CHAPTER II
MATERIAL AND METHOD

2.1 Equipments and reagents

2.1.1 Instruments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Automatic micropipettes P10, P20, P100 and P1000 (Gilson Medical Electrical S.A., France)
- A –20 °C Freezer
- A –80 °C Freezer
- Filter paper (Whatman No. 1) (Whatman International Ltd., England)
- Fluorescent microscope BX50 (Olympus Optical., LTd., Japan)
- Gene pulser (Bio-Rad Laboratories,USA)
- Heating block Bd 1761G-26 (Sybron Thermermolyne Co.,USA)
- Incubator BM-600 (Memmert GambH, Germany)
- Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)
- PCR Thermal cycler : model 2400 (Perkin Elmer)
- PCR Thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)
- PCR Workstation : Model # P-036 (Scientific Co., USA)
- Pipette tips 10, 20, 100 and 1000 µl (Bio-Rad Laboratories, USA)
- Power supply : Power PAC 300 (Bio-RAD Laboratories, USA)
- Refrigerated microcentrifuge : Kubota 1300 (Kubota, Japan)
- Refrigerated centrifuge : Model J-21C (Beckman Instrument Inc., Japan)
- Spectrophotometer DU 650 (Beckman, USA)

2.1.2 Chemicals

- Absolute ethanol (Merck, Germany)
- Acetosyringone (Fluka, Switzerland)
- Agarose (FMC BioProducts, USA)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)

- Boric acid (Merck, Germany)
- 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) (CLONTECH Laboratories Inc., USA)
- Bromphenol blue (Merck, Germany)
- Cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co., USA)
- Ethidium bromide (Sigma Chemical Co., USA)
- Ethylene diamine tetraacetic acid, disodium salt dihydrate (Fluka, Switzerland)
- 100 mM dATP , dCTP , dGTP , dTTP (Promega Corporation Medison, Wisconsin)
- GeneAmp PCR core reagent (Promega Corporation Medison, Wisconsin)
  : 10x PCR buffer (100 mM Tris-HCL pH 8.3, 500 mM KCl)
  : 25 mM $MgCl_2$
- Isoamyl alcohol (Merck, Germany)
- Phenol, crystal (Fluka, Germany)
- Sodium acetate (Merck, Germany)
- Sodium dodecyl sulfate (Sigma Chemical Co., USA)
- Sodium hydroxide (Merck, Germany)
- Tris-(hydroxymethyl)-aminomethane (Fluka, Switzerland)

2.1.3 Antibiotic

- Cefotaxime (Merck, Germany)
- Hygromycin B (Sigma Chemical Co., USA)
- Kanamycin (Sigma Chemical Co., USA)
- Rifampicin (Sigma Chemical Co., USA)

2.1.4 Kit

- Qiaquick Gel Extraction kit (Qiagen, Germany)

2.1.5 Enzymes

- Ampli *Taq* DNA polymerase (Promega Corporation Medison, Wisconsin)
- *Eco*RI (New England Biolabs, USA)
- *Hin*dIII (New England Biolabs, USA)
- RNase A (Sigma Chemical Co., USA)
- T4 DNA ligase (Pharmacia, USA)

2.1.6 Bacterial strains

- *Escherichia coli* strain XL1 Blue
  (F$^{/}$ :: Tn*10* *proA*$^{+}$ *B*$^{+}$*lacI*^{q} Δ *(lacZ) M15/recAl endAl gyr A96(nal*^{r}*) thi hrd17* ($r_k^+ m_k^+$) *supE44 relAl lac*)
- *Agrobacterium tumefaciens* strain EHA105 (pEHA105); a hypervirulent, L,L-succinamopine helper strain. (pEHA105 is a DNA deletion derivative of pTiBo542, the hypervirulent Ti plasmid of *A. tumefaciens* strain281) (Hood et al., 1993)

2.1.7 Transformation vectors

The expression vector pBY520, which is a derivative of pBluescriptIIKS (+) (strategene), consists of two gene expression cassettes. One of the cassette gene, *hva*1 gene, is regulated by the *Act*1 promoter and the potato Pin2 3$^{/}$ region and the other one *bar* gene, which serves as the selectable marker in rice transformation, is controlled by the CaMV 35S promoter and the nopaline synthase gene 3$^{/}$ region.

