CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plants

Tobacco (*Nicotiana tabacum*) cultivar Virginia Coker

2.1.2 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Automatic micropipette: Pipetman P2, P20, P100, P1000 (Gilson Medical Electronics S.A., France)

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Biophotometer (Eppendorf, Germany)

Film cassette with intensifying screen: 35x43 cm^2 (Kodak, USA)

-20 °C Freezer (Sharp, Japan)

Gel document : Gel Doc™ (Syngene, England)

Gel mate 2000 (Toyobo, Japan)

Gene Pulser : Micropulser™ (Bio-RAD Laboratories, USA)

Hybridization oven : Hybaid shake 'n' Stack (Thermo Scientfic, USA)

Incubator: BM-600 (Memmert Gambh, Germany)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific, UK)

Laminar flow: HS-124 (International Scientific Supply Co., Ltd., USA)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Magnetic stirrer and heater: Cerastir (Clifton, USA)

Mastercycler gradient PCR system (Eppendorf, Germany)

Microcentrifuge: PMC-880 (Tomy Kogyo Co., Ltd., Japan)

Microwave oven (Panasonic, Japan)

Orbital shaker (Labinco, Taiwan)

pH meter: pH900 (Precisa, Germany)

PCR workstation Model#P-036 (Scientific Co., USA)

Power supply: Power PAC 1000 (Bio-RAD Laboratories, USA)

Refrigerated centrifuge : 5804R (Eppendorf, Germany)

Refrigerated centrifuge : 5417R (Eppendorf, Germany)

Spectrophotometer: DU®640 (Beckman Coulter, USA)

UV chamber : GS Gene Linker™ (Bio-RAD Laboratories, USA)

UV transilluminator: 2001 microvue (San Gabriel California, USA)

Vacuum blotter : model 785 (Bio-RAD Laboratories, USA)

Vacuum pump (Bio-RAD Laboratories, USA)

Vortex mixer: Model K 550-GE (Scientific Inc., USA)

Waterbath: Isotemp210 (Fisher Scientific, USA)

2.1.3 Materials

Filter paper: Whatman No.1 (Whatman International Ltd., England)

KODAK BioMax MS film: 18x24 cm^2 (Kodak, USA)

Microcentrifuge tube 0.6 and 1.5 ml (Axygen Hayward, USA)

0.22µm Millipore membrane filter (Millipore, USA)

Nipro disposable syringe (Nissho, Japan)

PCR thin wall microcentrifuge tube 0.2 ml (Eppendorf, Germany)

Pipette tips10, 100, 1000 µl (Axygen Hayward, USA)

Hybond-N^+ membrane (Amersham Biosciences Inc., USA)

2.1.4 Chemicals and reagents

Absolute ethanol (BDH, England)

Acetic acid glacial (BDH, England)

Agar (Merck, Germany)

Agarose: Seakem LE Agarose (FMC Bioproducts, USA)

Ammonium nitrate (Sigma Chemical Co., USA)

Bacto tryptone (Difco, USA)

Bacto yeast extract (Difco, USA)

6-Benzylaminopurine (BAP) (Sigma Chemical Co., USA)

Beta-mercaptoethanol (Fluka, Switzerland)

Boric acid (Merck, Germany)

Bovine Serum Albumin (Sigma Chemical Co., USA)

5-Bromo-4-chloro-3-indole- β -D-galactopyranoside; X-gal (Sigma Chemical co., USA)

5-Bromo-4-chloro-3-indolyl-β-D-glucoronic acid; X-GLUC (*Phyto*Technology Laboratories, Inc., USA)

Bromophenol blue (Merck, Germany)

Calcium chloride (Carlo Erba Reagenti, Italy)

Calf thymus DNA (Sigma Chemical Co., USA)

Cetyltrimethyammonium bromide (CTAB) (Sigma Chemical Co., USA)

Chloroform (Merck, Germany)

Cobalt chloride (Fluka, Switzerland)

Copper sulfate (Carlo Erba Reagenti, Italy)

dATP, dCTP, dGTP, and dTTP (Fermentas Inc., USA)

Dithiothreitol (Sigma Chemical Co., USA)

Diethyl pyrocarbonate: DEPC (Sigma Chemical Co., USA)

Ethidium Bromide (Sigma Chemical Co., USA)

Ethylene diamine tetraacetic acid (EDTA) (Carlo Erba Reagenti, Italy)

Ferrous sulfate (Carlo Erba Reagenti, Italy)

Formamide (Fluka, Switzerland)

Formaldehyde (Sigma Chemical Co., USA)

Ficoll type 400 (Sigma Chemical Co., USA)

GBX developer solution (Kodak, USA)

GBX fixer solution (Kodak, USA)

Glycerol (BDH, England)

Glycine (Sigma Chemical Co., USA)

Glacial acetic acid (Carlo Erba Reagenti, Italy)

Hydrochloric acid (Merck, Germany)

Isoamylalcohol (Merck, Germany)

Isopropanol (Merck, Germany)

Iso-1-thio-β-D-thiogalactopyranoside: IPTG (Serva, Germany)

Lambda DNA (Promega Co., USA)

Manganese sulfate (Ajax Finechem, Australia)

Magnesium sulfate (Sigma Chemical Co., USA)

Methanol (Merck, Germany)

Methylene blue (Carlo Erba Reagenti, Italy)

Nicotinic acid (Sigma Chemical Co., USA)

Naphtalene acetic acid (Duchefa, Netherland)

Phenol crystal (BDH, England)

Phenylmethylsulfonyl fluoride: PMSF (USB, USA)

Polyvinyl pyrrolidone (Sigma Chemical Co., USA)

Potassium acetate (Merck, Germany)

Potassium Ferricyanide (BDH, England)

Potassium Ferrocyanide (BDH, England)

Potassium iodide (Mallinckrodt, USA)

Potassium nitrate (BDH, England)

Pyridoxine hydrochloride (Duchefa, Netherland)

Random hexamers (Promega Co., USA)

Sodium acetate (Carlo Erba Reagenti, Italy)

Sodium chloride (Carlo Erba Reagenti, Italy)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium dihydrogen orthophosphate (Carlo Erba Reagenti, Italy)

di-Sodium dihydrogen orthophosphate anhydrous (Carlo Erba Reagenti, Italy)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

Sodium molybdate (Carlo Erba Reagenti, Italy)

Sugar (Mitr Phol Sugar crop., Ltd., Thailand)

Thiamine hydrochloride (Duchefa, Netherland)

Triethanolamine (Sigma Chemical co., USA)

Tri-Sodium citrate (Scharlau chemie S.A., Spain)

Tri-Reagent® (Molecular research center, Inc., USA)

Tris-(hydroxyl methyl)-aminomethane (Fluka, Switzerland)

Triton X-100 (Merck, Germany)

Tween 20 (Bio-RAD Laboratories, USA)

Xylene Cyanol FF (Sigma Chemical co., USA)

Zinc sulphate (Ajax Finechem, New Zealand)

2.1.5 Enzymes

DNA polymerase, Large (Klenow) fragment (New England Biolabs, Inc., USA)

Restriction endonucleases: *Bam*HI, *Eco*RI, *Hin*dIII (Fermentas, Inc., USA), *Asc*I, *Msp*I, *Nco*I, *Pst*I, *Spe*I, *Swa*I, *Xba*I (New England Biolabs, Inc., USA)

RNaseA (Promega Co., USA)

T4 DNA ligase (New England Biolabs Inc., USA)

Taq DNA Polymerase (Fermentus, Inc., USA)

2.1.6 Kits and Plasmids

QIAquick™ Gel Extraction kit (Qiagen, Germany)

QIAprep Miniprep kit (Qiagen, Germany)

pGEM®-T vector system I (Promega Co., USA) (Appendix A)

pCAMBIA1301

pCAMBIA2300 (Appendix A)

pFGC5941 (Appendix A)

2.1.7 Radioactive

[α-^{32}P] dCTP (1000-3000 μCi/mmole) (Amersham Biosciences Biotech)

2.1.8 Antibiotics

Ampicillin (Sigma Chemical Co., USA)

Cefotaxime (UTOPIAN Co., Ltd., Thailand)

Hygromycin B (*Phyto*Technology Laboratories, Inc., USA)

Kanamycin (Sigma Chemical Co., USA)

Rifampicin (Sigma Chemical Co., USA)

2.1.9 Oligonucleotide primers

The oligonucleotide primers were synthesized by Operon, Germany.

