Chapter 2

Materials and Methods

2.1 Materials used:

2.1.1 Bacterial strains and vector used:

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Reference / Source</th>
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</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>BRL</td>
</tr>
<tr>
<td>E. coli MC1061</td>
<td>Amersham</td>
</tr>
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</table>

2.1.2 Vectors:

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Source</th>
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<tbody>
<tr>
<td>pBI 121</td>
<td>Jefferson et al. (1987)</td>
</tr>
<tr>
<td>pUEX-1</td>
<td>Amersham</td>
</tr>
<tr>
<td>pBluescript SK+</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

2.1.3 Antibiotics used:

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Abbreviation</th>
<th>Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Ap</td>
<td>40</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Sm</td>
<td>100</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Km</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Cb</td>
<td>100</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Cm</td>
<td>200</td>
</tr>
</tbody>
</table>

Cowpea seeds were obtained from local sources.
2.2 Purification of cowpea trypsin inhibitor (CPTI):

2.2.1 Protein Extraction

Seeds were soaked in water for 30 min. The seed coat was removed and the seeds were ground in extraction buffer (0.1 M Sodium Acetate buffer (pH 4.0) containing 0.3M NaCl, 0.01M CaCl$_2$) in a mortar pestle on ice and incubated at 4°C with gentle shaking for 16h. The extract was centrifuged at 8,000 rpm for 30 min. The supernatant contains crude protein extract of cowpea.

2.2.2 Partial Purification by Ammonium Sulphate precipitation

- The volume of the crude extract was measured.

- 30% ammonium sulphate precipitation: Ammonium sulphate (176g/l) was added slowly to the extract which was kept on ice. After complete dissolution of the salt the extract was incubated for 30 min on ice. It was then centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was dissolved in minimum volume of extraction buffer.

- 60% ammonium sulphate precipitation: The volume of the supernatant was measured. Additionally, ammonium sulphate (198g/l) was added slowly to the extract as described before. It was then centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was dissolved in minimum volume of extraction buffer.

- 90% ammonium sulphate precipitation: The same experiment was repeated with additional ammonium sulphate (227g/l).

2.2.3 Affinity chromatography

Activation of the column:

1. 20 ml packed bed volume of sepharose 4B (LKB-Pharmacia) was taken in a separating funnel and was washed with 200ml of the following solution:
2. The gel was weighed, transferred to a flask and suspended in 60% acetone (with w/v ratio = 1.1).

3. Solution of (30mg/g of Gel) CNBr (1M in acetone) was added to the gel and stirred continuously.

4. Triethylamine (1.5M in 60% acetone) was added dropwise.

5. 200 ml of washing solution was added (acetone:0.1N HCl – 1:1). This mixture is stable for one hour.

6. The activation of the gel was tested as described below:
   
   (a) Test solution: In a stoppered 25ml measuring cylinder, 25mg of thioburbutiric acid was suspended in 7ml of pyridine and 3ml of water. It was shaken well to dissolve.
   
   (b) A little gel slurry was dried in a filter paper and transferred to a test tube containing 1ml test solution.
   
   (c) The solution turned red-purple which confirmed activation.

Washing:

The CNBr-activated sepharose 4B was washed on a separating funnel with 60% acetone followed by 30% acetone, followed by 0.1M NaHCO₃ (pH 8.5).

Binding of trypsin to activated sepharose 4B:

Trypsin (Sigma) was dissolved (10mg/ml) 0.1M NaHCO₃.

- Stock trypsin solution was diluted with 900μl 0.1M NaHCO₃.
Table 2.1: Assay of trypsin

<table>
<thead>
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<th></th>
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<th>2</th>
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<th>5</th>
</tr>
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<tbody>
<tr>
<td>Azocasein (2.5%)</td>
<td>250μl</td>
<td>250μl</td>
<td>250μl</td>
<td>250μl</td>
<td>250μl</td>
</tr>
<tr>
<td>pH 8.3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1M Tris HCl</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
<td>50μl</td>
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<td>pH 8.0</td>
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<tr>
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<tr>
<td>Trypsin(50μg/ml)</td>
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<tr>
<td>Trypsin-gel</td>
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<td>-</td>
<td>-</td>
<td>2mg</td>
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</tr>
</tbody>
</table>

- O.D. was checked at 320nm, 280nm and 260nm.
- Total O.D. of the stock solution was calculated. From that value, concentration of the trypsin solution was measured. Trypsin solution was taken in a conical flask. Activated Sepharose 4B was added and incubated overnight at 10°C-15°C. It was transferred to a sintered separating funnel and was washed with 0.1M NaHCO₃. O.D. of the washings were checked at 320nm, 280nm and 260nm, which gave the concentration of the unbound trypsin. From that the total bound trypsin in the gel was estimated.

Assay of trypsin bound to sepharose 4B using Azocasein:

The procedure given in Table 2.1 was followed. All the reaction mixtures were incubated at 37°C for 10 min. The reaction was stopped by adding 500μl of 10% TCA (Trichloroacetic acid) in each tube. All the samples were centrifuged at 10,000 rpm for 1 min. 600μl of the supernatant was taken from each tube and 600μl of 0.5N NaOH was added to each of them. They were kept for 5 min and O.D. was checked at 428nm. Comparing with the value that was obtained for known concentration, that of the bound trypsin was calculated.

Storing of the gel:

The gel was washed with 1M NaCl followed by water. It was then equilibrated with 1M Tris (pH 8.0). 15-20% of glycerol was added for storing at −20°C.
Purification of the cowpea trypsin inhibitor:

The fraction that gave highest trypsin inhibitory activity after ammonium sulphate precipitation was dialysed against the protein extraction buffer. The activated trypsin-sepharose 4B column was packed at 4°C. It was equilibrated with the extraction buffer. The sample solution with known protein concentration was loaded and was recycled thrice. The column was washed thoroughly with the extraction buffer to remove the unbound protein. The CPTI was eluted with 0.01M HCl containing 0.3M NaCl and 0.01M CaCl₂. 0.5ml fractions were collected in tubes containing 0.5ml of 100mM Tris (pH 7.0). Absorbence at 320nm, 280nm, 260nm was measured for each fraction. The fraction containing the CPTI was pooled and assayed for trypsin inhibitory activity. This purified protein was used in all experimental procedures.

Assay of trypsin inhibitor in the protein extracts:

Trypsin was assayed in each fraction using the protocol described in Table 2.2. As soon as the azoacaein was added to reaction mixtures the tubes were incubated at 37°C. After 10 min, the reaction was stopped by adding 500µl of 10% TCA in each tube. After brief centrifugation, 600µl of the supernatant was taken from each tube and 600µl of 0.5N NaOH was added to each of them. Each sample was incubated at room temperature for 5 min. Absorbence at 428nm was measured in each case (Tomarelli et al., 1949).

