Chapter-III

Materials and Methods
Protein Analysis

Protein Extraction

Plant tissue was homogenized in buffer 1 containing: 100 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM PMSF, 20% ethanol and 5% (w/v) insoluble polyvinylpolypyrrolidone. The mixture was centrifuged at 20,000 g for 30 minutes. The supernatant was brought to 30 % saturation with solid ammonium sulfate and stirred for 1 hour at 4°C. The resultant precipitate was removed by centrifugation at 20,000 g for 30 min. The supernatant was brought to 50% saturation with solid ammonium sulfate and stirred for another hour. The precipitate was collected and dissolved in buffer B containing 50 mM Tris-HCl pH 7.5, 50 mM KCl, 1mM DTT and 10% ethanol and dialysed for 8 hours at 4°C against the same buffer (at 1:50 ratio of protein: buffer).

Protein Assay

The protein was assayed by Bradford method (Bradford, 1976). 200 μl of 5X Bradford solution (BioRad Company) was diluted to 1 ml in distilled water. 2 μl of protein extract was added to the diluted Bradford solution and optical density (OD) was measured at 595 nm. The amount of protein was calculated taking bovine serum albumin (BSA) as standard.
Aspartate Kinase Assay

Aspartate kinase was assayed by hydroxamate method (Wilson et al, 1991). The assay was performed at different pH, ATP and substrate concentrations. The final assay mixture was composed of 100 mM Tris-HCl pH 8.0, 50 mM Hydroxamyl-HCl pH adjusted to 8.0, 20 mM Na₂ATP, 100 mM aspartate and 40 mM MgSO₄. The reaction was started by addition of 200 μl of enzyme solution and incubated at 35°C for 60 min. The reaction was terminated by addition of 500μl of FeCl₂ containing 0.37 M HCl and 20% (w/v) TCA. After centrifugation to remove protein precipitate, the absorbance of the supernatant was read at 505 nm. The amount of enzyme unit was estimated tasking aspartyl hydroxamate as standard. The extinction coefficient for aspartyl hydroxamate at 505 nm was 750 cm⁻¹ M⁻¹.

For substrate kinetics, AK was assayed following pyruvate-kinase lactate dehydrogenase coupled method (Zlotnick and Gottlieb 1986). The couple buffer (2X) was prepared containing 25 mM Tris HCl pH8.0, 15 mM MgCl₂, 300 mM KCl, 25% glycerol, 6 mM DTT, 0.426 mM NADH, 1.2 mM PEP, 11.5 U pyruvate kinase (Sigma, USA) and 32.5 U lactate dehydrogenase (Sigma, USA). Enzyme reaction was started in 1X couple buffer adding 20 mM ATP, various aspartate concentrations (0 - 25 mM) and protein mixture in a total volume of 1 ml. The change of absorbance at 340 nm was measured spectrophotometrically and velocity of enzyme reaction was defined as the change of OD at 340 nm per minute.
Glutathione S-transferase (GST) Assay

GST was assayed according to the procedure of Gronwald and Plaisance (1998) with some modifications. The reaction mixture contained 0.1 M potassium phosphate buffer pH 6.5, 1.0 mM GSH (reduced glutathione), 1.0 mM CDNB (1-chloro 2,4-dinitrobenzene, in 95% ethanol), 1% absolute ethanol and 100µl of protein extract in a total volume of 1.0 ml. The reaction was initiated by the addition of CDNB and GSH and incubated for 5 min. The change of absorbance at 340 nm was monitored for 180 sec in a spectrophotometer. One unit of enzyme (IU) is defined as the formation of 1µmol CDNB-GSH conjugate min\(^{-1}\) at 25\(^{\circ}\)C (extinction coefficient at 340 nm = 9.6 mM\(^{-1}\) cm\(^{-1}\)). The specific activity of enzyme is defined as units of enzyme per mg protein.