2.1.10 Microorganisms

Escherichia coli

strain Top10F′: F′{*lacI*^{q} *Tn*10(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G

strain XL1-Blue: F′::*Tn*10(Tet^{R}) *pro*A^{+}B+ *lac*I^{q} *lacZ*ΔM15 *rec*A1 *end*A1 *gyr*A96 (Nal^{R}) *thi*-1 *hsd*R17 (r_k^- m_k^-) *gln*V44 *rel*A1 *lac*

Agrobacterium tumefaciens

strain EHA105 (pEHA105); a hypervirulent, L,L-succinamopine helper strain. (pEHA105 is a T-DNA deletion derivative of pTibo542, the hypervirulent Ti plasmid of *A. tumefaciens* strain A281) (Hood *et al.*, 1993).

2.2 Bacterial growth medium

Luria-Bertani broth (LB medium) (Maniatis *et al.*, 1982)

LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.2 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121° C, 1.2 kg/cm^2. If needed, a selected antibiotic drug was then supplemented.

2.3 Methods

2.3.1 Preparation of *gus* fragment

2.3.1.1 Primer design

Two oligonucleotide primers used for amplification of *gus* fragment were designed by Oligo 4.0 based on the *gus* coding sequence in pCAMBIA1301 plasmid (see in Appendix A) which was obtained from NCBI database (Accession No. AF234297). The *gus* coding sequence and its deduced amino acid sequence are shown in Figure 2.1. The designed forward and reverse primers were named GUS-F and GUS-R, respectively. GUS-F was designed with *Xba*I and *Asc*I restriction sites engineered at the 5′ end and GUS-R was designed with *Bam*HI and *Swa*I restriction sites engineered at the 5′ end. The sequence of forward and reverse primers used for the PCR amplification is as follows:

GUS-F(*Xba*I/*Asc*I)

5′- TGTCTAGAGGCGCGCCAGACTGTAACCACGCGTC -3′
*Xba*I *Asc*I

GUS-R(*Bam*HI/*Swa*I)

5′- CAGGATCCATTTAAATAATCACCACGATGCCATG -3′
*Bam*HI *Swa*I

2.3.1.2 PCR amplification

The *gus* coding sequence was amplified by PCR from the pCAMBIA1301 plasmid. The amplification reaction was performed in a 50-µl reaction containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 50-100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase. PCR amplification was performed as follows: pre-denaturation at 94 °C for 5 minutes,

```
5'-ATGGTAGATCTGAGGAACCGACGACTCGTCCGTCCTGTAGAAACCCCAACCCGTGAAATC
  M  V  D  L  R  N  R  R  L  V  R  P  V  E  T  P  T  R  E  I
AAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAG
  K  K  L  D  G  L  W  A  F  S  L  D  R  E  N  C  G  I  D  Q
CGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGTTTTAAC
  R  W  W  E  S  A  L  Q  E  S  R  A  I  A  V  P  G  S  F  N
GATCAGTTCGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAA
  D  Q  F  A  D  A  D  I  R  N  Y  A  G  N  V  W  Y  Q  R  E
GTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACT
  V  F  I  P  K  G  W  A  G  Q  R  I  V  L  R  F  D  A  V  T
CATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCAGGGCGGCTATACG
  H  Y  G  K  V  W  V  N  N  Q  E  V  M  E  H  Q  G  G  Y  T
CCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTT
  P  F  E  A  D  V  T  P  Y  V  I  A  G  K  S  V  R  I  T  V
TGTGTGAACAACGAACTGAACTGGCAGACTATCCCGCCGGGAATGGTGATTACCGACGAA
  C  V  N  N  E  L  N  W  Q  T  I  P  P  G  M  V  I  T  D  E
AACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGAATCCATCGC
  N  G  K  K  K  Q  S  Y  F  H  D  F  F  N  Y  A  G  I  H  R
AGCGTAATGCTCTACACCACGCCGAACACCTGGGTGGACGATATCACCGTGGTGACGCAT
  S  V  M  L  Y  T  T  P  N  T  W  V  D  D  I  T  V  V  T  H
GTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTC
  V  A  Q  D  C  N  H  A  S  V  D  W  Q  V  V  A  N  G  D  V
AGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAGGCACTAGCGGG
  S  V  E  L  R  D  A  D  Q  Q  V  V  A  T  G  Q  G  T  S  G
ACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTATGAACTC
  T  L  Q  V  V  N  P  H  L  W  Q  P  G  E  G  Y  L  Y  E  L
GAAGTCACAGCCAAAAGCCAGACAGAGTCTGATATCTACCCGCTTCGCGTCGGCATCCGG
  E  V  T  A  K  S  Q  T  E  S  D  I  Y  P  L  R  V  G  I  R
TCAGTGGCAGTGAAGGGCCAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGC
  S  V  A  V  K  G  Q  Q  F  L  I  N  H  K  P  F  Y  F  T  G
TTTGGTCGTCATGAAGATGCGGACTTACGTGGCAAAGGATTCGATAACGTGCTGATGGTG
  F  G  R  H  E  D  A  D  L  R  G  K  G  F  D  N  V  L  M  V
CACGACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGCATTACCCT
  H  D  H  A  L  M  D  W  I  G  A  N  S  Y  R  T  S  H  Y  P
TACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTGGTGATTGATGAAACT
  Y  A  E  E  M  L  D  W  A  D  E  H  G  I  V  V  I  D  E  T
GCTGCTGTCGGCTTTCAGCTGTCTTTAGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAA
  A  A  V  G  F  Q  L  S  L  G  I  G  F  E  A  G  N  K  P  K
GAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACTCAGCAAGCGCACTTACAGGCGATT
  E  L  Y  S  E  E  A  V  N  G  E  T  Q  Q  A  H  L  Q  A  I
AAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGCGTGGTGATGTGGAGTATTGCCAAC
  K  E  L  I  A  R  D  K  N  H  P  S  V  V  M  W  S  I  A  N
GAACCGGATACCCGTCCGCAAGGTGCACGGGAATATTTCGCGCCACTGGCGGAAGCAACG
  E  P  D  T  R  P  Q  G  A  R  E  Y  F  A  P  L  A  E  A  T
CGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCAC
  R  K  L  D  P  T  R  P  I  T  C  V  N  V  M  F  C  D  A  H
```

(continued)

Figure 2.1 The coding sequence and the deduced amino acid sequence of *gus* gene. The initiation codon was in bold letters, the stop codon is indicated by asterisk and His tag was underlined.

(continued)

```
ACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAACCGTTATTACGGATGGTAT
 T  D  T  I  S  D  L  F  D  V  L  C  L  N  R  Y  Y  G  W  Y
GTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGG
 V  Q  S  G  D  L  E  T  A  E  K  V  L  E  K  E  L  L  A  W
CAGGAGAAACTGCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGTTAGCCGGG
 Q  E  K  L  H  Q  P  I  I  I  T  E  Y  G  V  D  T  L  A  G
CTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATG
 L  H  S  M  Y  T  D  M  W  S  E  E  Y  Q  C  A  W  L  D  M
TATCACCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTTCGCC
 Y  H  R  V  F  D  R  V  S  A  V  V  G  E  Q  V  W  N  F  A
GATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATCTTCACT
 D  F  A  T  S  Q  G  I  L  R  V  G  G  N  K  K  G  I  F  T
CGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCAAAAACGCTGGACTGGCATGAAC
 R  D  R  K  P  K  S  A  A  F  L  L  Q  K  R  W  T  G  M  N
TTCGGTGAAAAACCGCAGCAGGGAGGCAAACAAGCTAGCCACCACCACCACCACCACGTG
 F  G  E  K  P  Q  Q  G  G  K  Q  A  S  H  H  H  H  H  H  V
TGA-3′
 *
```

Figure 2.1 The coding sequence and the deduced amino acid sequence of *gus* gene. The initiation codon was in bold letters, the stop codon is indicated by asterisk and His tag was underlined.