The transformation vector, pCAMBIA5305 , contains the synthetic green fluorescent protein (sGFP) with optimal human codon as a reporter gene, the hygromycin-resistant gene (*hpt*) with an intron as a plant selectable marker within the left and right border of T-DNA (Appendix C). Each gene was under the control of an 35S promoter from cauliflower mosaic virus (CaMV). The *npt*II gene encoding resistance to kanamycin was used as a bacterial selectable marker. This transformation vector has a wide-host-range origin of replication from the *Pseudomonas* plasmid pVS1; the pBR322 origin (pMB9-type) to allow high-yielding

DNA preparation in *E.coli* and the T-DNA borders. Intron from castor bean catalase eliminates any possibility of read through or inappropriate *hpt* production in prokaryotes, such as *Agrobacterium.* This intron is, however, efficiently spliced in dicots and monocots.

2.2 Preparation of pCAMBIA5305*hva*1

2.2.1 Digestion of pBY520 with *Xho*1 and *Eco*R1

Plasmid digestion with various restriction enzymes for this study were performed using the buffer provided with enzymes under the conditions recommended by the manufacturers. Five micrograms of pBY520 were digested 5 units of *Xho*I in the 100 µl reaction mixture. The reaction was incubated at 37°C, overnight. The DNA was precipitated with equal volumn of absolute ethanol and dissolved in 20 µl of sterilize deionized water. Total DNA was partial digested with *Eco*RI and separated on a 1% agarose gel in 1XTBE buffer. The gel segment corresponding to the DNA fragment size 5 kb was cut out, and the DNA fragments in the gel were recovered by using QIA quick Gel Extraction Kit.

2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used for DNA fragments on the basis of their molecular weight and used for rough estimation of DNA on the basis of its direct relationship between the amount of DNA and the level of the fluorescences after ethidium bromide staining. The DNA was run on 1% agarose gel in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric and 2.5 mM EDTA, pH 8.3). The gel was prepared by adding 0.5 g of agarose to 50 ml of 1X TBE buffer. 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 a preset well-forming comb. Ten microlitres of digested DNA sample were mixed with 2 µl of the loading dye (0.25% bromophenol blue and 25% Ficoll 400) before loading into the well of gel which was submerged in the TBE buffer . The 100 bp and λ / *Hin*dIII were used as standard DNA markers. Electrophoresis was operated at 5 volts/cm with bromophenol blue

moved to approximately 0.5 cm from the bottom of the gel. The electrophoresed gel was stopped and stained with a 2.5 μg/ml ethidium bromide for 5 minutes and subsequently destained in an appropriate amount with gently shaking for minutes to remove unbound ethidium bromide from agarose gels. The DNA fragments were visualized as fluorescent bands under a UV transilluminator and photographed through a red filter with Kodak Tri-X-Pan 400 film.

2.2.3 DNA fragment elution

The 6.0 kb fragment was recovered from the agarose gel by using QIAquick Gel Extraction Kit (QIAGEN, Germany). After eletrophoresis, the desired DNA fragment was excised as gel slice from the 1% agarose gel using a scalpel and placed in a preweighed microcentrifuge tube. Three volumes of the buffer QG (supplied by the manufacture) were added and incubated at 50°C for 10 minutes or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The mixture should be in yellow after the gel was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube centrifuged at 12,000 rpm for 90 seconds. The flow through solution was discarded. An another 500 μl of buffer QG was added to the QIAquick column and recentrifuged for 90 seconds. After this step, a 750 μl of buffer PE (supplied by the manufacturer) was added to the QIAquick column and centrifuged. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace amount of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microfuge tube. DNA was eluted by an addition of 30 μl of buffer EB (10mM Tris-HCl. PH 8.5) or H_2O to the center of the QIAquick membrane and let the column standing for 5 minutes, before centrifugation at 12,000 rpm for 90 seconds.

2.2.4 Preparation of vector DNA by digesting of pCAMBIA5305 with *Xho*I and *Eco*RI

Five micrograms of pCAMBIA5305 were digested with *Xho*I and *EcoR*I in the 100 µl reaction mixture using the condition recommended by the manufacturer. The restricted product was then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The DNA was precipitated with equal volume of absolute ethanol and dissolved in 20 µl of TE buffer.

2.2.5 Ligation of two gene expression cassettes to pCAMBIA5305

The 6.0 kb cassette DNA of *hva*I gene was ligated to pCAMBIA5305 ,which was cut with *E.coR*I and *Xho*I, in 20 µl reaction containing 500 ng of DNA fragment, 100 ng of digested pCAMBIA5305, 1X T_4 DNA ligase buffer (10mM Tris-acetate, pH 7.5), 10 mM magnesium acetate and 50 mM potassium acetate), 1 mM of ATP, 5.5 weiss units of T_4 DNA ligase (Pharmacia). The reaction mixture was incubated at 14°C, overnight.

