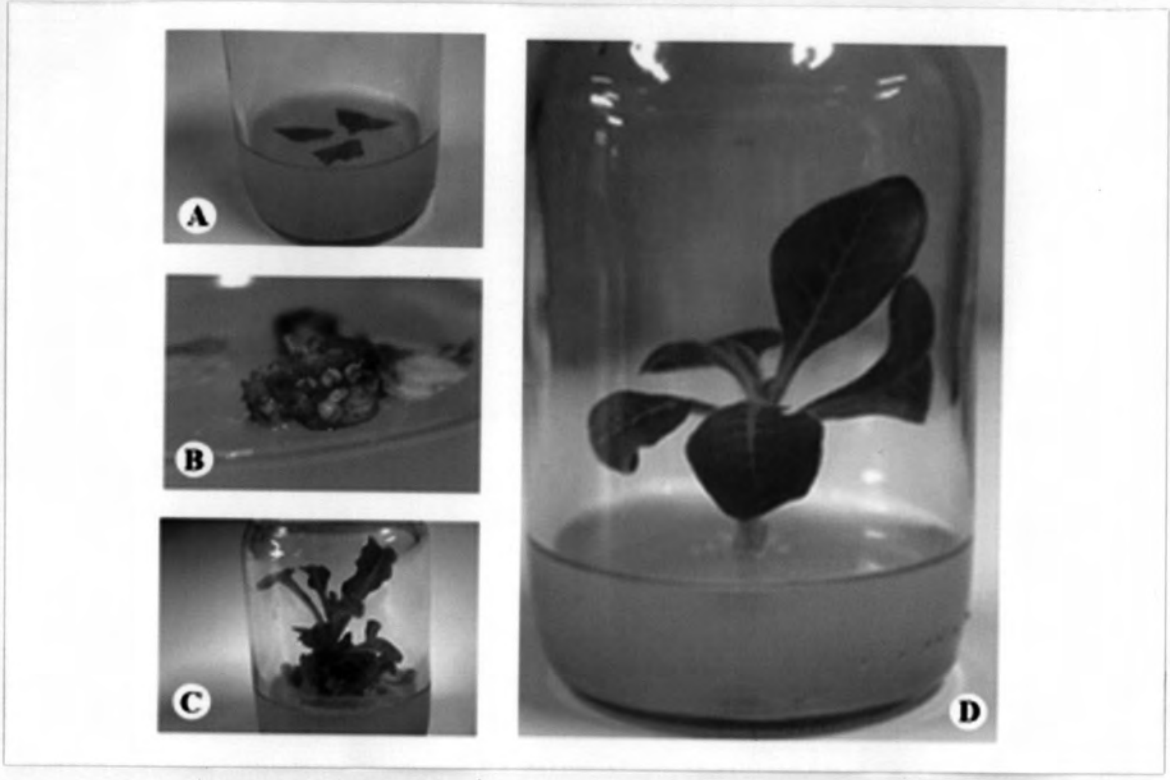


Figure 3.14 Leaf discs transformation and regeneration of *Nicotiana tabacum* L. Virginia Coker. (A) Leaf discs after co-cultivaion with *Agrobacterium* (B) Regenerated shoots obtained from leaf explants (C) Elongation of regenerated shoots (D) Transgenic tobacco plantlet *in vitro*

Parts of the 35SCaMV-*gus* gene and the hpGUS construct, respectively. First, genomic DNA was isolated from leaves of all putative independent lines using a modified CTAB extraction method. DNA concentrations were spectrophotometrically determined by measuring the absorbance at 260 nm (A_{260}). An A_{260} of 1.0 corresponds to a concentration of 50 μg double-stranded DNA/ml. Approximately 3-5 μg of nucleic acid were obtained from 100 mg of plant tissue. The ratio of A_{260}/A_{280} was 1.8-2.0 which indicated that relatively clean DNA was obtained.

Existence of the *gus* gene and the hpGUS construct in the chromosomal DNA of the three putative independent transgenic tobacco plants in each group was examined by PCR analysis using GUS check-F and GUS check-R primers; and hpGUS check-F and hpGUS check-R primers, respectively. PCR products of 983 bp or 318 bp were expected from DNA of the transgenic tobacco plants to which the 35SCaMV-*gus* gene or the hpGUS construct had been transferred, respectively. The results showed that bands of approximately 970 and 300 bp were detected in all putative transgenic tobacco lines of the hpGUS-expressing double transformants (Figure 3.15). In the case of transgenic tobacco plants double-transformed with pCAMBIA2300 alone, the specific 983-bp bands of the 35SCaMV-*gus* gene PCR product were detected whereas the 318-bp band of the hpGUS PCR product was not obtained.

