I. AGROBACTERIUM TUMEFACIENS TRANSFORMATION

The LBA4404 strain of *Argrobacterium tumefaciens* (LBA 4404) carrying either of the genes constructs plasmids were used to transform tobacco (*Nicotiana.tabacum* cv. xanthi) and tomato (*Lycopersicon estculentum* cv. Money maker, Cheery tomato) following the standard procedure described by Horsch *et al*., (1985).

Surface-sterilized leaf discs or other axenic explants were infected with the *A. tumefaciens* carrying the vector of choice and co-cultured on regeneration medium for 2 to 3 days without any selection pressure. After the co-cultivation, the explants were transferred to regeneration/selection medium. This contained 500 mg/L carbenicillin (or 250 mg/l cefotaxime) to kill the bacteria and the appropriate antibiotic (hygromycin / kanamycin depending on the construct used) to inhibit untransformed plant cells. During the next 3 weeks, the transformed cells grew into callus or differentiated into shoots via organogenesis. Within 3 to 6 weeks, the shoots developed enough to remove them from the explants and induce rooting in preparation for transfer to soil.

For tomato, seeds were sterilized by soaking in bleach solution for 20-30 minutes followed by at least three rinses in sterile water, germinated on RM medium and grew under moderate (room) light and temperature until cotyledons were fully expanded. Cotyledons were removed by cutting at their petiole, and then cut in half to increase the wound edge. Explants were precultured for 1 or 2 days upside down on RMOP medium to allow initial growth and to eliminate those that were damaged during sterilization or handling. *Agrobacterium tumefaciens* culture was grown overnight in LB with appropriate antibiotics to select for the vector. The culture was prepared for inoculation of explants by taking an overnight culture and diluting 1 to 10 times with liquid RM medium to get O.D around 0.6. Explants were inoculated by immersion in the culture of *Agrobacterium tumefaciens* and blotted dry gently as soon as all wounded edges have contact the inoculums. The explants were placed upside down

on culture plates and incubated for two to three days. Transferred explants to RMOP selection medium and incubated under light for 4 to 6 weeks. After 2-3 weeks, cut explants to separate clearly independent sites of transformation and transfer to fresh selection medium. Transferred entire explants to RM rooting medium as shoots appeared, even if not suitable for removal of individual shoots from the explants. When rooted, agar from base of plantlets was washed off and planted in sterile soil in pots. Placed pots in transparent boxes and closed tightly to retain humidity. After 7 to 10 days, slowly crack opened to reduce the humidity gradually until plants are acclimatised to the ambient humidity. Fertilized and grow under standard plant growth conditions.

II. PARTICLE GUN MEDIATED TRANSFORMATION

Preparation of gold particles for shooting

Taken 50 mg of tungsten powder in a microcentrifuge tube, added 1 ml of freshly-opened absolute ethanol, set water bath at 95^0C and kept the tungsten containing microcentrifuge tube for 2 hours with intermittent inversion and tapping. Centrifuged for 10 seconds at high speed in a microcentrifuge, discarded the supernatant. Resuspended the tungsten particles in 1 ml of fresh ethanol and transferred into 15 ml round bottom falcon plastic tube. Sonicated 3 times for 5 minutes each while keeping on ice, transferred the tungsten suspension to an eppendorf tube and centrifuged 10 seconds at high speed. Discarded the supernatant and washed tungsten with sterile double distilled water three times and resuspended the tungsten finally in 1 ml water.

Sample preparation

Mixed the below components maintaining the order with slow vortexing before adding the next component

1. 50µl tungsten solution
2. 10µl DNA in TE buffer (1µg/µl)

3. 50µl of $CaCl_2$ (2.5M)
4. 20µl spermidine free base (0.1M)

Vortexed for 30 minutes in cold room and proceeded further with the sample preparation (Keept the sample on ice throughout the preparation period). Added 200 µl ethanol, centrifuged 10 secs at 10,000 rpm and removed supernatant. Added 200µl ethanol and resuspended very well by pipetting or mild vortexing, centrifuged 10 secs and replaced the ethanol and repeated the above process 3 times. Resuspended the final DNA coated tungsten in 30µl of ethanol. Used 5µl sample for shooting each leaf or embryogenic calli

Coating DNA to microcarriers

Aliquoted out 5 µl of DNA coated tungsten suspension onto macrocarrier disc and allowed alcohol to evaporate in a sterile environment (a laminar flow hood).