Estimation of the protein:

Protein concentration was determined as described by Bradford (Bradford, 1976). Bio-rad protein assay reagent (200µl) was added to 800µl of the sample solution and was incubated for 10 min at room temperature. The absorbence was measured at 595nm. Protein concentration was deduced from the standard curve made with known concentration of BSA.
<table>
<thead>
<tr>
<th>Sample no.</th>
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<th>2</th>
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Incubate for 5 to 10 min.

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<th>Water</th>
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<th>100μl</th>
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<tbody>
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<td></td>
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<tr>
<td>Azocasein</td>
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<td>250μl</td>
<td>250μl</td>
<td>250μl</td>
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<td>250μl</td>
</tr>
<tr>
<td>(2.5%)</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>pH 8.3</td>
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</tbody>
</table>

Table 2.2: Assay of trypsin inhibitor
SDS–Polyacrylamide gel electrophoresis (SDS–PAGE):

(Reference Laemmli, 1970)

Reservoir gel buffer: 0.025M Tris. HCl (pH 8.3), 0.192M Glycine, 0.1% SDS

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Resolving Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide:Bis (30:0.8)</td>
<td>10.00 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>3M Tris HCl (pH 8.8)</td>
<td>3.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris HCl (pH 6.8)</td>
<td>-</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.40 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>1.5% APS</td>
<td>1.50 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Water</td>
<td>14.20 ml</td>
<td>10.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15μl</td>
<td>15μl</td>
</tr>
</tbody>
</table>

1. The gel plates were assembled with the spacers and 30 ml of resolving gel mixture was poured in and 0.5 ml of water saturated butanol was overlaid and the gel was allowed to polymerise for 20-30 min.

2. Excess of butanol was removed by filter paper and 20 ml of stacking gel mixture was poured and the comb was inserted.

3. After polymerisation, the comb was removed and the wells were rinsed with the reservoir buffer.

4. The gel was fitted to the apparatus and filled with the reservoir buffer, the protein samples were loaded carefully and electrophoresed at 5V/cm until the dye front is 1cm from the bottom of the gel.

5. The gel was removed and either stained or processed further for Western blotting.

Staining of the protein gel:

The polyacrylamide gel was stained in 0.2% Coomassie Brilliant blue, 10% acetic acid, 40% methanol for 30 - 90 min and destained several times with 10% acetic acid, 40% methanol.
Raising polyclonal antibodies in rabbits and mice:

Male rabbits of 1.5kg were used in all experiments. Sub-cutaneous and intramuscular injections were adopted for immunisation. 1.0mg of antigen in PBS was mixed with equal volume of Freunds Complete Adjuvant (FCA) until it formed a thick emulsion and injected. An equal antigen concentration in Freunds Incomplete Adjuvant (FIA) was injected on the 21st day. Serum was collected from the marginal ear vein on 31st day and tested for antibody titre by indirect-ELISA. 6-8 weeks old female mice were given 100μg of CPTI by intraperitoneal injection with equal volume of FCA (Sigma). On 7th, 14th, and 21st day the mice were reinjected with 100μg of the same antigen in FIA (Sigma). On 36th day test tail bleed was performed and the titre of the serum was determined by indirect-ELISA.

Indirect-Enzyme linked immunosorbent assay (Indirect-ELISA):

PBS: 10mM KH₂PO₄, 10mM K₂HPO₄(pH 7.5)
PBST: PBS + 0.05% Tween 20
Blocking solution: 1% BSA in PBS
Substrate Solution for Horse radish peroxidase (HRP): O'Phenyl diamine (OPD) 1mg/ml dissolved in 0.1M citrate buffer (pH 4.5), H₂O₂ 0.4μl/ml.

1. 100μl of the protein (10μg/ml) in PBS was coated in 96-well microtitre plates and incubated at 4°C overnight.

2. The plates were washed thrice with PBS and the wells were blocked by adding 150μl of blocking solution and incubated at 37°C for 1 h.

3. The plates were washed thrice with PBST.

4. 100μl of either one of the following primary antibodies were added and incubated at 37°C for 1h.

   (a) Rabbit polyclonal antibodies.

   (b) Mouse polyclonal antibodies.
5. The plates were washed thrice with PBST.

6. 100μl of the secondary antibody conjugate (goat anti-species enzyme conjugate) was added and incubated at 37°C for 1 h.

7. The plates were washed six times with PBST.

8. 100μl of the substrate solution was added and incubated at room temperature for 20 min.

9. The absorbence was determined at 450nm in an ELISA reader (Anthos).

### 2.3 Isolation and cloning of cowpea trypsin inhibitor gene:

#### 2.3.1 Total RNA extraction:

Total RNA was extracted from cowpea seeds following “Hot phenol method” as described by Gelvin (1988). All the glasswares were baked at 250°C for 6–8h. All the centrifuge tubes and tips were siliconised. All the solutions and distilled water were treated with DEPC followed by autoclaving.

Solutions:
- RNA extraction buffer: 100mM LiCl, 1% SDS, 100mM Tris. NaOH (pH 9.0), 10mM EDTA.

The material was harvested with liquid nitrogen. Equal volume of RNA extraction buffer and phenol with hydroxyquinoline were mixed and equilibrated the previous day. The mixture was heated to 90°C in a water bath in a fume hood. The material was ground in liquid nitrogen in a precooled mortar with a pestle until a fine homogeneous powder was obtained. The frozen powder was transferred to a conical flask kept in ice–salt mixture, precooled at −80°C with the help of a spatula cooled in liquid nitrogen.

2 ml of mixed phenol/extraction buffer was added per gram of fresh weight of seed. The flask was swirled vigourously until a milky suspension was obtained that was devoid of lumps of frozen material. The final temperature of the mixture was kept at 25-30°C.
The mixture was shaken at 300 rpm on a gyratory shaker for 5 min at room temperature, 1ml of chloroform was added per gram of fresh weight and shaking was continued for 15-30 min at room temperature. The suspension was transferred to a tube and centrifuged at 15,000 rpm for 15 min at 25°C. The upper aqueous layer was transferred to a flask and 1ml of chloroform was added per gram of fresh weight and the mixture was shaken at 300 rpm for 15 min.

The mixture was centrifuged at 15,000 rpm for 15 min at 25°C. The upper aqueous phase was collected in a centrifuge tube and 1/3 vol. of 8M LiCl was added and mixed thoroughly. RNA was allowed to precipitate for 16-48h at 4°C. It was centrifuged at 10,000 rpm for 15 min at 4°C and the RNA pellet was washed with 2M LiCl at 4°C and twice with 80% ethanol and finally was dried. The pellet was dissolved in sterile double distilled water and stored at —20°C in small aliquots.