Homoserine Dehydrogenase (HSDH) Assay

Assay of the enzyme was carried out for the reverse direction. The reaction mixture contained 100 mM Tris HCl pH9.0, 150 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM NADP\(^+\), 20 mM homoserine in 1 ml reaction volume. The rate of NADP\(^+\) reduction at 340 nm was recorded in absence of homoserine. The increase of absorbance was monitored for 5-10 min after addition of homoserine. An increase of absorbance of 0.1 min\(^{-1}\) corresponds to 0.268 nKat (Wilson et al, 1991)
Protein Purification

1. **DE52 Ion Exchange Chromatography**

   Ammonium sulfate fraction was loaded on pre-equilibrated DE-52 column (5 mg/ml). Loading was done at a flow rate of 1 ml/min. After binding the column was washed with the same buffer (2 bed volume). Unbound protein was eluted with the same buffer; bound protein was eluted with a linear gradient of 50-500 mM KCl. Five ml fractions were collected from the gradient and every third fraction was subjected to AK assay. The active fractions were pooled and were brought to 50% saturation of ammonium sulphate. The resultant precipitate was resuspended in buffer 2 and dialysed against the same buffer (1:50 ratio).

2. **Aspartate Sepharose Column**

   Preparation of Aspartate-Sepharose affinity matrix (Porath, 1974).
   Aspartate-Sepharose matrix was prepared in the process as follows:
   1. preparation of Cyanogen bromide (CNBr)- 100 mg/ml of distilled water. The solution was stirred for 30 minutes.
   2. activation of Sepharose with CNBr
      a) equilibrate Sepharose CL-6B (Pharmacia) with phosphate buffer (2M, pH 12.1) by suspending 40 ml swollen matrix in 15ml buffer for 30 min,
      b) equilibrate with 3 M phosphate buffer (pH12.1) for 30 min,
      c) equilibrate with 5 M phosphate buffer (pH12.1) for 30 min,
      d) cyanogen bromide (6ml/100g gel) was added to the slurry in small aliquots with constant stirring over 2 min followed by stirring for another 8 min,
MATERIALS AND METHODS

e) immediately the gel was transferred to sintered glass filter mounted on a suction conical flask. The matrix was washed thoroughly with cold distilled water,

f) 1-2 ml activated sepharose was mixed with 2 ml qualitative reagent (12 ml pyridine, 2.5 ml conc. HCl and 0.5 g barbituric acid in 20 ml volume). The activated matrix gave purple colour

3. The activated Sepharose was suspended in one volume of cold saturated solution of adipic acid dihydrazide. The reaction was allowed to proceed at 4°C for 16 hour on a rotary shaker in the dark.

4. The matrix was washed thoroughly with cold distilled water followed by the addition of 0.2 M NaCl to remove unbound dihydrazide. Finally the matrix was suspended in 0.1 M NaHCO₃ pH8.9 and mixed with 25 ml of aspartate (10 g aspartic acid pH adjusted to 8.9 with KOH pellet). The mixture was kept for 24 hours under continuous stirring.

5. The Matrix was then washed thoroughly with cold distilled water and stored at 4°C.

Aspartate- Sepharose column (10 ml) was packed and equilibrated with buffer 2. The DE52 purified protein was loaded onto the column and the column was washed thoroughly with 10 volume of the same buffer. Aspartate kinase was eluted with 20 ml buffer B containing 300 mM of KCl. The eluted protein was put in a dialysis tube and concentrated in cold PEG 8000. The resultant protein was suspended in buffer B and dialysed for 6 hours against excess of the same buffer B.
Polyacrylamide Gel Electrophoresis (PAGE) of Protein

Protein samples were analysed by polyacrylamide gel electrophoresis. The gel was run in 1X Tris-glycine buffer (1 litre 5X buffer contained 25 mM Tris-base, 250 mM glycine and 0.1% SDS (for denaturing gel). Separating gel (6% or 8%) was cast accordingly, and above the separating gel a stacking gel was cast and appropriate comb was placed. The protein sample was loaded with gel-loading buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS was omitted when run the nondenaturing gel, 0.1% bromophenol blue and 10% glycerol). The gel was run in 1X Tris-glycine buffer at constant voltage ranging from 100 to 200 volts as required. The protein band was visualised with Coomassie Blue G-250.

Fixation

The protein gel was fixed in 12.5% TCA for an hour and rinsed with distilled water.