Preparing rice embryogenic calli for bombardment

Dehulled the mature seeds and sterilized in 70% ethyl alcohol for 1 min and then in 45% chlorox (5-25% Na-hypochlorite solution) with 1-2 drops tween-20 for 30 minutes. Rinsed seeds with sterile distilled water 4-5 times and inoculated sterile seeds in callus introduction medium RM with 2 mg 2,4 D/l. After 6-7 days, separated the callus from scutellar tissue from the endosperm and inoculated them on callus introduction medium. After 1 month collected callus and incubated in a gyratory shaker at 90-120 rpm, subcutured the callus every week for first month and every two weeks for next 5-6 months. Collected embryogenic callus and arranged these in a 1-inch diameter circle at the centre of a Petri dish containing bombardment medium and 2 sheet of Whatman #4 circles paper (sterilized), then incubated the samples overnight in dark.

Shooting rice embryogenic calli

Biorad shooting kit was used through out the study (500 Standard pressure kit cat No. 165-2283). Before shooting, sterilized the number of discs and stopping screens that were need in a Petri dish containing absolute ethanol (100%) for 10 – 15 min.. The discs and screens are dried by standing them up along the side of a sterile Petri dish and allowed them to air-dry under sterile conditions in the laminar flow hood. Bombarded the calli as per the procedure described in the Biorad PDS-1000 He manual.

Transferred bombarded explants to RM2D (RM medium with 2,4-D 2 mg /l) containing 50 mg hygromycin/l. Incubated in dark at 25^{o}C for 3 weeks. Transferred surviving embryogenic calli to RM2D containing the same concentration of antibiotic, selected 3 times at 2 weeks interval. Transferred only the embryogenic calli that showed fresh growth to the next step. Transferred developing pro-embryos to generation medium with or without selection. Transferred the plantlets to rooting medium (without hormones and antibiotic).

III. CONSTRUCTION OF PLASMID VECTORS AND DNA PURIFICATION

1. Plasmid DNA isolation (alkali method)

This method was adopted from Sambrook *et al.* (1989). Picked single colonies of bacteria harboring the plasmid DNA of interest into a Flask containing of LB media supplemented with the appropriate antibiotic and incubated at 37^{o}C for overnight with shaking at 250 rpm. Harvested the cells by centrifugation at 5000 rpm for 5 minutes and decanted the supernatant. The pellet was suspended in 1.5 ml of solusion I (25mM Tris-HCL, pH 8.0, 10mM EDTA, 50 mM Glucose) mixed, and incubated for 5 minutes on ice. Added 2 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS), mixed by inverting and incubated at room temperature for 5 minutes. Added 1.5 ml of

solution III (3M potassium Acetate, pH 4.8), mixed by inverting and incubated on ice for 20 minutes. Centrifuged at 12,000 x g for 10 minutes at 4^{o}C, the supernatant was collected and treated with RNase A (10 mg/ml) at 37^{o}C for 30 minutes. The supernatant containing plasmid was extracted twice with phenol: chloroform: isoamyl alcohol (24:24:1). Precipitated the DNA by adding 2 volumes of 95% ethanol or by 0.7 volume isopropanol and resuspended the dried DNA pellet in TE buffer.

2. Plasmid DNA purification by Qiagen column

After the cells were harvested by centrifugation at 5000 rpm for 5 minutes, resuspend the bacterial pellet in 4 ml of buffer P1 premixed with RNase A. Added 4 ml of buffer P2, mixed gently by inverting 4-6 times and incubated at room temperature for 5 minutes. Added 4 ml of buffer P3, mixed immediately but gently and incubated on ice for 15 minutes. Centrifuged 12,000 rpm for 30 minutes at 4^{o}C. Equilibrated a QIAGEN-tip 100 by applying 4 ml of buffer QBT and allowed the column to empty by gravity flow. Applied the supernatant from centrifuge to the QIAGEN-tip and entered the resin by gravity flow. Washed the QIAGEN-tip with 2 x10 ml of buffer QC. Eluted DNA with 5 ml of buffer QF and precipitated DNA with 0.7 volumes of room temperature isopropanol. Centrifuged immediately at 12,000-15,000 x g for 30 minutes at 4^{o}C and carefully removed the supernatant. Washed DNA with 2 ml of 70 % ethanol, air dried for 5 minutes and redissolved in a suitable volume of TE buffer.

3. Restriction digestions

Restriction enzyme digestions are performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier, and at the optimal temperature for that specific enzyme. Typical digestions included a unit of enzyme per microgram of starting DNA for 1 hour to overnight depend on enzyme to insure complete digestion. The volume of the reaction depended on the amount and size of the DNA being digested. Larger

DNA amounts were digested in larger volumes (between 50-100 µl). For double digestion, a suitable buffer was used based on suppliers instructions.

