MATERIALS AND METHODS

Materials:

Tobacco seed Havana petit SR1, *Agrobacterium tumefaciens*, MP90, *E. coli* strain MC1061 and plasmids pGSC1703A, pAT2S3, pAT2S21, were obtained from Prof. Marc Van Montagu, University of Gent, Belgium. *E. coli* strains BHB 3565 (pBI121), pRK 2013 were obtained from Prof. B. Hohn, Switzerland. Restriction enzymes and other enzymes were obtained either from BRL, Maryland or from USB, Ohio, USA. Nylon membranes and nitrocellulose membranes were purchased either from Schleicher & Schuell or from Boehringer Mannheim, Germany. Radio isotopes were purchased from BARC, Bombay, India. X-ray films were purchased from ORWO, Germany. (P 81) paper was purchased from Whatmann International Ltd., Madison, England. Reagents or chemicals were purchased either from Sigma Chemical Company, St. Louis, MO, USA or from local market.

Plant Growth Media and Culture Condition:

Sterile explants were grown either in B5 or Gamborg's basal medium (Gamborg et al., 1968) for germination. Inoculums were grown either on B5 basal medium (Micro-elements: sodium dihydrogen phosphate 150 mg/l, magnesium sulphate heptahydrate 134 mg/l, calcium chloride dihydrate 150 mg/l, ferrous sulphate heptahydrate 27.8 mg/l, sodium-EDTA 37.3 mg/l and potassium nitrate 3000 mg/l; Organic compounds : Myo inositol 100 mg/l, nicotinic acid 1 mg/l, thiamine-HCL 10 mg/l, pyridoxine 1 mg/l, sucrose 30 gm/l; Micro-elements: boric acid 3 mg/l, manganese sulphate monohydrate 10

mg/l, zinc sulphate heptahydrate 2 mg/l, sodium molybdate dihydrate 0.25 mg/l, calcium chloride hexahydrate 0.025 mg/l, potassium iodide 0.75 mg/l, calcium sulphate pentahydrate 0.025 mg/l, pH 5.5 and Agar 8 gm/l), Murashige and Skoog's basal (Murashige and Skoog, 1962) medium (Macro-element: ammonium nitrate 1650 mg/l, potassium nitrate 1900 mg/l, calcium chloride with two molecules of water 440 mg/l, magnesium sulphate with seven molecules of water 370 mg/l, potassium dihydrogen phosphate 170 mg/l; and macro-elements: boric acid 6.2 mg/l, manganese sulphate monohydrate 22.3 mg/l, zinc sulphate quadrohydrate 8.6 mg/l, potassium iodide 0.83 mg/l, sodium molybdate dihydrate 0.25 mg/l, copper sulphate pentahydrate 0.025 mg/l, cobalt chloride hexahydrate 0.025 mg/l; Fe-EDTA, Na-EDTA 37.2 mg/l and ferreous sulphate heptahydrate 27.8 mg/l; Inositol 100 mg/l, sucrose 20 gm/l, Agar 8 gm/l, vitamin: thiamine 0.4 mg/l), with nutrient media formulated with addition of 2,4D, NAA, kinetin, BAP and Zeatin. These growth hormones were used either singly, or in combination with concentration ranging from 1 μM to 12 μM. Cultures were maintained at 25°C ± 1°C under photoperiodic conditions of 16 hour light and 8 hour dark at an illumination of 1.2 W/m. The humidity of the culture room was maintained in the range of 75%. The subcultures were performed at an interval of 28 days.

Growth of *Agrobacterium* and *E. coli* strains:

Agrobacterium tumefaciens were grown in YEB (Bacto beef extract 0.5%, Bacto yeast extract 0.1%, Bacto peptone 0.5%, Sucrose 0.5%, $MgSO_4$

0.002 M), *E. coli* in LB (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) medium. Solid media were prepared by adding 1.5%-2.0% agar agar. Incubations were performed at 28°C. *Agrobacterium* C58 CIGmr (pMP 90) (Debaere et al., 1985) and the transconjugants out of the recipients of this *Agrobacterium* strains that is, pMP 90 : pMP constructs were grown in presence of gentamycin 40 µg/ml, streptomycin 300 µg/ml and spectinomycin 100 µg/ml respectively. *E. coli* strains DH5α and MC 1061 were grown in LB without any antibiotic; HB 101 (pRK 2013) (Fagurski and Helinski, 1979) was grown in LB containing 30 µg/ml of Kanamycin (Kn).