2.3.2 Purification of mRNA:

The mRNA was purified by passing the total RNA through oligo dT cellulose column as described by Sambrook et al (1989).

Solutions:
1x column loading buffer: 20mM Tris. HCl (pH 7.6), 0.5M NaCl, 1mM EDTA (pH 8.0), 1% sodium lauryl sarcosinate.
Elution buffer: 10mM Tris. HCl (pH 7.6), 1mM EDTA (pH 8.0), 0.05% SDS.

0.5-1.0 g of oligo dT cellulose was suspended in 0.1 N NaOH. A column of oligo dT cellulose was prepared in a sterile Dispocolumn. The column was washed with three volumes of sterile water and then with 1x column loading buffer. The RNA was dissolved in sterile water and was heated to 65°C for 5 min. The RNA solution was cooled to room temperature quickly and equal volume of 2x column loading buffer was added. The solution was applied to the column and immediately the eluate was collected in a sterile tube. When all the RNA solution had entered the column, one column volume of 1x column loading buffer was added and the collection of the eluate was continued. When all the solution had eluted, the collected fractions were heated to 65°C for 5 min. and reapplied to the column. Again the material flowing through it was collected. The
column was washed with 5-10 column volumes of 1x column loading buffer, collecting 1ml fractions. The absorbence of each fraction was measured at 260nm. The poly(A)+ RNA was eluted from the oligo (dT)-cellulose with 2-3 column volumes of sterile, RNase free elution buffer. The fractions were collected equivalent in size to 1/3 to 1/2 of the column volume. The absorbence of the fractions were measured at 260nm (the cuvettes were soaked in concentrated HCl:methanol (1:1) for 1h and then washed extensively in DEPC treated water). The fractions were pooled. 3M sodium acetate (pH 5.2) was added to it to make a final concentration of 0.3M, mixed well. 2.5 volumes of ice-chilled ethanol was added and mixed and stored for 30 min on ice. The poly(A)+ RNA was recovered by centrifugation at 10,000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol and was allowed to dry in air. The RNA was redissolved in a small amount of sterile water. Absorbence at 260nm was measured. 3 volumes of ethanol was added and stored at —70°C until it was needed. To recover RNA, 3M sodium acetate (pH 5.2) was added to a final concentration of 0.3M, mixed well and centrifuged at 4°C for 15 min at 10,000 rpm.

2.3.3 Agarose gel electrophoresis of RNA:

Solutions:
Gel running buffer(5x): 0.1M MOPS (pH 7.0), 40mM sodium acetate, 5mM EDTA (pH 8.0)
Gel loading buffer: 0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 1mM EDTA (pH 8.0), 50% glycerol.
The agarose was melted in water and cooled to 60°C. 5x gel running buffer and formalde­hyde were added to a final concentration of 1x and 2.2M respectively. The gel was casted in a chemical hood and was allowed to set for at least 30 min at room temperature.

The samples were prepared by mixing the solutions in a microfuge tube, as described in Table 2.3. The samples were incubated at 65°C for 15 min and then chilled on ice. 2μl of gel loading buffer was added. Before loading the samples, the gel was prerun for 5 min at 5V/cm. Immediately the samples were loaded. The RNA was separated at 3-4V/cm using 1x gel running buffer for 2-3 h. The gel was washed with several volumes
Table 2.3: Preparation of Samples in RNA Electrophoresis

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (up to 30 µg)</td>
<td>4.5 µl</td>
</tr>
<tr>
<td>5x Formaldehyde Gel running Buffer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>Formamide</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

of DEPC treated water to remove the formaldehyde and stained with EtBr (0.5µm/ml in 0.1M ammonium acetate) for 30-45 min and photographed under UV light.

2.3.4 Synthesis of cDNA

Synthesis of first strand:

Reverse transcriptase buffer (RTase buffer) (5x):
250mM Tris.HCl (pH 8.3), 40mM MgCl₂, 250mM KCl

The solutions were mixed as described in Table 2.4. The RNA was heated to 65°C for 10 min. Quickly a pre-mix was made except the RTase. It was incubated at 42°C for 10 min and transferred to 37°C water bath and the enzyme was added. To measure the incorporation in the first strand synthesis, in a control tube, to 10 µl of this mixture 1µl (10µCi) of α³²P dCTP was added. Both the tubes were incubated at 37°C for 60–90 min. Both the tubes were then transferred to ice. 1 µl of the α³²P product was spotted on a Whatman filter paper to obtain the total cpm.

Second strand synthesis:

The solutions were assembled on ice as described in Table 2.5. 40µl of the mixture was taken out and 2µl (20 µCi) of α³²P dCTP was added to it. Both the tubes were incubated at 15°C for 4h. Aliquots were taken to obtain total cpm. EDTA was added to a final concentration of 10mM. It was heated at 70°C for 10 min and transferred to a 37°C water bath. 10 µm of DNase free RNase A was added and kept for 30 min. The solution was extracted with phenol–chloroform followed by chloroform. Sodium acetate was added to
Table 2.4: Composition of Solution for Synthesis of First Strand

<table>
<thead>
<tr>
<th>Cowpea Poly (A)^+ mRNA</th>
<th>20 µl (approx. 10–13 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x RTase buffer</td>
<td>12 µl</td>
</tr>
<tr>
<td>100mM DTT</td>
<td>6 µl</td>
</tr>
<tr>
<td>Oligo dT (1µg/µl)</td>
<td>3 µl</td>
</tr>
<tr>
<td>25mM dNTPs</td>
<td>1.2 µl</td>
</tr>
<tr>
<td>3H dCTP (24Ci/mmole)</td>
<td>5µl(20 µCi)</td>
</tr>
<tr>
<td>AMV RTase (200U/µl)</td>
<td>8µl</td>
</tr>
<tr>
<td>RNasin (100U/µl)</td>
<td>2µl</td>
</tr>
<tr>
<td>Water</td>
<td>3µl</td>
</tr>
</tbody>
</table>

Table 2.5: Composition of Solution for Synthesis of Second Strand

<table>
<thead>
<tr>
<th>First Strand Reaction Mix</th>
<th>50 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second strand synthesis buffer (5x)</td>
<td>80 µl</td>
</tr>
<tr>
<td>100mM DTT</td>
<td>15 µl</td>
</tr>
<tr>
<td>25mM dNTPs</td>
<td>3 µl</td>
</tr>
<tr>
<td>10mM β-NAD</td>
<td>10 µl</td>
</tr>
<tr>
<td>RNaseH/ E.coli ligase</td>
<td>20 µl</td>
</tr>
<tr>
<td>DNA polymerase I (10U/µl)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Water</td>
<td>12µl</td>
</tr>
</tbody>
</table>

a final concentration of 0.3M and equal volume of isopropanol was used to precipitate the DNA. After 30 min of incubation on ice it was spun at 12,000 rpm for 30 min at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 20 µl of 10:1 TE.

