Material and Methods
Plant materials

Twenty-four different tomato genotypes were considered for the study. The *Lycopersicon esculentum* cultivated varieties were purchased from the seed companies as indicated in Table 1, while the *L. esculentum* accessions were obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. The other tomato species *L. peruvianum, L. cerasiformae,* and *L. pimpinellifolium* were obtained from the Indian Institute of Horticultural Research (IIHR), Bangalore, India. The fungal strain *Fusarium oxysporum* f.sp. *lycopersici* Race 2 Fol007 and the identified resistant cultivar C295 was obtained as a generous gift from Prof. M. Rep of the Swammerdam Institute of Life Sciences, Amsterdam.

Plant infection

The fungus *F. o. lycopersici* Race 2 was grown on Potato Dextrose Agar (Himedia) plate. After seven days of culture, conidia were scrapped from the surface with a sterile scalpel and resuspended in water. The spore count was estimated with a haemocytometer and the suspension adjusted to a concentration of $2 \times 10^5$ spores per ml prior to plant infection. Tomato seeds were germinated and subsequently grown in sterile soil rite at $25^\circ$C under controlled temperature and humidity in a BOD. Two weeks old plants in 3 true-leaved stage were taken for infection with *F. o. lycopersici* by the root dip method as described by Simons *et al.* (1998) or by direct leaf infection. Five-microlitre spore suspension was spotted on the abaxial surface of the terminal leaflet of the second true leaf of the plant for leaf infection. For infection through roots, the roots were washed thoroughly to remove remnant soil rite particles, washed in sterile double distilled water and soaked within folds of sterile tissue paper. The roots were then transferred into a spore suspension containing $2 \times 10^5$ spores/ml, for
Material and Methods

10 min and planted in sterile soil rite again. The plants were infected through roots in all cases except one occasion (mentioned in relevant text). Plants were kept in dark for two days under similar condition to ensure infection and then in 16 h light and 8 h dark under the same conditions of temperature and humidity.

Scoring of disease development

Disease progression was monitored morphologically by monitoring leaf chlorosis and abscission upon infection and the overall stunting of the plant. The disease evaluation was carried out by counting the numbers of plants survived and died upon infection out of fifty plants in triplicates taken together from each of the twenty-four genotypes.

Trypan Blue Staining

To monitor the extent of plant infection Trypan Blue staining was done. The infected leaves were washed and destained by boiling for 1 min in 95% ethanol, soaked in alcoholic lactophenol (25% Lactic acid, 25% phenol, 25% glycerol and 25% double distilled water-1 part and 2 parts 95% ethanol) and stained with 2.5mg/ml Trypan Blue. The leaves were subsequently destained in lactophenol (25% Lactic acid, 25% phenol, 25% glycerol and 25% water), and mounted in 50% glycerol.

Detached leaf assay

Healthy leaves from 1-month-old plants were cut carefully at the petiole and placed in a sterile petridish with a filter paper soaked in half strength of MS salts. An agar block with Fusarium spores was placed on the leaf surface and observed for lesion formation and hypersensitive response (if any) for a period of 48 hours with respect to
Material and Methods

control. Trypan Blue staining was done with an interval of 12 hours to determine the extent of fungal progression.

Scanning Electron Microscopy (SEM)

For the purpose of observation of morphological characters by scanning electron microscopy, infected leaves were fixed in 1:1:10 Formalin: Acetic Acid: 30% alcohol (FAA) overnight. The samples were then dehydrated. Dehydration was done by passing the samples through alcohol grades 20%, 30%, 50%, 70%, 80%, 90%, 95% and absolute alcohol, keeping in each for 3h, except in 70% and absolute alcohol, where the time was extended overnight. The samples were then subjected to various grades of alcohol: isoamyl acetate grades 3:1,1:1 and 1:3 for 3h each, followed by two subsequent changes in pure isoamyl acetate for 2h. The samples were dried to critical point and then mounted on stubs with stick tape followed by sputter coating with gold (7mA). Surface structures were studied at different magnifications in a JEOL Scanning Electron Microscope (Model JSM 5200) and photomicrographs were taken.

Root-stem serial sectioning

Plants were infected through roots as described earlier. On the 8th and 15th day after infection, serial transverse sections were cut starting from root through the region of the root-stem transition and up to the stem for the study of disease progression. The sections of different time points were stained with Trypan Blue (Grant et al. 2003) to determine the extent of infection by microscopic observation of xylem vessels with hyphal growth.
Material and Methods

Phloroglucinol-HCl staining
Sections were stained with Phloroglucinol-HCl (1% w/v phloroglucinol in 6N HCl) for 15 min, for observation of differential tissue lignification due to infection.

Aniline Blue staining
Plant material was immersed in 5 ml alcoholic lactophenol (1 volume of phenol: glycerol: lactic acid: water (1:1:1:1) and 2 volumes of ethanol) for 2h. Auto fluorescence was observed in the cleared leaf samples mounted in 50% glycerol by epifluorescent illumination (365 nm excitation filter, 395 nm chromatic beam splitter, 420nm barrier filter) on an Olympus Microscope. To detect callose, cleared leaves were rinsed in 50% ethanol, rinsed in water and then stained for 30 min in 150 mM di-Sodium Phosphate buffer (pH 9.5) containing 0.01% Toluidine Blue to quench the auto fluorescence and subsequently with 0.01% Aniline blue. Samples were mounted in 50% glycerol for observation.

Transmission Electron Microscopy (TEM)
Roots of infected and uninfected (Control) plants of the varieties C295 and Moneymaker were washed thoroughly under running tap water to remove remnants of soil rite and subsequently in double distilled water. One mm cube tissue was then cut on a clean slide immersed in 2-3 drops of 3% glutaraldehyde solution, in 0.2M Sodium Phosphate buffer pH 7.2, using a sharp scalpel.

Fixation
The samples were transferred to 1ml 3% glutaraldehyde solution in ultra clean glass vials and kept at room temperature for 5h. The samples were subsequently washed
with 0.2M phosphate buffer thrice for 30 min each and kept overnight at 4°C. The tissue was post fixed for 3h in 1% Osmium tetraoxide at room temperature and washed thoroughly in distilled water.

**Dehydration**

Dehydration was done by passing the samples through differential grades of acetone 30%, 50%, 70%, 80%, 90%, 95% and absolute acetone with 2 washes at each grade for a period of 15 min each at room temperature, except in 50%, where the samples were kept at 4°C for overnight.

**Infiltration and embedding**

Infiltration was carried out at room temperature with the medium that was subsequently used for embedding the samples. The medium constituted of

- **Resin:** Araldite G 212 10 ml
- **Hardener:** Dodecyl Succinic Anhydride (DDSA) 10 ml
- **Accelerator:** Tri-dimethyl-amino-methyl-Phenol (DMP) 0.4 ml
- **Plasticizer:** Dibutylphthalate 1 ml

The Araldite was stirred with the accelerator thoroughly and the hardener was gradually added in an ultra clean beaker constantly stirring with a glass rod. The medium was kept at room temperature for 2-3h to remove the entrapped bubbles.