Conjugation or Mobilisation of Binary Vectors to *Agrobacterium tumefaciens*:

This was done as per Van Haute et. al., (1983). *Agrobacterium* strains, *E. coli* strains MC1061 or BHB3565 containing the desired constructs and helper strain HB101 (pRK2013) (Fagurski and Helinski, 1979) were grown in LB medium containing appropriate concentration of required antibiotic(s) as discussed in the previous section, and grown upto log phase at 37°C (*E. coli*) and at 28°C (*Agrobacterium*). Cells were harvested, washed with 0.9% NaCl to remove antibiotics and mixed in equal proportion and dropped on Lauria agar (without any antibiotic) and incubated at 28°C overnight. After that cells were suspended in 0.9% NaCl and plated in appropriate dilution (generally 10^{-6}) on appropriate concentration of antibiotic described previously and desired transconjugants were obtained.

***Nicotiana tabacum* Leaf Disc Transformation:**

Transformation of tobacco was done as per Horsch et al., (1985). Tobacco seeds cv. *Havana petit* SR1 were grown asceptically in B5 or MS Agar medium. The leaves of these asceptically grown plants were used for making discs. The medium used in different purposes were described below. The leaf disc infections were done by inserting leaf discs in infection medium at 25-27°C under fluorescent white light for 48 hours with 1 ml of log phase *Agrobacterium* culture (without antibiotic) per 20 ml of infection medium (B5 medium with 250 mg/l ammonium nitrate, 3% sucrose and 0.5 g/l MES pH 5.5). After 48 hours the leaf discs were washed with sterile water and after soaking in sterile filter paper, inoculated in callus inducing medium (B5 medium with 250 mg/l ammonium nitrate, 2% glucose, 0.5 g/l MES (pH 5.7), 40 mg/l adenine, 0.8% agar, 50 μmoles IAA, 4.4 μmoles BAP, 100 mg/l cefotaxime and 100 mg/l Kn). After calli were produced (about 21 days), these were transferred on to shoot inducing medium (same as callus inducing medium without IAA). The shoots were then transferred on to root inducing medium (MS medium without any supplement). The reported plants before being transferred into pot was gently washed to remove the agar medium sticking to the roots. The plants were kept into small plastic cup (with a small perforation below) containing sterile sea sand. The cup was then mounted on a petri-dish containing half-strength MS salt solution. This whole system was then covered with a beaker in order to maintain a relatively high humidity and kept at room temperature for 7-10

days. This is the hardening period required for transferring the transgenic plant to pot and this method has been successfully persued in our laboratory with a very high survival rate.

The transformed plant has been maintained in the greenhouse condition and minimal hardening has been required in case of tobacco in the present situation.

Estimation of Protein:

Protein was estimated according to the method of Lowry et al., (1951). An aliquot of the protein sample (1-2 µg) was diluted to 0.25 ml and 2.5 ml protein reagent (3% Na-carbonate ; 4% Na-K-tartarate ; 2% CuSO4 ; 100 : 1:1) was added, kept at room temperature for 10 min., 0.25 ml Folin Ciocalteau reagent was added, incubated at room temperature for 30 min and absorption was measured at 660 nm against appropriate blank. Standard curve was drawn assaying known amount of BSA. The quantitation of the sample was done by comparing with the calibration curve.

β-D Glucuronidase Assay:

This was done according to Jefferson, (1987). There are two methods to estimate the enzyme.

i) **By Histochemical Method:**

The plant tissue was placed in 200 µl of 50 mM sodium phosphate buffer pH 7.0 with x-gluc (5-bromo 4-chloro 3-indolyl β-D glucuronide), 50 ug/ml and incubated at 37°C overnight. Histochemical staining was also done in the 0.1M phosphate buffer, 0.3% titron X-100, 20% methanol, 0.5 mM ferrocyanide, 0.5 mM ferricyanide with 1.5 mM x-gluc (Reinold et al., 1993). The incubation was done at 37°C as required.