2.3.5 Cloning of cDNA

Ligation of Adaptors to cDNA and Vector:
cDNA (less than 1µg) was mixed with 6µl pUEX-1 (3µg)(cut with BamHI). The final volume was adjusted to 20µl. 1µl of the mixture was kept separately for negative control, after adding 4µl of 10:1 TE. 5µl of the adaptors (500 pmoles) was added to the sample solutions and both the mixtures were incubated at 65°C for 5 minutes and allowed to cool
to room temperature. To the sample solution, 4\mu l of 10x reaction buffer and 2.5 units T4 DNA ligase was added and the final volume was made up to 40\mu l with water. To the negative control, 1\mu l of 8x adaptor ligation mixture and 1\mu l of 0.5 unit DNA ligase was added. The final volume was made up to 8\mu l. Both the samples were incubated at 14°C overnight. The adaptors were removed using spun-column chromatography.

**Ligation and Kinasing of the pooled material:**

900\mu l of the adaptor-free ligation mixture was mixed with 100\mu l of 10x reaction buffer (Amersham) and 200 units of T4 polynucleotide Kinase were added. 24\mu l was kept aside as an unligated control. 2.5 units of T4 DNA ligase was added to the reaction mixture and incubated at 30°C for a minimum of 2 hours. Transformation of the cDNA was carried out as described later in this section.

**Competent cell preparation and transformation of E. coli:**

1. SOC medium: 2% Tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgSO_4, 20mM Glucose (pH 7.5).

2. 0.5ml of the overnight-grown culture was inoculated in 50 ml of SOC medium and incubated at 37°C for 2-3h until the O.D. reached 0.4 at 600nm.

3. The culture was chilled in ice and centrifuged at 5000 rpm for 5 min at 4°C and the bacterial pellet after washing once with cold 75mM CaCl_2 was resuspended in 0.5 volume of 75mM CaCl_2.

4. After incubation in ice for 30 min and centrifugation at 5000 rpm for 5 min at 4°C, the pellet was resuspended in 0.1 volume of 75mM CaCl_2.

5. 0.2ml of the cells were mixed with 0.1ml of DNA (0.1 - 0.5\mu g) in ice for 5 min. The mixture was then incubated at 42°C for 45 sec and kept on ice for 15 min.

6. 1ml of SOC medium was added and incubated at 30°C for 2h and then plated on SOC medium containing appropriate antibiotics.
7. The plates were incubated at 30°C overnight.

2.3.6 Expression screening of cDNA libraries:

Induction of the expression of the proteins

*E. Coli* MC1061 (transformed with cDNA) culture was diluted in LB to about $2.5 \times 10^4$ CFU/ml and spread over L- agar plate containing ampicillin. The plates were incubated at 30°C for overnight. A dried nitrocellulose paper was marked and placed over the plate. The filter was gripped by the top corners and peeled off in one movement with colonies attached. It was transferred to a fresh agar plate with colony side up. Air bubbles were avoided. It was incubated at 42°C for 3-4h to induce the expression. The master plate was reincubated at 30°C to allow the colonies to regrow.

SDS lysis of the colonies

Electrophoresis buffer:
2.5g Tris. base, 12g Glycine in 1 l water.
Wash buffer: PBS containing triton X-100, 0.5% gelatin.
The filters were transferred over a layer of Whatman 3MM paper impregnated with 5% SDS. It was covered with a lid and incubated at 95°C in an oven for 30 min. The colonies were allowed to stand for a few min at room temperature and subjected to electrophoresis for a minimum of 30 min at 5V/cm using 1x electrophoresis buffer with colony side of the filter towards the negative electrode. This was done to immobilise the protein onto the membrane. The filter was washed with the washing buffer. The membrane was incubated with the wash buffer having 10μg/ml DNase. The filters were stored sandwiched between Whatman 3MM paper in a plastic wallet at 4°C until they were used for immunological screening.
Immunological screening

PBS: 10mM KH$_2$PO$_4$, 10mM K$_2$HPO$_4$ (pH 7.5)
PBST: PBS + 0.05% Tween 20
Blocking solution: 1% BSA in PBS
Substrate Solution for Horse radish peroxidase (HRP): 0.5 mg/ml of DAB in 50mM Tris. HCl (pH 7.6), 4µl of 30% H$_2$O$_2$ for 5ml of buffer.

1. The nitrocellulose was incubated in 50 ml of blocking solution overnight at 4°C or for 2h at 37°C.
2. The filter was washed with PBS and incubated with the primary antibody for 1h.
3. The filter was washed with PBST thrice and incubated with secondary antibody solution (1:3000 dilution) for 1h.
4. The filter was washed six times with PBST and incubated with the substrate solution until the colour developed.
5. The colour reaction was stopped by washing in PBST and the membrane was dried.

Extraction of the protein from *E. coli*

1. The *E. coli* strain containing the cDNA was grown in LB with ampicillin at 30°C.
2. The cells after reaching a O.D. of 0.4 at 600nm were incubated at 42°C for 3–4h
3. The cells were pelleted and resuspended in 10mM Tris.HCl (pH 8.0), 50mM EDTA, 15% (mass/volume) sucrose and frozen at −20°C.
4. After thawing, the cell suspension was treated with lysozyme (2mg/ml) and incubated in ice for 30 min. In some cases, the cells were sonicated.
5. The cells were sonicated in ice until the cells were completely lysed and mixed with SDS-gel loading dye and boiled for 3 min in a boiling water bath.
6. The sample was centrifuged for 1 min in microcentrifuge and the protein in supernatant was separated in SDS-PAGE.

**Western blot**

The method adopted has been as that of Towbin et al. (1979).

Transfer buffer: 0.3% Tris. HCl, 1.44% Glycine, 20% methanol.

1. The gel was equilibrated in transfer buffer for 5-10 min.

2. The blotting paper and the Nitrocellulose paper were soaked in transfer buffer. The gel was kept on the pre-soaked blotting paper and the nitrocellulose was placed over the gel carefully. The nitrocellulose paper was further stacked with pre-soaked blotting paper. Care was taken to avoid the presence of any air bubble.

3. Transfer of the protein to the nitrocellulose was carried in a semi-dry blotting apparatus (ATTO) at a constant current of 175 mAmps for 5-6h (anode towards the nitrocellulose).