The samples were passed through differential mixtures of the media and acetone for proper embedding; 1:3 medium: absolute acetone, 1:1, 3:1 and finally the media keeping at each grade for a period of 2h changing twice, except in the grade 1:1, where the samples were kept for overnight.

**Moulding and Polymerization**

The samples were carefully placed within the mould and gradually polymerized at 50°C for 24h, 60°C for 24h and 70°C for 48h respectively.
**Material and Methods**

**Ultramicrotomy and observation**

The embedded samples were finally fitted to a Leica ultramicrotome. The resin blocks were trimmed and the sections of 60mm thickness were collected in a pool of distilled water in compatible troughs. The sections were rinsed in chloroform and transferred to 0.01mm copper grids. The sections were subsequently stained in uranyl acetate for 10min and subsequently for 5min in lead citrate. The stained grids were dried and the sections observed in the transmission electron microscope (JEOL JEM 200cx). Photomicrographs were subsequently taken.

**Large scale preparation of plant DNA using Cetyl triethylammonium bromide (CTAB)**

Principle: Nucleic acids form stable soluble complexes with the detergent CTAB under high salt conditions (0.7 M Sodium chloride). When the salt concentration is reduced below 0.4M Sodium chloride, the CTAB nucleic acid complex starts precipitating leaving the majority of polysaccharides in solution (Murray and Thompson 1980). DNA was isolated from freeze-dried plant material using 2X extraction buffer.

*Reagents used:*

1. 2X CTAB Extraction Buffer (pH: 8.0)
   - 100mM TRIS
   - 20mM EDTA
   - 1.4 M NaCl
   - 2% w/v CTAB
   - 1% PVP

2. 10% CTAB Solution
   - 10% CTAB
   - 0.7M NaCl
Material and Methods

3. CTAB Precipitation Buffer (pH:8.0)
   1% CTAB
   50mM TRIS
   10mM EDTA

4. 3M Sodium Acetate solution (pH: 5.5)
   3M Sodium Acetate

5. High Salt TE Buffer (pH:8)
   10mM TRIS
   1mM EDTA
   1M NaCl

6. Chloroform: Isoamyl alcohol (24:1)

7. Phenol:Chloroform
   Mixture of equal volume of phenol:chloroform saturated with 10:1 TE buffer.

Procedure

1. Five gm of fresh plant tissue was ground to a fine powder in a mortar after freezing with liquid nitrogen. Five ml of CTAB extraction buffer was then added and the macerate transferred to a 50mL polypropylene centrifuge tube.

2. Capped tubes were incubated in a water bath at 56° C for 20 min, agitating the tube occasionally.

3. The incubated product was allowed to cool to room temperature and an equal amount of phenol: chloroform was added. The solution was mixed by gentle inversion until a single-phase emulsion was formed.

4. The debris was pelleted and the organic and aqueous phases separated out by centrifugation at 10,000 rpm for 15 minutes.

5. The aqueous phase was removed into a clean 50mL tube and an equal volume of chloroform: iso amyl alcohol added . The solution was then mixed gently as described earlier, and centrifuged at 10,000 rpm for 15 minutes.
Material and Methods

6. The upper aqueous phase was taken into a clean tube and 1/10th volume of 10% CTAB solution was added. The solution was mixed thoroughly until a clear white fluid was formed, and the chloroform: iso amyl extraction was repeated.

7. Avoiding the debris at the interphase, the aqueous phase was removed to a fresh tube and three times the volume of CTAB precipitation buffer was added, mixed gently and left to stand at room temperature for three hours.

8. The precipitate was pelleted upon centrifugation at 10,000 rpm for 10 min at room temperature. The supernatant was discarded and to the pellet 1ml of high salt TE buffer was added. The tube was then transferred to a water bath set at 55°C, until the pellet had dissolved completely.

9. An equal volume of phenol: chloroform was added, mixed gently as described earlier and centrifuged at 10,000 rpm for 5 minutes at room temperature.

10. The aqueous phase was taken into a fresh tube and phenol: chloroform extraction repeated once again.

11. The aqueous upper phase was taken in a fresh tube and the DNA precipitated with two volumes of chilled ethanol. The precipitate was pelleted upon centrifugation at 10,000 rpm for 15 minutes at 4°C.

The pellet was washed once with 70% ethanol, dried thoroughly and dissolved in 10:1 TE buffer for further RNase treatment.

RNase treatment

Preparation of RNase solution

RNase A (Sigma) was dissolved in a solution containing 10mM TRIS, 1mM EDTA and 20mM sodium chloride set at pH 7.5 at a concentration of 10mg/ml. The solution
was then placed in a boiling water bath for 15 min, cooled and stored as aliquots at -20 °C for future use.

Procedure
To the extracted DNA, 20μl RNase A (Stock 10 mg/ml) was added and the tube incubated at 37°C for 2 hours, at the end of which the DNA was extracted with phenol: chloroform twice and finally precipitated with one tenth volume of 3M sodium acetate and two volumes of chilled ethanol. The precipitate was pelleted upon centrifugation at 10,000rpm for 15 min, washed with 70% ethanol, dried thoroughly and finally dissolved in sterile HPLC water.

Agarose gel electrophoresis

Preparation of stock solutions
50X TAE (TRIS- Acetate, 0.001M EDTA; 1000ml)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Base</td>
<td></td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td></td>
<td>57.1 ml</td>
</tr>
<tr>
<td>0.5M EDTA (pH 8.0)</td>
<td></td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Loading Dye (6X buffer)

- 0.25% Bromophenol Blue
- 0.25% Xylene cyanol
- 40% (w/v) sucrose

DNA was electrophoresed in 1-1.8% agarose gel by dissolving required amount of agarose in 1X TAE buffer upon heating and then cooled to room temperature. Ethidium bromide was then added in an appropriate amount and the gel poured gently avoiding bubbles. DNA was mixed with gel loading dye prior to loading in the lanes of agarose gel. Electrophoresis was done with 1XTAE buffer in a submerged horizontal gel.
Material and Methods

Polyacrylamide Gel Electrophoresis (PAGE)

Preparation of stock solutions

Solution A: Acrylamide stock solution

Acrylamide 29.2 gm
N, N' Methylene bis acrylamide 0.8 gm
Volume made up to 100 ml

Solution B: 5x TBE

TRIS Base 54g
Boric acid 27.5g
0.5M EDTA (pH 8) 20ml

Solution C: Ammonium persulphate (APS, 10% w/v)

APS 0.10 gm
Volume made up to 1 ml

The solution was prepared prior to use.