ii) **Fluoremetric Assay:**

Plant tissues about 100 mg were ground in a mortar and pestle with 0.5 ml GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM DTT, 1 mM EDTA, 0.1% Sodium lauroyl sacrosine, 0.1% triton X-100). The supernatant was used for the assay of the enzyme. To start the fluorogenic assay 0.5 ml of 1 mM solution of MUG (made in GUS extraction buffer) was prewarmed at 37°C for 10 min, then about 30 µg of crude protein was added to the MUG solution (at 37°C) and mixed, after 10-20 min 100 µl of the reaction mixture was withdrawn and added to 0.9 ml of 0.2 M Na_2CO_3 solution to stop the reaction. This was noted as zero time. Then 100 µl of reaction mix was withdrawn at regular intervals (10-20 min as desired in specific assays) and mixed with 0.9 ml of Na_2CO_3 taken in 1.5 ml eppendorf tubes properly marked. Usually reaction mixtures were withdrawn at 3-4 time points. The fluorescence of the solution to determine the 4-MU concentration were measured by excitation at

365 nm and emission at 455 nm. Before taking reading of experimental sample, the instrument was calibrated with standard solution of 4-MU.

Plant DNA Isolation:

Plant DNA was isolated according to Murray, (1979) with modification i.e. homogenising 1 gram of tissue in mortar and pestle in the homogenising buffer (10 mM Tris. pH 8.0,100 mM NaCl, 10 mM EDTA, 2.5% SDS), treated with proteinase K at 65°C for 1 hour, the supernatant was treated with phenol, phenol : chloroform : isoamylalcohol (1:1:1/24) and chloroform with isoamyl alcohol (24:1). The nucleic acid was precipitated with isopropanol (0.6 volume) or with 2.5 volume ethanol. The nucleic acid was spooled with pipette tip, washed with 70% alcohol, dried and resuspended in 0.5 ml TE (10 mM Tris and 1 mM EDTA, pH 8.0), added potassium acetate and SDS to the final concentration of 1.25% and 1% respectively kept on ice for 1 hour, centrifuged at 10000 rpm in SS34 rotor at 4°C in Sorvall RC5B for 10 min to collect the supernatant. It was then precipitated with 2 vol. of ethanol, centrifuged, the pellet was washed with 1 ml of 70% alcohol and finally the dry pellet was resuspended in 0.5 ml TE. The DNA solution was treated with RNAase (DNase free) (50 µg/ml), PEG and NaCl were added to the final concentration of 10% and 0.5M respectively. After keeping on ice for 2 hours the nucleic acid was collected after centrifugation and resuspended in 0.25 ml TE (10mM Tris and 1mM EDTA, pH 8.0). The DNA solution was finally treated once with phenol, phenol:chloroform and chloroform and precipitated with 2.5 vol. ethanol

with 0.1 volume 3(M) Na-acetate pH 5.2 and after washing with 70% ethanol, the dried pellet was finally resuspended in 0.2 ml of sterile water.

Isolation of Plasmid DNA:

This was done by alkali-SDS method as described by Birnboim and Doly, (1979).

Plasmid DNA Mini Preparation:

0.7 ml over night culture was centrifuged, the supernatant was aspirated with pipette tip. Resuspended the pellet in 100 µl of solution I (25 mM Tris, pH 8.0, 10 mM EDTA and 50 mM glucose); added 200 µl of solution II (0.2 M NaOH and 1% SDS); mixed gently but thoroughly followed by addition of 150 µl of solution III (3 M sodium acetate, pH 6.0) mixed thoroughly, kept on ice for 10 min, centrifuged for 5 min at 12,000 rpm in eppendorf centrifuge. The supernatant was withdrawn in a fresh tube, added 1 ml dehydrated alcohol, mixed, centrifuged at room temperature for 5 min, the supernatant was discarded completely, washed the pellet with 1 ml 70% ethanol. Finally the dried pellet was resuspended in 50 µl of sterile water.

Large Scale Plasmid Preparation:

Overnight grown *E. coli* culture (250 ml) was centrifuged in Sorvall GS3 rotor for 10 min at 5000 rpm. The pellet was resuspended in 5 ml of solution I. Added 10 ml of solution II, then mixed thoroughly, added 200 µl of