4. Agarose gel electrophoresis

Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel or used for membrane transfer. Prior to gel casting, dried agarose is dissolved in buffer by heating and the warm gel solution then is poured into a mold, which is fitted with a well-forming comb. The percentage of agarose in the gel varied. Although 0.7% agarose gels (in 1X TEA or TEB buffer) typically are used, in cases where the accurate size fractionation of DNA molecules smaller than 1 kb is required, a 1, 1.5, or 2% agarose gel is prepared, depending on the expected size(s) of the fragment(s). Ethidium bromide is included in the gel matrix to enable fluorescent visualization of the DNA fragments under UV light. Agarose gels are submerged in electrophoresis buffer in a horizontal electrophoresis apparatus. The DNA samples are mixed with gel tracking dye and loaded into the sample wells. Electrophoresis usually is at 70 - 100 volts for 0.5-1 hour at room temperature, depending on the desired separation. Size markers were co-electrophoresed with DNA samples, often λ DNA digested with *HindIII*, was used when appropriate for fragment size determination. After electrophoresis, the gel was placed on a UV light box and a picture of the fluorescent ethidium bromide-stained DNA separation pattern was photographed with a camera (gel documentation system).

5. Elution of DNA fragments using Qiaquick gel extraction columns

The agarose gel piece containing DNA fragment was taken in a microcentrifuge tube, added 3 volumes of QG buffer as supplied with QIAQUICK gel extraction kit and dissolved by heating 60^{o}C for 15 minutes. The mixture was loaded on to QIAQUICK spin column and spun briefly. The flow through was discarded and the column was

washed twice with buffer PE. The purified DNA fragment was eluted with 50 µl of TE buffer.

6. DNA ligation

DNA ligations were performed by incubating DNA fragments with appropriate linearized cloning vector in the presence of a buffer and T4 DNA ligase. For blunt end ligations or single digestion, dephosphorylated double-stranded DNA linearized vector with Alkaline Phosphatase (AP) was used. These usually included parallel ligations in the absence of insert DNA to determine the background clones arising from self-ligation of inefficiently phosphatased vector. Parallel ligations also were performed with a known blunt-ended insert to insure that the blunt-ended ligation reaction would yield sufficient insert containing clones, independent of the repair process. Combined the following reagents in a microcentrifuge tube and incubated overnight at 4^{o}C to 16^{o}C. A typical reaction included the following:

DNA fragments	100-1000 ng
Cloning vector	2 µl (10 ng/µl)
10X ligation buffer	1 l (or 2 µl)
T4 DNA ligase (NEB 202L)	1 µl (400 U/µl)
sterile ddH2O to	10 µl (or 20 µl)

Depend on concentration of vector DNA and fragment, volume was adjusted for ligation reaction.

7. *E.coli* competent cell preparation and transfer of plasmid DNA

For the calcium chloride method, a glycerol cell culture stock of the respective *E. coli* strain DH5α was thawed and added to 5 ml of liquid media. This culture then was preincubated at 37^{o}C for overnight, transferred to flask containing 50 ml LB and incubated at 37^{o}C on incubator-shaker for 2-3 hours. The cells were pelleted by centrifugation, resuspended in calcium chloride solution, and incubated in an ice-

water bath. After another centrifugation step, the resulting cell pellet again was resuspended in calcium chloride with 12.5 % glycerol to yield the final competent cell suspension. Competent cells are stored at -80^{o}C.

In detail, thawed a frozen glycerol stock of the appropriate strain of *E. coli*, added it to a falcon containing 5 ml of LB media, and pre-incubated at 37^{o}C overnight with shaking. Inoculated 5ml of overnight culture in Erlenmeyer flask containing 50 ml LB for 2-4 hours incubated at 37^{o}C with shaking at 250 rpm. Transferred 40 ml of the cells to a sterile 50 ml polypropylene centrifuge tube, and collected the cells by centrifugation at 3000 rpm for 10 minutes at 4^{o}C. After centrifugation, decanted the supernatant and resuspended the cell pellet in 20 ml of cold, sterile 50 mM calcium chloride, incubated in an ice-water bath for 20 minutes, and centrifuged at 3000 rpm for 10 minutes at 4^{o}C. Decanted the supernatant and gently resuspended the cell pellet in 4 ml of cold, sterile 50 mM calcium chloride (with 12.5% glycerol) to yield the final competent cell suspension. The competent cells then should be placed at -70^{o}C and can be stored indefinitely

To use competent cells for transformation, removed from freezer and thawed for a few minutes at 37^{o}C, placed on ice, added plasmid DNA or ligation reaction and incubated for 15-20 minutes. Heat shock was given at 42^{o}C for 2 minutes and cooled briefly, added 0.7 ml of LB and incubated for 1 hour at 37^{o}C before spreading on selection LB plates with supplement of appropriate antibiotic.