4. After the transfer, the nitrocellulose was rinsed in PBS and processed for immunological screening.

**2.3.7 Isolation of the trypsin inhibitor gene from the cDNA clones:**

All recombinant DNA techniques were followed according to Sambrook et al., (1989).

**Miniscale preparation of *E.coli* plasmid DNA**

Solution I: 50mM Tris.HCl (pH 8.0), 10mM EDTA, 50mM Dextrose.

Solution II: 0.2N NaOH, 1% SDS.

Solution III: 3M Potassium acetate (pH 5.6)

1. *E.coli* were grown in LB media containing appropriate antibiotics at appropriate temperature overnight.
2. The cells were pelleted at 10,000 rpm for 1 min.

3. The cell pellet was washed once with 50:20 TE.

4. The cell pellet was resuspended in 100 μl of Solution I containing 2 mg/ml of lysozyme and incubated at room temperature for 5 min.

5. 200 μl of Solution II was added, mixed gently and incubated on ice for 5 min.

6. 150 μl of Solution III was added, mixed well and incubated on ice for 15 min.

7. The mixture was centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to a fresh eppendorf tube and Phenol extracted as described later in this section.

Large Scale Preparation of Plasmid DNA from *E. Coli*

The following solutions are used.

Luria Broth (LB):
1% Tryptone, 1% NaCl, 0.5 % Yeast Extract pH 7.5.

10:1TE:
10 mM Tris. HCl (pH 8.0), 1 mM EDTA.

50:20 TE:
50 mM Tris. HCl (pH 8.0), 20 mM EDTA.

Method

1. Bacteria was pelleted from the 1 liter overnight culture in LB medium at 4000 rpm for 5 minutes at 4°C.

2. The cell pellet was washed once with cold 50:20 TE at 4°C.

3. The pellet was resuspended in Solution I containing lysozyme (2 mg/ml) and kept in room temperature for 20 minutes.

4. 10% SDS was added to a final concentration of 1% and kept on ice for 10 minutes.
5. The pH was increased to 12.3 by adding 4N NaOH drop by drop and the solution was kept in ice for 10 minutes.

6. The pH was reduced to 8.3 by adding 2M Tris. HCl (pH 7.0) and the solution was kept in ice for 10 minutes.

7. 1/4 th volume of 5M NaCl was added to the above solution. It was mixed well and incubated at 4°C for 2 hours.

8. The mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was poured into a fresh tube and 1/4 th volume of 50% PEG was added, mixed well and incubated in ice for 2 hours.

9. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes at 4°C and the plasmid DNA was further purified by equilibrium centrifugation in CsCl-ethidium bromide density-gradient.

Purification of Plasmid DNA

The plasmid DNA was separated by Caesium Chloride density gradient centrifugation.

1. Plasmid DNA was dissolved in 4.5ml of 50:20 TE and 5.0gm of Caesium Chloride and 360 µl of ethidium bromide was added.

2. The sample was sealed in a quick seal tube and centrifuged at 38,000 rpm (using a Beckman Ultra Centrifuge) at 20°C for 16 hours.

3. Plasmid band was collected from the tubes with the help of a 20-gauge needle and a 1ml syringe.

4. The EtBr was removed by adding an equal volume of n-butanol and mixing well. The butanol layer containing the EtBr was discarded and the process was repeated until the ethidium bromide was removed.

5. The DNA sample was dialysed in 10:1 TE overnight at 4°C.
Phenol Extraction of DNA

1. Equal volume of Phenol:Chloroform (1:1) saturated in 10:1 TE was added to the DNA sample, mixed well for 3 minutes and centrifuged for 5 minutes in a microcentrifuge.

2. The upper aqueous phase was transferred to a fresh Eppendorf tube and mixed with equal volume of chloroform : iso-amyl alcohol (24:1)

3. After centrifugation for 5 minutes, the upper aqueous phase was transferred to a fresh Eppendorf tube and 0.1 volume of 3M Sodium acetate (pH 5.6) and 2.0 volume of chilled ethanol was added.

4. After incubating either for 15 minutes at \(-70^\circ\text{C}\) or overnight at \(-20^\circ\text{C}\), the DNA was pelleted in a microcentrifuge for 10 minutes, washed with 70% ethanol and after lyophilisation the DNA was dissolved in 10:1 TE.

Restriction Digestion of DNA

Restriction digestion of DNA was carried out according to the instruction of the manufacturers.

Agarose Gel Electrophoresis

Solutions:

1x TBE:

0.089M Tris. HCl (pH 8.0), 0.089M Boric Acid, 0.0002M EDTA.

6x DNA Loading Dye:

0.25% Bromophenol Blue, 0.25% Xylene Cynol, 40% Sucrose.

Method:

1. Agarose Gel (0.7% - 1.0%) was prepared in 1x TBE buffer by heating.

2. It was cooled and poured into the gel-casting apparatus.
3. The DNA was mixed with the gel-loading dye to a final concentration of 1x.

4. The DNA was loaded in the respective lanes.

5. The DNA was electrophoresed in 1x TBE buffer with a constant voltage gradient of 8V/cm for 2-3 hours.

6. After staining the gel in EtBr, the DNA was visualised on an Ultraviolet transilluminator.

Recovery of DNA from Agarose Gel

1. DNA was digested with a suitable restriction enzyme.

2. DNA was electrophoresed in 0.7% - 1% low melting point Agarose at 4°C.

3. The Gel was stained with EtBr and the desired band was cut with a sterile blade.

4. The band was transferred to a fresh Eppendorf tube.

5. Five volumes of 10:1 TE was added.

6. The sample was incubated at 60°C until the agarose melted.

7. Phenol-Chloroform extraction was performed.

8. The DNA was precipitated by ethanol.

Dephosphorylation of the vector DNA

Solution(s):

10x CIAP Buffer:

0.5M Tris. HCl (pH 8.0), 10mM MgCl₂, 1mM ZnCl₂, 10mM Spermidine.

Method:

1. The vector DNA was digested with a suitable restriction enzyme.
2. It was resuspended in 44μl of sterile water and 5μl of 10x CIAP buffer.

3. 0.01U/μmol of calf intestine alkaline phosphatase (CIAP) was added.

4. The sample was incubated at 37°C for 30 minutes.

5. An additional 0.01U/μmol of calf intestine alkaline phosphatase (CIAP) was added.

6. The sample was re-incubated at 37°C for 30 minutes.

7. 2μl of 0.5M EDTA was added to stop the reaction.