Solution D: N, N, N', N'-tetramethylethylenediamine (TEMED)

Used as supplied.

Solution E: Reservoir buffer

1x TBE buffer
Volume made up to 1000 ml

Recipe for gel preparation using Non Dissociating Continuous buffer system

Resolving gel (6%)(Volume for 100ml)

Solution A 20
Solution B 20
Solution C 0.7
dd H2O 59.3

The volumes indicated in the above table were used for gels of dimension 17.5x22.5 cm gels.
Material and Methods

Procedure and Precautions

(i) To ensure that the gel plates were completely clean, the glass plates were soaked in chromic acid overnight, rinsed with water and then with ethanol and air-dried.

(ii) Thin teflon spacers (1mm thickness) were used between the plates to obtain gels of uniform thickness.

(iii) The resolving gel mixture was prepared by adding all the components in proper proportions. TEMED was added just prior to pouring the gel solution. A Perspex comb was immediately inserted between the glass plates taking care that no air bubble was trapped beneath the comb. The assembly was left undisturbed for proper polymerization.

(iv) After polymerization (10-20 min), as evidenced by the presence of a sharp interface between the polymerized gel and the overlay, the assembly was tilted to pour off the overlay.

(v) After polymerization, the comb was carefully removed to expose the sample wells, which were rinsed with reservoir buffer to remove un polymerized gel pieces.

(vi) The gel was loaded with equivalent amount of DNA, 8μl in each well. The samples were mixed with 2μl 6x gel loading dye prior to loading. Care was taken to avoid mixing of the samples in adjacent wells. The reservoir buffer was then put in the upper and lower chamber of the gel apparatus very slowly.

Running of gel

Electrophoresis was carried out for 4-4.5 h at 2mA per lane (constant current). The migration of the tracking dye was monitored for completion of gel run. After the
run was over, the gels were stained with 5μl ethidium bromide (10mg/ml) in 50 ml dd water, for 15 min. The gel was subsequently washed with double distilled water and viewed on a transilluminator.

**Nucleotide Binding Site (NBS) profile analysis**

Degenerated primers of the NBS region were designed according to Pan *et al.* (2000a) (Table 2). All the forward primers were designed from the conserved P-loop domain, while only the REV primer was designed from the conserved GLPL motif. Reverse primers specific for both NBS Group II and I were considered for amplification with combination of the different forward primers. All the primers were obtained from MWG Biotech.

Plant genomic DNA of all the varieties was extracted by Cetyltrimethylammonium bromide (CTAB, SIGMA) method as described earlier (Murray and Thompson 1980). Template DNA (500ng) was amplified with 1U Taq DNA Polymerase (NEB), in a 50μl volume, with an initial denaturation of 4 min at 94 °C followed by 35 cycles consisting of 1 min at 94°C, 1 min at 40°C and 1 min at 72°C, with a final extension of 7 min at 72°C in a Peltier effect Thermal Cycler PTC100 (MJ Research).

**I2 Specific Profile Analysis**

Since the *I2* gene has been identified to be responsible for the resistance of tomato to *F. oxysporum f.sp.lycopersici* race 2, specific primers for the amplification of the *I2* gene alone were considered. Previous studies on the functional *I2* gene revealed an allele specific gene structure and coding sequence is responsible for the resistance phenotype. Based on which primers FP71: 5′ GCT GAC CTT CCA CCT TAAG
**Material and Methods**

3' and PF91: 5' GCA AAT TAC TCT TAA CCT TCA G 3', designed earlier by Mes et al. (2000) from the intron of the 5' UTR of the functional I2 gene, were used with the aim to identify the presence of functional I2 from different genotypes of tomato. PCR was performed with the 24 different DNA samples under similar conditions at 58°C annealing temperature to ensure specific amplification.

Apart from that, for understanding of the distribution of the I2 class of genes within the germplasms under consideration, degenerated primers were designed from the conserved LRR regions of the identified I2 and the I2 homologues I2C1, I2C2, I2C3, I2C4 and I2C5, aligned (Fig. 1) using the CLUSTAL W software. Both the published full lengths as well as partial sequences were considered, since genomic DNA was used for PCR mediated amplification. Primers were designed from the regions of the LRR showing maximal conservation within the homologues of the I2 gene and distinctly different from LRRs of any other R genes so as to eliminate chances of amplification of any other class of R genes. The primer pair I2LRF 5'CAA GGC CA(G/A) (C/A)TT TCC TCC TTT TCT CAC 3' and I2LRR 5'TG(T/G) CCA GTA TTC CCC CTT 3' was used for amplification.

**Agromorphological parameters**

Ten different agromorphological characters were considered from 40 plants of each variety. Plant habit, plant height, the number of primary branches, presence of stipule, trichome abundance was considered along with fruit characteristics consisting of the number of fruits per plant, fruit size, fruit weight, color of the pericarp and shape (Fig.2).
Figure 1. Multiple sequence alignment of amino acid sequences of C terminal region of the genes I2 (Accession AF118127) and I2C homologs, I2C1 (Accession AF004878), I2C2 (Accession AF004879), I2C3 (Accession AF004880), I2C4 (Simons et al., 1998), and I2C5 (Accession AF 408704). Shading shows consensus sequences above 60%. Numbers at right indicate the positions of the residues in the protein sequences. *indicates the unknown amino acid residue number of I2C4 (since the sequence was derived from Simons et al., 1998). (-) indicates gaps incorporated for alignment. Arrows indicate regions from where primers were designed. Lower bar indicates the 23 amino acid repeat sequence.
Figure 2. Polymorphism in size, shape and pericarp color of fruits of a few representative tomato genotypes: (a) *Lycopersicon peruvianum*, (b) *L. pimpinellifolium*, and few of the *L. esculentum* genotypes viz. (c) G, (d) N5, (e) M, (f) SK, (g) D, (h) N14, (i) K2, (j) K, (k) C3, (l) PC (Abbreviations as stated in Table 1).
Data Analysis

Phenograms based on the banding patterns were constructed using the Numerical Taxonomy System (NTSys-pc) version 2.02e (Rohlf 1998). Only the major bands were considered. The presence of the band was coded as 1 and its absence as 0. Percent polymorphism was scored for all the bands polymorphic against the total number of bands amplified for each genotype. A similarity matrix based on the simple matching coefficient was generated by the SIMQUAL program, and cluster analysis performed with the Unweighted Pair Group Method with Average (UPGMA) in the SAHN (Sequential Agglomerative Hierarchical and Nonoverlapping) program. The statistical stability of the branches in the cluster was estimated by bootstrap analysis with 1000 replicates using the WINBOOT software.