Staining & Destaining

The protein gel was soaked overnight in staining solution (0.25% Coomassie Blue G-250) and then destained with destaining solution (7% acetic acid and 5% methanol).

Plasmid Engineering

Minipreps

Plasmid DNA transformants were isolated by the alkaline lysis method of Sambrook et al (1989) with modifications.
Cultures were grown in tubes containing 1.5 ml LB with appropriate antibiotics. The cells were pelleted down by centrifugation at 5000 rpm for 2 minutes at 4°C. The supernatant was discarded and the pellet was suspended in 100 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, pH 8.0) and 5μl of RNase (20 mg/ml). Freshly prepared 200μl lysis buffer (0.2N NaOH, 1% SDS) was added, mixed slowly and incubated on ice for 5min. Then 150 ml ice cold 3M potassium acetate pH 5.5 was mixed and the mixture was kept in ice for 10 minutes.

The white precipitate containing genomic DNA and proteins was pelleted down by spinning at 12000 g for 20 minutes at 4°C. The supernatant was carefully taken and mixed with equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1). The mixture was centrifuged at 12000 g for 10 minutes and the upper aqueous layer was taken and extracted with a mixture of chloroform: isoamylalcohol (24:1). Isopropanol (0.6 volume) was added and incubated for 10 minutes at room temperature. The precipitated DNA was pelleted at 12,000 g for 15 minutes at room temperature. The pellet was washed with 70% ethanol and vacuum dried. Plasmid DNA was dissolved in 50 ml TE buffer and stored at 4°C.

Maxipreps

Medium scale isolation and purification of plasmid DNA (100μg) for restriction digestion, PCR, sequencing, etc. was carried out using "Qiagen Column" (Germany)
Cells were grown in 50 ml Luria Broth (LB) with appropriate antibiotics. The cell pellet was resuspended in 4.0 ml P1 with RNase (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). 4.0 ml P2 buffer (200 mM NaOH, 1% SDS) was added, mixed gently, and incubated at room temperature for 5 min. 4.0 ml of chilled P3 buffer (3.0 M potassium acetate, pH 5.5) was added, mixed immediately but gently and incubated on ice for 15 min.

The precipitate containing genomic DNA and proteins was centrifuged at 12,000 at 4°C for 20 minutes. The supernatant was promptly decanted and subjected to further centrifugation to remove the residual precipitate at the same speed.

Qiagen midi-Column was equilibrated with 4.0 ml of equilibration buffer, QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100). The supernatant was loaded onto the column and allowed to pass. The column was washed with wash buffer, QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol). Finally DNA was eluted with 5 ml of elution buffer, QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol).

The plasmid DNA was precipitated by adding 0.6 volume of isopropanol. The precipitate was pelleted down by centrifugation at 15,000 rpm at room temperature for 10 minutes. The pellet was washed with 70% ethanol, vacuum dried and dissolved in 200 µl of TE buffer.

**Agarose Gel Electrophoresis**

DNA samples were analysed by electrophoresis on 0.7-1.0 % agarose gel as required. The gel was run in 1X TAE buffer (1 litre 10X buffer contained
MATERIALS AND METHODS

48.4 gm Tris-base, 11.4 ml glacial acetic acid and 20 ml 0.5 M EDTA pH 8.0). Agarose was dissolved by heating in 1X TAE buffer and ethidium bromide was added at a concentration of 0.5 mg/ml after cooling. The gel slabs were prepared by pouring the dissolved agarose into the required casting tray fitted with appropriate comb. The samples were loaded after mixing with 0.1 volume of 6X gel loading buffer (30% glycerol, 0.25 % xylene cyanol and bromophenol blue). The gel was run in horizontal electrophoresis tank in 1X TAE buffer at constant voltage ranging from 50 to 100 volts as required. The DNA band was visualised under UV transilluminator (Photodyne) and photographed.

Plasmid DNA Digestion

Plasmid DNA was digested with restriction enzyme bought from New England Biolabs or Boehringer Mannheim USA. 2-5 U of appropriate enzyme was used for 1 µg plasmid DNA digestion for 4 hours at 37°C. The buffer used was supplied by the respective company. BSA was supplemented when required. The digestion was stopped either by heat inactivation or by adding 1-5 µl of 0.5 M EDTA to the reaction mixtures.