8. DNA was purified by spun-column chromatography followed by phenol extraction and ethanol precipitation.

**Spun Column Chromatography**

1. The tip of an 1ml disposable syringe was plugged with glass wool.

2. Sephadex G-50 equilibrated in 10:1 TE was added up to 1ml.

3. The column was spun within a centrifuge at 1600×g for 5 minutes.

4. 0.2ml of 10:1 TE was added to the column.

5. The column was centrifuged as described earlier.

6. The DNA sample was loaded on the column and spun at 1600×g for 5 minutes.

7. The eluent was collected in a decapped Eppendorf tube.

**Ligation of DNA**

Ligation was carried out with the molar concentration of 1:1 to 1:3 of the vector DNA and the insert DNA. The following procedure was used:

1. The vector DNA and the insert DNA were co-precipitated with chilled ethanol.

2. The DNA pellet was washed with 70% chilled ethanol.
Table 2.6: Solution for DNA Ligation

<table>
<thead>
<tr>
<th>Chemical Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA(insert and vector)(1:1)</td>
<td>13.5μl</td>
</tr>
<tr>
<td>10x Ligation Buffer</td>
<td>2.0μl</td>
</tr>
<tr>
<td>100mM Dithiothetol</td>
<td>2.0μl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>2.0μl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1.0 Weiss Unit</td>
</tr>
</tbody>
</table>

3. The DNA was lyophilised.

4. The DNA was dissolved in water.

5. Ligation was done with the solution whose composition is given in Table 2.6.

6. The sample was mixed and incubated at 16°C for 12-16 hours.

**PCR Amplification**

**Solutions:**

10x Amplification Buffer:

0.5M KCl, 0.1M Tris. HCl (pH 8.3), 15mM MgCl₂, 0.1% Gelatin.

**Procedure:**

- In a sterile 0.5ml Eppendorf tube, the solution described in Table 2.7 is taken.

- The reaction mixture was denatured for 5 minutes at 94°C.

- 0.5μl of Taq DNA Polymerase (5U/μl) (From Perkin Elmer Cetus, Catalogue Number N801-0046) was added.

- 100μl of mineral oil was overlayed and amplification was carried out for 24 cycles. The details of the amplification schedule are shown in Table 2.8.

- After the amplification, an aliquot of the sample was analyzed using agarose gel electrophoresis.
### Table 2.7: Solution used in PCR Amplification

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Water</td>
<td>30 μl</td>
</tr>
<tr>
<td>10x Amplification Buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>dNTPs (1.25mM each)</td>
<td>4μl</td>
</tr>
<tr>
<td>Primer 1 (100 pmoles)</td>
<td>5μl</td>
</tr>
<tr>
<td>Primer 2 (100 pmoles)</td>
<td>5μl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1μg</td>
</tr>
</tbody>
</table>

Water was added to make final volume 100μl

### Table 2.8: Amplification Schedule

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Denaturation Conditions</th>
<th>Annealing Conditions</th>
<th>Polymerisation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Cycle</td>
<td>94°C</td>
<td>50°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>1 minute</td>
<td>1 minute</td>
<td>2.0 minutes</td>
</tr>
<tr>
<td>Intermediate Cycles</td>
<td>94°C</td>
<td>50°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>1 minute</td>
<td>1 minute</td>
<td>1.5 minute</td>
</tr>
<tr>
<td>Last Cycle</td>
<td>94°C</td>
<td>50°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>1 minute</td>
<td>1 minute</td>
<td>10.0 minutes</td>
</tr>
</tbody>
</table>

Table 2.8: Amplification Schedule
DNA Sequencing

Reagents:
Annealing Mix: DNA 7µl, Reaction Buffer 2µl, Primer 1µl
Sequenase Buffer (5x): 200mM Tris. HCl, pH 7.5 100mM MgCl₂ 250mM NaCl
DTT
Labelling Mix(dGTP) 5x: 7.5µM dGTP 7.5µM dCTP 7.5µM dTTP
ddG Termination Mix: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl
ddA Termination Mix: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl
ddT Termination Mix: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl
ddC Termination Mix: 80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl
Stop Solution: 95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF
Enzyme Dilution Buffer: 10mM Tris. HCl, pH 7.5, 5mM DTT, 0.5mg/ml BSA
Labelled dATP:[α-³⁵S]dATP
Gel Recipe(6%): Acrylamide/Bisacrylamide 5.7g/0.3g, Urea 42-50g, 10x TBE 10ml, water made upto 100ml
When ready to pour, 1ml of 10% ammonium persulphate and 25µl TEMED was added. Gel was prepared 2-10h prior to use and pre-run for 15-60 minutes.

Method:

1. Double stranded DNA was denatured.

2. DNA was annealed by heating the annealing mixture for two minutes at 65°C and then cooled slowly to less than 35°C over 15-30 minutes. It was centrifuged briefly and chilled on ice for use in step 6.

3. While cooling, tubes were labelled, filled with 2.5µl of each termination mixture (G,
A, T, C). They were kept covered at room temperature for steps 5 and 7.

4. Labelling Mix was diluted five-fold to working concentration, volume made up to 10μl.

5. Four termination tubes were pre-warmed, from step3 in a 37°C water bath.

6. To ice-cold, annealed DNA mixture the following reagents were added:
   - DTT, 0.1M - 1μl
   - Diluted Lab. Mix - 2μl
   - (α–35S)dATP - 0.5 μl
   - Diluted Sequenase Polymerase - 2μl

   It was mixed and incubated at room temperature for 2-3 minutes.

7. 3.5μl of labelling reaction mixture was transferred to each termination tube, mixed and incubation was continued at 37°C for 5 minutes.

8. The reaction was stopped by adding 4μl of stop solution.

9. Samples were heated to 75°C for 2 minutes immediately before loading onto the sequencing Gel. 2-3 μl was loaded in each lane.