The results of PCR analysis certainly confirm that the three putative independent transgenic tobacco lines of the hpGUS-expressing double transformants contain the hpGUS construct in their genomes but the control double transformants did not.

3.6 Southern blot analysis of hpGUS-expressing double transformants

Southern blot analysis was further used (1) to determine the integration of the transgene into the putative transgenic tobacco plants' genome (Zhen *et al.*, 2004)

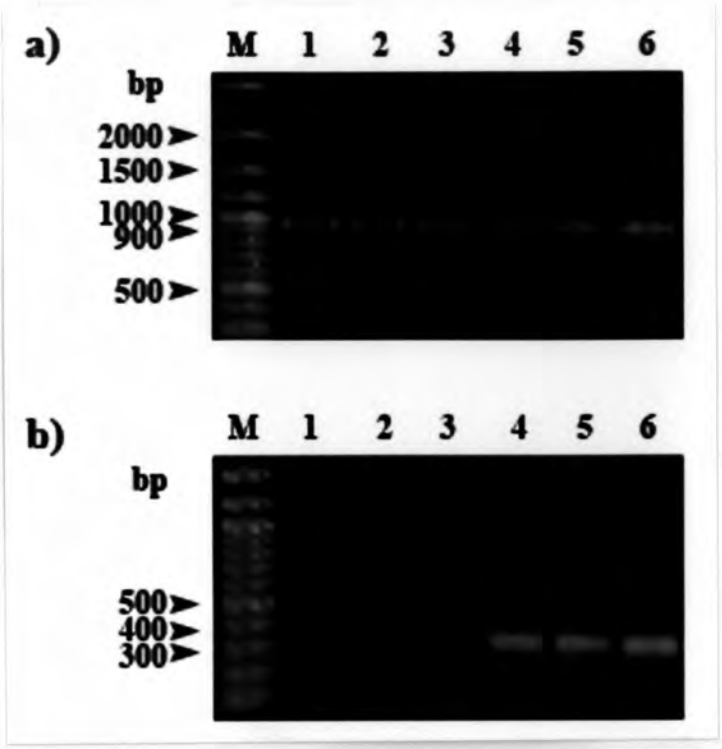


Figure 3.15 PCR analysis of *gus* gene and hpGUS construct insertion in the genome of six putative independent lines of the hpGUS-expressing and the control double transformants. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

a) The amplification of 35SCaMV-*gus* gene

Lane M 100 bp plus DNA ladder

Lane 1-3 control plants double transformed with pCAMBIA2300 without the hpGUS construct lines 1, 2 and 3 respectively

Lane 4-7 hpGUS-expressing plants lines 1, 2 and 3, respectively

b) The amplification of hpGUS construct

Lane M 100 bp plus DNA ladder

Lane 1-3 control plants double transformed with pCAMBIA2300 without the hpGUS construct lines 1, 2 and 3 respectively

Lane 4-7 hpGUS-expressing plants lines 1, 2 and 3, respectively

(2) to determine whether the transgenic tobacco lines, previously isolated from screening of the kanamycin-resistant cells, were independent lines and (3) to estimate the copy number of insertion in independent transgenic lines (Tijen and Nermin, 1999). In total, three putative PCR-positive transgenic tobacco lines of hpGUS-expressing double transformants were analyzed by Southern blot hybridization. The genomic DNA was isolated from leaves of all putative independent lines which were double-transformed with pCAMBIA2300-hpGUS. Thirty micrograms of genomic DNA from each putative line were completely digested with *Hin*dIII, fractionated on 0.8% agarose gel electrophoresis and transferred to a positively charged nylon membrane. The blot was hybridized with a probe prepared from the 601-bp *npt*II gene fragment which was generated from PCR amplification of the *npt*II gene in pCAMBIA2300 using NptII-F and NptII-R primers (Figure 3.16). Three micrograms of the purified DNA product were labeled using DIG-dUTP. After Southern hybridization with the probe was carried out at 40°C overnight, the blot was washed with 2X SSC 0.1% SDS twice, with 0.5X SSC 0.1% SDS twice, blocked and incubated with DIG antibody. Positive hybridizating bands were detected by colorimetric method. The result shows that one hybridizating band of different size was detected in each putative PCR-positive transgenic tobacco line (Lanes 1-3). Lines 1, 2 and 3 produced a band of approximately 16.3 kb, 9 kb and 10.5 kb band, respectively (Figure 3.17).