lysozyme (10 mg/ml of 10 mM Tris, pH 8.0), mixed, kept in room temperature for 10 min and added 7.5 ml of solution III, mixed gently and thoroughly, incubated in ice for 20 min, centrifuged at 4°C in Sorvall SS34 rotor for 30 min at 12000 rpm. The supernatant was collected in a fresh polypropylene tube, added equal volume of isopropanol, mixed, kept standing in room temperature for 10 min followed by centrifugation at 10000 rpm for 10 min. The pellet was washed with 1 ml 70% ethanol, decanted the liquid completely, resuspended the pellet in 2 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA). Added to it 2 ml of chilled 5 M lithium chloride, incubated in ice for 10 min, centrifuged, the supernatant was collected and added to it 0.1 volume 3M Na acetate (pH 5.2), and 2.5 volume of dehydrated alcohol. The pellet collected was resuspended in 0.5 ml TE, incubated with RNase (finally 50 µg/ml) for 30 min at room temperature, added PEG 6000 and sodium chloride to the final concentration of 10% and 0.5M. Kept in ice for 2 hours, centrifuged, the pellet was resuspended in 0.5 ml TE, extracted with phenol, phenol/chloroform and chloroform. Finally alcohol precipitated. The pellet was washed with 70% alcohol, dried and resuspended in 200 µl of sterile water.

Isolation of Plant RNA:

RNA was isolated according to Beachy et al., (1985). Plant tissues (approx. 1 gm) were ground in a mortar and pestle with 5 ml NTES buffer (0.1M NaCl, 0.1 M Tris-HCl, pH 8.3, 50 mM EDTA, 2% SDS) with equal volume of phenol,

phenol/chloroform/isoamyl alcohol (1:1:1/24), centrifuged and collected the supernatant, of which the total nucleic acids were precipitated with 2 volume of ethanol in presence of 0.1 M Sodium acetate (pH 6.0). The precipitate was washed with 1 ml of 70% ethanol and resuspended in 1 ml of DEPC treated water. Then equal volume of 4 M LiCl was added and kept on ice for at least 3 hours. The pellet was resuspended in 0.25 ml of water, precipitated with 2.5 volume ethanol and finally resuspended in 0.1 ml of water. The polyA+RNA was isolated according to Sambrook et al., (1989).

Yeast DNA and RNA isolation:

Total yeast DNA was prepared as described by Davis et al. (1980). Total RNA was isolated from yeast cells grown to a density of 10^7 cells/ml as described by Schmitt et al. (1990).

Electrophoresis of Nucleic Acids:

Plasmid DNAs were run in 0.8-1.5% submerged agarose gel in Tris-borate buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA). RNA was fractionated in horizontal submerged 1%-1.4% agarose gel with formaldehyde as denaturing agent in 0.05 M morpholinopropanesulfonic acid (MOPS), pH 7.0. Glass plates were heat sterilized and gel solutions and tank buffers used were DEPC treated and autoclaved (MOPS buffer was passed through millipore). RNA was denatured in 50% deionized formamide (millipore), 2.2 M formaldehyde (millipore), 10 mM Na-phosphate buffer, pH 7.0 and 1 mM EDTA,

at 65°C for 5 min, chilled in ice, made 1 x in loading buffer (5% glycerol, 25 mM EDTA, 0.5% SDS and 0.08% bromophenol blue) before loading. Electophoresis was carried out at 3-5 V/amp for 2-5 hours in room temperature.

Transfer of Nucleic Acids to Nitrocellulose/Nylon Membrane:

DNA was transferred to nitrocellulose according to the method of Southern, (1975). Agarose gel after run was transferred to a glass tray containing 0.25 M HCl, sufficient to cover the gel and incubated at room temperature (25-28°C) for 10-15 min. This acid treatment was done when transfer of >10 Kb DNA is required. If the gel was exposed too long (>5 min) to UV as sometimes needed to take photograph of the gel, acid treatment is not essential. Acid solution was then removed with a 50 ml syringe, the gel was rinsed with water and DNA was denatured by soaking in 0.5 M NaOH and 1.5 M NaCl for 15 min. at room temperature with occasional shaking. This treatment was repeated once more. After 30 min gel was rinsed with water and neutralized in 0.5 M Tris, pH 7.4 and 1.5 M NaCl for 30 min (2 x 15 min). A piece of plexiglass (one inch in thickness) wrapped with Whatmann 3 MM paper was placed in a glass tray and the tray was then filled with 5 x SSPE (1 x SSPE is 150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, pH 7.4) almost to the top of the plexiglass. Air bubbles were removed with a smooth cylindrical surface and the gel was placed on the support. Strips of exposed but rejected X-ray films was placed on the support around the gel to prevent short circuiting. Nitrocellulose paper or nylon membrane, initially soaked in water for

a brief period and then in 5 x SSPE, was placed on the gel carefully, the air bubbles if any trapped inside were removed with a smooth glass rod. Relatively old nitrocellulose paper sometimes do not wet evenly. Floating of such nitrocellulose in 70-80°C water wet evenly and can be safely used. Two pieces of 3 mm paper of gel size was placed on nitrocellulose paper followed by a stack of blotting paper 1-2 inches thickness. Above this stack a glass plate was placed on which 500 g weight was kept. Blotting was done 12-14 hour depending on the thickness of the gel.