For analysis of agro morphological characters, data of forty plants were considered for each trait. Standard errors of the means were calculated. Similarity matrix was constructed based on the simple matching coefficient. The similarity matrix ultimately was used for cluster analysis performed with UPGMA in the SAHN module, and the tree ultimately generated, using the NTSYSpc software ver 2.02.

General tissue culture conditions

Calli of the tomato genotype C295 were derived from the cotyledonary leaves of aseptically germinated seedlings maintaining physical and chemical conditions of standard tissue cultural practices. The temperature of the culture room was maintained at the range of 25°C±2°C while a fixed relative humidity (78%) and photoperiod (16h light and 8h dark) was also maintained. For callus induction and maintenance, the cultures were kept under complete darkness. All tissue cultural operations were carried out in a laminar flow-hood chamber (Klenzaid India Ltd.). pH of the basal
medium (5.6 in case of MS) was adjusted carefully with 1(N) KOH or 1(N) HCl solution and checked with a digital pH meter (Delta T-MS30).

Cultures were induced and maintained in semisolid (agar-gelled 0.8% w/v) media with different chemicals depending on the experimental requirements. All the chemicals used were of AnalaR (analytical reagent) grade purchased from standard companies (viz. Sigma, SRL, BDH, E Merck, etc.). Double glass-distilled water was used for the preparation of all solutions for the preparation of media.

Stock solutions of individual chemicals were added sequentially to a measured volume of water, mixed thoroughly and finally the required volume was made up.

Desired amount of sucrose (20 gm L\(^{-1}\)) and bacto agar (8 gm L\(^{-1}\) in case of semi solid media) were added. After melting the agar, plant growth regulators at precise amount were finally added to the media and mixed thoroughly. Growth regulators used were initially converted to their respective water-soluble salts.

The melted media was immediately distributed in culture tubes or conical flasks per requirement and plugged with non-absorbent cotton. For the purpose of cell suspension culture, liquid ½ strength of MS media was used. Media was steam-sterilized in an autoclave (15lbs/sq. inch for 20 min).

**Induction of callus**

Tomato seeds were surface sterilized with 0.1% Mercuric chloride in combination with 0.01% TritonX-100 for 5 minutes followed by repeated washes in sterile double distilled water four times at a duration of 10 minutes per wash. The seeds were then germinated and seven-day-old cotyledonary leaves and hypocotyls used as explants for callus induction.
Material and Methods

Composition of basal media taken for standardization of the culture technique.

<table>
<thead>
<tr>
<th>Compounds (mg/L)</th>
<th>MS</th>
<th>½ MS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄ .7H₂O</td>
<td>370</td>
<td>185</td>
</tr>
<tr>
<td>FeSO₄ .7H₂O</td>
<td>27.85</td>
<td>13.925</td>
</tr>
<tr>
<td>Na₂EDTA</td>
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</tr>
<tr>
<td>CaCl₂ .2H₂O</td>
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</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
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<td>85</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>3.1</td>
</tr>
<tr>
<td>MnSO₄ .4H₂O</td>
<td>22.3</td>
<td>11.15</td>
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<tr>
<td>ZnSO₄ .7H₂O</td>
<td>8.6</td>
<td>4.3</td>
</tr>
<tr>
<td>CuSO₄ .5H₂O</td>
<td>0.025</td>
<td>0.0125</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
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<td>Na₂MoO₄ .2H₂O</td>
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<td>0.0125</td>
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<tr>
<td><strong>Organics</strong></td>
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</tr>
<tr>
<td>Glycine</td>
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<td>1.0</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
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<td>0.25</td>
</tr>
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<td>Thiamine-HCl</td>
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<td>0.05</td>
</tr>
<tr>
<td>Inositol</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Calcium Pantothenate</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 gm L⁻¹</td>
<td>15 gm L⁻¹</td>
</tr>
</tbody>
</table>

MS = Murashige and Skoog (1962)
½ MS = ½ strength MS media.

Proliferation and maintenance of callus

Callus of the tomato variety C295 was induced from cotyledon and hypocotyl explants in Murashige and Skoog’s medium supplemented with NAA (5 mg L⁻¹) and BAP (2 mg L⁻¹). After three cycles of subculture for a period of 21 days in each culture, photoautotrophic cell suspension culture was established in half concentration.
Material and Methods

of Murashige and Skoog medium supplemented with NAA (0.5mg L⁻¹) and BAP (0.2 mgL⁻¹). Cells of exponential growth phase were used for various experiments. To ensure uniform experimental conditions, the cultures were mixed one day prior to treatment. An initial cell concentration of 2x 10⁵ cells ml⁻¹ was maintained for all subsequent experiments.

Preparation of elicitor from *Fusarium oxysporum f.sp. lycopersici*

*F. oxysporum f.sp. lycopersici*, Fol1007, Race 2 was cultured in a medium containing Glucose (50g L⁻¹), casamino acid (8g L⁻¹), yeast extract (0.5g L⁻¹), Magnesium sulphate and Ferrous sulphate (0.2g L⁻¹ each), Calcium chloride (20mg L⁻¹) and Manganese sulphate and Sodium molybdate (1.5mg L⁻¹ each), in 25nM potassium phosphate buffer at pH 7.5. After 4 days of shaking at 29°C the culture was reautoclaved to kill the fungus, washed several times with dd water, and lyophilized. The elicitor stored at −20°C was used at a concentration of 150 mg per one liter of tomato cell suspension culture.

Determination of tissue viability

Cell suspension culture (100ml) was distributed in aliquots of 5ml each to maintain uniform experimental conditions. Elicitor was treated to the individual 5ml cell suspension at various time points according to experimental requirements. The suspension was equally distributed into two aliquots for cell population studies by flow cytometry and subsequent microscopic examination of the same.
Material and Methods

**Fluorescein diacetate (FDA)**

The cell suspension was centrifuged at 2,000 rpm for 5 min. A stock solution of FDA at 5 mg/ml of acetone was diluted to a 2% staining solution with 10 mM Potassium phosphate buffer (pH 6.4). The cells were incubated in the staining solution for 5 min at room temperature and then monitored against control (untreated) in a flow cytometer (FACS Calibur; Becton Dickinson, San Jose CA). FDA, a hydrophobic molecule, enters cells passively and is then cleaved by intracellular lipases, becoming fluorescent at green wavelengths and charged, such that it is unable to leave the cells. Excitation of FDA was monitored at 488 nm and emission was detected at 530±15 nm as FL1 fluorescence.

Results were obtained in three replicates using high flow rates. List mode data were converted from Hewlett Packard to Apple (Macintosh) computer format using the program FACS-Convert (Becton Dickinson, San Jose, CA). Data were then analyzed using the CellQuest software (version 3.3; Becton Dickinson) and expressed as percentage of cells fluorescing in the FL-1 range.