8. *Agrobacterium* competent cell preparation and transfer of plasmid DNA

Inoculated colony in 2 ml YEM with streptomycin (25mg/l) and incubated overnight at 30^{o}C shaker. Transferred culture to 200 ml YEM in a sterile 500ml flask and kept at 30^{o}C with shaking at 250 rpm until the OD reached 0.3 (4-5 hours). Spun in sterile cap tubes 3000 rpm at 4^{o}C for 10 minutes, checked to make sure cells are pelleted, if not repeated at higher speed. Aspirated supernatant, resuspended pellet in 20ml ice cold 0.1M calcium chloride solution (filter sterile), respun and resuspended pellet in

2ml ice cold sterile 0.1M calcium chloride (with 12.5% glycerol). Dispensed in 50µl aliquots in pre-chilled, sterile microcentrifuge tubes, frozen in liquid nitrogen and stored at -70^{o}C.

To use competent cells for transformation, removed from freezer and thawed for a few minutes at 37^{o}C, placed on ice, added plasmid DNA and incubated was given for 15-20 minutes as in the standard transformation procedure. Cool shock was given in liquid nitrogen for 2 minutes, 5 minutes at 37^{o}C and cooled briefly on ice, added 0.7 ml of LB and incubated for 1 hour at 30^{o}C before spreading on selection LB plates with supplement of appropriate antibiotic.

IV. GUS ASSAY – HISTOCHEMICAL

Solutions

1. 0.5 M MES (2-[N-Morpholino] ethanesulfonic acid) pH 5.6
   For 100 ml: dissolve 9.76 g of MES (Sigma) in 80 ml double H_2O. Adjusted pH to 5.6 with NaOH and made up the volume. Stored at room temperature.
2. 50 mM Sodium phosphate buffer (Na PO_4) pH 7.0
   57.7 ml of Na_2HPO_4 (1 M stock)
   42.3 ml of NaH_2PO_4 (1 M stock)
   Distilled water to 2000 ml
3. Histochemical reagent [2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 50 mM $NaPO_4$, pH 7.0]
   For 10 ml: Dissolved 10 mg of X-Gluc in 100 µl of dimethyl formamide in a pyrex tube. Made up to 10 ml with 50 mM $NaPO_4$ pH 7.0. Made up fresh when required. Stored solid X-Gluc-desiccated at -20^{o}C or -70^{o}C.
4. 70% Ethanol

Placed the tissue in fixation solution (formaldehyde 0.2% in 50 mM NaPO4 pH 7.0) and vacuum infiltrated tissue briefly (2 min) and incubated for 4-5 min at room

temperature followed by several washes (3 times) in 50 mM $NaPO_4$ pH 7.0. Added 100 µl of X-gluc and incubated for 2 to 4 hours (or overnight) at 37^{o}C. After staining, rinsed sections in 70% ethanol for 5 minutes and then mounted on microscope slides or visualized directly in a Petri plate under miscropse.

V. GUS ASSAY-SPECTROFLUOMETER

GUS extraction buffer

50 mM Na_2HPO_4, pH 7.0

10 mM beta-mercaptoethanol

10 mM EDTA

Assay buffer (MUG)

1mM MUG in extraction buffer (Dissolve 22 mg 4-methyl-umbelliferyl-beta-D-glucuronide in 50 ml GUS extraction buffer).

Stop Buffer

0.2 M Na_2CO_3. Dissolve 21.2 gm Na_2CO_3 in 1L of distilled water.

Calibration standards

1 mM methylumbelliferone (MU). Dissolved 19.8 mg methylumbelliferone in 100 ml distilled water and wraped bottle in aluminium foil and stored at 4^{o}C.

+ 100 µM MU: add 100 µl of 1 mM stock solution to 900 µl stop buffer, mix well.

+ 10 µM MU: add 100 µl of 100µM to 900µl stop buffer, mix well.

+ 1µM MU: add 100 µl of 10 µM MU to 900µl stop buffer, mix well.

Extraction and assay

Homogenized tissue (100 mg) in 300 µl of GUS extraction buffer and centrifuged at 12,000 rpm. Quantified the protein. Added 1-5 µg of protein to 1 ml assay buffer. Mixed thoroughly with pipet or vortexed. At defined time intervals (0, 15, 30, 60, 90

min), removed 200 µl aliquots into Eppendorf tubes containing 800 ml stop buffer. Read fluorescence with excitation at 365 nm emission at 455 nm in DNA FLUOROMETER (model TKO100).

Calibration

Took 1 ml stop buffer and set the reading to `0' (zero). Added 1 ml of 1000 nM MU into cuvette and set the reading to 1000. Read the sample. The value get was expressed as nM of MU due to GUS activity in the sample.