30 cycles of denaturation at 94°C for 3 minutes, annealing at 59°C for 1 minute and extension at 72°C for 1 minute. The final extension step was performed at 72 °C for 10 minutes. The PCR product was separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used for separation of DNA fragments on the basis of their molecular weights and the rough estimation of DNA on the basis of its direct relationship between the amount of DNA and the intensity level of the fluorescence after ethidium bromide staining. The resulting *gus* fragment was run on 1.0% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer. The gel was prepared by adding 1 g of agarose to 100 ml of 1X TAE. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50-60°C before pouring into a plastic gel former with the presence of a well-forming comb.

The size of separated DNA fragments was estimated from the standard curve of molecular weight markers, i.e., fragments of Lambda DNA digested with *Hin*dIII and fragments of pBR322 digested with *Msp*I. The calibration curve was plotted between logarithmic molecular mass and their relative mobilities.

The DNA was mixed with 1/10 volumes of DNA gel loading buffer (0.1M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of bromophenol blue). The mixture was loaded into slots of the gel which was submerged in the electrophoretic chamber filled with TAE. Electrophoresis was carried out at constant 100 volts. The duration of the running times depended on the size of DNA. Generally, the gel was run until the bromophenol blue moved to 1 cm from the bottom of the gel. After electrophoresis, the gel was

stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 3-5 minutes and was destained with an appropriate amount of water with gentle shaking for 10 minutes to remove unbound ethidium bromide from the agarose gel. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed.

2.3.1.4 Extraction of DNA fragment from agarose gel

The amplified product was extracted from agarose gel using QIAquick gel extraction kit protocol (QIAGEN, Germany). After electrophoresis, the desired DNA fragment was excised as gel slice from an agarose gel using a scalpel and transferred to a microcentrifuge tube. Three volumes of buffer QG (supplied by manufacturer) were added and the gel was incubated for 10 minutes at 50°C or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The gel mixture should be in yellow after the gel is completely dissolved. The mixture was then transferred into a QIAquick column and centrifuged at 12,000 rpm for 1 minute. The flowthrough solution was discarded. 750 μl of buffer PE (supplied by manufacturer) was added to the QIAquick column and the column was centrifuged at 12,000 rpm for 1 minute. The flowthrough solution was discarded. The QIAquick column was centrifuged to remove a trace element of the washing solution. The QIAquick column was placed into a sterile 1.5-ml microcentrifuge tube. DNA was eluted by an addition of 10-20 μl of steriled water to the center of the QIAquick column. The column was let standing for 1 minute, and then centrifuged at 12,000 rpm for 1 minute. The concentration of the eluted DNA was determined by agarose gel electrophoresis.

2.3.2 Cloning of *gus* fragment into pGEM®-T vector

2.3.2.1 Ligation of *gus* fragment to pGEM®-T vector

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in a ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The *gus* fragment purified by the QIAquick Gel Extraction Kit (see in 2.3.1.4) was ligated to the pGEM®-T vector (see in Appendix A) according to the plasmid supplier's recommendation. The ligation reaction was performed in the total volume of 10 µl containing 50 ng of the PCR product, 50 ng pGEM®-T vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 2 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG 8000), 3 weiss units of T4 DNA ligase. All components were mixed and incubated at 4 °C overnight. The ligation product was electrotransformed into *E. coli* Top10 cells.

2.3.2.2 Transformation of *E. coli* host cells with the ligated products by electroporation

a) Preparation of *E. coli* strain Top10 competent cells

Competent *E. coli* strain Top10 was prepared according to the method of Sambrook *et al.* (1989). The glycerol stock of *E. coli* strain Top10 was streaked onto an LB agar plate (see in 2.2) and the plate was incubated at 37 °C overnight. Ten ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) was inoculated with a single colony of *E. coli* strain Top10 and incubated at 37 °C with shaking at 250 rpm overnight. One litre of LB broth was inoculated with the starting culture and incubated at 37 °C with shaking at 250 rpm for 3-4 hours until the optical

density at 600 nm (OD_{600}) of cells reached 0.4-0.6. The cells were chilled on ice for 15-30 minutes and harvested by centrifugation at 8,000 rpm at 4 °C for 15 minutes. The supernatant was removed as much as possible. The cell pellet was washed with 100 ml of cold sterile deionized water, resuspended by gentle mixing and centrifuged at 8,000 rpm at 4 °C for 15 minutes. The supernatant was discarded. The cells were resuspended and washed further with 50 ml cold sterile deionized water, followed by 20 ml of cold steriled 10% glycerol twice. Finally, the cells were resuspended in 300 µl of cold 10% glycerol. The cell suspension was devided into 45 µl aliquots and stored at -80 °C for later use.

b) Electroporation of recombinant DNA

The competent cells were gently thawed on ice. Forty five microlitres of competent cells were mixed well with 1 to 3 µl of the ligation mixture (2.3.2.1), and then placed on ice for 1 minute. The cells were transferred to a cold cuvette, chilled on ice previously and transformed by setting the GENE pulser apparatus (Bio-RAD) as follows: 25 F, 200 Ω of the pulse controller unit, and 2.50 kV. After that, 1 ml of LB broth was added immediately to the cuvette and quickly resuspended with a pasture pipette. The cell suspension was transferred to a new tube and incubated at 37 °C with shaking at 250 rpm for 60 minutes. Finally, the cell suspension was spread on LB agar plates which contained 100 µg/ml ampicillin and 10 µl of 25 mg/ml Iso-1-thio-β-D-thiogalactopyranoside (IPTG), and 70µl of 25mg/ml 5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside (X-gal) previously spread on top of the plates and incubated at 37 °C overnight. The recombinant clones containing inserted DNA were identified as white colony, while those without inserted DNA were blue. The white colonies containing potential recombinant plasmids were selected.

2.3.2.3 Plasmid DNA isolation by alkaline lysis method

Single colonies of *E. coli* harboring the recombinant plasmids (2.3.2.2 b) was cultured in 20 ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing 100 μg/ml of ampicillin at 37 °C with shaking at 250 rpm overnight. Plasmid DNA was isolated by alkaline lysis method. The cells were spun in a microcentrifuge at 14,000×g for 1 minute at 4 °C. The cells were resuspended in Lysis buffer (50 mM of Tris base, 10 mM of $Na_2EDTA.H_2O$ and 100 μg/ml of RNaseA) and mixed by pipetting up and down. The suspension was allowed to stand at room temperature for 5 minutes, then 300 μl of Alkaline-SDS solution (200 mM NaOH and 1% SDS) was added. The suspension was inverted several times to mix and allowed to stand on ice of 5 minutes. 300 μl of High salt solution (3 M of potassium acetate) was added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 14,000 rpm at 4 °C for 15 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was precipitated by adding 480 μl (0.6 volumes) of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 30 minutes to collect the precipitated DNA. The pellet was resuspended in 90 μl of sterile water and the suspension was vortexed gently. 10 μl of 3 M sodium acetate, pH 7.0 and 300 μl of cold absolute ethanol were added to the mixture. The mixture was mixed and chilled on ice. DNA was collected by centrifuging at 14,000×g for 20 minutes at 4 °C. The pellet was rinsed with 300 μl of 70% ethanol and allowed to dry for 10-15 minutes. Plasmid DNA was resuspended with 50 μl of sterile water and stored at -20 °C. DNA concentration was estimated by measuring the absorbance at 260 nm, and calculated

in μg/ml unit, using the following equation:

$$[DNA] = A_{260} \times \text{dilution factor} \times 50^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the DNA of approximately 50 μg/ml (Sambrook *et al.*, 1989).

