3.0 Materials and Methods

3.1 Plant tissue culture

3.1.1 Maintenance and subculturing of tobacco (*Nicotiana benthamiana*) explants

Healthy plantlets of *Nicotiana benthamiana* was kindly provided by the Plant Biotechnology Incubator Unit, University of Malaya (PBIU, UM). Plantlets were subcultured on Murashige and Skoog (1962) (MS) (**Appendix 1**) medium supplemented with 3% (w/v) sucrose and 0.2% (w/v) phytagel (System ® ChemAR ®, USA) every 2 weeks to obtain sufficient explant materials for transformation. The pH of the medium was adjusted to 5.8 with hydrochloric acid (HCl) and sodium hydroxide (NaOH) prior to autoclaving at 121 °C for 20 minutes at 101 kPa. All cultures were prepared under aseptic conditions and grown at 26 °C under 16 hours light: 8 hours dark photoperiod condition with a light intensity of 31.4 µmol $m^{-2}s^{-1}$ provided by cool fluorescent lamp.

Explant materials used in transformation experiments were excised from 14 – 20 days old nodal cultures. Leaf explants of approximately 0.5cm X 0.5cm in size were cut and then cultured on basal MS medium supplemented with 3% (w/v) sucrose, 0.2% (w/v) phytagel, and 0.1 mg/ ml of α-

Napthaleneacetic Acid (NAA) and 1.0 mg/ ml of 6- Benzyladenine (6-BA) for callus induction. Induced callus were then transferred to MS medium containing 1 mg/ L of 2, 4 – dichlorophenoxyacetic acid (2, 4 – D) and 0.02 mg/ L of Kinetin to induce friable callus (Young *et al*., 2004).

3.1.2 Determination of the best media for shoot induction of *C. willisii*

The basal MS media with 3% (w/v) sucrose, 0.2% (w/v) phytagel, pH 5.8 supplemented with different concentration of 6-BA (2, 4, 6, 8, 10 mg/ L) were tested for *C. willisii* shoot induction. Shoot tip explants were cut and placed onto media with different concentration of 6-BA in 60 ml disposable universal containers. Induced shoots were then transferred to basal MS0 with 3% (w/v) sucrose, 0.2% (w/v) phytagel, pH 5.8 for rooting. Experiments were done in 5 replicates. The *C. willisii* explants used in the experiments were kindly provided by the Department of Fisheries, Malaysia.

3.1.2.1 Determination of hygromycin concentration for selecting of transformed *C. willisii*

Explants were subcultured on solid media supplemented with different concentration of hygromycin. The best concentration was then selected for selecting successful transformed plant. Experiments were done in 5 replicates.

3.2 Constructs and vectors

The plasmid pCAMBIA1304 (CSIRO, Australia) kindly provided by Department of Genetics and Molecular Biology, University of Malaya was used for transformation of *Agrobacterium tumefaciens* strain LBA4404. Constructs and vectors were verified with Polymerase Chain Reaction (PCR) (as described in **Section 2.5.3**) prior to the experiment.

3.2.1 Growth of *Agrobacterium tumefaciens* harbouring binary system LBA4404 and pCAMBIA1304

The *Agrobacterium tumefaciens* culture was grown on selection medium containing 100 mg/L Kanamycin and 25 mg/L Streptomycin. A single bacterial colony was used as inoculum for 10 ml of Yeast Extract Broth (YEB) medium (**Appendix 2**) supplemented with the same selection antibiotics. Bacterial cultures were then incubated at 28 °C on rotary shaker (250 rpm) in darkness.

The optical density (OD) of *Agrobacterium tumefaciens* cultures was measured at the wavelength of 550 nm (OD_{550}) using spectrophotometer (Pharmacia Biotech Novaspec II). Reading was taken at every 1 hour interval for 40 hours until a stationary phase of bacterial growth was obtained.

Bacterial growth curve was plotted according to the value of optical density obtained. Cell density at different growth phase was then determined by using serial dilution spread-plate method. Colony forming unit (CFU) of viable bacteria cells were counted and recorded.

3.2.2 Preparation of *Agrobacterium* glycerol stock

Single colony inoculum of *Agrobacterium tumefaciens* broth culture was grown to exponential phase of growth. Fresh bacteria culture was used to prepare glycerol stock. Glycerol stocks containing 850 µl of bacteria broth and 150 µl of sterilized glycerol in microcentrifuge tubes were then stored at -80°C.