Quantitation of proteins (Bradford method)

Materials

1. 0.5 mg/ml bovine serum albumin (BSA)
2. 0.15 M NaCl
3. Bradford reagent from BioRad

Aliquoted 0, 5, 10, 15 and 20 µl of 0.5 mg/ml BSA and added 0.15 M NaCl to bring the volume to 100 µl in each tube. Added 1 ml Bradford reagent (five times diluted) and vortexed, waited 2 min at room temperature. Determined the OD at 595 nm using spectrophotometer and made a standard curve by plotting absorbance versus protein concentration. Determined the absorbance for the unknown and used the standard curve to determine the concentration of protein in the unknown sample.

VI. PCR ANALYSIS

Standard procedures were followed for PCR amplification of DNA fragments. The general procedure followed is given below:

A. Template DNA	2 µl (contained about 0.5 µg of DNA)
B. Taq Buffer (10 X)	5 µl
C. dNTPs (1mM)	5 µl
D. Primer GUS 5'	1 µl (10-25 pmoles)

E. Primer GUS 3'	1 µl (10-25 pmoles)
F. Taq polymerase	1 µl (2 –2.5 Units)
G. Water	35 µl
Total	**50 µl**

PCR parameters

Temp (°C)	Time sec	Temp (°C)	Time sec	Temp (°C)	Time sec	Cycles No.
94	30	65	30	72	120	2
94	30	63	30	72	120	2
94	30	61	30	72	120	2
94	30	59	30	72	120	2
94	30	57	30	72	120	2
94	30	55	30	72	120	2
94	30	53	30	72	120	2
94	30	51	30	72	120	2
94	30	49	30	72	120	25
94	30	49	30	72	600	1

Analysed the PCR products on Agarose gel.

VII. NUCLEIC ACID ANALYSIS

1. Isolation of genomic DNA from plant tissue

Small piece of leaf tissue (1 cm x 1 cm) was ground with blue micropistil in an Eppendorf tube. Added 400 µl of extraction buffer and ground with pistil, repeating this process until it became a homogenous paste. Incubated at 60°C in a water bath for 30 minutes and extracted twice with 700 µl $HCCl_3$/Isoamyl alcohol (24:1). Took the supernatant and precipitated the DNA with 2/3 volume Isopropanol. Incubated for

30 minutes at -20^{o}C, spun for 3 minutes with full speed in microcentrifuge at room temperature. Washed the pellet with 70% ethanol 3 times. Resuspended the DNA in 30-50 µl of T.E buffer or H_2O.

Extraction Buffer	**100 ml**
2% CTAB	2 grams
1.4 M NaCl	(28 ml of 5M)
20 mM EDTA (pH8)	(4 ml of 0.5M)
100 mM Tris/HCl (pH8)	(10 ml of 1M)
100 mM bME (fresh)	1ml neat

2. Isolation of total RNA from plant tissue

All the plastic and glass ware was treated with DEPC and autoclaved to eliminate possible contamination of RNAse. Powdered 0.25g of leaf tissue in a mortar pestle in liquid nitrogen, added 0.5 ml extraction buffer and allowed frozen tissue to thaw and transferred into an eppendorf tube. Added 0.5ml phenol preheated to 70^{o}C to the powdered tissue, vortexed for 30 seconds. Centrifuged for 5 min at 14,000 rpm to separate phenol. Transfer the supernatant to a clean eppendorf tube. Re-extracted phenol with 0.5 ml extraction buffer. Centrifuge for 5 minutes at 14,000 rpm to separate phenol from extraction buffer. Combined supernatants in a clean tube from the two extraction steps. Added 0.5 ml of phenol-chloroform to the supernatant. Vortexed 20 seconds and separated phases by centrifugation for 5 minutes at 4^{o}C. Transferred the supernatant to a fresh eppendorf tube and added 1/3 volume of 10M LiCl (Ratios of supernatant: 10M LiCl 600 µl: 200 µl; 700 µl: 233 µl; 800 µl: 267 µl), Mixed and precipitated overnight at 4^{o}C. Centrifuged at 14,000 rpm for 10 min at 4^{o}C, washed the pellet with 2.5M LiCl solution at 4^{o}C. Washed the pellet with 70% ethanol (made in DEPC treated water) at 4^{o}C. Vacuum dried the RNA and dissolved in sterile DEPC treated water (0.1 ml).