10. After running the Gel, it was soaked in 5% acetic acid, 15% methanol to remove urea.

11. Drying was carried out at moderate temperature (80°C) to preserve resolution.

12. X-ray film was exposed overnight.
Table 2.9: Composition of the Plant Medium

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>MS</th>
<th>K3</th>
<th>H</th>
<th>LS</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>1.9g</td>
<td>2.5g</td>
<td>1.9g</td>
<td>1.9g</td>
<td>1.01g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1.65g</td>
<td>0.25g</td>
<td>0.6g</td>
<td>1.65g</td>
<td>0.8g</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>-</td>
<td>0.15g</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.44g</td>
<td>0.9g</td>
<td>0.6g</td>
<td>0.44g</td>
<td>0.44g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.37g</td>
<td>0.25g</td>
<td>0.3g</td>
<td>0.37g</td>
<td>0.74g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.17g</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>0.134g</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na₄EDTA</td>
<td>-</td>
<td>74.6mg</td>
<td>74.6mg</td>
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<td>74.6mg</td>
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<tr>
<td>FeCl₃.6H₂O</td>
<td>-</td>
<td>27mg</td>
<td>27mg</td>
<td>27mg</td>
<td>27mg</td>
</tr>
<tr>
<td>Fe. EDTA</td>
<td>36.7mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>22.0mg</td>
<td>10mg</td>
<td>10mg</td>
<td>16.9mg</td>
<td>10mg</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>8.6mg</td>
<td>2mg</td>
<td>2mg</td>
<td>8.6mg</td>
<td>2mg</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>6.2mg</td>
<td>3mg</td>
<td>3mg</td>
<td>6.2mg</td>
<td>3mg</td>
</tr>
<tr>
<td>KI</td>
<td>0.83mg</td>
<td>0.75mg</td>
<td>0.75mg</td>
<td>0.83mg</td>
<td>0.75mg</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25mg</td>
<td>0.25mg</td>
<td>0.25mg</td>
<td>0.25mg</td>
<td>0.25mg</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025mg</td>
<td>0.025mg</td>
<td>0.025mg</td>
<td>-</td>
<td>0.025mg</td>
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<tr>
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</table>

The pH was adjusted to 5.8 and volume made to 1 litre.
2.4 Plant Transformation Studies:

2.4.1 Preparation of Protoplast

Solutions:

In general, all solutions and media were sterilised by filtration through 0.22μm filters. Simple salt solutions were sterilised by autoclaving. Compositions of different media has been charted in Table 2.9.

Mannitol Solution: 0.4M Mannitol, 6mM MgCl₂, 0.5% MES (pH 5.6 with KOH)

Enzyme Solution: 1.2% Cellulase, 0.4% Macerozyme in K3 medium with 0.4M sucrose instead of 0.3M, 0.6M sucrose containing 0.5% MES (pH 5.6 with KOH), 0.17M CaCl₂ solution

Method:

1. Three fully grown leaves of the shoot culture were taken under sterile condition. They were placed on a petri-dish and wet thoroughly with enzyme solution. The mid-ribs were removed and the leaves were cut into several small pieces and wet both sides with enzyme solution.

2. The cut pieces were floated with the bottom side down in a petri-dish containing the enzyme solution. It was sealed with parafilm and incubated at 26°C in the dark.

3. The mixture was gently agitated and further incubated for four hours for complete digestion. The solution was filtered through a 100 μm stainless steel mesh sieve and washed with one half volume of the 0.6M sucrose solution.

4. The protoplast suspension was mixed gently and distributed into two capped centrifuge tubes. It was centrifuged for 10 minutes at 600 rpm in a clinical centrifuge. The protoplast collected at the upper surface of the solution was taken out.

5. It was resuspended in K3 medium containing 0.4M sucrose and the centrifugation and resuspension steps were repeated using fresh medium two times. 0.1ml sample
was taken for counting before the last centrifugation, and it was diluted in 0.9ml of 0.17M CaCl$_2$ solution.

6. The protoplast was resuspended in 0.4M Mannitol, containing 6mM MgCl$_2$, to stabilise the protoplast at cell density of $1.6 \times 10^6$/ml.

2.4.2 Stable transformation of protoplasts with PEG uptake:

Solutions:
0.5M Mannitol containing 15mM MgCl$_2$, 0.1% MES, pH 5.6 with KOH.
PEG solution: 40% w/v PEG 4000 (Merck) in 0.4M Mannitol, 0.1M Ca(NO$_3$)$_2$.4H$_2$O, pH 8 with KOH.
W5 solution: 154mM NaCl, 125 mM CaCl$_2$, 5mM KCl, 5mM glucose (pH 6.0 with KOH)
K3/H: See Table 2.9.

Method:

1. The protoplast was prepared following the above protocol (2.4.1) till step 5. Instead of washing by K3 medium, they were washed two times by resuspension in W5 solution and centrifugation at 600 rpm for 5 min. followed by counting.

2. They were resuspended at a density of $1.6 \times 10^6$/ml in the Mannitol/Magnesium solution. They were subjected to heat shock for 5 min at 45°C followed by cooling to room temperature. They were distributed into 0.3ml aliquots.

3. 30µl of the DNA solution was added to each of them and mixed. 30µl of the PEG solution was added and incubated for 25-30 minutes at room temperature with occasional shaking.

4. 10ml of W5 solution was added very slowly and then it was centrifuged for 10 min. at 600 rpm.

5. Protoplast was resuspended in 1ml of K3 medium and transferred to a sterile petri-dish. 7ml of a 1:1 mixture of K3 and H media containing 0.6% w/v Seaplaque agarose was added and allowed to set.
2.4.3 Protoplast Culture, Selection of transformed plants and regeneration:

1. The dishes containing the protoplast were sealed with parafilm and incubated at 24°C for one day in the dark followed by six days in continuous dim light.

2. The agarose gel was cut containing the protoplast into quadrants and placed in 50ml of A medium containing 50mg/l Kanamycin Sulphate for selection of transformants and kept at 24°C in continuous dim light.

3. Resistant clones were first seen 3-4 weeks after the start of the experiment. After a total time of 5-6 weeks, they were transferred to A medium containing 0.7% agar with 30g/l Mannitol and 50mg/l Kanamycin sulphate.

4. After a further 2-4 weeks of growth, colonies were transferred to the same media with no Mannitol.

5. After 2-3 weeks, shoot regeneration was induced by culture on the medium of step 4 with only 20g/l sucrose and 0.25 mg/l BAP. These were incubated in the dark for one week and thereafter in light.

6. Shoots arising from the callus on the regeneration medium were excised when 1-2 cms long and placed on LS medium without hormones for root induction.

2.4.4 Preparation of Plant Extract for assay of CPT1 gene Expression:

1. The plant tissue was frozen in liquid nitrogen and allowed to thaw by adding 100mM PBS (pH 7.0), 2% β-mercaptoethanol.

2. The tissue was homogenised using a hand homogeniser on ice and incubated at 4°C overnight.

3. The extract was centrifuged at 15,000 rpm for 15 minutes at 4°C and the clear supernatant was transferred to a fresh tube.
4. This plant extract was used for protein estimation, Sandwich ELISA and Western Blotting.

2.4.5 Sandwich Enzyme Linked Immunosorbent Assay

The following method was used:

1. 100μl of rabbit polyclonal antibodies against the protein was added to each well of microtitre plates and incubated at 4°C overnight.

2. The plates were washed thrice with PBS.

3. The wells were blocked by adding 200μl of blocking solution and incubated at 37°C for one hour.

4. The plates were washed thrice with PBS.

5. Known amounts of antigen (1μg to 100pg) were added to each well (5 replicas each) and incubated at 37°C for one hour.

6. The plates were washed thrice with PBST.

7. 100μl of mouse polyclonal antibodies was added to each well of microtitre plates and incubated at 37°C for one hour.