3.2.3 Minimal inhibitory concentration (MIC) of *Agrobacterium*

This experiment was carried out to determine the minimal concentration of antibiotics for the elimination of *Agrobacterium tumefaciens*. Different concentrations and combinations of cefotaxime and carbenicilin (**Appendix 2**) were incorporated in YEB broth medium and bacterial growth was observed.

Cultures without visible cell growth were then plated onto antibiotics-free YEB medium and incubated at 28 °C on rotary shaker (250 rpm) in darkness for at least 1 week. Growth/ inhibition of bacteria was then examined.

3.2.4 Preparation of plasmid DNA from *Agrobacterium*

Plasmid DNA was prepared using the method of alkaline lysis as described by Sambrook *et al*. (1989). One ml of overnight cultured bacterial cells was aliquoted into sterile microcentrifuge tubes. The cells were then centrifuged at 13,000 rpm for 1 min with centrifuge machine (Hettich Zentrifuge EBA 12 R, USA). Pellet of bacterial cells was then resuspended in 100 µl of pre-cold Solution I (**Appendix 3**) and incubated on ice for 5 mins after brief vortexing. After 5 mins, 200 µl of freshly-prepared Solution II (**Appendix 3**) was added. The contents were then inverted gently 4 – 5 times followed by 5 mins incubation on ice. An aliquot of 150 µl of pre-cold Solution III (**Appendix 3**) was then added and inverted for 5 times. The mixtures were then placed on ice for 10 mins.

The mixtures were then centrifuged at 14,000 rotations per minute (rpm) for 4 mins. Approximately 400 µl of the supernatant were retrieved and transferred to a new microcentrifuge tube. An equal volume of phenol was added in fume cupboard and mixed well. The mixtures were then centrifuged at 14,000 rpm for 2 mins. The aqueous phase (upper part) was then transferred to a new tube and an equal volume of chloroform was added. The mixtures were then centrifuged at 14,000 rpm for 2 mins after mixed well.

Two volumes of cold absolute ethanol (EtOH) were added to the supernatant and transferred into new tubes. The mixtures were then left on ice for 10 mins and later centrifuged at 13,000 rpm, 4 °C for 8 mins. Then, 1000 µl of 70 % (v/v) EtOH was added after draining away all the absolute EtOH inside the tube. The mixtures were then centrifuged at 13,000 rpm, 4 °C for 3 mins after mixed well. Finally, the pellet was dried completely by inverting on the paper towel and 50 µl of Tris-EDTA (TE) buffer (pH 8.0) was added to resuspend it. One µl of 20 µg/ ml ribonuclease (RNase) was pipetted carefully into the final product and incubated at room temperature for 20 mins.

Gel electrophoresis analysis (as described in **Section 2.5.4**) was then carried out to check the integrity of the plasmid extracted.

3.3 *Agrobacterium*-mediated plant transformation

Mid-log phase cultures of *Agrobacterium* were used for plant transformation experiments. Meanwhile, tobacco leaf explants and cut rhizome shoot base of *C. willisii* were used as target tissues for transformation experiments.

C. willisii explants were submerged in liquid cultures of *Agrobacterium* for infection (2, 4, 6, 8, and 10 mins) and then blotted-dry. The explants were then placed on MSO for co-cultivation (1, 2, 3, and 4 day) at 28 °C in darkness. Each treatment was done in duplicate and experiment was repeated. Two explants dipped in liquid broth without bacteria were used as negative control.

For the transformation of tobacco plants, standard protocol was employed (Kim *et al.*, 2003).

3.3.1 Post – Cocultivation

After the co-cultivation period, *C. willisii* explants were soaked in 300 mg/ L Cefotaxime washing solution for 10 mins to eliminate *Agrobacterium*. The explants were mixed well by shaking in the washing solution to rinse off all

the bacteria. After that, the explants were then transferred to antibiotic-containing (Cefotaxime and Carbenicilin) medium to eliminate further growth of *Agrobacterium.* Explants were then incubated at 26 $^{\circ}$C under 16 hours light: and 8 hours dark photoperiod with a light intensity of 31.4 µmol $m^{-2}s^{-1}$ in the growth room. Five mg/ L of hygromycin was incorporated into the regeneration medium for selection of transformed plants. The putative transformed plants were then subjected for further verification and confirmation to eliminate escapes.

3.4 Verification of putative transformants

3.4.1 Fluorescent microscopic visualization of green fluorescent proteins (GFP)

Putative transformed explants were subjected to fluorescent microscopic assessment after 14 – 20 days of transformation. Explants were exposed to blue ultraviolet light at excitation bandpass of 400 nm with fluorescent inverted microscope (Olympus IX71, Japan) equipped with narrowband filter of 460 – 490 nm. Pictures of explants were then captured using Olympus camera with Analyser (Olympus, Japan) software.