2.3.2.4 Restriction enzyme analysis

The recombinant plasmids were digested with *Nco*I and *Spe*I using the condition recommended by the manufacturer. The digestion was performed in the 50-μl reaction containing 5 μg of plasmid DNA, 1X enzyme reaction buffer, 1x BSA and 5 units of each restriction enzyme. The reaction was incubated at 37 °C overnight. The digestion products were analyzed by 1% agarose gel electrophoresis and the size of DNA insert was compared with Lambda DNA/*Hin*dIII ladder.

2.3.3 Construction of *gus* inverted repeat

2.3.3.1 The first construction step

a) Preparation of *gus* fragment for the sense cloning orientation

The first *gus* sequence to be inserted into pFGC5941 was prepared by digesting the recombinant plasmid (2.3.2.4) at the inner restriction sites: *Swa*I and *Asc*I as indicated in Figure 2.2. First, the recombinant plasmid (2.3.2.4) was isolated by alkaline lysis method (2.3.2.3) and digested using the condition recommended by the manufacturer. The reaction contained about 5 μg of DNA in a 50-μl reaction which included 1X enzyme reaction buffer, 1x BSA and 5 units of restriction enzyme. The reaction was incubated at 37 °C overnight. The restriction products were fractionated by 1% agarose gel electrophoresis and the size of the digested *gus* fragment was estimated by comparing with Lambda DNA/*Hin*dIII ladder. The expected

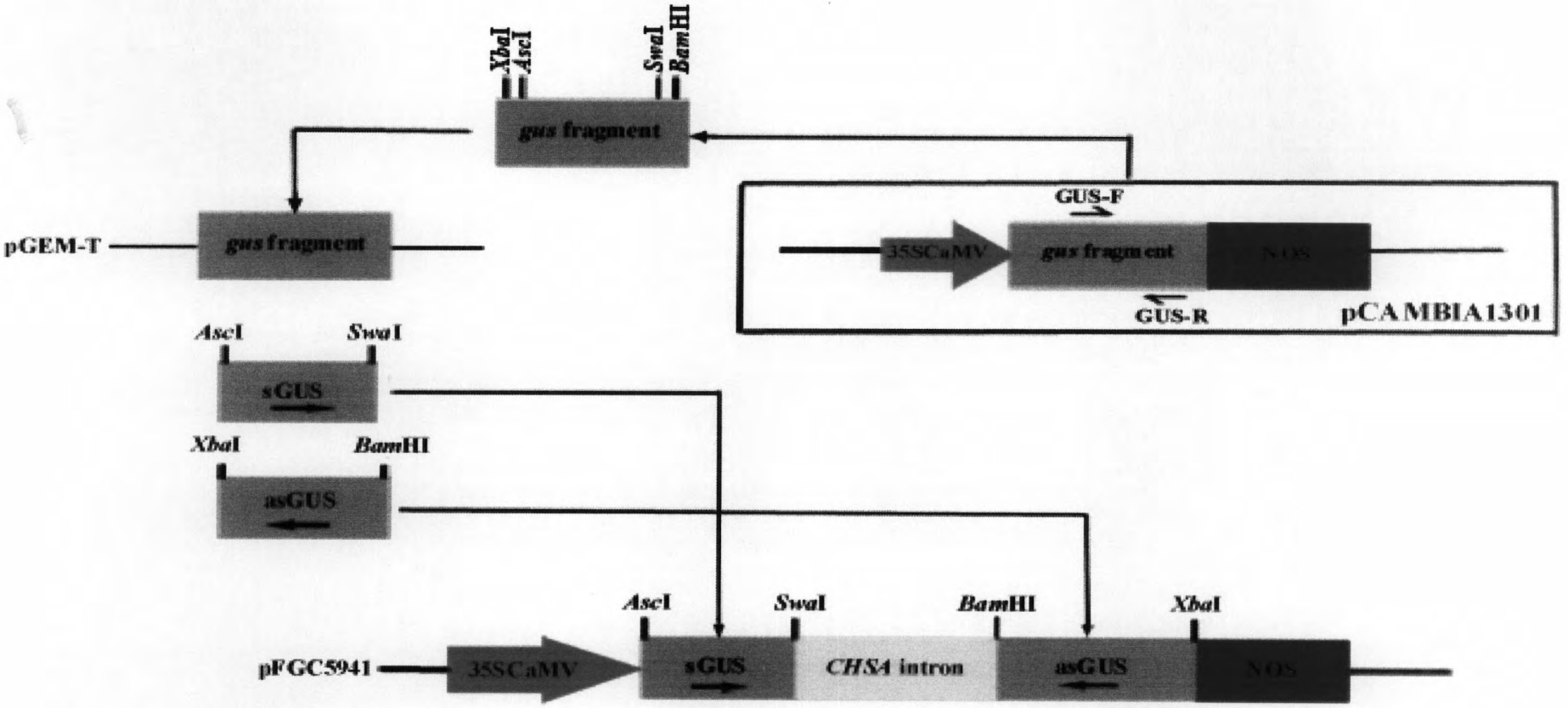

Figure 2.2 Production of the hpGUS RNAi cassette. One pair of primers were used to PCR-amplify a portion of the 495 nucleotides *gus* gene, yielding fragments with *Asc*I/*Swa*I and *Xba*I/*Bam*HI ends. These fragments were directionally cloned into the RNAi vector pFGC5941. Shown are the *gus* sense and antisense amplification fragments.

fragment was extracted from the agarose gel by using QIAquick gel extraction Kit (2.3.1.4) and dissolved in 20 µl of sterile water.

b) Preparation of pFGC5941 for the sense cloning orientation

Five micrograms of pFGC5941 (see in Appendix A) were digested with *Swa*I and *Asc*I in the total reaction volume of 50 µl using the condition as described in section 2.3.3.1 a. To get rid of proteins, phenol:chloroform (50:50) was added to the digested product and spun at 13,000 rpm for 1 minute. The top aqueous layer was transferred to a new tube and the extracted was repeated with chloroform only. Then the top aqueous layer was transferred to a new tube and the plasmid DNA was precipitated with 0.1 volume of 3 M sodium acetate pH 7.0 and 2.5 volumes of ice cold absolute ethanol. The sample was incubated on ice for 20-30 minutes and centrifuged at 13,000 rpm at 4 °C for 10-20 minutes. The supernatant was removed and the pellet was rinsed with 200 µl of 70% ethanol. The plasmid was collected by centrifuging at 13,000 rpm for 10-15 minutes and allowed to dry. The plasmid DNA was dissolved in 20 µl of sterile water and stored at -20 °C

c) Ligation of the first *gus* sequence into pFGC5941

The first *gus* fragment was ligated at the *Swa*I and *Asc*I sites of pFGC5941 vector in a 20-µl ligation reaction containing 674 ng of *gus* fragment, 168 ng of the digested pFGC5941 vector, 1X T4 DNA ligase buffer (50mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA), 5.5 weiss units of T4 DNA ligase. All components were mixed and incubated at 18 °C overnight.

d) Transformation of *E. coli* host cells with the ligation mixture by electroporation

1) Preparation of *E. coli* competent cells

The glycerol stock of *E. coli* strain XL1-Blue was streaked onto LB agar plate (see in 2.2) and incubated at 37 °C overnight. Competent cells were prepared as describe in 2.3.2.2 a.