RNA was transferred to nitrocellulose according to the method of Thomas, (1980). After electrophoresis in formaldehyde gel, the gel was washed with the DEPC treated water several time to remove the formaldehyde, the gel was transferred on a one inch thick plexiglass wrapped with Whatmann 3 mm paper and placed in a tray filled with 20 x SSPE almost to the top of plexiglass. Nitrocellulose paper, presoaked first in water and then in 20 x SSPE, was placed on the gel in bubble free condition, the two pieces of 3 MM paper and a stack of blotting paper of gel size was placed. Above the stack a glass plate and on it 500 g weight was placed and kept 12-14 hours.

In vitro labelling of DNA:

DNA was labelled in vitro by nick translation according to Sambrook et al., (1989). A typical reaction mixture contains 0.5-0.8 µg linearised plasmid DNAs or inserts (100-200 ng). 25 µM unlabelled each of three dNTPs except the labelled one, 50 µCi (3000 Ci/nmole) alpha 32p labelled fourth dNTP, nick-

translation buffer (50 mM Tris, 10 mM $CaCl_2$ and 0.1 mM DTT), DNase 1 (40 pg for DNA > 1 Kb and 20 pg for DNA < 1 Kb) and DNA polymerase 1 (5 U). The reaction was carried out at 15°C for 2-2.5 hours, then stopped by EDTA (25 mM final), reaction vol. was increased to 100 µl by TE buffer (pH 7.4), extracted once with equal volume of water saturated phenol and labelled DNAs were then separated from free 32p dATP labelled nucleotide by exclusion chromatography over Sephadex G-75 (Pharmacia) in TE buffer pH 7.4. The specific activity of labelled DNA usually obtained was 1-2 x 10^8 cpm/µg.

In some cases DNA was also labelled using non radioactive method (ECL direct nucleic acid labelling and detection kit from Amersham).

Southern Hybridization:

This was done according to Southern, (1975) and Sambrook et al., (1989). This prehybridization and hybridization was done in 5 x SSC or SSPE, 2x Denhardt's reagent and 0.1% SDS at 65°C or with 50% formamide at 42°C.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting:

Western blotting was done according to Howard and Lane, (1989). About 100 µg of crude proteins were separated in 12% SDS polyacrylamide gel using 25 mM Tris and 192 mM glycine with 0.1% SDS as running buffer until the dye front reaches the bottom of the gel. The gel was washed with 25 mM Tris and 292 mM glycine, 20% methanol as transfer buffer. The transfer of nitrocellulose or nitran membrane was carried out using HICON transfer

apparatus at 25V/175 mA for 3-4 hour. Membrane was washed with 10 mM Tris pH 7.4 and blocked in 3% BSA in PBS overnight. Next day, the membrane was washed with PBS buffer containing tween 20 (0.05%) thrice each at 5 min intervals. Then the membrane was incubated with specific antibody at 1:200 dilution for 1 hour at 37°C. It was washed with PBS containing tween-20 for 5 times each at 5 min interval and incubated with the peroxidase conjugate (Goat anti rabbit IgG linked with horse raddish peroxidase, 1:5000, Sigma) dilution at 37°C for 1 hour. Then after washing with the PBS tween-20 for 3 times, the filter was incubated with the substrate 3,3' diaminobenzidene (Sigma) in 10 mM tris and 200 mM NaCl at concentration of 1 mg/ml. The reaction was stopped by distilled water.

Vector Construction and Bacterial Transformation (Cloning):

Cloning was done as per the basic protocols described in Sambrook et al., (1989) and Ausubel et al., (1989). DNA fragments were purified from 0.8% low melting agarose (USB) in 1x TAE running buffer at 4°C. Agarose gel blocks were remelted in TE (10 mM Tris, 1 mM EDTA pH 8.0) at 65°C for 10 min. Extracted with phenol, phenol/chloroform and chloroform. Nucleic acids were precipitated with 0.6 volume isopropanol or 2.5 volume ethanol in presence of 0.3 M sodium acetate pH 5.2. Ligation reactions were performed in 10 µl reaction volume with 1 unit of T4 DNA ligase in 1 x ligation buffer (BRL). For sticky ends ligations inserts and vectors were ligated in the ratio of 2:1 moles. And for blunt end ligations vector and inserts were ligated in 1:4 molar ratio.