3.4.2 GUS assessments

3.4.2.1 Histochemical staining

Putative transformed explants tissues were stained in histochemical reagent (**Appendix 4**) which contained 0.1 M phosphate buffer, 0.5mM ferricyanide, 0.5 mM ferrocyanide, 0.1 % (v/v) Triton X- 100, 10.0 mM EDTA, 20 % (v/v) methanol and 1.0 mM 5-Bromo-3-indolyl-glucuronide (X-gluc) (Fermentas) after 2 weeks of transformation.

Explants were incubated at 37 $^{\circ}$C in darkness for 4 – 24 hours until colour developed. Stained samples were then transferred and washed in 70 %

(v/v) EtOH. Finally, stained samples were transferred and fixed in Formalin/ Acetic/ Alcohol (FAA) solution (**Appendix 4**) (Jefferson *et al.*, 1987). The GUS stains were then examined under contrast phase light microscope (Stemi SV 6, Zeiss) and photographed with Canon camera (PowerShot A650 IS) attached with the microscope.

3.4.2.2 GUS fluorometric assay

Fluorometric assay was done to quantify hydrolysis rate of GUS enzyme activity in transformed *Cryptocoryne willisii* plantlets using fluorogenic substrate 4- methylumbelliferyl β-D-glucuronide (MUG) (Sigma) as described by Jefferson (1987). GUS enzyme activities toward the MUG substrates will give rise to fluorescent 4- methylumbelliferone (4-MU). Fluoroscent signals were measured with DyNA quant 200 Fluorometer (Hoefer Scientific Instruments) at wavelength of 295nm. A calibration curve was prepared prior to samples analysis by using known concentration of commercial 4-MU (Hoefer, USA) (**Appendix 5**).

Approximately 0.2 g of *Cryptocoryne willisii* leaves samples were ground in 200 µl of GUS Extraction Buffer (GEB) (50 mM $NaHPO_4$, pH 7.0, 10 mM 2–mercaptoethanol, 10 mM Na_2EDTA, 0.1% Sodium lauryl sarcosine, 0.1% Triton X-100) (**Appendix 4**) and vortexed to mix well. Samples were

kept on ice until all of the samples have been ground. Ground samples were then centrifuged at 12,000 rpm for 5 mins at 4 °C. One hundred µl of the supernatant was taken from each cell extracts and 700 µl of GEB was added in a new tube. The mixtures were then incubated at 37 °C for 5 mins. The remaining supernatant was kept for Bradford protein assay.

After 5 mins, 200 µl of GUS Assay Buffer (GAB) (**Appendix 4**) was added to the mixtures and samples were further incubated at 37 °C for various time points (30, 60, and 90 mins). Then, 100 µl of the samples were transferred to a new microcentrifuge tube and 900 µl of 0.2 M Carbonate Stop Buffer (CSB) (**Appendix 4**) was added before the samples were assayed for fluorescent signal.

For Bradford protein assay, 5 µl of crude cell extracts were mixed with 200 µl of Bradford's reagent (Sigma) and 795 µl of sterile distilled water (dsH_20). The mixtures were then vortexed to mix well and incubated at room temperature for 10 mins in the dark. The samples were read with spectrophotometer (Pharmacia Biotech Novaspec II) at the wavelength of 595 nm (OD_{595}) for the protein contents. The OD readings were then compared to the calibration graph prepared earlier using Bovine Serum Albumin (BSA) as a standard (**Appendix 6**).

Data were expressed as pmoles of 4MU min^{-1} mg^{-1} and background fluorescent based on fluorometric measurement of negative control explants was subtracted from all of the fluorometric assay values obtained. Next, the data were subjected to analysis of variance ANOVA (SPSS, Inc).

3.5 Confirmation of transformation /Molecular assessment

3.5.1 Plant DNA (Deoxyribonucleic acid) extraction and quantification

Plant DNA was extracted with modified Doyle & Doyle method (1987). Approximately 2 g of leaf materials were ground in the presence of liquid nitrogen (N_2) using mortal and pestle.

Ground powder of plant materials were then transferred into Falcon tubes containing 10 ml of CTAB homogenization buffer (**Appendix 7**). Homogenates were then incubated in water bath at 65 $^{\circ}C$ for 60 mins. Ten ml of Chloroform: Isoamyalcohol (24: 1) were then added and the mixtures were inverted gently for 10 mins. The mixtures were then centrifuged at 6,000 rpm for 15 mins.