2) Electroporation of recombinant DNA

The competent *E. coli* strain XL1-Blue were transformed with the ligation mixture (2.3.3.1 d) by electrotransformation as described in section 2.3.2.2 b. After electroporation, one ml of LB broth was immediately added to the electroporated cells and the cell suspension was removed from the cuvette to a new tube. The cell suspension was incubated at 37 °C with shaking at 250 rpm for 60 minutes. Finally, the cell suspension was spread on LB agar plates which contained 50 μg/ml kanamycin and incubated at 37 °C overnight. On the next day, colonies of *E. coli* strain XL1-Blue were randomly selected. The plasmids were extracted and digested with *Swa*I and *Asc*I. The restricted products were analyzed by 1% agarose gel electrophoresis.

2.3.3.2 The second construction step

a) Preparation of *gus* fragment for the antisense cloning orientation

The second *gus* sequence to be cloned into pFGC5941 was prepared by digesting the recombinant plasmid (2.3.2.2 b) at the outer sites: *Xba*I and *Bam*HI as indicated in Figure 2.2. First, a single colony of *E. coli* harboring the recombinant plasmid (2.3.2.4) was cultured and the plasmid was isolated by alkaline lysis method as described in section 2.3.2.3. The recombinant plasmid was digested with *Xba*I and

*Bam*HI using the condition recommended by the manufacturer. The reaction contained about 5 µg of DNA in a 50-µl reaction which included 1X enzyme reaction buffer and 5 units of each restriction enzyme. The reaction was incubated at 37 °C overnight. The restriction products were fractionated by 1% agarose gel electrophoresis and the size of the digested *gus* fragment was estimated by comparing with Lambda DNA/*Hin*dIII ladder. The expected fragment was extracted from the agarose gel by using QIAquick gel extraction Kit (2.3.1.4) and dissolved in 20 µl of sterile water.

b) Preparation of pFGC5941 for the antisense cloning orientation

Five micrograms of pFGC5941 that contained the inserted first *gus* sequence (2.3.3.1 d) were digested with *Xba*I and *Bam*HI in the total reaction volume of 50 µl using the condition as described in section 2.3.3.2 a. To get rid of proteins, phenol:chloroform (50:50) was added to the restricted product and the mixture was spun at 13,000 rpm for 1 minute. The top aqueous layer was transferred to a new tube and the extraction was repeated with chloroform only. Then the top aqueous layer was transferred to a new tube and the plasmid DNA was precipitated with 0.1 volume of 3 M sodium acetate pH 7.0 and 2.5 volumes of ice cold absolute ethanol. The sample was incubated on ice for 20-30 minutes and centrifuged at 13,000 rpm at 4 °C for 10-20 minutes. The supernatant was removed and the pellet was rinsed with 200 µl of 70% ethanol. The plasmid was collected by centrifuging at 13,000 rpm for 10-15 minutes and allowed to dry. The plasmid DNA was dissolved in 20 µl of sterile water and stored at -20 °C

c) Ligation of the second *gus* sequence into pFGC5941

The second *gus* fragment was ligated at the *Xba*I and *Bam*HI sites of pFGC5941 from 2.3.3.2 b in a 20-µl ligation reaction containing 705 ng of DNA fragment, 174 ng of the digested pFGC5941 that contained the inserted first *gus* sequence, 1X T4 DNA ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA), 5.5 weiss units of T4 DNA ligase. All components were mixed and incubated at 18 °C overnight.

d) Transformation of *E. coli* host cells with the ligation mixture by electroporation

1) Preparation of *E. coli* competent cells (see in 2.3.3.1 d)

2) Electroporation of recombinant DNA

The competent *E. coli* strain XL1-Blue were transformed with the ligation mixture (2.3.3.2 c) by electrotransformation as described in section 2.3.2.2 b. One ml of LB broth was immediately added to the electroporated cells and the cell suspension was removed from the cuvette to a new tube. The cell suspension was incubated at 37 °C with shaking at 250 rpm for 60 minutes. Finally, the cell suspension was spread on LB agar plates which contained 50 µg/ml kanamycin and incubated at 37 °C overnight. On the next day, colonies of *E. coli* strain XL1-Blue were randomly selected. The plasmids were extracted and digested with *Xba*I and *Bam*HI. The restricted products were analyzed by 1% agarose gel electrophoresis.

2.3.3.3 Restriction analysis of pFGC5941-hpGUS

The recombinant plasmid (2.3.3.2 d) isolated by alkaline lysis method (2.3.2.3) was analyzed to confirm the insertion of *gus* sequences by digestion with appropriate restriction endonucleases. For simplification, the fragment that includes

the first and the second *gus* sequences on sides the 3′ and the 5′ of the intron sequence, respectively was called hairpin GUS (hpGUS). The hpGUS recombinant plasmid was digested with *Pst*I and *Eco*RI using the condition recommended by the manufacturer. The reaction contained about 5 μg of DNA in a 20-μl reaction which contained 1X enzyme reaction buffer and 5 units of each restriction enzyme at 37 °C overnight. The retricted products were analyzed by 1% agarose gel electrophoresis. The size of the DNA fragments was estimated by comparing with Lambda DNA/*Hin*dIII ladder.

2.3.3.4 Sequence analysis of pFGC5941-hpGUS

The pFGC5941-hpGUS recombinant plasmid was extracted by QAIprep Miniprep kit (Qiagen, Germany) and the nucleotide sequence of the hpGUS was determined. DNA sequencing was carried out at Macrogen, Korea. The sequences of the forward and reverse primers used for sequencing is as follows:

sGUS	5′- CTACCTTCCCACAATTCGTC -3′
asGUS	5′- GGAGTTTATGTTTTAGTGTT -3′

2.3.4 Construction of plant expression vector for silencing *gus* gene

The pCAMBIA2300 plasmid (see in Appendix A) was employed for plant transformation. It has neomycin phosphotransferase gene (*npt*II) and LacZ alpha within the T-DNA region. The *npt*II gene was under the control of 35SCaMV promoter and was used as a plant selectable marker, which confers resistance to kanamycin. In addition, this vector harbors the pUC18 MCS polylinker within the LacZ alpha fragment allowed blue/white screening of clones in *E. coli* cloning work.

The hpGUS construct was extracted from pFGC5941-hpGUS by digestion with *Pst*I and *Eco*RI and ligated to the binary vector pCAMBIA2300 which digest with the same restriction enzymes. The ligation reaction was performed in the total volume of 20 µl containing the ratio between pCAMBIA2300 vector and hpGUS construct of 1:4, 1X T4 DNA ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA), 5.5 weiss units of T4 DNA ligase. The reaction mixture was incubated at 18°C overnight. The ligation mixture was electrotransformed into *E. coli* XL1-Blue. The white colonies were selected. The potential recombinant plasmids were extracted and analyzed by *Pst*I and *Eco*RI digestion. The restricted products were fractionated by 1% agarose gel electrophoresis. The resulting recombinant plasmid was called pCAMBIA2300-hpGUS which contained the expression cassette of the *gus* sequences in the sense and antisense orientation on sides the 5′ and the 3′ of the intron sequence, respectively under the control of the 35SCaMV promoter and the OCS3′ terminator.

2.3.5 Plant transformation

2.3.5.1 Preparation of Tobacco leaf discs

Transgenic tobacco plants (*Nicotiana tabacum* cv. Virginia Coker) harboring the T-DNA region of pCAMBIA1301 were culturally grown on MS medium (Murashige and Skoog, 1962) containing 250 µg/ml cefotaxime and 50 µg/ml hygromycin and subcultured to fresh MS basal medium every 3-4 weeks. The cultures were incubated at 25 °C under a 16/8 hours light/dark photoperiod. To prepare leaf discs, leaves of tobacco were detached from *in vitro* plantlets and placed on a Petri dish. The leaves were cut off as square sections of approximately 1.0x1.0 cm^2 and 1-2 mm of wounds made were on the square leaves by a scalpel.