- **RNA Extraction Buffer** (Final Concentrations)
  0.2M Sodium Acetate (pH 5.2)
  1% SDS
  0.01M EDTA (pH 8.0)

- **Composition of RNA extraction Buffer** (500 ml)
  40.67 ml Sterile Water
  3.33 ml 3M Sodium Acetate (pH 5.2)
  5.0 ml 10% SDS
  1.0 ml 0.5M EDTA (pH 8.0)
- Phenol-saturated with Tris.Cl (pH 8.0)
- Phenol-chloroform
- 10M LiCl sterilized by autoclaving
- 2.5M LiCl sterilized by autoclaving

Using TRIzol Reagent method (Gibco BRL)

Homogenized tissue samples in 1ml of TRIzol Reagent per 50-100 mg of tissue using glass homogenizer. Following homogenization, insoluble material from the homogenate was removed by centrifugation at 12,000 x g for minutes at 2 to 8^{o}C. The supernatant contained RNA. Incubated the homogenized samples for 5 minutes at 15 to 30^{o}C to permit the complete dissociation of nucleoprotein complexes. Added 0.2 ml of chloroform per 1ml of TRIzol Regeant. Shaken tubes vigorously by hand for 15 seconds and incubated them at 15 to 30^{o}C for 2-3 minutes. Centrifuged the samples at no more than 12,000 x g for 15 minutes at 2-8^{o}C. Transferred the aqueous phase to the fresh tube, precipitated the RNA from the aqueous phase by mixing with isopropyl alcohol (0.5 ml for 1ml TRIzol Regeant). Removed the supernatant and washed the RNA pellet once with 75% ethanol, mixed the sample by vortexing and centrifuged at 7,500 x g for 5 minutes at 2 to 8 ^{o}C.

3. Spectrophotometric estimation of nucleic acids

The quantity and quality of DNA and RNA were determined by measure the absorbance at 260 nm and 280 nm. It was calculated OD=1 for 50 µg/ml for DNA and 40 µg/ml for RNA (A_{260}). The purity of DNA and RNA were determined by calculating the ratio of A_{260}/A_{280}.

4. Southern hybridization

The genomic DNA (30 µg) was digested overnight with restriction enzymes. The digested products were run on a 0.8 % agarose gel in 1X TBE, pH 8.3 containing ethidium bromide (0.5 µg/l) at 40 volts for approximately 10-15 hours. The gel was visualized and photographed. For breakage of large DNA in gel by depurination, placed the gel in a tray and added 250 ml of 0.2 N HCl at room temperature. Rocked occasionally for 15 minutes, decanted the acid, and repeated in two times. Rinsed with water briefly and proceeded immediately to the next step of alkaline denaturation: Added 250 ml of 0.5 M NaOH and 1.5 M NaCl and gently agitated for 15 minutes. Decanted alkali and repeated one more time. Neutralized gel with 250 ml of 1 M ammonium acetate for 10 minutes, repeated one more time and transferred to membrane.

5. Northern blotting

The agarose is melted by boiling in 10 mM sodium phosphate buffer, pH 6.8 containing 1 µl of 10 mg/ml ethidium bromide per 100 ml of buffer, then cooled to 60^{o} C and poured in the casting tray. In a 1.5 ml sterile Eppendorf tube 30 µl of the RNA dissolved in sterile H_2O was mixed with 2 µl of sterile 6X loading buffer.

6X loading buffer

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

30% (w/v) glycerol

1.2% SDS

60 mM sodium phosphate (pH 6.8)

The mixture was incubated at 75^{o}C for 5 minutes followed by immediate loading of the sample to a submarine gel. When analyzing many samples, the denatured RNA can be placed on ice before loading on a gel. The gel was electrophoresed at 3 to 7 V/cm in 10 mM sodium phosphate buffer, pH 6.8, containing 1 µl of 10 mg/ml ethidium bromide per 100 ml of buffer. Because the buffering capacity of the electrophoretic buffer was relatively weak due to its low ionic strength, constant recirculation of the buffer was maintained to prevent the formation of an undesirable pH gradient which can lead to degradation of the RNA during electrophoresis. The migrating RNA in the gel was visualised with medium-wave UV light to verify the migration and integrity of the RNA.

Pre hybridization

Placed DNA bound gene screen plus paper in a heat sealable plastic bag (hybridization bag). Added approximately 20 ml (~70 µl/cm^2) of hybridization buffer.

Prehybridization buffer (1000 ml)

20 X SSC	250 ml
50% Dextran sulphate	100 ml
1.0 M sodium phosphate pH 7.2	50 ml
50 X Denhardts solution	100 ml
0.5 M EDTA	5 ml
20% SDS	20 ml
Distilled water	475 ml
	1000 ml

Divided into 100 ml aliquots and store at –20^{o}C

Added 200 µl (5 mg/ml) heat denatured salmon sperm DNA to the hybridization bag, mixed well and sealed the bag. Removed all air bubbles before sealing the bag and prehybridized at 65^{o} C for 3-8 hrs.