Gel Elution of DNA Fragment

The required DNA fragment was purified from agarose gel by phenol/chloroform method. The digested sample was run on 0.7% agarose gel, the desired band was cut out and transferred to microfuge tube. TE-saturated phenol (approx. ½ volume of agarose piece) was added to the tube, vortex to get a suspension and kept at - 80°C for an hour. The tube was centrifuged at 14,000 rpm for 15 min. To the upper aqueous layer half volume of TE-saturated phenol and equal volume of chloroform : isoamyl
alcohol (24:1) were added and the mixture was centrifuged at 12,000 rpm for 10 min. The upper aqueous layer was collected and subjected to another chloroform/isoamyl alcohol extraction. To the aqueous layer 0.2 volume of 10 M ammonium acetate, 2 volume of 100 % ethanol were added, and kept at -80°C for an hour. The DNA precipitate was then collected after centrifugation at 14,000rpm for 15 min, washed with 70% alcohol and vacuum dried. DNA was dissolved in required amount of TE.

Dephosphorylation of Vector DNA

The linear pUC18 vector containing EcoRI sticky ends was dephosphorylated using Shrimp alkaline phosphatase (SAP, NEB). This enzyme removes 5' phosphate group from linear DNA which is required by ligase, thus preventing self ligation of the vector. The DNA sample was incubated with SAP (5U) in 1X SAP buffer at 37°C for 10 minutes. The enzyme was inactivated by heating at 65°C for 15 minutes.

Ligation of DNA

Ligation of the vector with insert was carried out in 1:10 molar ratio in presence of T4 DNA ligase from NEB. The dephosphorylated pUC18 or pGemT vector was purified and mixed with 10 U of enzyme in 1X buffer and kept at 14°C for 16 hours.

Microbiological Procedures

Preparation of Competent Cells

The protocol followed for making bacterial competent cells is given below (Sambrook et al, 1989). The host cell culture (DH5α) was streaked on a
LB plate containing nalidixic acid (15 µg / ml) from a frozen glycerol stock at -70 °C. A single colony was inoculated in 2 ml LB containing nalidixic acid (15 µg / ml). Overnight culture was inoculated to 50 ml LB and allowed to grow for 2-3 hours until OD_{600} nm reached to 0.3- 0.4. The flask was then kept on ice for 15-30 minutes.

Cells were then transferred aseptically in a sterile ice cold tube and centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 25 ml ice-cold 0.05 M CaCl₂ and kept on ice for 30 minutes.

The cells were recovered by centrifuging at 5000 rpm for 5 minutes at 4°C. The supernatant was decanted completely and the pellet was resuspended in a total volume of 4 ml ice cold 0.05 M CaCl₂ and 1 ml 75% ice cold sterile glycerol.

A sample aliquot of 200 µl was transformed for checking the viability, contamination and efficiency of transformation. Rest of the suspension was kept at 4 °C for 6 hours to increase the efficiency of transformation. Once the competent cells were observed to be all right, these cells were dispensed in 200 µl aliquots and stored at -70°C for transformation.

**Transformation**

Transformation protocol was followed according to Sambrook et al. (1989). The aliquot of 200 µl competent cells was thawed on ice, to which 50% of the ligated DNA was added, mixed by tapping slowly and kept on ice for 30 minutes. The tube was given heat shock at 42 °C for 90 seconds and rapidly returned to ice for 2 minutes. LB (1 ml) was added and the cells were
incubated at 37°C for an hour with slow shaking. The cells were pelleted down by centrifugation at 5000 rpm for 5 minutes. The concentrated cell suspension was plated on the antibiotic selection plate (ampicillin 100 mg/ml).

For blue white screening, 8 μl of 100 mg/ml IPTG in H2O and 40 μl of 20 mg/ml X-gal in DMF was plated on the plate before spreading the transformants. The plate was kept at 37°C for 12-16 hours.