2.2.6 Transformation of ligated products to *E coli* host cells by electroporation

2.2.6.1 Preparation of competent *E coli* cells

A single colony of *E coli* XL-1 BLUE was innoculated in 10 ml of LB-broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl) and incubated with vigorous shaking at 37°C, overnight. The starting culture was incubated into 1 liter of L-broth and continued culture at 37°C with vigorous shaking to the OD_{600} of 0.5 to 0.8. The cells were chilled briefly on ice for 15 to 30 minutes, and harvested by centrifugation in a prechilled rotor at 4,000 g for 15 minutes at 4°C. The pellet were resuspended in 1 liter of cold water and centrifuged as above. After the pellet was resuspended in 0.5 liter of cold water, the suspension was centrifuged and the pellet was resuspended in 20 ml of 10% glycerol. The cell were recentrifuged, and finally resuspended in 2 to 3 ml of 10% glycerol. This concentrated cell suspension was

divided into 45 µl aliquots. These cells could be used immediately or stored at -80°C for later used.

2.2.6.2 Electrotransformation of recombinant DNA to *E.coli* host cell

The competent cells were thawed on ice for 5 minutes. One or two microlitres of the ligation mixture was added and gently mixed by pipetting. The mixture was left on ice for approximately 1 minute. The mixture was eletroporated in a prechilled 0.2 cm cuvette using a Gene pulser (Bio-Rad) with the setting paramaters of 25 µF 200Ω and 2.5 KV. After electroporation, the mixture were immediately removed from the cuvette and added to a new tube containing 1 ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) or LB broth. The cell suspension was incubated with shaking at 37°C for 1 hours. Approximately 10-50 µl of this were spreaded on a selective LB agar plates containing 50 µg/ml of kanamycin, and further incubated at 37°C, overnight. The recombinant clones containing inserted DNA were white whereas those without inserted DNA were blue.

2.2.6.3 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated using a modification of the alkaline lysis DNA method (Li et al., 1997). A white colony was incubated into a sterile tube containing 5 ml of LB broth supplement with 50 µg/ml of kanamycin and incubated with shaking at 37°C, overnight. The culture was transferred into a new 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 30 seconds. The supernatant was carefully decanted. One hundred microlitres of solution 1 (50 mM glucose, 10 mM EDTA, pH 8.0, 25 mM Tris-HCl, pH 8.0) was added to the cell pellet and vortexed. Two hundred microlitres of solution 2 (0.2 N NaOH and 1% SDS) was added and gently mixed by inversion of the tube. One hundred and fifty microlitres of solution 3 (3 M sodium acetate, pH 4.8) was added and mixed by trapping of the tube. The tube was centrifuged at 10,000 rpm for 30 seconds to pellet cell debris. The supernatant was transferred into a new microcentrifuge tube. An equal volumn of cold absolute ethanol was added and mixed by inversion following by centrifugation at 12,000 rpm

for 10 minutes. The pellet was dried in vacuum for 5 minutes. The pellet was resuspended in 50 µl of TE buffer. RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The reaction mixture was incubated at 37°C for 30-60 minutes. Plasmid DNA was stored at -20°C

2.2.6.4 Detection of recombinant plasmid

The existence of the insert DNA fragment was examined by digestion of recombinant DNA with *Xho*I and *Eco*RI. The reaction was carried out in a 20 µl standard mixture at 37°C, overnight.

At the end of digestion period, the resulting product was eletrophoretically analyzed by 1% agarose gel. The size of DNA insert was compared with that of a λ / *Hin*dIII and 100 bp DNA ladder. This recombinant plasmid was named pCAMBIA5305*hva*I and will be used in rice transformation experiment.

2.2.7 Transformation of pCAMBIA5305 and pCAMBIA5305*hva*I to *A. tumefaciens* EHA105 host cells by electroporation

2.2.7.1 Preparation of competent *A. tumefaciens* EHA105 cells (Gelvin and Schilperoort, 1994)

A. tumefaciens EHA105 was streaked on solid LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl and 1.5% Bacto agar) supplemented with 25 µg/ml rifampicin and incubated at 28°C for 2 days. A well-separated colony was innoculated in 10 ml of LB-broth supplemented with 25 µg/ml rifampicin and incubated with vigorous shaking at 28°C for 8 hours. The starting culture was inoculated into 1 liter of L-broth and continued culture at 28°C with vigorous shaking to the OD_{600} of 1.0 –1.5. The cells were harvested by centrifugation in a cold rotor at 4,000 g for 15 minutes. The pellet were resuspended in 1 liter of cold water and centrifuged as above. The supernatnat was carefully poured off. After resuspension in 0.5 liter of cold water, the pellet was centrifuged and resuspended in 20 ml of 10% glycerol. The cells were recentrifuged, and finally resuspended in 1 ml of 10% glycerol. Usually, cells can be resuspended in the 10% glycerol that remained in the