The different integration patterns can be explained in terms of stable genetic integration either in different chromosomes or the copy number of insertion that have been delivered into a plant genome (Valdez-Ortiz *et al.*, 2005). This analysis indicates that (1) the RNAi construct of *gus* gene was successfully integrated into the tobacco genome, (2) the three putative PCR-positive transgenic tobacco lines which obtained previously from the regeneration of kanamycin resistant cells, are independent

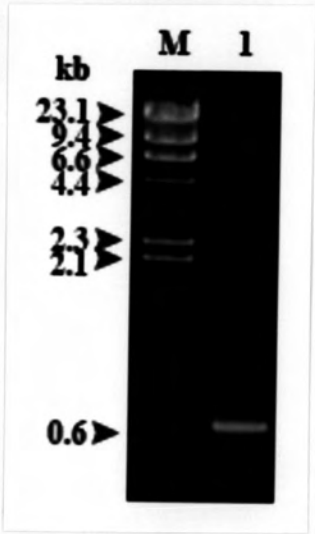


Figure 3.16 Agarose gel electrophoresis of the amplified fragments of the *npt*II gene

The PCR product was separated on a 1.0 % agarose gel and visualized by ethidium bromide staining.

Lane M λ/*Hin*dIII standard marker

Lane 1 *npt*II fragment

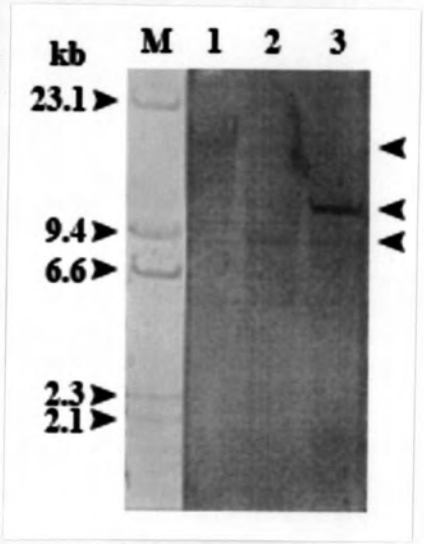


Figure 3.17 Genomic DNA blot analysis of three putative independent lines of the hpGUS-expessing double transformants. Tobacco genomic DNA was digested with *Hin*dIII and hybridized with part of the *npt*II gene. Positive hybridizing bands were detected by colorimetric method.

Lane M EtBr-stained λ/*Hin*dIII standard marker

Lane 1, 2, 3 the hpGUS-expessing putative independent lines 1, 2 and 3, respectively

transgenic tobacco lines and (3) each independent transgenic tobacco line probably has one copy of insert in their genome. Although the transgene was integrated into the plant genomes, we cannot know the number copy of insert for sure because the genomic DNA was digested with only one restriction enzyme, therefore the information on the copy number of insertion is not conclusive.

3.7 Northern blot analysis of transgenic tobacco plants

To compare the *gus* expression level between the three independent lines of the hpGUS-expressing double transformants that harbor the *gus* gene and the hpGUS construct regulated by 35SCaMV promoter and the control double transformants that harbor only the *gus* gene which is regulated by the same promoter, northern blot analysis was performed. Total RNA was isolated from leaves of all transgenic lines and its concentration was spectrophotometrically determined by measuring the absorbance at 260 nm (A_{260}). An A_{260} of 1.0 corresponds to a concentration of 40 μg RNA/ml. Approximately 4-5 μg of RNA were obtained from 100 mg of plant tissue. The ratio of A_{260}/A_{280} was ~2.0 which indicated that relatively clean RNA was obtained. Twenty micrograms of total RNA were fractionated on 1.5% formaldehyde agarose gel electrophoresis and transferred to a positively charged nylon membrane. The blot was hybridized with a labelled probe prepared from the 495-bp *gus* gene fragment which was generated from PCR amplification of the *gus* gene in pCAMBIA1301 using GUS-F and GUS-R primers (Figure 3.1). The DNA product was purified and labelled with [α-^{32}P]dCTP and used in northern hybridization which was carried out at 40°C for 16 hours. The blot was washed with 2X SSPE, 0.1% SDS twice and then with 1X SSPE, 0.1% SDS once at room temperature. Positive hybridizing bands were detected by autoradiography.