**Colony PCR**

The recombinant bacteria were analysed by colony PCR. A master PCR mixture was prepared without template DNA with 200 mM dNTPs, 100 nM of each primers, 1X buffer, IU Taq Polymerase. The putative recombinant colony was then picked up with a tooth pick and suspended in the reaction mixture.

The reaction mixture was then subjected to following thermo cycles: in the first cycle denaturation temperature was kept for 5 min and the last cycle extension was for 7 min at 72 °C and all other cycles had 94°C for 1 min (denaturation temperature), 55°C for 1 min (annealing temperature) and 72°C for 2 min (extension temperature). The reaction was carried out for 30 cycles.

**Plating and Transferring Bacteriophage Library**

**Plating Bacteria**

We used LE392 (F⁻ hsdR514, supE44, supF58, lacY1 or Δ (lacIZY)6, galK2, galT22, metB1, trpR55, ì⁻) as plating bacteria. A single colony was inoculated in LB medium in presence of 0.2% maltose and 0.01 M MgSO₄ and grown overnight.
MATERIALS AND METHODS

Number of Bacteriophage λ Plaque

0.2 ml of plating bacteria was added to 3.0 ml of soft agarose medium (0.6% agarose in LB medium) at 55 °C and the soft agarose-bacterial suspension was poured onto a plate containing LB bottom agar. The plate was then dried in a laminar hood for half an hour.

The phage lysate was serially diluted in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl pH 7.5 and 0.01% gelatin) as 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10² and 10 µl was spotted on the above plate. The plate was incubated at 37 °C for 6 hours and number of plaques were counted at different dilutions.

Screening Bacteriophage λ Plaque

1. An aliquot of Bacteriophage λ (library) containing 3,000 recombinant clones was mixed with 200 µl of LE392 and incubated at 37 °C for 20 minutes. The mixture was then added to 3.0 ml molten top agarose at 55°C and poured onto a plate containing hard agar. The plate was dried and incubated at 37 °C until a near confluent layer was obtained.

2. When the near confluent layer was achieved the plate was kept at 4°C for one hour. A nylon membrane (82 mm, Amersham, USA) was placed on the top agarose and marked by piercing the needle through the membrane at three asymmetric positions with one, two and three dot markings respectively.

3. The membrane was taken out very carefully and placed (plaque side up) onto a 5 mm Whatman paper soaked with denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 5 minutes.

4. The nylon membrane was overlaid onto a 5 mm Whatman paper wetted in a neutralizing solution (1.5 M NaCl and 0.5 M Tris-HCl pH 8.0) for another 7
MATERIALS AND METHODS

minutes and subsequently rinsed with 2X SSPE (1 litre 20X SSPE containing 174 g NaCl, 27.6 g NaH₂PO₄ and 7.4 g EDTA) to remove debris.

5. The membranes were then crosslinked and hybridized as described in the section DNA analysis.

**Bacteriophage DNA Isolation**

1. The bacteriophage was mixed with plating bacteria (~10⁵) and incubated at 37°C for 20 min.

2. The plate was incubated for 8-12 hours at 37°C without inversion.

3. When confluent layer was achieved the soft agar was scraped into a sterile centrifuge tube. SM buffer (5ml) was added to the plate to rinse off any remaining agarose.

4. Chloroform (0.1ml) was added and mixed by rotation for 15 min at 37°C.

5. The supernatant was recovered by centrifugation (4,000 rpm, 10 min).

6. In 50 ml supernatant 100 µl of Buffer L1 (300mM NaCl, 100 mM Tris-HCl pH 7.5, 10 mM EDTA) was added with RNase and DNase and incubated at 37°C for 30 min.

7. 10 ml of Ice-cold buffer L2 (30 % polyethylene glycol, 3M NaCl) was added, mixed gently and incubated on ice for 60 min.

8. Treated supernatant was centrifuged at 15, 000g for 15 min and the pellet was resuspended in 3 ml of buffer L3 (100 mM NaCl, 100 mM Tris-HCl pH 7.5, 25 mM EDTA).