**Fluorescein diacetate (FDA) and Propidium iodide (PI) double staining**

Modified double staining with FDA and PI for determination of viability was used. A stock solution of PI (1 mg ml⁻¹) was prepared and used at a working concentration of 20 μg ml⁻¹ in cell culture. PI enters cells whose plasma membranes have altered permeability and stains nucleic acids, emitting red fluorescence. The cells were incubated in the staining solution consisting of FDA and PI at the concentrations as indicated for 5 min at room temperature and then observed on a hemocytometer, counting the number of red and green fluorescent cells using an epifluorescent microscope. A total of 400 cells were counted for 10 optical fields and cell viability...
was expressed as the percent of viable cells (green fluorescing) to the number of dead cells (red fluorescing) counted per plane.

**Measurement of Cytosolic Free Calcium**

The cytosolic free calcium was measured using FURA 2 fluorescence according to Grynkiewicz et al. (1985). The ratiometric dye, Fura 2AM, an acetoxymethyl ester derivative of Fura 2, was dissolved in DMSO as 1mM stock solution and stored at –20°C. Fura 2AM, being lipophilic entered the cells readily. Cell suspension was loaded with 50μM Fura 2AM and incubated at 37°C in dark for 1 h in 1/2 MS media set at pH 5.5. The cells were then washed with the media twice at 2,000 rpm for 5 min and transferred to a 3ml capacity quartz cuvette of Hitachi spectrofluorometer (model 3010) equipped with a thermostated cuvette holder, stirring apparatus and chart recorder. The cells were continuously stirred in the cuvette with a magnetic stirrer and equilibrated for at least two min before the addition of elicitor to the cuvette. Fluorescence was measured at 37°C with excitation at 380nm and emission at 510nm, with both excitation and emission slits set at 5 nm. Fluorescence at 510nm (F) in these conditions was dependent upon average levels of endogenous free calcium in all cells. Treatment of Fura-2 loaded cells with 5μM Calcium ionophore A23187 caused rapid influx of saturating levels of calcium from the external medium, leading to an increase in fluorescence to a maximum (F$_{max}$), which reflected the efficiency of Fura 2 loading. Subsequently, the addition of 300 μM Mn$^{2+}$ caused quenching of intracellular calcium Fura-2 fluorescence (F$_{min}$). The concentration of cytosolic free calcium was then obtained from the values of F, F$_{max}$ and F$_{min}$ by using the following formula (Gryniewicz et al.1985)

$$(\text{Ca}^{2+})_{cyt} = K_d (F - F_{min}) / (F_{max} - F),$$
where, $K_d$ was the association constant of calcium with Fura 2 and set at 224nm at physiological pH. Autofluorescence was measured in cells treated in similar fashion but loaded with DMSO instead of FURA 2AM, and the values subtracted from the values obtained from the experimental samples. For studies with metabolic inhibitors, all inhibitors were used at concentrations as indicated, 10 min prior to elicitor addition.

**Estimation of K$^+$ content**

Callus (100mg) was weighed aseptically and transferred to 2ml of suspension media in small 10ml capacity conical flasks for individual treatment sets. Elicitor was treated at various time points prior to harvesting and estimation in a uniform condition. The calli were harvested by suspending in 10ml of HPLC water. After 10h incubation, the calli suspension (in deionised water) was centrifuged at 10,000 rpm for 10min. Potassium content of both the elicitor treated calli and suspended solutions were assayed (Watad *et al.*1986) to quantify the total amount of potassium (amount retained and leached by the calli). The clear supernatant was considered as the source of potassium leached out from the calli, while the pellet (calli) was washed thrice with deionised HPLC water, macerated with measured amount of water and centrifuged again at 10,000 rpm for 2 min. The supernatant was considered as the source of potassium retained within the tissue. The previous washes were pooled and added to that quantified from the tissue extract.

The potassium content was finally quantified by means of an Atomic Absorption spectrophotometer (Varian AA-575 series) and expressed as $\mu$ mole $K^+$ g$^{-1}$ callus fresh weight. Newmann Keul's *post hoc* test was done to check the level of significance.
Material and Methods

Extracellular pH measurements
Extracellular pH was measured directly in the medium of cell suspension (Bouizagarne et al. 2006). The experiments were run simultaneously in three sets containing 2g tissue per 5 ml of culture, under continuous shaking, using three different pH meters for reproducible comparison. For each condition, the pH of the medium at the start of the experiment was between 5.6 to 5.8. The elicitor was added when a stable pH was obtained and the values were monitored for a period of 35 min.

Measurement of Intracellular Reactive Oxygen Species (ROS)
Cell suspension (100ml) was dispensed into small 25ml capacity conical flasks at 5ml each for individual treatment. Elicitor was treated at a concentration of 150mg ml\(^{-1}\) at various time points according to experimental requirement so that the cells were harvested at a particular time point to ensure uniform experimental conditions. For measurement of reactive oxygen species, cells were incubated in dark at 37\(^0\)C for 1h in the presence of 50\(\mu\)M \(\text{H}_2\text{DCFDA}\) (dihydro dichloro fluorescein diacetate) in 0.1M phosphate buffered saline (PBS, pH 7.0). Stock solution of \(\text{H}_2\text{DCFDA}\) was prepared in dimethylsulfoxide (DMSO) and stored at \(-20^0\)C. This non-fluorescent compound enters the cell and is converted to highly fluorescent and non-permeant DCF (dichlorofluorescein) by intracellular esterases and peroxides such as \(\text{H}_2\text{O}_2\) and lipid hydroperoxides. Intracellular ROS generation was monitored in a cell population of 10,000 cells, against control in a flow cytometer (FACS Calibur; Becton Dickinson, San Jose CA). Excitation of DCF was monitored at 488nm and emission was detected at 530±15 nm as FL1 fluorescence. For a comparative estimate of the degree of ROS generation, the Mean Fluorescence Intensity (MFI) at the FL1 region was calculated as M1 beyond the logarithmic scale of 10\(^1\), to minimize the interference of
the cellular auto fluorescence at the FL1 range. Newmann Keul’s post hoc test was
done to check the level of significance.

All flow cytometry results were obtained in three replicates using high flow
rates. List mode data were converted from Hewlett Packard to Apple (Macintosh)
computer format using the program FACS-Convert (Becton Dickinson, San Jose,
CA). Data were then analyzed using the CellQuest software (version 3.3; Becton
Dickinson).