Hybridization

Added ~10^{8} dpm denatured DNA probe (nick-translated ^{32}p following the Amersham DNA labelling kit) heat sealed the bag. Placed sealed bag into a second bag and heat sealed. Wet paper towel placed in the second bag helped prevent drying of the membrane.

Washing of membrane

Hybridized for 8-24 hours at 65^{o} C in a water bath shaker. Cut open bag and removed membrane. Washed 2 times for 10 minutes in 250 ml of 2X SSC + 0.1% SDS at room temperature. Washed 2 times for 15 minutes in 250 ml of 0.5X SSC + 0.1% SDS at 65^{o}C in a water bath shaker. Wash 2 times for 8 minutes in 250 ml of 2X SSC at room temperature.

20X SSC (1000 ml)

NaCl	175.3 g
Sodium citrate	88.2 g

Dissolved in 800 ml of water. Adjusted pH to 7 with NaOH or HCl and made up the volume to 1000 ml.

Autoradiography of ^{32}P on membrane

Covered the membrane in Saranwrap and exposed to X-ray film over night by placing in side a cassette at –70 ^{o} C. To develop the film, brought the cassette to room temperature before developing. This was to prevent condensation on film and damage to intensifying screen. Developed the X-ray film: 1 minute in Kodak GBX 20%

developer solution, rinse in water and 3 minute in Kodak GBX 20% fixer solution. Hung firm to dry.

6. cDNA for primer extension and Real time PCR (Invitrogen kit)

A 20 µl reaction volume can be used for 1ng to 5µg of total RNA or 1ng-500 ng of RNA. Added the following components to a nuclease-free microcentrifuge tube: 1µl Oligo (dT)12-18 or gene specific primer, 1 ng to 5 µg total RNA, 1µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH) and distilled water to make up the volume to 12µl. Heated mixture to 65^{o}C for 5 minutes and quick chilled on ice. Collected the contents of the tube by brief centrifugation and added: 4µl 5X First-Strand Buffer, 2µl 0.1 M DTT and 1 µl RNAseOUT Recombinant Ribonuclease Inhibitor (40 Units/µl). Mixed contents of the tube gently and incubated at 42 ^{o}C for 2 minutes. Added 1 µl (200 Units) of SuperScript II (Reverse transcriptase), mixed by pipetting gently up and down. Incubated 50 minutes at 42^{o}C. Inactivated the reaction by heating at 70^{o}C for 15 minutes. The cDNA can now be used as a template for amplification in PCR. However, amplification of some PCR targets (>1kb) could require the removal of RNA complementary to the cDNA. To remove complementary RNA, added 1 µl (2 units) of *E.coli* RNase H and incubated at 37^{o}C for 20 minutes.

7. Transcription initiation analysis

Primer extension was performed using preamplification kit (Invitrogen). Reaction was carried out with 10 µg of total RNA using the GUS internal primer. Primer was labeled with (gamma ^{32}P) ATP using T4 polynucleotide kinase (Promega). The size of the extension product was determined by comparison with the DNA sequence generated using the same primer and pITB450 DNA (Sequenase II kit, USB).

8. Induction and analysis of GUS expression

Tetracycline (Tc,1 mg/L) was used for the induction of GUS expression in single leaves and in *in vitro* grown plants. Kinetics of induction was followed by real time PCR and by quantifying the GUS activity in the Tc-treated and untreated leaf samples at defined time periods.

9. Real time PCR

The RNA isolated from various explants were converted into cDNA using Preamplification Superscript kit (Invitrogen) and poly dT oligonucleotide. GUS specific beacon primers and cDNA as a template was used to detect the GUS expression. Amplification of actin as a control for cDNA amounts was also included in the same PCR reactions.

10. DNA sequencing.

Sequencing of recombinant clones was done either by sequenase version 2.0 kit (USB-Amersham, Life Sciences), which was based on Sanger's dideoxy chain termination methods. Plasmid DNA was denatured with 0.2 M NaOH and 0.2 mM EDTA at room temperature for 10 minutes. The denatured plasmid was purified by passing it through TE-equilibrated G-50 column made in a 500 µl effendorf tube and centrifuged at 3000 rpm for 3 minutes at room temperature. Annealing of the primers to the denatured DNA was done at 37 ^{o}C for 15 minutes. This was followed by a labelling reaction, which was performed essentially as described by the supplier (USB-Amersham, USA) in the presence of S^{35}-dATP and incubated at 37^{o}C for 10 minutes. Termination of the reaction was done by the addition of respective dideoxynuclotides and further incubation at 37^{o}C for 5 minutes. Stop buffer was added and the reaction mix was boiled, cooled and loaded onto 6% urea-acrylamide TBE gel pre-warmed to 55^{o}C. Electrophoresis was carried out at constant power (70

watts) to maintain the gel temperature at 55^{o}C. Three loading were done to completely resolve the nucleotide sequence. After completion of the run, the gel was transferred to a Whatman sheet and dried in vacuum heating drier. The dried gel was exposed to autoradiography film at room temperature.