9. Three ml of buffer L4 (4 % SDS) was added, mixed gently and incubated at 70°C for 20 min.

10. Three ml of buffer L5 (3 M potassium acetate) was added, mixed immediately, but gently and centrifuged at 15, 000g to get a clear particle free lysate. Qiagen column (Germany) was used to purify DNA as described in plasmid purification.
DNA Analysis

Plant DNA Isolation

Plant DNA was isolated by CTAB (Ausubel et al, 1995) method as follows:

1) In CTAB extraction solution (2% CTAB, 100 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0 and 1.4 M NaCl) 2-ME was added to give a final concentration of 2% (v/v). This solution and CTAB/NaCl (10 % CTAB in 0.7 M NaCl) solution were heated to 65°C.

2) Plant tissue was pulverised to a fine powder by liquid nitrogen and the frozen tissue was transferred to an organic solvent-resistant test tube.

3) 2-ME/CTAB extraction solution was added to the pulverised tissue and mixed to wet it thoroughly. Incubation was done for 60 min with occasional mixing at 65°C.

4) The homogenate was extracted with equal volume of 24:1 chloroform:isoamyl alcohol and centrifuged for 5 min at 7500 g.

5) The supernatant was collected and 1/10 volume of 65°C CTAB/NaCl solution was added and mixed by inversion.

6) The upper aqueous layer was collected, equal volume of chloroform/isoamyl alcohol was added and centrifuged at 7000g.

7) Top aqueous layer was collected to which equal volume of CTAB precipitation solution (1 % w/v CTAB, 50 mM Tris-HCl pH8.0, 10 mM EDTA pH 8.0) was added. The mixture was incubated for 30 min at 65°C.

8) The precipitate was collected after centrifugation at 3000 rpm for 5 min.
9) The precipitate was resuspended in 1 ml high salt TE (10mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0 and 1M NaCl).

10) The DNA was finally precipitated by adding 0.6 volume of isopropanol, mixed well and centrifuged at 10,000 rpm for 15 min at 4°C.

11) The pellet was washed in 70 % alcohol, vacuum dried and dissolved in TE. DNA was used for southern hybridization or PCR.

Southern Blotting

DNA was digested with various restriction enzymes and run in 1 % agarose gel with appropriate DNA marker. The gel was stained with ethidium bromide. The gel, before blotting onto the membrane, was subjected to the following treatment: I) it was soaked in 0.25 M HCl for 30 min with gentle shaking (depurination) II) following depurination, the gel was rinsed with distilled water and soaked in denaturing solution containing 1.5 M NaCl and 0.5 M NaOH for 40 min with gentle shaking (denaturation). III) After denaturation the gel was treated with neutralizing solution containing 1.5 N NaCl and 0.5 M Tris-HCl pH 7.0 for 40 min. After neutralization the gel was blotted onto the membrane as follows: 1) A blotting sheet (3 mm) was placed on a glass plate and soaked with 20x SSC, 2) The gel was placed on it, and 3) a nylon membrane of exactly the same size of the gel was soaked with 2X SSC and placed on the gel, 4) Two Whatman 3 mm papers followed by paper towels were placed on the membrane and weighed 5) The whole assembly was kept for 16 hours. The membrane was taken out, soaked in 2X SSC and UV crosslinked (Stratagene).
Southern Hybridization

Hybridization was done as follows (Sambrook et al, 1989):
1) The membrane was placed in hybridization bottle and rinsed with water. Excess water was removed. 2) Hybridization solution containing 5X SSC, 5X Denhardt solution, 1%SDS, 100g/ml denatured salmon sperm DNA (1 ml/cm² of the membrane) was added in the bottle and prehybridization was carried out at 42°C for an hour. 3) Fresh hybridization solution was replaced and heated at 42°C, to which denatured probe was added and the hybridization was carried out at 42°C for 16h.

PCR by Degenerate Primer

Four degenerate primers were designed from the reported sequence of aspartate kinase as follows: AK1, AK2, AK3 and AK4. The PCR reaction was carried out employing different primer combinations and changing different PCR components like template DNA (genomic DNA), primer concentrations, Mg²⁺ concentrations and annealing temperatures.