Fatty acid profile analysis

Lipid extraction

Cell suspension (200ml) treated with elicitor at the concentration of 150mg ml⁻¹ was
harvested at 0, 15, 30 and 60min after treatment. The cell suspension culture was
sieved and 5g (cell fresh weight) were blotted dry within the folds of a sterile filter
paper, to remove excess culture media. The cells were crushed in liquid nitrogen and
extracted in 2:1 methanol: chloroform. Lipid extraction of all the sets was considered
simultaneously to ensure uniform experimental conditions. The resultant suspension
was kept overnight at room temperature. The suspension was further filtered and the
residue re extracted in 2:1:0.8 methanol: chloroform: water, with vigorous shaking.
The process was repeated twice from the residue to ensure complete extraction. The
filtrate was pooled and an equal volume of 1% Sodium chloride, chloroform (1:1) was
added and thoroughly mixed in a separating funnel by vigorous shaking. The flask
was left for separation and the lower layer separated in a conical flask. Oven dried
anhydrous sodium sulphate was added in a requisite amount to remove the traces of
water and kept overnight at 4°C. Next day, the sample was decanted in a round-
bottomed flask and dried in a rotary vacuum evaporator at 34°C and 76mm pressure to
ensure complete dehydration. Benzene was added next and dried under similar conditions, and the residue was extracted finally in chloroform. The resultant fluid was taken into a preweighed 5ml capacity conical flask. The fluid was dried under a flush of nitrogen and the final weight determined.

For separation of the neutral and phospholipid fractions, the dried fluid was first resuspended in chloroform and petroleum ether (1:1). The extracts were spotted on 1mm thick 14x20 thin layer chromatography (TLC) plates. The glass plates were coated with silica gel G and activated at 110°C prior to use, and finally run in 1:3 acetone: ether solvent system. The plates were air dried after the run was complete and developed in an iodine chamber. Two distinct zones were clearly visible which were marked separately and the zones were scrapped individually and kept in two separate conical flasks. To confirm the lower, slow migrating zone as phospholipid moiety, staining was carried out by spraying molybdenum blue reagent in a separate experiment. The lipid fractions were extracted separately with 2:1 methanol: chloroform and the silica gel was separated on centrifugation and stored at 4°C overnight. The extracts were dried in a rotary evaporator and finally resuspended in chloroform, methanol (1:2) until completely dissolved, and transferred to preweighed 5ml capacity conical flasks. To determine the weight of the neutral and phospholipid fractions of the lipid extracted, the solvent was evaporated in a flush of nitrogen and the conical flasks weighed carefully.

Preparation of molybdenum blue reagent

Reagent I

10.3g molybdenum oxide+25ml 25(N) H$_2$SO$_4$ was boiled till molybdenum oxide had dissolved completely.
Reagent II

0.54g of molybdenum powder was added to 150ml of reagent I and the mixture was boiled gently for 15 min. The solution was finally cooled and decanted.

100ml of reagent I and 100ml of reagent II were mixed with two volumes of water.

Identification of phospholipids by TLC

The different phospholipids namely phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), and phosphatidic acid (PA) was separated after spotting on 1mm thick TLC plate coated with Silica Gel H with solvent CHCl₃:CH₃OH:H₂O (65:25:4) and the spots were developed by spraying molybdenum blue. Individual phospholipids were identified by comparing the Rf values of the extracted phospholipids with those of the standard phospholipids separated in the same silica gel plate.

Preparation of Methyl Ester

The neutral lipid fraction was extracted in distilled methanol in a round-bottomed flask. About 3-4 drops of concentrated sulphuric acid was added to the methanolic sample till the solution was acidic (checked with pH paper), and the resultant solution refluxed for 4h on a water bath. After that methanol was nearly evaporated and cooled to room temperature. The evaporated samples were then dissolved in chloroform and double distilled water was added until the phases were separated. The chloroform layer was collected; using a separating funnel and anhydrous sodium sulphate was added for complete dehydration and kept overnight. The solvent was finally decanted and evaporated by flushing nitrogen gas, and the methyl ester separated from unesterified derivatives by TLC using petroleum ether: ether (90:10) as the solvent.
system. The methyl ester was identified on comparison of the Rf with standard methyl ester of capric acid (Sigma).

Gas Liquid Chromatography

The composition of the fatty acid methyl esters was determined by Gas Liquid Chromatography (GLC) using a dual column analytical gas chromatograph with dual flame ionization detector (F&M Model 700R) and a linear programmer (F&M Model 240 Hewlett Packard, Avondale, Pennsylvania, USA). The gas chromatographic column used constituted of 15% Diethyl Glycol Succinate (DEGS) on 100-120-mesh gaschrom P, packed into 6ft x 0.125-inch tube. The column was conditioned under a stream of nitrogen for 16-20h at 200°C prior to experimentation.

Samples (0.2-0.5μl) were injected by means of 10μl Hamilton syringes through the injection port and separation was carried out at a detection temperature of 50°C, above that of the column oven temperature. The oven temperature was maintained at 165°C. The carrier gas used was pure nitrogen having flow rate of 30-35ml min⁻¹ at a pressure of 20-lbs sq. inch⁻¹. A mixture of dry hydrogen and compressed air was used to produce the flame. The operating pressure of hydrogen and air was 25 and 40 lbs/sq.inch, with flow rates of 40 and 80 ml min⁻¹ respectively.

The individual fatty acids were identified on comparison of the retention time of the standard methyl ester used, consisting of fatty acid methyl ester mix (FAME) of C8:0 to C22: 1 (Supelco, Bellefonte, PA, USA). The quantity of the individual fatty acids was represented as percentage of the total peak area of the detectable individual components, as obtained from the linear programmer (F&M Model 240 Hewlett Packard, Avondale, Pennsylvania, USA). Variation of an individual fatty acid content was represented as percent variation of the component.
**Material and Methods**

**Variation in enzyme activity upon elicitation.**

Cell suspension (100ml) was treated with elicitor at a concentration of 150mg ml\(^{-1}\) up to 5 days after elicitor treatment, at an interval 24h. Protein was extracted individually and stored at -20\(^{\circ}\)C. Protein estimation and subsequent activity assays were carried out together to ensure uniform experimental conditions.

*Extraction of total protein from cell suspension for enzymatic assay*

The cell suspension was filtered and tissue of 1g of fresh weight was crushed in a prechilled mortar and pestle with liquid nitrogen to ensure maximum disruption of cell wall with 2 ml of 0.1M TRIS HCl buffer (pH 8.0) containing 0.5M sucrose, 0.1% ascorbic acid and 0.056M 2 β mercaptoethanol. The macerate was centrifuged at 12,500 rpm for 20min at 4\(^{\circ}\)C, and the supernatant used as the source of protein/enzyme for all subsequent enzyme analysis.

*Estimation of total protein content*

Buffer soluble protein content was estimated by the modified Lowry procedure (Markwell et al. 1978).

Reagents used

**Reagent A**

- Na\(_2\)CO\(_3\) 2%
- NaOH 0.4%
- Na-K-tartarate 0.16%
- SDS 1%

**Reagent B**

- CuSO\(_4\).5H\(_2\)O 4%

**Reagent C**

- Reagent A: Reagent B (100:1v/v)
Reagent D
Folin Ciocalteu phenol reagent (2N)-diluted with ddH₂O (1:1, v/v) on the day of use.