Our result shows that no hybridizing band was detected in lines 1 and 2 of the hpGUS expressing double transformants (Figure 3.18, lane 4-5) whereas a single 1.8-kb band was detected in line 3 of the hpGUS-expressing double transformants (Figure 3.18, lane 6) and the three putative independent lines of control plant (Figure 3.18, lane 1-3). However, the band intensity in line 3 of hpGUS-expressing double transformants is much weaker than that of the control plants. In previous study, Sasha *et al.* (2007) reported that the differences of expression were probably related to the transgene position and Bestor and Tycko (1996) reported that transgene inserts that integrate into transcriptionally inactive heterochromatin region which is a tightly packed form of DNA to which transcription factors are difficult to bind were immediately silenced, therefore, differences in *gus* mRNA level in the independent lines of the hpGUS-expressing double transformants may have resulted from the hpGUS construct in line 3 was integrated into a heterochromatin region. Nonetheless, the level of *gus* mRNA found in the independent lines 3 of the hpGUS-expressing double transformants was considerably lower than those observed in the three putative independent lines of the controls indicating a significant decrease in *gus* expression in the hpGUS-expressing double transformant (Figure 3.18). These results indicate that transformation with the pCAMBIA2300-hpGUS plasmid silenced the constitutive expression of the *gus* gene, as expected. This event probably have resulted from the hpGUS that generated a double-stranded RNA (dsRNA) by complementary sequences of the *gus* sense and antisense fragments in the hpGUS RNA. These dsRNAs were probably cleaved by Dicer into 21-25 nucleotides small RNA fragments called small interfering RNA (siRNA), and siRNA was then incorporated with RNA induced silencing complex (RISC) guiding the RISC to degrade *gus* mRNA.

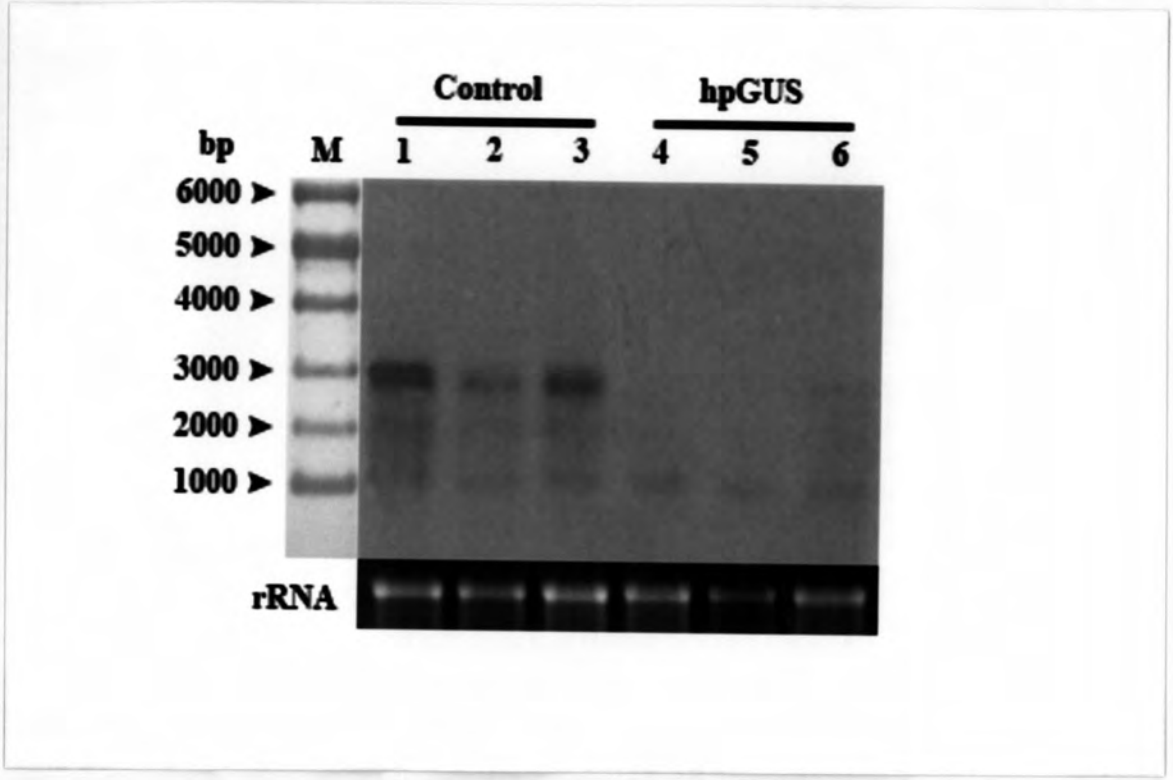


Figure 3.18 RNA blot analysis of *gus* gene in three putative independent lines of the control double transformants and three independent lines of the hpGUS-expressing double transformants. Twenty micrograms of total RNA isolated from leaves were analyzed by gel blot hybridization with a denatured ^{32}P-labeled *gus* probe. An ethidium bromide-staining gel of each analysis is shown under its corresponding autoradiography.