RNA Analysis

RNA Isolation

Plant RNA was isolated by guanidium thiocyanate method as follows (Sambrook et al, 1989). 1) Plant tissue (1 g) was pulverised to a fine powder by liquid nitrogen 2) 12 ml of cold denaturing solution (4 M guanidium thiocyanate and 33 ml CSB buffer containing 42 mM sodium citrate, 0.83% N-sarcosine and 0.2 mM 2-ME) was added to the pulverised tissue 3) 2 M sodium acetate was
added, mixed thoroughly and chilled on ice for 15 min, 4) after centrifugation at 10,000 rpm for 20 min the supernatant was collected to which equal volume of isopropyl alcohol was added and the mixture was incubated at -20°C for one hour to precipitate the RNA, 5) RNA pellet was resuspended in 5 ml of denaturing solution at 65°C and precipitated by adding equal volume of isopropyl alcohol at -20°C for 30 min, 6) RNA was washed in 70% alcohol, vacuum dried for 15 min and finally dissolved in DEPC treated water. The RNA was used immediately or stored at -80°C in ethanol (1 ml of RNA in DEPC water, 100 μl 3 M sodium acetate and 2 ml ethanol).

RNA Dot-Blot

RNA samples (20 μg) were denatured in presence of 6 % formaldehyde and 50 % formamide at 55°C for 30 min and were blotted on Hybond-N membrane (Amersham, USA) by dot-blot apparatus (S&S, USA). The membrane was crosslinked and used for hybridization.

Gel Electrophoresis of RNA

The RNA was analysed on 1% denaturing gel containing 1 g of agarose melted in 72 ml of water and cooled to 65°C, in a solution of 10 ml of MOPS buffer (0.4 M MOPS pH 7.0; 0.1 M sodium acetate, 0.01 M EDTA) and 18 ml of formaldehyde.

Preparation of RNA before Loading

RNA (5 to 30 μg), MOPS (10X), formaldehyde (1.75 μl) and formamide (5 μl) were mixed together to 10 μl final volume. The mixture was then heated at 55°C for 15 min, 1μl of dye (1mMEDTA, 0.025 g BPB, 50% glycerol) was
added, spinned briefly and loaded into the gel. The gel was run in 1X MOPS buffer.

Staining & Destaining

After running the RNA gel to ¾ distance, the gel was rinsed with RNase free water. Formaldehyde was removed by soaking the gel in 0.5 M ammonium acetate for 20 min. The gel was stained with 5 μg/ml ethidium bromide in 0.5 M ammonium acetate for 40 min. Destaining was done in RNase free water to remove the excess stain.

RNA Blotting

The RNA gel was subjected to the following treatment: 1) the gel was soaked in an excess of 50 mM NaOH and 10 mM NaCl for 45 min at RT, 2) the gel was neutralised by soaking in 0.1 M Tris-HCl pH 7.5 for another 45 min, 3) finally the gel was soaked in 20X SSC for one hour. The RNA was transferred to the nylon membrane as follows: 1) A blotting sheet (3 mm) was placed on a glass plate and soaked with 20X SC, 2) The gel was placed on it, 3) A nylon membrane of exactly the same size of the gel was soaked with 2X SSC and placed onto the gel, 4) Two Whatman 3 mm papers followed by paper towels were placed on membrane and weighed down, 5) The transfer assembly was kept for 16 hours. The membrane was taken out, soaked in 2X SSC and was UV crosslinked.

Northern Hybridization

Hybridization was done as follows (Sambrook et al, 1989): 1) The membrane was placed in hybridization bottle and rinsed with water. Excess water was removed, 2) Hybridization solution containing 5X SSC,
50%(w/v) formamide, 1% (w/v) SDS, 5X Denhardt solution and 100μg/ml denatured salmon sperm DNA (1 ml /cm² of the membrane) was added in the bottle and prehybridization was carried out at 42 °C for 3 hours, 3) Fresh hybridization solution was replaced and heated to 42 °C, to which denatured probe was added and the hybridization was carried out for 16 hours. The membrane was washed after hybridization as follows: 1) The hybridization solution was removed and the membrane was washed twice with 2X SSC and 0.1 % SDS for 5 min at RT, 2) For low stringency, the membrane was washed with 0.2 x SSC and 0.1 % SDS for 5 min at RT, 3) For medium stringency, washing was carried out with 0.2 x SSC and 1% SDS at 42 °C. After washing the membrane was exposed to X-ray film.