VIII. WESTERN BLOTTING AND SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (PDS-PAGE)

Homogenized leaf tissue (100 mg) in 300 µl of GUS extraction buffer as described in GUS assay protocol. Quantified the protein and loaded 20-50 µg of protein per sample.

Preparation of separating gel

In a 25-ml sidearm flask, mixed 30% acrylamide/0.8% bisacrylamide solution, 4xTris-Cl/SDS, pH 8.8 (see reagents, below), and 3.05 ml H_2O. Degassed under vacuum 10 to 15 min. Added 0.025 ml of 10% ammonium persulfate and 0.005 ml TEMED. Swirled gently to mix and use immediately.

Reagents used in gels

30% acrylamide/0.8% bisacrylamide

Mixed 30.0 g acrylamide and 0.8 N,N'-methylene-bisacrylamide in a total volume of 100 ml H_2O. Filtered the solution through a 0.45-mm filter and stored at 4^{o}C in the dark.

4xTris-Cl/SDS, pH 6.8 (0.5 Tris-Cl containing 0.4% SDS)

Dissolved 6.05 g Tris base and 0.4 g SDS in 40 ml H_2O. Adjusted to pH 6.8 with 1 N HCl, added H_2O to 100 ml total volume and filtered the solution through a 0.45-mm filter and stored at 4^{o}C.

4xTris-Cl/SDS, pH 8.8 (1.5 M Tris-Cl containing 0.4% SDS)

Dissolved (1 g Tris base and 2 g SDS in 300 ml H_2O. Adjusted to pH 8.8 with 1 N HCl. Added H_2O to 500 ml total volume. Filter the solution through a 0.45-mm filter and store at 4^oC.

Bromphenol blue solution

20 ml glycerol

4 mg Bromphenol blue

Add H_2O to 100 ml

5xSDS/electrophoresis buffer

15.1 g Tris base

72.0 g glycine

5.0 g SDS

Add H_2O to 1000 ml

Do not adjust the pH of the stock solution, since the pH is 8.3 when the solution is diluted to 1x for use in the protocol.

4X Sample Loading Buffer

To 20 ml H_2O add:

1.52 g Tris Base

20 mL Glycerol

2.0 SDS

2.0 mL β-mercaptoethanol

adjusted pH 6.8 with HCl and add H_2O to 50 ml

The gel was made and then run until the Bromphenol Blue tracking dye has reached the bottom of the separating gel.

Transfer of proteins to hybond C+ membrane

Cut two pieces of Whatman 3MM filter paper, cut to the same size as the gel and prewetted with electroblotting buffer. Placed the prewetted filter, gel, nitrocellulose membrane and again filters sequentially from the cathode. Care was taken to remove air bubbles after each step by gently rolling a glass tube on the set-up. Placed this sandwich into a plastic support. Placed the support containing the sandwich into the electroblotting apparatus in the correct orientation. Filled the tank with electroblotting buffer. Connected the leads of the power supply to the corresponding and cathode sides of the electroblotting apparatus. Electrophoretically transferred the proteins from the gel to the nitrocellulose at 100 mA constant current 1 hour.

Western blotting analysis

Following transfer of proteins (Western) onto nitrocellulose or nylon membrane, washed the membrane in 40 ml of TSW buffer for 1 hour. Repeated the washing for 30 minutes. To the 40 ml buffer, added 50 µl of rabbit anti-β-glucoronidase serum. Incubated with gentle shaking for 1 hour. Decanted solution and wash with 20 ml of TSW buffer for 30 minutes. To 50 ml of TSW buffer, add 5 µl goat anti-rabbit alkaline phosphatase conjugate as a secondary antibody (concentration: 1 mg/ml). Incubate with gentle shaking for 30 minutes. Decanted solution and washed with 20 ml of TSW buffer for 30 minutes. Replaced TSW buffer with the following buffer; 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. Added 33 µl NBT (Nitro blue tetrazolium, 50 mg/ml in 70% Dimethylformamide) and 16.5 µl BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 50 mg/ml in Dimethylformamide) in 5 ml of the TSW buffer. Full colour development should occur within 30 minutes.

Composition of TSW buffer: 10 mM Tris-HCl, pH 7.4
0.9% NaCl
0.25% Gelatin
0.1% Triton X-100
0.02% SDS