2.3.5.2 Transformation of *A. tumefaciens* EHA105 host cells with pCAMBIA2300 and pCAMBIA2300-hpGUS by electroporation

a) Making competent *A. tumefaciens* strain EHA105 cells

The glycerol stock of *A. tumefaciens* strain EHA105 was streaked on LB ager plate (2.2) containing 25 µg/ml rifampicin and incubated at 28 °C for 2 days. Ten ml of LB-broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) supplemented with 25 µg/ml rifampicin was inoculated with a single colony of *A. tumefaciens* strain EHA105 and incubated at 28 °C by shaking for 8 hours. One liter of LB broth was inoculated with the starting culture and incubated at 28 °C with shaking until the OD_{600} of cells reached 1.0-1.5. The cells were harvested by centrifugation in a cold rotor at 5,000 rpm for 15 minutes. The pellet was resuspended in 1 liter of cold sterile distilled water and centrifuged as above. The supernatant was carefully discarded. The cell pellet was resuspended in 500 ml of cold sterile distilled water and centrifuged again. Then, the pellet was resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and the supernatant was removed. Finally, the cell pellet was resuspended 1 ml of 10% glycerol. The cell suspension was divided to 45 µl aliquots. These cells could be used immediately or kept at -80 °C for 6 months.

b) Electrotransfomation of *A. tumefaciens* EHA105

The competent *A. tumefaciens* strain EHA105 cells were transformed with the pCAMBIA2300 plasmid and pCAMBIA2300-hpGUS recombinant plasmid (2.3.4) by electrotransformation as described in section 2.3.2.2 b. After electroporation, one ml of LB broth was immediately added to the electroporated cells and the cell suspension was removed from the cuvette to a new tube. The cell suspension was incubated at 28 °C with shaking for 2 hours. Approximately 25-50 µl of the cell

suspension was spread on LB agar plates which contained 50 μg/ml kanamycin, 25 μg/ml rifampicin and incubated at 28 °C for 2 days. After transformation, colonies of *A. tumefaciens* strain EHA105 were randomly selected.

2.3.5.3 Co-cultivation of tobacco leaf discs

A. tumefaciens strain EHA105 harboring pCAMBIA2300 without the hpGUS and pCAMBIA2300-hpGUS were used as a transformation vehicle to mediate gene transfer into tobacco for generating control and transformed tobacco, respectively. The glycerol stock of *A. tumefaciens* strain EHA105 harboring pCAMBIA2300 and pCAMBIA2300-hpGUS were streaked on LB ager plates containing 50 μg/ml kanamycin, 25 μg/ml rifampicin and incubated at 28 °C for 2 days. Ten ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) supplemented with 50 μg/ml kanamycin, 25 μg/ml rifampicin was inoculated with a single colony and incubated at 28 °C for 2 days. Then, the cell suspension was diluted with equal volume of sterile liquid MS medium containing 0.5 mg/l MES. Leaves discs (2.3.5.1) were immersed in the cell suspension culture for 5 minutes. After that, the leaves were transferred onto CM medium (MS basal medium supplemented with 1 mg/l of BA and 1 mg/l of NAA) and incubated at 25 °C under a 16/8 hours light/dark photoperiod for 2 days.

2.3.5.4 Selection and regeneration of transformed tobacco

After 2 days, the co-cultivated leaves were transferred to selection medium (CM medium supplemented with 50 mg/l of kanamycin and 250 mg/l cefotaxime) and incubated at 25 °C under a 16/8 hours light/dark photoperiod for 7 days. Then, these leaf explants were transferred to shoot induction medium (MS basal medium

supplemented with 1 mg/l of BA and 250 mg/l cefotaxime) and subcultured to fresh medium every 2 weeks until shoots appeared. The healthy shoots were separated from the rest of leaf explants and transferred to MS basal medium for stimulation of roots. The resulting transgenic tobacco plants were subcultured on free medium every 3 weeks.

2.3.6 Molecular analysis of transformed plants

2.3.6.1 Analysis of genomic DNA of the transformed tobacco plants by PCR

a) Genomic DNA extraction

Genomic DNA was isolated from leaves of transformed tobacco plants by the CTAB method (Doyle, 1989). Plant tissues were harvested and ground to a fine powder in liquid nitrogen using chilled mortars and pestles. The frozen powder was immediately transferred to 1.5 ml microcentrifuge tubes containing 700 µl of preheated extraction buffer (100 mM Tris-HCl (pH 8.0), 40 mM EDTA (pH 7.5), 1.4 M NaCl, and 2% (w/v) CTAB, at 60 °C) and 3 µl of 2-mercaptoethanol. Then, the mixture was inverted several times to mix and incubated at 60 °C for 30 minutes. During the incubation time, the mixture was inverted gently every 10 minutes to ensure efficient extraction. After that, the mixture was left at room temperature for 5 minutes before adding 500 µl of chloroform:isoamylalcohol (24:1) and mixed for 5 minutes followed by centrifuging at 14,000 rpm for 10 minutes at 4 °C. The upper aqueous phase was transferred to a new tube. Genomic DNA was precipitated by the addition of 0.1 volumes of 3 M NaOAc and 0.6 volumes of isopropanol. The suspension was mixed and incubated at -20 °C for 30 minutes. The DNA pellet was collected by centrifugation at 14,000 rpm for 10 minutes at 4 °C and air-dried for 10-15 minutes. The genomic DNA was resuspended with 100 µl of TE buffer (10 mM

Tris-HCl, pH 7.4 and 1 mM EDTA). RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA and the mixture was incubated at 37 °C for 1 hour. After that, an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added. The sample was mixed gently and centrifuged for 1 minute. The upper aqueous phase was transferred to a fresh tube and extracted once with an equal volume of chloroform-isoamyl alcohol (24:1). The mixture was centrifuged at 14,000 rpm for 1 minute at 4 °C and the top aqueous phase was collected. 0.1 volume of 3 M NaOAc and 2.5 volumes of cold absolute ethanol were added before mixing gently. The suspension was allowed to stand for 30 minutes at -20 °C and then centrifuged at 14,000 rpm for 10 minutes to collect the genomic DNA. The pellet was rinsed with 100 µl of 70% ethanol and centrifuged at 14,000 rpm for 5 minutes. The genomic DNA was air-dried and resuspended in 50 µl of sterile water. The DNA was analyzed by agarose gel electrophoresis and the concentration was estimated by measuring the absorbance at 260 nm as described in (2.3.2.3).

b) PCR amplification of *gus* and hpGUS in the transformed tobacco plants

The putative transformants were screened using GUS check-F and GUS check-R primers for insertion of *gus* gene while that of hpGUS was screened with hpGUS check-F and hpGUS check-R primers. The sequences of forward and reverse primers used for PCR amplification is as follows:

GUS check-F	5′- TGAAGATGCCTCTGCCGACAGTGGT -3′
GUS check-R	5′- CAGGTGTTCGGCGTGGTGTAGAGCA -3′
hpGUS check-F	5′- CATGAAGATGCGGACTTACG -3′
hpGUS check-R	5′- CTACCTTCCCACAATTCGTC -3′

The amplification reactions were performed in a 50-µl reaction containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase. The reaction was pre-denatured at 94 °C for 5 minutes following by 30 cycles of denaturation at 94°C for 3 minutes, annealing at 63°C for 1 minute and extension at 72°C for 1 minute. The final extension was performed at 72°C for 10 minutes for *gus* gene and a program of 94°C for 3 minutes, 64°C for 1 minute and 72°C for 1 minute for hpGUS. After that, PCR products of the *gus* gene and hpGUS were analyzed and compared with 100 bp DNA ladder (Fermentas, USA) by 1% agarose gel electrophoresis, respectively.