Procedure
Appropriate aliquot (20-30μl) for the estimation of protein was initially standardized. Volume of all the extracts was made up to 1ml with the extraction buffer keeping one tube as blank without any extract. Reagent C (3ml) was then added and incubated at room temperature for 60 min. Reagent D (300μl) was added and the reaction mixtures shaken, again incubated for 45 min at room temperature and the absorbance was measured at 660 nm with a UV-VIS recording spectrophotometer (Shimadzu UV-160A).

A daily reference curve of standard protein (Bovine Serum Albumin, BSA, SIGMA) was prepared and the buffer soluble protein content of the tissues was calculated from the reference curve. Protein content was expressed as mg/100mg fresh weight of tissue.

Determination of Peroxidase (PO) activity
Assay mixture consisted of 0.1M sodium phosphate buffer pH 6.5, 300 microliter 1mg ml⁻¹ O-Dianisidine and 30 microliter enzyme extract. The reaction was started by adding 25 microliters 10%(w/v) hydrogen peroxide in the cuvette and the increase in absorbance noted at 430 nm. Enzyme unit was expressed as the increase in absorbance min⁻¹ mg⁻¹ protein.
Material and Methods

Assay of Catalase (CAT) Activity

Hydrogen peroxide phosphate buffer was prepared by diluting 10% hydrogen peroxide to an appropriate volume of 0.067M-phosphate buffer (pH 7), so as the initial OD was 0.5 at 240 nm. Adding the enzyme to the buffer started the reaction and the time required for the decrease in OD by 0.05 was noted. The activity of the enzyme was expressed in unit min⁻¹mg⁻¹ protein.

Assay of Polyphenol Oxidase (PPO) activity

The reaction mixture consisted of 100 microliter enzyme extract and 1.8ml 0.1M sodium phosphate buffer (pH 6.5). To start the reaction, 200 microliters 0.01M catechol was added and the activity expressed as changes in absorbance at 495nm min⁻¹mg⁻¹protein.

Assay of Phenylalanine ammonia Lyase (PAL) activity

Activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm spectrophotometrically after addition of 1ml 30mM L-Phenylalanine dissolved in appropriate amount of 50mM Tris HCl (pH 8.6) to 2ml of the supernatant (protein content adjusted to 1mg/ml). Absorbance at 290 nm was measured in Shimadzu UV160A spectrophotometer after 2h incubation at 30°C. The activity was expressed as the amount of Cinnamic acid produced min⁻¹ mg⁻¹protein.

Data for all enzymatic reactions were taken in triplicates; mean and standard errors were calculated and one-way ANOVA was performed using Newman-Keuls Multiple Comparison Test using the PRISM software ver.2.01.
In gel analysis of Iso-peroxidase activity

Electrophoresis

General procedure followed was as described by Hames (1981) with necessary modifications.

Preparation of stock solutions

Solution A: Acrylamide stock solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.2 gm</td>
</tr>
<tr>
<td>N, N’ Methylene bis acrylamide</td>
<td>0.8 gm</td>
</tr>
<tr>
<td>Volume made up to 100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Solution B: Resolving (Running) gel buffer (1.5 M TRIS HCl, pH 8.8)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>18.17 gm</td>
</tr>
<tr>
<td>Volume made up to 100 ml</td>
<td></td>
</tr>
<tr>
<td>pH was adjusted to 8.8 with 6N HCl</td>
<td></td>
</tr>
</tbody>
</table>

Solution C: Stacking gel buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>6.050 gm</td>
</tr>
<tr>
<td>Volume made up to 100 ml</td>
<td></td>
</tr>
<tr>
<td>pH was Adjusted to 6.8 with 6N HCl</td>
<td></td>
</tr>
</tbody>
</table>

Solution D: Ammonium persulphate (APS, 1.5% w/v)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>0.15 gm</td>
</tr>
<tr>
<td>Volume made up to 10 ml</td>
<td></td>
</tr>
<tr>
<td>The solution was prepared prior to use.</td>
<td></td>
</tr>
</tbody>
</table>

Solution E: N, N, N’, N”-tetramethylethylenediamine (TEMED)

Used as supplied.

Solution F: Reservoir buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Glycine (pH 8.3)</td>
<td></td>
</tr>
<tr>
<td>TRIS (25mM)</td>
<td>3.025 gm</td>
</tr>
</tbody>
</table>
Material and Methods

Glycine (192 mM) 14.413 gm
Volume made up to 1000 ml

Recipe for gel preparation using Native PAGE Discontinuous buffer system

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel (10%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>10.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Solution B</td>
<td>3.75</td>
<td>-</td>
</tr>
<tr>
<td>Solution C</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>Solution D</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Solution E</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>ddH2O</td>
<td>16.0</td>
<td>7.2</td>
</tr>
</tbody>
</table>

The volumes indicated in the above table were used for mini gels.

The gel was casted taking into consideration all possible precautions and loaded with equivalent amount of protein, 50μg in each well. The protein samples were mixed with 2μl bromophenol blue (Glycerol: bromophenol blue, 1:1) prior to loading. Care was taken to avoid mixing of the samples in adjacent wells. The reservoir buffer was then put in the upper and lower chamber of the gel apparatus very slowly.

Running of gel

Electrophoresis was carried out for 1-1.5 h at 2mA; per lane basis (constant current). The migration of the tracking dye was monitored for completion of gel run. After the run was over, the gels were stained for isozymic analysis.

 Peroxidase Reaction mixture

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>O- dianisidine</td>
<td>100mg</td>
</tr>
<tr>
<td>Glacial Acetic acid</td>
<td>1ml</td>
</tr>
<tr>
<td>30% Hydrogen peroxide</td>
<td>0.2ml</td>
</tr>
</tbody>
</table>

Volume made up to 100 ml with dd H2O.
O-dianisidine was first dissolved in glacial acetic acid and the volume was made up to 100 ml with double distilled water. The gel was put in this solution and hydrogen peroxide added quickly. The brown colored bands became visible very rapidly and as soon as the background of the gel started to take color, the solution was poured off and the gel washed with dd water for 2-3 times. The gel was subsequently fixed for 10 min in a fixing solution containing methanol, glacial acetic acid and dd H₂O (4:1:15) and finally stored in ddH₂O and kept in the refrigerator at 4°C. Photographs of the gels were taken as early as possible. The relative mobility factor (R_{mf}) of the individual bands was calculated for representation as the ratio of migration of an individual band to migration of the dye front.

**Isolation of plasmid DNA from *E.coli***

**Small-scale isolation**

1. A single colony of *E.coli* was transferred into 10 ml liquid LB media with the appropriate antibiotic and incubated overnight at 37°C with shaking.