3.8 β-glucuronidase (GUS) activity of transgenic tobacco plants

The GUS reporter gene was chosen for this study because β-glucuronidase (GUS) enzyme is easily and sensitively assayed spectrophotometrically in *vitro* and can also be histochemically assayed to localize GUS activity in plant cells and tissues. In addition, GUS is stable and shows high level of activity after a prolonged storage.

3.8.1 Histochemical analysis of GUS expression in double transformants

Histochemical GUS analysis is usually done to verify that genetic transformation takes place cell in the genome of plant cells. The activity of β-glucuronidase enzyme can be observed as blue stain by incubating the transformed tissues with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). In this research, the tobacco plants which contain the β-glucuronidase (*gus*) gene as a reporter gene from the first transformation was double transformed with the binary plasmid pCAMBIA2300-hpGUS or pCAMBIA2300 alone to be used as a control. Both were subjected to analysis for GUS activity.

Three putative independent lines of the control double transformants and three independent lines of the hpGUS-expressing double transformants were histochemically analysed for GUS activity. Leaves and roots of all plants were cut and stained with solution containing the substrate X-Gluc. After incubation at 37 °C overnight, blue color formation occurred in the expression sites where X-gluc was catabolized by the GUS gene product (β-glucuronidase). Both leaves and roots from all putative transgenic lines of the control plants exhibited a high level of GUS activity which was observed when the color of the staining solution and the leaves and roots turned blue. For the hpGUS-expressing plants, the leaves of line 1 and 2 showed little GUS activity. The color of the staining solution remained unchanged and the leaves and

roots did not display intense blue color as observed in the control plants. On the contrary, line 3 has a higher level of GUS activity than line 1 and line 2 and showed similar results to the control plants (Figure 3.19). With the exception of line 3 the results indicate that the hpGUS-expressing double transformants likely produced the hpGUS to silence expression of the *gus* gene at the protein level.

3.8.2 GUS spectrophotometric analysis in double transformants

GUS spectrophotometric assay was further used to analyze expression of the *gus* gene at the protein level for determining the efficiency of gene silencing. This method is more reliable than the histochemical assay because it shows the result as quantitative data. Hence, leaves of all transgenic tobacco plants used in the histochemical assay were spectrophotometrically assayed for GUS activity. Crude proteins were extracted from leaves of the three putative independent lines of the control plants and three independent lines of the hpGUS-expressing transgenic tobacco plants. The protein concentration of each sample was determined by Bradford's method. One hundred of crude extract was incubated with PNPG at 37 °C for 60 minutes. Two hundred microliters of each reaction were collected every 15 minutes during the incubation period and the liberated *p*-nitrophenol was monitored by measuring absorbance at 415 nm. The resulting absorbance values were used to generate a curve which its data were used to calculate GUS activity. The concentration of *p*-nitrophenol was calculated through Beer's Law using a molar extinction coefficient of 14000 l mol^{-1} cm^{-1} for *p*-nitrophenol. One unit (U) of enzyme is defined as the amount of enzyme that releases one pmol of *p*-nitrophenol in one minute. Specific activity is expressed as units per milligram of protein. The results can be described as follows:

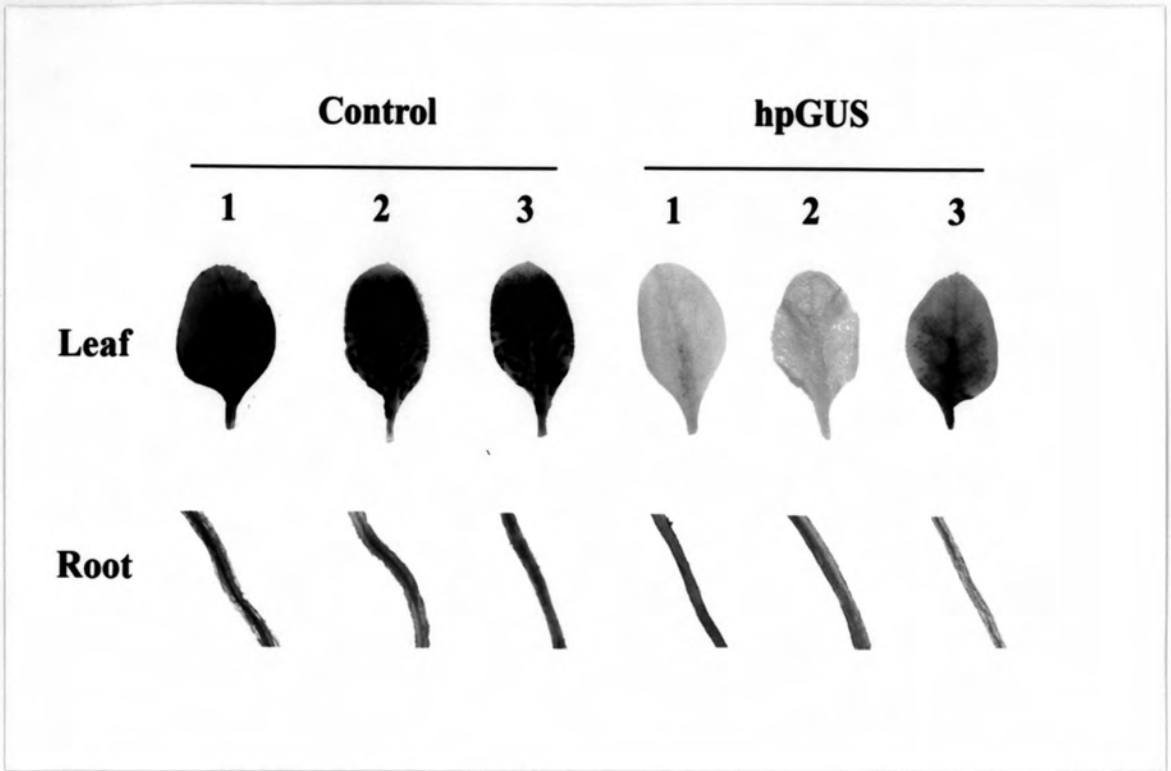


Figure 3.19 Histochemical analysis of GUS activity in leaf and root of the hpGUS-expressing double transformants. The transgenic tobacco plants containing pCAMBIA2300 alone was used as a control.

(1) Hydrolysis of PNPG in extracts from all transgenic tobacco leaves increased between 0 to 60 minutes of incubation with the substrate and graphs between the absorbance at 415 nm and the incubation time were linear. The slopes were approximately 0.0167, 0.0041 and 0.0057 min^{-1} in lines 1, 2 and 3 of the control plants, respectively and 0.0004, 0.0009 and 0.0086 min^{-1} in lines 1, 2 and 3 of the hpGUS-expressing double transformants, respectively (Figure 3.20).

(2) Total activity of GUS in extracts from all transgenic tobacco leaves was calculated using the slopes shown in Table 3.1. GUS activity in the control lines 1, 2 and 3 averages 311, 77 and 106 pmol/min, respectively and in hpGUS-expressing lines 1, 2 and 3 averages 7, 16 and 160 pmol/min, respectively.

(3) Specific activity of GUS in extracts from all transgenic tobacco leaves were shown in Table 3.1 and Figure 3.21. Specific activity in the control lines 1, 2 and 3 averages 529, 130 and 173 U/mg protein, respectively and in the hpGUS expressing lines 1, 2 and 3 averages 10, 23 and 310 U/mg protein, respectively. Variability in the level of GUS activity which may have resulted from difference in size and age of leaves (Laurian *et al.*, 1989) between three replications of each sample was minimal (Figure 3.21). Among the results, we found that specific activity in the control line 1 is the highest while that in the hpGUS-expressing line 1 is the lowest. These results show that most specific activity values of the hpGUS-expressing lines are lower than those of the control plants, except the hpGUS-expressing line 3 which has higher GUS activity than the control lines 2 and 3.

(4) Comparison of the specific activity between the control and the hpGUS expressing double transformants were displayed as % inhibition of GUS activity. Specific activity in the three control transgenic lines averages at 278 U/mg protein. Specific activity of the hpGUS-expressing lines 1 and 2 were approximately 96 % and