2.3.6.2 Southern Blot Analysis

a) Digestion of genomic DNA with restriction enzymes

The extracted genomic DNA (2.3.6.1 a) was digested with *Hin*dIII. The reaction mixture contained 50 µg of genomic DNA, 1X enzyme reaction buffer and 50 units of restriction enzyme in final volume 40 µl. All components were mixed and incubated at 37 °C overnight.

b) Agarose gel electrophoresis

Thirty micrograms of the digested genomic DNA were mixed with 10% (v/v) of DNA gel loading buffer (0.1 M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of bromophenol blue). and loaded into a 0.8% (w/v) TAE agarose gel. Electrophoresis was carried out at constant 80 volts. The gel was run until the bromophenol blue reached the bottom of the gel.

c) Southern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 5 gel volumes of denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 20 minutes with gentle agitation and in 5 gel volumes of neutralization solution (0.5 M Trisma base, 3 M NaCl) for 15 minutes twice with gentle agitation. In the meantime, a tray was filled with 20xSSPE (3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 20 mM $Na_2EDTA.2H_2O$ and NaOH) and a supporting platform was placed on the tray. A piece of nylon membrane was cut to the dimensions of the gel and pre-wetted with distilled water. Filter paper were cut into three sheets in the same size of the gel and one long shut to be used as a wick. The platform was covered with a wick so that the edges were submerged in the transfer buffer. One sheet of filter paper in the same size of the gel was placed on top of the wick. The gel was then laid face down on the filter paper. Any air bubbles between the gel and the wet filter paper were removed. The gel was immediately overlaid with the nylon membrane and any air bubbles were again removed. Strips of saran wrap were cut and laid on each edge of the membrane and draped over the side of the tray. Another sheet of filter paper was placed on the nylon membrane. Paper towels were cut in the same size as the membrane and stacked on top of the filter to the height of about 10 cm. Then a weigh was placed on top of the stack. When three-day transfer was completed, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The DNA was immobilized on the blotted membrane by UV cross-linking in Bio-Rad GS Gene Linker™ UV chamber. After cross-linking, the membrane was stained by shaking for 3-5 minutes with methylene blue (0.015% (w/v) methylene blue, 0.3M NaOAc pH 5.2) and destained with water for 3 minutes.

d) Preparation of probes

A DNA probe for Southern blot hybridization was prepared from a purified PCR product of the *npt*II gene which amplified from pCAMBIA2300 plasmid using NptII-F and NptII-R primers. The sequences of forward and reverse primers used for preparing the probe is as follows:

NptII -F 5′- CGCAGAAGGCAATGTCATAC -3′

NptII -R 5′- GGAATGTCTCCTGCTAAGGT -3′

The amplification reactions were performed in a 50-µl reaction containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase. The reaction was pre-denatured at 94 °C for 5 minutes following by 30 cycles of denaturation at 94 °C for 3 minutes, annealing at 59 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes. After that, PCR product was analyzed and compared with Lambda DNA/*Hin*dIII ladder by 1% agarose gel electrophoresis. The amplified product was extracted from the agarose gel using QIAquick gel extraction Kit (2.3.1.4) and dissolved in 10 µl sterile water.

DIG label probe for southern blot hybridization was prepared according to manufacturer's instruction of DIG High Prime DNA labeling and Detection Starter KitI (Roche, Germany). Approximately 3 µg of the extracted DNA (used as template) were made up to 16 µl with sterile water and denatured in boiling water for 10 minutes. The denatured DNA was immediately chilled on ice for 30 seconds, and 4 µl of 5X DIG-High Prime (random primer, nucleotides, DIG-dUTP (alkali-labile), Klenow enzyme and buffer) was then added to the DNA. The reaction mixture was

incubated at 37 °C overnight and stopped by heating to 65 °C for 10 minutes or adding 2 µl of 0.2M EDTA.

e) Non-radioactive DIG hybridization

This Non-radioactive DIG hybridization and immunological detection steps were carried out using DIG High Prime DNA labeling and Detection Starter KitI (Roche, Germany). The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the DIG Easy Hyb Granules solution was prepared and warmed to 40 °C. The DIG Easy Hyb Granules solution was added to the blotted membrane placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at 40 °C for 30 minutes. When prehybridization was completed, the prehybridization solution was discarded and replaced with flesh DIG Easy Hyb Granules solution previously equilibrated to 40 °C. In the meantime, the DIG label probe was denatured in boiling water for at least 5 minutes and immediately transferred to ice. The DIG label probe was then added to flesh DIG Easy Hyb Granules solution. After removing bubbles, the bag was sealed using a heat-sealer and incubated overnight at 40 °C (the desired temperature). When hybridization was completed, the membrane was removed and immediately washed in 2X SSC (3 M NaCl and 0.3 M Sodium citrate), 0.1% SDS twice for 5 minutes each time and 0.5X SSC, 0.1% SDS twice for 15 minutes each time in a plastic box. After that, the membrane was rinsed with washing buffer (0.1 M Maleic acid, 0.15 M NaCl and 0.3% (v/v) Tween 20) for 5 minutes and the washing buffer was then replaced with 1X Blocking solution for 30 minutes. The 1X Blocking solution was discarded and replaced with Antibody solution for 30 minutes. The membrane was washed by washing buffer for 15 minutes twice and

equilibrated with Detection buffer (0.1 M Tris/HCl and 0.1 M NaCl, pH 9.5). Finally, the membrane was stained with Color-substrate solution (18.75 mg/ml nitroblue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 67% (v/v) DMSO) overnight in a plastic bag. The reaction was stopped by washing the membrane for 5 minutes with sterile water or 1X TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0).

The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be calculated using the following equation:

$T_m = 49.82 + 0.41$ (% G + C)-(600/n) ; (n = length of hybrid in base pairs)

$T_{opt} = Tm - 20$ to 25 °C

When the oligonucleotide is used as a probe, hybridization is usually carried out at 20-25 °C below the calculated T_m, and prehybridization is usually performed at the same temperature.

2.3.6.3 Northern blot analysis

The transcription of *gus* gene in transformed tobacco plants was examined using northern blot analysis.

a) Total RNA extraction

Total RNA was extracted from 50-100 mg of leaves of transformed tobacco plants using Tri-Reagent®. Plant tissues were ground to a fine powder in liquid nitrogen using chilled mortars and pestles, and immediately transferred to microcentrifuge tubes containing 1 ml of Tri-Reagent®. The mixture was homogenized by vortexing and incubated at room temperature for 5 minutes. Consequently, 200 µl of chloroform was added and vortexed again. The mixture was left at room

temperature for 15 minutes and centrifuged at 12,000 rpm at 4 °C for 15 minutes. The upper aqueous phase was transferred to a new tube. The total RNA was recovered by adding 500 µl of isopropanal and mixing thoroughly. The mixture was left at room temperature for 5-10 minutes and then centrifuged at 12,000 rpm at 4 °C for 8 minutes. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7,500 rpm at 4 °C for 5 minutes. The RNA pellet was air-dried and dissolved in diethylpyrocarbonate (DEPC)-treated water by incubating at 55-60 °C for 10 minutes. The concentration of total RNA was estimated by measuring the optical density at 260 nm, and calculating in µg/ml unit, using the following equation:

$$[\text{RNA}] = A_{260} \times \text{dilution factor} \times 40^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook et al., 2001).

b) Formaldehyde-agarose gel electrophoresis

Formaldehyde-agarose gel electrophoresis was used to analyze RNA. A 1.5% (w/v) formaldehyde agarose gel was prepared as follows. A 1.5 g of agarose was mixed with 2.5 ml of 40X Gel buffer (1.6 M Triethanolamine, 80 mM $Na_2EDTA.2H_2O$), 90 ml of H_2O and was boiled until complete solubilization. The melted agarose gel was allowed to cool to 50 °C and mixed with 8.4 ml formaldehyde in 100 ml agarose gel and poured into a chamber set which was previously treated with diethylpyrocarbonate solution and washed with DEPC-treated water.