Sequencing
Sequencing was performed WITH USB SEQUENASE DNA SEQUENCING KIT

Preparation of Alkali-Denatured DNA

3-5 mg of RNA-free DNA (~1 pmol) was incubated in 0.2 M NaOH, 0.2 mM EDTA at 37 °C for 30 minutes. The DNA was precipitated with 0.1 volume of 3 M NaOAc and 3-4 volumes of ethanol, and kept at -70 °C for 30 minutes. The DNA was recovered by centrifugation at 14,000 rpm for 10 min, washed with 70% ethanol, air dried and dissolved in 7 ml water.

Primers Used

M13 / pUC18 forward primer. 5’ GTT TTC CCA GTC ACG AC 3’
M13 / pUC 18 reverse primer : 5’- TCA CAC AGG AAA CAG CTA TGA- c 3’
T7 promoter primer : 5’- TAA TAC GAC TCA CTA TAG GG -3’
T3 promoter primer : 5’- ATT AAC CCT CAC TAA AGG GA-3’
MATERIALS AND METHODS

Annealing of Primer to Template

The following components were added to the DNA solution (7 ml)

- Primer (0.5 pmol / ml) 1 ml
- Reaction buffer (5X) 2 ml

and heated at 65 °C for 2 minutes. The mixture was cooled slowly to 35 °C (~15-30 min) and kept on ice.

Labelling Reaction

Labelling mixture (1 in 5 in ddH₂O) and sequenase enzyme (1 in 8 in sequenase buffer) was diluted. The following components were added

- Template-primer 10.0 ml
- DTT 1.0 ml
- Diluted labelling mix 2.0 ml
- $35^\text{dATP}$ 0.5 ml
- Diluted sequenase 2.0 ml (to start reaction)

and incubated 2-5 minutes at room temperature or 22 °C.

Termination Reaction

2.5 ml each of ddGTP, ddATP, ddTTP and ddCTP were taken in four different eppendorfs and preheated these tubes at 37 °C. After 2 minutes of labelling reaction 3.5 ml of labelling reaction mixture was added to each of the four termination tubes, mixed and quickly returned to 37 °C for 5 minutes. Reaction was terminated by adding 4 ml of stop solution.

Preparation of Sequencing Gel

The plates were cleaned with ethanol and the notch plate was anti-siliconised with dimethyl dichloro silane (SIGMA) (5% in chloroform) and simultaneously spreaded the binding silane (30 ml methanol, 100 ml glacial
acetic acid and 30 ml binding silane; Pharmacia) on the other plate to stick the gel on the plate.

**Preparation of 6% Gel Mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>22.0 grams (7 M)</td>
</tr>
<tr>
<td>30% acrylamide solution</td>
<td>10.5 ml</td>
</tr>
<tr>
<td>TBE (10X)</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Made the volume of the mixture to 50 ml with ddH₂O.

To start polymerisation reaction, TEMED (20 μl) and 20% APS (200 μl) were added. The solution was mixed and cast the gel immediately with the sequencing comb.

When the gel was set, the tape was removed and then placed the plates in an electrophoresis apparatus. Filled the top and the bottom tank of the electrophoresis apparatus with 1X TBE (10X; 500 ml; 54 gm Tris base, 27.5 boric acid, 20 ml 0.5M EDTA, pH 8.0). The comb was removed and the wells were rinsed with 1X TBE to remove bubbles and urea. Gave a pre run (1250 V or 50 W) before loading DNA samples.

**Sample Loading**

Heated the samples at 75-90 °C for 2 minutes. Rinsed the wells to remove any bubbles and urea. Loaded 3 ml of reaction mixture in the order GATC or ACGT.

**Fixing the Gel and Drying**

After the gel was run, it was soaked in fixing solution (5% acetic acid, 15 % methanol in H₂O) for 20-25 minutes to fix the DNA and to remove urea. After fixing the gel was dried for 4 hours under vacuum maintaining temperature at 70 °C. Autoradiographs were taken.