Ligation reactions were carried out at 8-12°C overnight. *E. coli* strain DH5α (BRL) and MC 1061 (Meissner et al., 1987) were made competent using $CaCl_2$ method (Mandel & Higa, 1970). 50 ml LB was reinoculated with 100 µl of saturated culture and grown upto OD about 0.3, chilled the culture on ice for 1 hour, centrifuged at 2000 rpm/10 min in SS34 rotor at 4°C (the rotor was precooled to 4°C). The pellet was resuspended mildly in 20 ml of prechilled 0.1 M $CaCl_2$, kept on ice for 30 min, recentrifuged at the similar condition as before. The pellet was resuspended in 0.5 ml of 0.1 M $CaCl_2$ (prechilled) and kept in ice for at least 1 hour, 100 µl of this competent cells were used for transformation. For transformation, 5-10 µl of ligation mixture was added to the 100 µl of competent cell. Incubated at 37°C water bath for 5 min, chilled quickly in ice for 5 min, added 0.9 ml LB and incubated at 37°C for 1 hour, 100 µl of this cells were plated on selection plates and incubated at 37°C for overnight to get the transformants. The transformants were confirmed by mini preparation of plasmid DNA and restriction digestion. Transformants were selected on Luria agar plates containing 60 µg/ml ampicilin, or 100 µg/ml spectinomycin and 300 µg/ml streptomycin.

Transformation of Yeast:

There are two methods of transforming yeast cells.

i) Protoplast fusion (Beggs, 1978).

ii) Transformation of yeast using lithium salts (Ito et al., 1983).

The second method requires fewer steps and chemicals and it is simpler to perform. The drawback is the lower efficiency of transformation. However, for routine work which does not demand a large number of transformants, we have used this second method only.

Yeast strains BJ2168 and BJ5457 were grown in YEPD medium upto OD (650 nm) of 2 to 5 i.e. about 2 to 5 x 10^7 cells/ml (mid log phase). Cells were harvested and then subsequently washed with distilled water, resuspended in 0.1 M LiCl (or in 0.1 M lithium acetate) in TE to about 5.0 x 10^8 cells/ml which was then incubated with agitation at 30°C for 1 to 24 hours. The cells were then subsequently divided into centrifuge tubes, each of which should ideally contain 100 µl of cells, 10-50 µg of carrier DNA (approx. 10 µl), 0.1-2.0 µg of transforming DNA, & then incubated at 30°C for 30 minutes. 0.7 ml Li-PEG in TE was then added to each tube & incubated again at 30°C for a further period of 30 minutes. Heat shock was then applied for 5 min at 42°C in water bath, centrifuged for 2 seconds, washed twice in TE (microfuging 1-2 secs only) & resuspended in TE (100 ml) for subsequent spreading on selective plates.

Construction Strategy of pMP24, pMP25 & pMP26:

pMP24: The CaMVp35S-GUS chimeric gene was isolated from pBI121 as 3.0Kb HindIII/EcoRI fragment and cloned in the same site of YIP5 to produce this construct (8.5 Kb).

pMP25: The GUS gene isolated from pBI121 was cloned in pBS (Stratagene) as a BamHI/EcoRI fragment to get pMP8. The BamHI linearised pMP8 was made blunt ended with PolIK and redigested with HindIII to isolate the GUS insert as 2.2 Kb fragment. The NcoI linearised pMP9 (contains the entire At2S3 gene as 3.5 Kb BamHI/HindIII fragment in the same site of pBS) was made blunt ended with PolIK, redigested with BamHI to isolate the 1.5 Kb pAT2S3. Then these 2.2 Kb GUS and the 1.5 Kb AT2S3p fragments were cloned in the BamHI/HindIII site of pBS to yield pMP14a. The pMP14a was linearised with NcoI, treated with mung bean nuclease to remove the 5' overhang, religated and cloned to get pMP14. The pMP14 was digested with BamHI/HindIII to isolate the 3.7 Kb At2S3pGUS fusion and cloned in the same site of YIP5 to get this construct (8.9 Kb).

pMP26: The Pykp as 0.45 Kb EcoRI/XbaI fragment from Pyk (YIP5) and GUS as 2.2 Kb XbaI/HindIII fragment from pMP8 were isolated and then these two fragments were cloned in the EcoRI/HindIII site of YIP5 to get this plasmid (8.1 Kb).