Supernatants were retrieved and transferred into new tubes. 2/3 volume of pre-cold Isopropanol was added into the supernatant and mixtures were kept in – 20 $^{\circ}C$ overnight to precipitate DNA. Then, the supernatants were

discarded after 15 mins of centrifugation at 6,000 rpm. Resulting pellet was then washed with 70 % (v/v) EtOH and transferred into new 1.5 microcentrifuge tube. Washed pellet was dried after centrifugation at 13,000 rpm for 10 mins.

Finally, 500 µl of TE buffer and 2 µl of RNase were added to the DNA samples. DNA samples were then incubated at room temperature for RNase to work before storing in – 20 °C.

3.5.2 DNA Quantification

Extracted DNA samples were quantified using Biophotometer (Eppendorf, USA) at wavelength of 260 nm (OD_{260}) and 280 nm (OD_{280}). Concentration of DNA samples were then calculated as equation below:

$$\textbf{DNA Concentration (µg/ µl)} = \frac{OD_{260} \times \text{Dilution Factor} \times 50\ \mu g / ml}{1000}$$

Purity of DNA samples were indicated by the ratio of OD_{260} to OD_{280} (OD_{260} / OD_{280}). Integrity of DNA samples were checked by gel electrophoresis as described in **Section 2.5.4**.

3.5.3 Polymerase chain reaction (PCR)

3.5.3.1 Primer design for *mgfp5* gene

Pair of primers was for *mgfp5* gene was designed using the software Primer3 Input 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences of *mgfp5* gene were retrieved from the database of National Centre of Bioinfomatics (NCBI) (http://ncbi.nlm.nih.org). Query was then submitted to Primer3 Input 0.4.0 in order to pick the best primers. Resulted short oligonucleotides were then subjected to primer test PCR using the software AmplifX1.4 (http://ifrjr.norp.univ-mrs.fr/AmplifX?lang=en).

3.5.3.2 PCR condition and system

The PCR reaction mixtures were prepared as described in **Table 3.1**. Master mixture was prepared with inclusion of one negative control and one positive control.

No.	Particulars	Volume
1.	10 x Reaction Buffer	2.5 µl
2.	25 mM Magnesium Chloride ($MgCl_2$)	0.75 µl
3.	10 mM dNTP mix	0.25 µl
4.	m*gfp5* Forward primer (10 µM)	0.5 µl
5.	m*gfp5* Reverse primer (10 µM)	0.5 µl
6.	*gus* Forward primer (10 µM)	0.5 µl
7.	*gus* Reverse primer (10 µM)	0.5 µl
8.	Taq Polymerase (5 U/ µl)	0.5 µl
9.	DNA template (100 ng/ µl)	2.5 µl
10.	sdH_2O	16.5 µl
Total volume per reaction:		**25 µl**

Table 3.1: Components of PCR reaction mixture used for amplification of m*gfp5* gene

3.5.3.3 Optimization of m*gfp5* genes amplification

Gradient PCR was carried out using Mastercycler Gradient thermacycler (Model 5330, Eppendorf, USA) to obtain optimum annealing temperature for m*gfp5* gene primers. Other factors that influenced specificity and sensitivity of PCR system were also taken into consideration, for instance, concentration of $MgCl_2$, primers, dNTP mix and DNA template.

3.5.4 Agarose gel electrophoresis

Extracted plasmid DNA, plant DNA and PCR products were analyzed and visualized with Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA) on different concentration of agarose gel electrophoresis as indicated in **Table 2.2**.

Samples	**Percentage (w/v) of Agarose Used for Gel Electrophoresis (%)**
Plasmid DNA	0.8 %
Plant DNA	0.7 %
PCR products	1.0 %

Table 3.2: Different concentrations of agarose gel used in electrophoresis for different types of DNA samples

Agarose gel was prepared by dissolving appropriate amount (as in Table) of agarose powder in 40 ml of 0.5X Tris-Acetate EDTA (TAE) buffer (**Appendix 7**) (Sambrook *et al*, 1982). DNA samples to be analysed were mixed with 6X loading dye (Promega, USA) (**Appendix 7**) at a ratio of 4 vol. DNA: 1 vol. of Loading dye before being loaded into the wells.

Agarose gel electrophoresis was then executed at 80 Volts for 45 minutes. Agarose gel was then viewed, photographed and analysed using Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA).