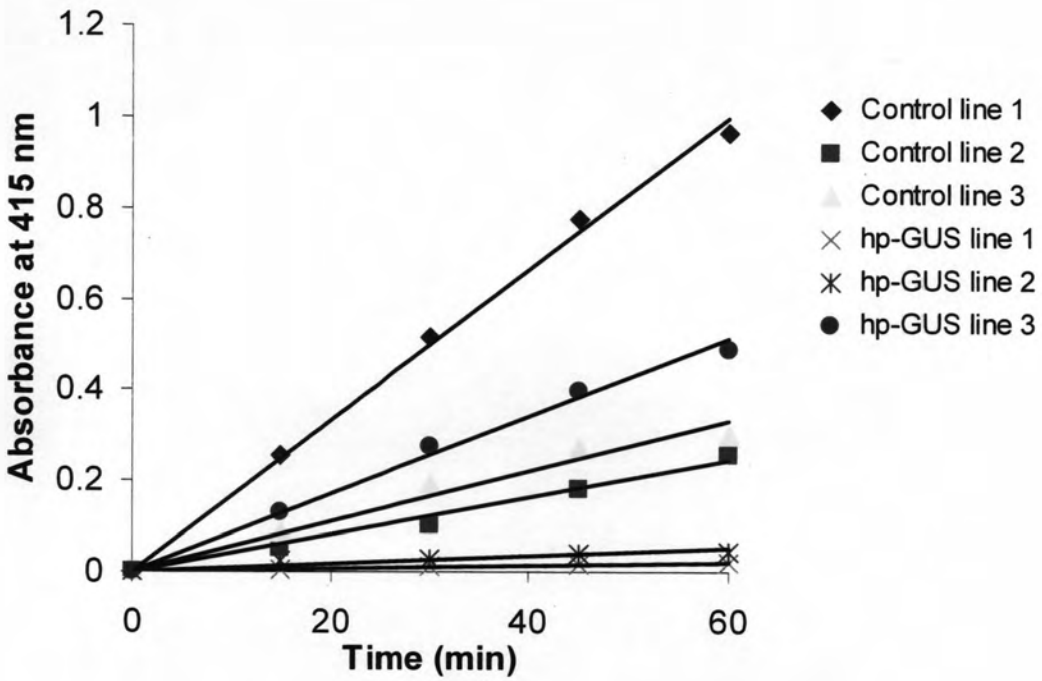
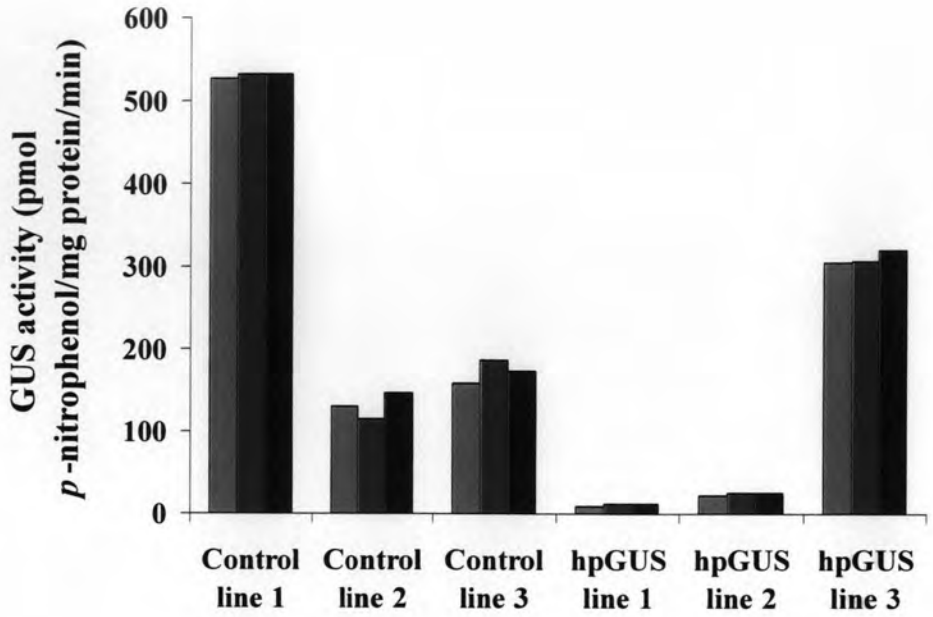


Figure 3.20 Graphs between absorbance at 415 nm monitoring the release of *p*-nitrophenol and the incubation time of the extract from each of the six transgenic tobacco leaves and the substrate PNPG.

Table 3.1 GUS activity from crude extract of the control and the hpGUS-expressing double transformants

Source	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg protein)	Average specific activity (U/mg protein)
Control line 1	308.28	0.585	526.58	529
	312.00	0.587	531.35	
	312.00	0.587	531.97	
Control line 2	74.29	0.573	129.71	130
	76.14	0.665	114.42	
	79.86	0.543	147.00	
Control line 3	102.14	0.642	159.15	173
	105.86	0.566	187.08	
	109.57	0.633	173.04	
hpGUS line 1	5.57	0.651	8.56	10
	7.43	0.644	11.53	
	7.43	0.606	12.27	
hpGUS line 2	14.86	0.691	21.50	23
	16.71	0.683	24.46	
	16.71	0.668	25.03	
hpGUS line 3	157.86	0.516	305.71	310
	161.57	0.527	306.77	
	161.57	0.505	319.82	

a)



b)