Primer Extension Experiment:

20 to 50 µg of total RNA was mixed with 10^4 to 10^5 cpm of ^{32}PdATP labelled oligo, the solution was lyophilised or precipitated. To the pellet dNTPs were added to the concentration of 2.5 mM each so that the volume of the

sample becomes 10 µl, incubated the solution at 80°C for 15 min, transferred to 37°C for annealing. In the other microfuge tube 4 µl of 5X reverse ranscriptase (RT) buffer (USB), 2 µl of 0.7 mM DTT and 2 µl water were taken and preincubated at 37°C for 10 min (this was done simultaneously with putting the first tube in the 80°C for denaturation). The content of the preincubated mixture was then transferred to the annealing mixture followed by addition of 2 µl of the RT (400 unit of AMLV) and incubated at 37°C for 1-2 hours. Added to this, RNAse to the final concentration of 50 µg/ml and incubated at the same temperature for another 15 min. The reaction mixture was then extracted with the phenol/chloroform and alcohol precipitated. After centrifugation and 70% alcohol wash the dried pellet was resuspended in 2.5 µl of formamide gel loading buffer [80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue] and loaded the entire heat denatured (75°C for 5 min) sample in 6% denaturing polyacrylamide sequencing gel (Sambrook et al., 1989). The gel was dried and autoradiographed.

Identification of DNA Binding Protein(s) by South-Western Blotting:

In this method, a cell-free extract containing the DNA binding protein(s) of interest is first electrophoresed on SDS-polyacrylamide gel to resolve protein species according to the relative molecular mass. Since the strength of the final signal obtained by this technique is proportional to the

quantity of protein run on the gel, best results are obtained by running the maximum amount of extract that does not overload the gel. The protein(s) in the electrophoresed gel are then electrophoretically transferred onto a nitrocellulose membrane and subsequently renatured on the membrane by treatment with and then withdrawal of protein denaturants such as guanidinium chloride or urea. The nitrocellulose membrane is finally probed with a radiolabelled DNA fragment bearing the binding site of the protein. After washing off non-specifically bound DNA, the relative molecular mass of the protein is determined following exposure to X-ray film. Failure of this technique may indicate either that the DNA binding protein of interest has not been efficienly renatured, or that greater than one polypeptide species is required to reconstitute the binding activity.

In our efforts to elucidate DNA binding protein that binds with the *Arabidopsis thaliana* 2S3 promoter in the *Agrobacterium*, yeast & flowering stages of the tobacco plant, we have taken nuclear extract from tobacco flowers and yeast & whole cell extract from *Agrobacterium*. Nearly 100 µg of protein from each respective system were run on an SDS-polyacrylamide (12%) gel then transferred on to a nitrocellulose membrane. The transfer on nitrocellulose was carried out using HICON transfer apparatus at 25V/175 mA for 3-4 hours. The nitrocellulose filter was then subjected to washing with Z buffer (composition 25 nM HEPES-KOH pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P40, 0.1M KCl, 1 nM DTT) containing 6 M guanidinium chloride. After two subsequent washing with 6 M guanidinium chloride, half of

this solution was mixed with an equal volume of solution of X buffer (without guanidium chloride) so the effective concentration of guanidium chloride in the solution was reduced to 3 M. The nitrocellulose filter was then washed with this solution. This step was then followed subsequently five more times so that the guanidium chloride concentration will be gradually reduced to 0.1 M. Next, the filter was washed twice with Z buffer lacking guanidinium chloride (5 min each time) after which the membrane was incubated for 2 hrs. in blocking buffer (in buffer containing 3% non fat dry milk) to block non specific binding sites on the membrane, then washed for 5 min with the binding buffer (which is Z buffer containing 0.25% non fat dry milk). The binding buffer was then replaced by fresh binding buffer containing ^{32}P radiolabelled double stranded DNA probe bearing binding site of interest. To make the probe concentration as high as possible, volume of the buffer was restricted to the minimum to cover the membrane. This incubation of the membrane with labelled DNA was carried for 30 min, after which the solution containing the radioactive probe was disposed of & the membrane was washed with Z buffer three times for a total time of 15 min. This was then followed by washing the membrane with Z buffer containing 0.2 M KCl. This was done to reduce the non-specific binding of the probe to the membrane which might have resulted due to complexes between the probe and weakly interacting proteins. However, the salt concentration in this wash must not be sufficient to dissociate complexes between the probe and the

protein of interest. Next, the nitrocellulose membrane was exposed to X-ray filter for autoradiography.