3.5.5 Southern Blotting

For Southern Blot analysis, genomic DNA of transformed *C. willisii* were digested with the restriction enzyme *HindIII* (Vivantis) prior to gel electrophoresis. Separated fragments of DNA in 1% (w/v) agarose gel were depurinated in 0.2M HCl solution for 30 mins, denatured into single-stranded (ss) DNA in denaturation buffer (1.5M NaCl and 0.5N NaOH) for 30 mins (2 times) and then neutralized in 1M Tris pH7.4 and 1.5M NaCl with agitation for 30 mins. Separated ssDNA were then transferred onto nitrocellulose membrane (Hybond-N, Amersham, US) with the set-up illustrated in **Figure 3.1** for 16 – 48 hrs. Paper cuts were changed from time to time to provide better transfer capillary action from the agarose gel to the membrane.

Membrane was then briefly air-dried and UV cross-linked at 1.5J/ cm^3 for 3 mins.

Probes were biotin-labelled by random priming with exonuclease activity-free Klenow fragment according to manufacturer's description (PureExtreme^{TM}, Fermentas, US). Hybridization was then carried out by labelled-probes-containing hybridization buffer (6X SSC, 1% (w/v) SDS and 0.01M EDTA) at 42 ℃ in hybridization oven with agitation for 16 hrs. Membrane was washed twice with 2X SSC, 0.1% (w/v) SDS at room temperature for 10 mins followed by 20 mins high stringency wash with 0.1X SSC, 0.1% (w/v) SDS at 65 ℃ for 20 mins.

Chromogenic detection was done using Streptavidin-conjugated Alkaline Phosphatase which cleaves BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) to give an insoluble blue precipitate. Biotin-labelled probes with affinity to streptavidin bind to complementary ssDNA on the membrane and reacts and gives a defined band at the site. Membrane was incubated with BCIP-T substrates solution at room temperature in the dark until blue coloration forms.

Finally, reaction was stopped by removing the substrate solution and membrane was rinsed several times with dH_2O and blotted dry on tissue paper.

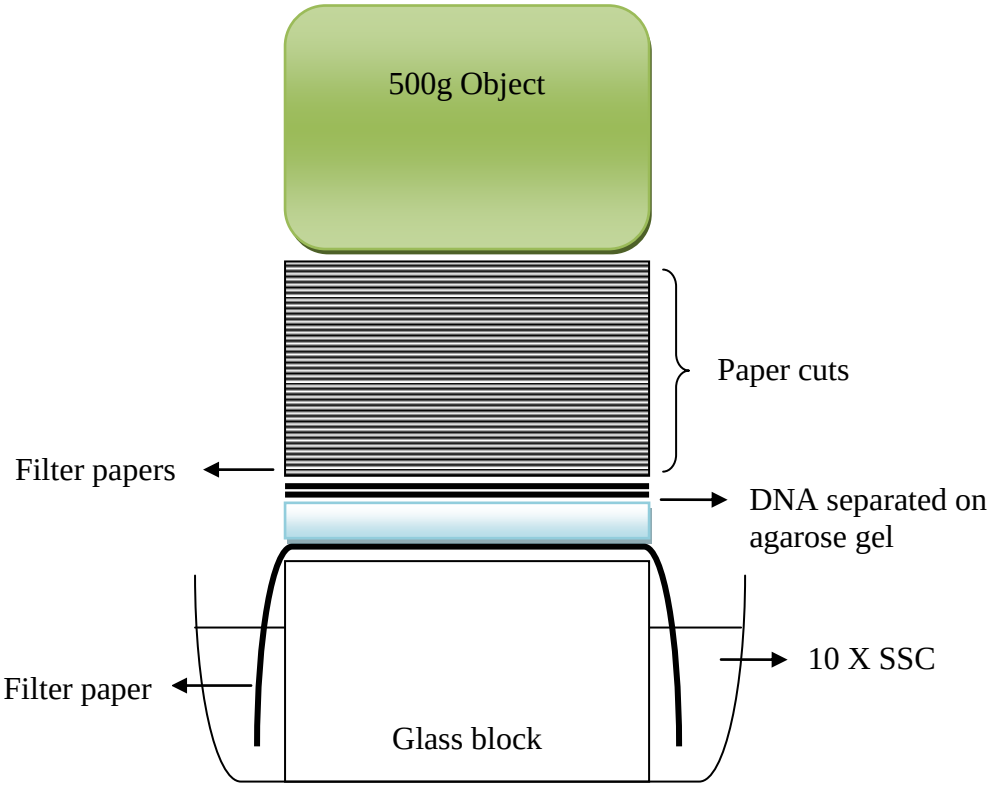

Figure 3.1: An illustration of set-up for Southern transfer