centrifuge bottle. This concentrated cell suspension was devided to 45 µl aliquots. These cells could be used immediatedly or frozen in a -80°C freezer for 6 months.

2.2.7.2 Electrotransfomation of each binary vector to *A. tumefaciens* EHA105

The transformation vectors, pCAMBIA5305 and pCAMBIA5305*hva*I were introduced into *A. tumefaciens* EHA105 by eletrotransformation using condition as described above in section 2.2.6.2. The electroporated cells were immediately removed from the cuvette and added to a new tube containing of LB-broth. The cell suspension was incubated at 28°C with shaking for 2 hours. Approximately 10-50 µl of the cell suspension were spreaded on a selective LB agar plates containing 50 µg/ml of kanamycin, 25 µg/ml rifampicin and incubated at 28°C for 2 days.

2.2.7.3 Detection of binary vector

A white colony of *Agrobacterium* was inoculated into a sterile tube containing 5 ml of LB broth supplemented with 50 µg/ml of kanamycin and 25 µg/ml rifampicin with vigorous shaking at 28°C for 2 days. The plasmid DNA was extracted by a modification of alkaline lysis method as described in section 2.2.6.3. The pCAMBIA 5305 and pCAMBIA5305*hva*I were digested with X*ho*I and *Eco*RI to determine the size of insert *hva*I cassette using the condition as describe above in section 2.2.6.4. These digested vectors were eletrophoretically analyzed by 1 % agarose gel.

2.3 Production of embryogenic calli from mature embryos

The *indica* rice cultivar (*Oryza sativa* L.) KDML 105 was obtained from the Bangkhen rice research center. Mature seeds were dehulled and first sterilized with 70% ethanol for 1 minute and then with 2.5% sodium hypochlorite for 40 minutes with shaking. The seeds were further rinsed 3 times with sterilize deionized water. These were cultured on NB (Li et al., 1993) (Appendix A) supplemented with 2 mg/l of 2,4-dinitrophenoxy acetic acid (2,4D) for callus induction. The cultures were

incubated in the dark at 28°C for 6 weeks. The embryogenic calli observed as being compact, yellowish and granular (Peterson and Smiyh, 1991) were separated with sterile scalpel and subcultured on fresh medium. Actively growing embryogenic calli (1-2 mm in diameter) were used for transformation experiments.

2.4 Transformation of binary vectors to embryogenic calli by co-cultivation with *Agrobacterium*

2.4.1 Co-cultivation of embryogenic calli

A. tumefaciens strain EHA105 habouring pCAMBIA5305 and *A. tumefaciens* EHA105 containing pCAMBIA5305*hva*1 were streaked on solid AB medium (Appendix A) supplemented with 50 mg/l kanamycin . The bacteria were incubated at 28°C for 2 days and collected by scraping from plates with platinum loop. The bacteria were resuspened in AAM medium (Appendix A) containing 100 μM of acetosyringone with vigorous shaking for a minute. The optical density of the bacterial suspension was adjusted to an OD_{600} of 0.01 by diluting with AAM medium. The embryogenic calli from section 2.3 were subcultured to fresh medium and incubated in the same condition for 4 days before using in co-cultivation. The four-day incubated embryogenic calli were immersed in bacterial suspension for 15 min with occasional shaking. The excess of bacteria was removed by decanting the liquid and calli were blotted dry on sterile filter papers (Whatman No. 1). The calli were then transferred to the co-cultivation medium (NB-AS; NB supplemented with 100 μM of acetosyringone, Appendix A) and incubated in the dark at 22°C for three days. After the co-cultivation, the calli were removed from the co-cultivation medium and blotted dry on strile filter papers whereas the *Agrobacterium* overgrowing calli were discarded.