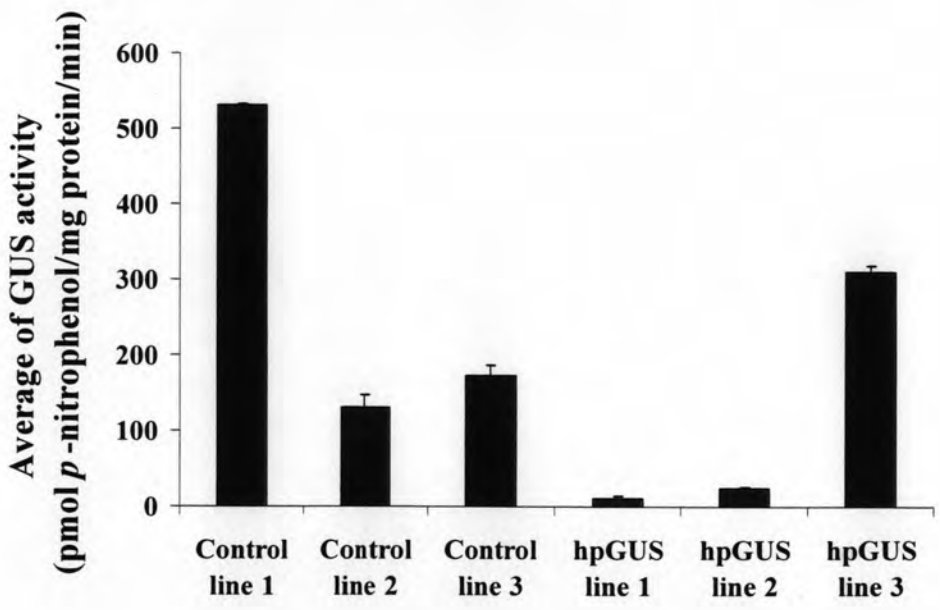


Figure 3.21 Histograms showing GUS activity levels of all six transgenic tobacco plants. (a) Comparison of the specific activity of GUS between three replications (displayed in green, red and blue) (b) The average of GUS specific activity from three replications and the results shown are mean values $\pm$ SD of independent analyses.

91%, respectively while that of the hpGUS-expressing line 3 does not show any reduction when compared with the average specific activity of the control plants. The decreased GUS activity level of the hpGUS expressing double transformants corresponds with the reduction of *gus* mRNA level in northern blot analysis and the reduction of GUS activity level of the hpGUS-expressing double transformants shown by histochemical analysis. Difference among hpGUS-expressing double transformants may have resulted from the transgene positional effect. It seems that transformant line 3 has no or little silencing of the GUS protein (Figure 3.22). Nonetheless, results from the hpGUS-expressing lines 1 and 2 indicate that the hpGUS construct can silence expression of the *gus* gene up to 96% indicating that gene silencing by production of hairpin RNA is a highly efficient system in inhibiting expression of a target gene.

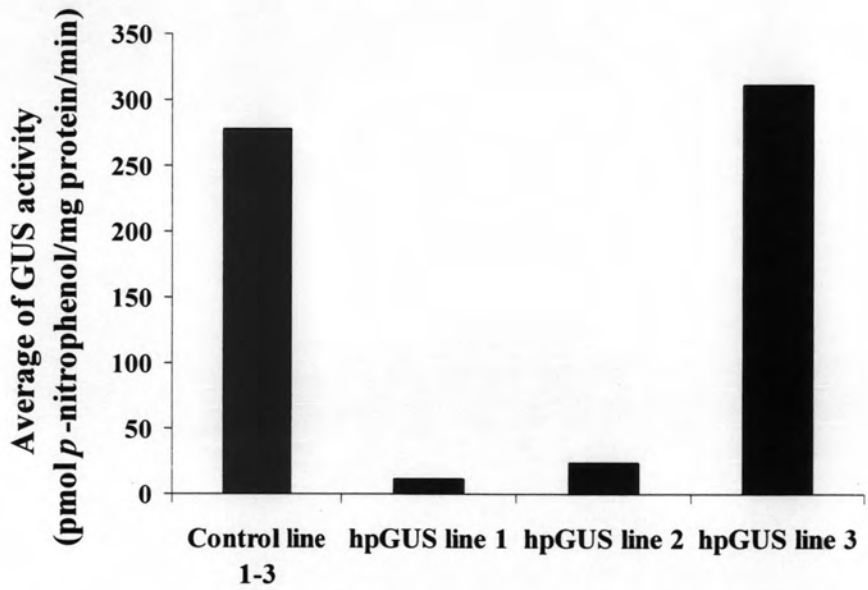


Figure 3.22 Comparison of the average GUS specific activity from all lines of the control plants (red) with each of the hpGUS-expressing lines (blue).