Twenty micrograms of the total RNA (2.3.6.3 a) were mixed with sample buffer and incubated at 65 °C for 10 minutes. The sample was loaded into the 1.5%

(w/v) formaldehyde agarose gel. The RNA Ladder (Fermentas, USA) was used as a standard RNA marker. Electrophoresis was carried out in reservoir buffer at 70 volts, until bromophenol blue reached approximately ¾ of the gel length. The total RNA was visualized as fluorescent bands using a UV transilluminator and photographed.

c) Northern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 250 ml of 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 twice for 20 minutes each time and then transferred to positively charged nylon membrane by the same method of southern blotting (see in section 2.3.6.2 c).

d) Preparation of probes

A *gus* probe for northern blot hybridization was prepared from the purified PCR product of the *gus* gene which was amplified from pCAMBIA1301 plasmid using GUS-F and GUS-R primers. The sequences of forward and reverse primers used for preparing the probe is as follows:

GUS-F 5′- TGTCTAGAGGCGCGCCAGACTGTAACCACGCGTC -3′

GUS-R 5′- CAGGATCCATTTAAATAATCACCACGATGCCATG -3′

The amplification reactions were performed in a 50-µl reaction containing 1X *Taq* polymerase buffer, 5 mM $MgCl_2$, 100 ng of DNA template, 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer and 5 units of *Taq* polymerase. The reaction was pre-denatured at 94 °C for 5 minutes following by 30 cycles of denaturation at 94 °C for 3 minutes, annealing at 59 °C for 1 minute and extension at 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes. After that, PCR product was analyzed and compared with pBR322/*Msp*I ladder by

1% agarose gel electrophoresis. The amplified product was extracted from the agarose gel using QIAquick gel extraction Kit (2.3.1.4) and dissolved in 10 µl sterile water.

Nine microliters of 200 ng of DNA were mixed with 1 µl of 60 ng/µl of random hexanucleotide in the final volume of 10 µl in a microcentrifuge tube. The mixture was incubated in boiling water for 5 minutes. Then, the denatured DNA was chilled on ice for 30 seconds, and any condensation was collected by a 2-second spin in a minicentrifuge. While the tube was held behind a plexiglass shield, the labeling was started by adding 2 µl of 1X klenow buffer, 5 µCi of [α-^{32}P]dCTP, 2 µl of H_2O and 1 µl of 5-6 units of klenow fragment of DNA polymerase. The mixture was incubated for 60 minutes at room temperature and the reaction was stopped with 25 mM Na_2EDTA. The solution was passed through a spin column to remove the unincorporated [α-^{32}P]dCTP. To prepare the Bio-Rad spin column, excess liquid was removed and the column was packed by spinning for 2 minutes at 2,500 rpm. Then, the sample was loaded to the center of the packed resin and collected by spinning for 4 minutes at 2,500 rpm. To prepare for hybridization, the probe was incubated in boiling water for at least 5 minutes, then immediately added to the hybridization mixture.

e) Northern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool down to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50% (v/v) deionized formamide, 5x SSPE/NaOH (pH 7.4), and 20 mM $Na_2EDTA.2H_2O$, 1X Denhardt's solution (100X Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone (PVP) at 2% (w/v) each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 µg/ml denatured

DNA stock (calf thymus DNA and TE buffer, pH 8.0) was prepared and warmed to 40 °C. The prehybridization solution was added to the blotted membrane placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at 40 °C overnight. When prehybridization was completed, the prehybridization solution was discarded and replaced with the hybridization solution (same as prehybridized solution, but without denatured DNA) previously equilibrated to 40 °C, then the prepared denatured ^{32}P-oligolabeled DNA probes was added. After removing bubbles the bag was sealed by a heat-sealer and incubated for at least 16 hours at 40 °C. When hybridization was completed, the membrane was removed and immediately washed in 2X SSPE, 0.1% SDS twice and once in 1X SSPE, 0.1% SDS at room temperature. If the general level of cpm was too high, washes would be repeated in 1X SSPE, 0.1% SDS at higher temperature. After that the damp membrane was wrapped in plastic wrap and placed in an x-ray cassette with a KODAK BioMax MS scientific imaging film. The film was exposed two days at -80 °C. For detection, the X-ray film was developed in a developer solution and a fixer solution for 1 minute each and air-dried. The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be calculated using the following equation:

$$T_m = 16.6(\log[Na^+]) + 0.41(\%\ G+C) + 81.5 - (0.7C)(\%\ \text{formamide}) - \%\ \text{mismatch} - 500/n;\ \text{if } n < 100$$

When the oligonucleotide is used as a probe, hybridization is usually carried out at 5-12 °C below the calculated T_m, and prehybridization is usually performed at the same temperature.

2.3.7 Assays of β-glucuronidase (GUS) activity

2.3.7.1 Histochemical analysis for β-Glucuronidase (GUS) activity

Histochemical GUS assays were made after co-cultivation of calli with EHA105 (pCAMBIA2300 and pCAMBIA2300-hpGUS) and after leaves and roots were regenerated. Transgenic tobacco tissues were placed in microcentrifuge tubes and 1 ml of histochemical (X-Gluc) straining solution composed of 1 mM of X-Gluc, 100 mM NaH_2PO_4/Na_2HPO_4, 0.5 mM K Ferrocyanide, 0.5 mM K Ferricyanide, 0.1% (v/v) Triton X-100 and 10 mM EDTA was added. The tissues were vacuum infiltrated for 60 minutes and incubated at 37 °C overnight. To facilitate detection of the blue color, 70% of ethanol was added to remove the chlorophyll and to detect the formation of blue color.

2.3.7.2 Spectrophotometric analysis for β-glucuronidase (GUS) activity

a) Crude enzyme extraction

Crude enzyme was extracted from 50-100 mg of the transformed tobacco leaves according to Angela *et al.* (1990). Plant tissues were harvested and ground to a fine powder in liquid nitrogen using chilled mortars and pestles. The frozen powder was immediately transferred to 1.5 ml microcentrifuge tubes containing 200 µl of cold GUS extraction buffer (50 mM phosphate buffer, 1 M DTT and 1 mM PMSF) and centrifuged at 12000 rpm, 4 °C for 5 minutes. The supernatant (crude enzyme) was collected to a new tube and used for protein concentration determination and activity assay.

b) Protein measurement

Protein concentration was determined according to Bradford's method

(Bradford, 1976). Two microliters of each sample were mixed with distilled water to make a total volume 100 µl. Subsequently, one ml of Bradford working buffer (0.1% (w/v) Serva Blue G, 10% (v/v) of 85% phosphoric acid and 5% (v/v) of 95% ethanol) was added to the diluted protein sample and the mixture was vortexed. After 5 minutes, the protein concentration was monitored by measuring the absorbance of the generated blue color at 595 nm and calculated from the standard curve of a standard protein (BSA).

c) Determination of β-glucuronidase activity

The activity of β-glucuronidase (GUS) was assayed by measuring hydrolysis of glucuronide. The reaction mixture of 1 ml consisting of 100 µl of crude extract, 0.2 M phosphate buffer and 50 mM *p*-nitrophenyl glucuronide (PNPG) substrate in 0.2 M phosphate buffer (pH 5.2) was incubated at 37 °C for 1 hour and collected every 15 minutes during the incubation period. The reaction was stopped by addition of 0.4 M Na_2CO_3. After that, the reaction was monitored by measuring the absorbance of the liberated *p*-nitrophenol at 415 nm. The resulting absorbance values of each sample were used to generate a curve and the GUS activity was calculated through Beer's Law using a molar extinction coefficient of 14,000 l mol^{-1} cm^{-1}. One unit (U) of enzyme is defined as the amount of enzyme that will releases one pmole of *p*-nitrophenol in one minute. Specific activity is expressed as units per milligram of protein.