Preparation of Plant Nuclear Extracts:

Plant material was frozen in liquid nitrogen and stored at -80°C until extracted. All operations were performed at 4°C. Frozen tissue was homogenized with a mortar and pestle in the presence of liquid nitrogen and resuspended at approximately 2 g of tissue/ml in 10 mM NaCl, 10 mM MES(pH 6.0), 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.6% Triton X-100, and 0.25 M sucrose (buffer A). The homogenate was filtered twice through miracloth and centrifuged for 10 min at 2000 g. The crude nuclear pellet was washed once in buffer A, resuspended in 0.5 ml to 1 ml of extraction buffer [20 mM Hepes (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT] and homogenized (10 strokes) in a Dounce homogenizer using a "B" pestle. The mixture was incubated for 1 h → 2 h, and nuclear debris was removed by centrifugation for 15 min at 15,000 g. Extracts containing nuclear proteins were dialyzed for 2 h against 50 volumes of 20 mM HEPES(pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Aliquots were frozen and stored at -80°C.

Preparation of Probes for Gel Retardation Assay:

For the G-box and its mutated probe, oligonucleotide was synthesised with an Applied Biosystems DNA synthesizer. Complementary pairs of oligonucleotide were annealed by boiling equimolar amounts in annealing buffer and then cooling to room temperature. Probes were then labelled by filling in the sticky ends with the Klenow fragment of DNA polymerase with ^{32}P dATP and ^{32}P dCTP.

Gel Retardation Assay:

Labelled DNA was incubated in a final volume of 20 µl containing the following : 4 µl of 5X assay buffer (binding), 250 ng of yeast tRNA, 10,000 cpm of labelled DNA, and varying amounts of nuclear protein extracts. BSA, cold homologous DNA and nonhomologous DNA were added as appropriate controls. The reaction mixture was incubated at 25°C for 15 min. They were then loaded immediately onto 5% polyacrylamide gel for electrophoresis in 4°C cold room using gel retardation electrophoresis buffer. The gel was fixed in 10% ethanol, 10% acetic acid and autoradiographed.

Preparation of yeast nuclear extract:

Growing cells were harvested and resuspended in spheroplasting buffer (1M sorbitol, 50 mM tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). The cells were removed by incubation at 30°C with 25-50 U/ml recombinant lyticase enzyme. The spheroplast were washed twice with spheroplasting buffer and then

resuspended in Ficoll buffer [18% Ficoll, 20 mM piperizine-N,N'-bis(2-ethanesulfonic acid), pH 6.3, 0.5 mM $CaCl_2$, 1 mM DTT, 1 mM EDTA] plus protease. The suspension was treated with 30 strokes of a tight pestle in a Dounce homogenizer and then unbroken cells were removed by two low speed spins. The nuclei were pelleted by a high speed spin and the cytosolic fraction was removed. The nuclei were resuspended in 100 µl of extraction buffer [10 mM HEPES, pH 7.5, 1 mM EDTA, NaCl as required] plus protease and phosphatase inhibitors and incubated on ice for 20 minutes. After another high speed spin, the nucleosolic fraction was removed and the nuclear pellet was resuspended in 100 µl of extraction buffer containing 0.5 M NaCl.

Sequencing of At2S3 promoter:

The sequencing of pAt2S3 upto 940 bases upstream of ATG was determined by the dideoxy chain termination method (Sanger et al. 1977) in a automated DNA sequencer (model 377, semiadaptive version 3.0) using double stranded plasmid DNA, Bluescript (containing the insert) and the unique forward and reverse primer of Bluescript according to the detailed method supplied. sequence of both the strands were determined.

Preparation of the cell extract of *Agrobacterium*:

Agrobacterium cell extract was prepared by harvesting the cells and then sonicated. The sonicated cells were subjected to centrifugation and the clear supernatant was taken.

Treatment of leaves with ABA:

For the treatment with abscisic acid (ABA-cis,trans, Sigma) detached leaves were floated on water containing 100 mM ABA for 16 hours. Protein synthesis inhibitor was applied as follows: detached leaves were incubated with 10 uM cycloheximide for 2 hours, followed by the addition of ABA for 16 hours.