2.5 Selection and regeneration of transformed calli

2.5.1 Co-cultivation by *A. tumefaciens* carrying pCAMBIA5305

The co-cultivation calli were transferred to selection medium (NB-CH; NB supplemented with 500 mg/l cefotaxime and 50 mg/l hygromycin; Appendix A) and incubated at 28°C for 4 weeks. The hygromycin resistant calli obtained after round of selection were subcultured for two cycles onto fresh selection mediun every two weeks. The hygromycin resistant calli were then transferred to regeneration medium without any antibiotics (NB4-RE; NB-RE containing 4 mg/l 6-benzylaminopurine (BAP), Appendix A) and incubated at 28°C under 16 hours light photoperiod for 3-4 weeks. Green shoots were observed after 4 week. When the transformed shoots become 2-3 cm. in length, they were transferred to hormone-free NB medium for stimulation of rooting and stem elongation for 4 weeks and transfer to soil for further growth.

2.5.2 Co-cultivation by *A. tumefaciens* carrying pCAMBIA5305*hva*1

The co-cultivation calli were transferred to selection medium (NB-0-0-0; NB without glutamine , casein hydrolysate and proline) supplemented with 500 mg/l cefotaxime and different concentration selection agent (6, 8, 10 mg/l ammonium glufosinate) at 28°C. All tissues are nonselectively subcultured without breaking every 12 days for 8 weeks. After the first subculture, the tissue is partially fattened on the medium to promote direct contact with herbicide. After 6-8 weeks on herbicide, resistant and positive GFP calli were selected and subcultured on fresh herbicide medium (NB-0-0; NB without glutamine , casein hydrolysate) supplemented with 500 mg/l cefotaxime and 8 mg/l ammonium glufosinate for 4 weeks. Ammonium glufosinate-resistance calli were then transferred to regenerate medium (NB4-RE-0-0; NB4-RE without glutamine , casein hydrolysate) supplemented with 6 mg/l glufosinate and incubated at 28°C under 16 hours light photoperiod for 3-4 weeks. Green buds/shoots were observed after 4 week. When the transfomed shoots become 2-3 cm.in length, they were transferred to hormone-free rooting medium (NB-R-0-0;

NB-R without hormone glutamine and casein hydrolysate) supplement with 3 mg/l ammonium glufosinate for 4 weeks.

2.6 Detection of GFP expression

2.6.1 Detection of GFP expression by fluorescent microscopy

Visualization of GFP fluorescence in leaves, roots and resistant calli was examined using an Olympus BX-50 fluorescent microscope fitted with a fluorescein isothiocyanate (FITC) filter set comprising excitation filter 450-490 nm, dichroic mirror 510 nm and barrier filter LP520 nm. The light source was provided by a HBO 50w high-pressure mercury bulb. Rice tissues were transferred to microscope slides and observed through 4X or 10X objectives. Photographs were taken using an Olympus automatic exposure photomicrographic system with Kodak Ektachrome 400 film.

2.7 Analysis of GFP, *npt*II and *hva*1 gene in total DNA of transformed rice

2.7.1 Rice DNA extraction using modified CTAB method (Weising et al., 1995)

Genomic DNA was extracted from control and putative transformed plants using the modified CTAB method.

The CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 0.2 M EDTA and 0.1 M Tris-HCl, pH 8.0) was preheated at 60°C. One gram of fresh plant tissue was harvested and grinded to a fine powder in liquid N_2 with mortar and pestle. The forzen powder was transferred to an organic solvent-resistant test tube. The 7.5 ml preheated CTAB extraction buffer was added to the tissue powder and mix throughly. The mixture was incubated at 65°C for 30 minutes with regularly swirling to ensure efficient extraction. The mixture was then extraction once with an equal volumn of chloroform-isoamyl alcohol (25:24:1) by mixed gently for 15 minutes and centrifuged at 5,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and 0.6 volumn of ice cold

isopropanol was added and the mixture was incubated at -80°C for 10 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed once with 70% ethanol. The pellet was air-dried and resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH7.4 and 1 mM EDTA). RNaseA was added to a final concentration of 200 µg/ml to digest contaminating RNA. The mixture was incubated at 37°C for 30 minutes. The DNA was then extracted once with an equal volumn of phenol-chloroform-isoamyl alcohol (25:24:1) by mixed gently for 15 minutes and centrifuged at 12,000 rpm for 10 minutes to separate phases. The upper aqueous phase was transferred to a new tube and further extracted twice with an equal volumn of chloroform-isoamyl alcohol. One-tenth volume of 3 M sodium acetate pH 5.5 was added. DNA was precipitated by an addition of two volumn of ice-cold absolute ethanol and incubated at -80°C for 15 minutes. The precipitated DNA pellet was recovered by centrifugation at 12,000 rpm for 15 minutes and briefly washed once with 70% ethanol. The pellet was air-dried and resuspended in 150 µl of TE buffer.