8. The plates were washed thrice with PBST.

9. 100μl of goat antimouse - HRP conjugate (1:3,000) dilution was added to each well and incubated at room temperature for one hour.

10. The plates were washed six times with PBST.

11. 100μl of the OPD solution was added and incubated at room temperature for 20 minutes.

12. The absorbence was determined at 450nm and a standard curve was prepared.

13. The concentration of the unknown samples were deduced from the standard curve.
2.4.6 Modified Sandwich ELISA:
The protocol followed was as that has been described in the previous section, except that trypsin (1mg/ml) was used to bind the CPTI antigen instead of capture antibodies.

2.4.7 Plant DNA Extraction:
Plant DNA was extracted as described by (Murray and Thompson, 1980).

Solutions:
1X CTAB Extraction Buffer: 50mM Tris. HCl (pH 8.0), 0.7M NaCl, 10mM EDTA, 1% CTAB, 20mM 2-mercaptoethanol
CTAB Precipitation Buffer: 50mM Tris. HCl (pH 8.0), 10mM EDTA, 1% CTAB
10% CTAB: 10% CTAB in 0.7M NaCl

Procedure:
1. The plant material was frozen in liquid nitrogen. It was powdered with a mortar and pestle. It was transferred to an Eppendorf tube.

2. Pre-warmed 1x CTAB extraction buffer was added, mixed and incubated at 56°C for 15-20 minutes.

3. An equal volume of chloroform:octanol (24:1) was added, mixed and centrifuged at 10,000 rpm for 5 minutes.

4. The upper aqueous phase was transferred to a fresh Eppendorf tube, and 0.1 volume of 10% CTAB was added.

5. The chloroform:octanol extraction was repeated and the upper aqueous phase was transferred to a fresh Eppendorf tube.

6. The sample was incubated with an equal volume of CTAB precipitation buffer for 20 minutes, at room temperature and spun at 10,000 rpm for 10 minutes in a microcentrifuge.

7. The DNA pellet was dissolved in 1M NaCl and incubated at —20°C overnight, with addition of 2 volumes of cold ethanol.
8. The DNA was pelleted at 10,000 rpm for 15 minutes, washed once with 70% alcohol and after lyophilisation, the DNA was dissolved in 10:1 TE.

2.4.8 Preparation of Radioactive DNA Probe:

The DNA probe was prepared using Prime IT Kit II (Stratagene) and the protocol was followed according to the manufacturer's instructions.

- The DNA (100ng) was dissolved in 26µl of water and 10µl of 5x primer mixtures were added and boiled in a water bath for 10 min. It was allowed to re-anneal at 37°C for 5 min. 10µl of 5x dCTP buffer was added and 3µl of α-32P dCTP (Amersham) was added. 0.8µl of Klenow enzyme was added and mixed well. It was incubated at 37°C for 10 min. and the labelled DNA was separated from the unincorporated dNTPs by spun-column chromatography.

2.4.9 DNA Dot Blot Hybridisation:

The blotting was carried out in a dot blot apparatus under vacuum.

Solutions:
20X SSC: 17.53% NaCl, 8.82% Sodium Citrate (pH 7.0).
Prehybridisation Solution: 50% Formamide, 10% Dextran Sulphate, 0.9M NaCl, 1% SDS, Denatured Salmon Sperm DNA (100µg/ml).

Procedure:
1. The pads and Hybond N+ (Amersham) membrane were soaked in 20x SSC and placed in the dot blot apparatus.
2. The denatured DNA samples were loaded in the individual wells and vacuum was applied.
3. The wells were rinsed with cold 20x SSC and the transfer was carried out for 10-15 minutes.
4. The membrane was removed and cross-linked in a UV cross-linker (Stratagene).
5. The filters were soaked in 6x SSC and incubated in prehybridisation solution at 42°C for 5 hours.

6. The DNA probe was added to the prehybridisation solution and it was further incubated at 42°C overnight.

7. The filters were then washed in the following solutions with several changes:
   (a) A mixture of 2x SSC, 0.1% SDS at room temperature for 15 minutes.
   (b) A mixture of 0.1x SSC, 0.1% SDS at 68°C for 30-45 minutes.

8. The filters were then exposed to X-ray in an X-ray film cassette containing intensifying screen at —70°C overnight.

2.4.10 Southern Hybridisation:

1. The DNA samples were separated in agarose gel and photographed after staining with EtBr.

2. The DNA was transferred to Hybond N+ membrane under vacuum in the “VacuGene” apparatus (Pharmacia). The membrane was soaked in 20x SSC and placed beneath the window of the apparatus. The gel was placed over the membrane and the pressure was set at 50 cm H₂O. The following solutions were poured over the gel and after incubation, the excess solutions were removed.
   (a) 0.25M HCl for 10 minutes
   (b) Denaturation solution for 30 minutes
   (c) Neutralisation solution for 30 minutes
   (d) 20x SSC for 60 minutes

3. The membrane was removed, washed briefly in 6x SSC and baked at 80°C for two hours in vacuum.
4. The filter was soaked in 6x SSC and incubated in the pre-hybridisation solution at 42°C for five hours.

5. The DNA probe was added to the pre-hybridisation solution and incubated overnight at 42°C. The filter was then washed in the following solutions with several changes.
   (a) 2x SSC and 0.1% SDS at room temperature for 15 minutes
   (b) 0.1x SSC and 0.1% SDS at 68°C for 30-45 minutes

6. An X-ray film was exposed to the membrane at −70°C overnight.

2.4.11 Northern Blotting

Solution 1. Formaldehyde mixture for denaturing RNA: 50% Formamide, 6% formaldehyde, 1X MOPS Buffer
Solution 2. 1% Agarose in 10mM sodium phosphate (pH 6.5), 1.1M formaldehyde
Solution 3. Loading Mixture: 0.005% (w/v) Bromophenol Blue, 1% (w/v) SDS, 50% Glycerol

Method:
1. The RNA samples were denatured with solution 1 at 65°C for 5 min.
2. 1µl of the loading dye was mixed with 4µl of the sample and loaded in the agarose gel.
3. The RNA samples were electrophoresed for the appropriate time in 10mM sodium phosphate (pH 6.5).
4. The Hybond N+ (Amersham) was presoaked in distilled water and then in 6x SSC.
5. The RNA was transferred to the membrane using vacuum transfer. (See section 2.4.10, step 2)
6. The membrane was dried and the RNA was fixed by UV crosslinking and used for hybridisation (see section 2.4.10, steps 4 to 6).