2.7.2 Measurement of DNA concentrations

The concentration of extracted DNA was spectrophotometrically measured at the optical density of 260 nanometre (OD_{260}). An OD_{260} of 1.0 corresponded to a concentration of 50 µg/ml double stranded DNA. Therefore, the concentration of each samples (in µg/ml) was calculated by the equation as the following:

$$[DNA] = OD_{260} \times \text{dilution factor} \times 50$$

The purity of DNA samples could be obtained by calculating a ratio of OD_{260}/OD_{280}. The ratio of 1.8 to 2.0 indicated pured DNA whereas much higher and lower values of this ratio indicated RNA or protein contamination of isolated DNA samples, respectively (Sambrook, 1989).

2.7.3 Analysis of GFP fragment from putative transformants by polymerase chain reaction (PCR)

The presence of the GFP gene in putative transformed plants was assessed by PCR. Forward and reverse primers of GFP and *npt*II were designed using the Oligo 4.0 program. PCR conditions were optimized.

2.7.3.1 PCR amplification of a *gfp* DNA fragment

The amplification reactions were performed in a 25 µl reaction volumn containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The PCR programme consisted of one cycle at 94°C for 2 min to completely melt the two-strand of genome DNA, then 30 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min and extension at 72°C for 1 min. After the last cycle, extension at 72°C for 5 min was performed to fulfill polymerization. At the end of the reaction, the PCR products were stored at 4°C.

2.7.3.2 PCR amplification of a *npt*II DNA fragment

The amplification reactions were performed in a 25 µl reaction volumn containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The PCR programme consisted in one cycle at 94°C for 2 min , then 30 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 30 sec. and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. At the end of the reaction, the PCR products were stored at 4°C.

Ten microliters of the amplification products were eletrophoresed through 1.2% agarose gel and visualized by ethidium bromide staining.

Table 2.1 Sequences of the primers used for PCR amplification of DNA fragments coding for sGFP gene, *npt*II gene and *hva*1 gene.

Gene	Sequence (5' → 3')	PCR product Size (bp)
sGFP gene	F primer : -ATG GTG AGC AAG GGC GAG GAG C- R primer : -TTA CTT GTA CAG CTC GTC CAT GCC-	720
npt II gene	F primer : -AAA ACT GAT CGA AAA ATA CCG CTG C- R primer : -TCC CCA GTA AGT CAA AAA ATA GCT C-	707
*hva*1 gene	F primer : -AGA CGA AGA TGG CCT CC- R primer : -GTC TAG TGA TTC CTG GT-	652

F primer = Forward primer

R primer = Reverse primer

2.8 RNA analysis

All solution used in RNA analysis were prepared in water treated with diethylpyrocarbonate (DEPC).

2.8.1 RNA isolate from plant tissue and synthesis of first-stand cDNA

Leaf sample 0.1 grams was powdered with pestle and mortar in the presence of liquid nitrogen and then transferred to eppendrof tube immediately. One ml of TRIZOL reagent (GIBCO-BRL) was added to powder tissue and incubated at room temperature for 5 min. The mixture was further added with 0.2 ml of chloroform and shaked vigorously for 15 sec then incubated at room temperature for another 3 min. The mixture was spun in refrigerature centrifuge at 12,000 rpm for 10 min. The gel like RNA pellet was collected by centrifugation at 12,000 rpm for 20 min at 4°C and washed once with 1 ml of cold 75% ethanol and recentrifuged at 12,000 rpm for 10 min at 4°C. The washed RNA pellet was air-dried until ethanol evaporated

completely and dissolved in sterile deionized water. One µl of RNA solution was measured the optical density at 260 nm, and the remain was stored at -20°C. For long term storage, total RNA was precipitated and kept in absolute ethanol, at -70°C.

First stand cDNA templates for PCR were prepared by using Omniscript Reverse Transcriptase (Qiagen)

2.8.2 PCR amplification of a *hva*1 DNA fragment from cDNA

The amplification reactions were performed in a 25 µl reaction volumn containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of each primer and 1.0 unit of Ampli *Taq* DNA polymerase. The PCR programme consisted of one cycle at 94°C for 2 min , then 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 30 sec. and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. At the end of the reaction, the PCR products were stored at 4°C.

Ten microliters of the amplification products were eletrophoresed through 1.2% agarose gel and visualized by ethidium bromide staining.