2. Next day, 1.5 ml liquid culture was poured into a microfuge tube and centrifuged at 10,000 rpm for 1 min. The supernatant was removed and the pellet resuspended in ice-cold solution 1 and vortexed to disperse the pellet.

*Solution 1*

- 50 mM Glucose
- 25 mM TRIS (pH 8.0)
- 10 mM EDTA (pH 8.0)

3. To the suspension, 200μl of freshly prepared solution 2 was added and incubated for 3-5 min.
Material and Methods

Solution 2
0.2N NaOH
1% SDS

4. To the suspension 150μl solution 3 was added and incubated on ice for half an hour, after which the precipitate was pelleted on centrifugation at 10,000rpm for 10 min at 4°C.

Solution 3
5M potassium acetate 60 ml
Glacial Acetic Acid 11.5 ml
Double distilled sterile Water 28.5 ml

5. The supernatant was carefully transferred to a fresh tube without disturbing the pellet and an equal volume of phenol: chloroform was added. The solution was then mixed by gentle inversion until a single-phase emulsion was formed.

6. The suspension was centrifuged at 10,000 rpm for 2 min and the upper aqueous phase was taken into a fresh tube. Isopropanol (0.6 volume) was then added and incubated at room temperature for 15 min followed by centrifugation at 10,000 rpm for 10 min at room temperature.

7. The pellet was washed with 70% ethanol, thoroughly dried at room temperature and dissolved in 10:1 TE buffer for further RNase treatment.

Large scale isolation

1. Cells were harvested from 50ml culture by centrifugation at 10,000rpm for 5 min at room temperature. The supernatant was discarded and to the pellet 5 ml ice-cold solution 1 was added and vortexed to disperse the pellet.

2. To the suspension, 10 ml of freshly prepared solution 2 was added, and mixed gently by inverting the tube occasionally.
Material and Methods

3. After 5 min of incubation in solution 2, solution 3 (7.5 ml) was added and incubated in ice for 30 min.

4. The precipitate formed was pelleted upon centrifugation at 10,000 rpm for 10 min, the supernatant carefully transferred to a fresh tube and an equal amount of phenol: chloroform was added. The solution was then mixed by gentle inversion until a single-phase emulsion was formed.

5. The suspension was centrifuged at 10,000 rpm for 3 min, the upper aqueous phase taken into a fresh tube avoiding the debris at the interphase and 0.6 volume of iso propanol was added, which was incubated at room temperature for 15 min followed by centrifugation at 10,000 rpm for 15 min.

6. The supernatant was discarded and the pellet washed with 70% ethanol, air dried thoroughly and finally dissolved in 10:1 TE buffer for further RNase treatment.

Purification and precipitation of DNA

DNA was purified upon requirement by extracting twice with an equal volume of phenol: chloroform and the aqueous phase taken into a fresh tube. The DNA was finally precipitated with one-tenth volume of 3M sodium acetate (pH 5.5) and two volumes of ethanol. The pellet was washed with 70% ethanol, air dried thoroughly and finally dissolved in sterile HPLC water.

Restriction digestion of DNA

DNA was digested with different restriction enzymes upon requirement by adding appropriate amount of SURECUT A, B, C or D (Roche). 10X restriction buffer dependent upon the combination of the enzyme used. The mixture was incubated at 37°C for 3 hours for digestion.
Recovery of DNA from agarose gel

Using low melting agarose followed by phenol extraction

DNA digested with restriction enzyme was electrophoresed on 1.2% low melting agarose gel and the desired DNA fragment was extracted from the gel using a sharp scalpel blade on the transilluminator, after staining the gel with ethidium bromide. Two volumes of STE buffer (0.1M NaCl, 10mM TRIS pH 8.0 and 1mM EDTA pH 8.0) was then added to the gel piece and incubated for 5 min at 65°C with occasional stirring. The mixture was then extracted once with phenol (saturated with 10 mM TRIS, 1 mM EDTA pH 8.0) and twice with phenol: chloroform. The DNA was finally recovered by ethanol precipitation as described earlier.

Using QIA quick gel extraction kit (QIAGEN)

To the gel slice taken in a colorless preweighed tube, three times buffer QG (w/v) (provided in the kit) was added to one volume of the gel and incubated at 50°C for 10 min, occasionally stirring to completely dissolve the gel. To the mixture one gel volume of isopropanol was added and mixed by gently inverting the tube. The resultant solution (650μl) was added to the QIAquick column (provided in the kit) and centrifuged at 7,500 rpm, repeating until the whole of the solution was exhausted. The flow through was discarded and the column spunned for an additional 1 min at 10,000 rpm. It was then placed in a clean 1.5 ml micro centrifuge tube and the DNA was eluted by adding 50μl HPLC water at the middle of the membrane, let to stand for one min and centrifuged at 10,000 rpm for 1 min.
Material and Methods

Ligation of the DNA fragments with different vector(s) using T4 DNA ligase

Ligation reaction was carried out in most cases with the ratio of 1:2 of the cleaved plasmid DNA: cleaved foreign DNA, depending upon the size and subsequently the copy number of the foreign DNA. The digested plasmid upon restriction with two different restriction enzymes and the foreign DNA similarly digested with the appropriate restriction enzyme was purified by extraction with phenol: chloroform as described earlier. The mixture of DNA was subsequently precipitated with ethanol and the DNA pellet was washed with 70% ethanol and thereafter air-dried thoroughly. To the dried pellet, appropriate amount of sterile HPLC water was added directly dissolving it completely, and ligation reaction carried out on adding 2µl T4 DNA ligase, 2µl 10X buffer to make up the final volume to 20µl.

Transformation of E. coli using Rubidium chloride

Preparation of competent cells

1. A single colony of E. coli was transferred to 10 ml of liquid LB media and incubated overnight at 37°C with shaking.

2. Next day, 50ml of liquid LB media was inoculated with 1ml of the overnight culture and incubated under similar conditions. After 2-3 hours, OD of the culture was checked to be between 0.3-0.4 at 600nm and was incubated on ice for 45 min and then centrifuged at 5,000 rpm for 5 min at 4°C

3. The pellet was gently resuspended in 25ml Solution 1 containing 10mM MOPS (pH 7) and 10mM Rubidium chloride; and centrifuged at 5,000 rpm for 5 min at 4°C.
Material and Methods

4. The pellet was gently resuspended in 25ml Solution 2 containing 10mM MOPS (pH 6.5), 10mM Rubidium chloride and 50mM calcium chloride; and incubated on ice for 45 min, after which the cells were precipitated once again by centrifugation at 5,000 rpm for 5 min at 4°C.

5. The pellet was resuspended in 8.5 ml Solution 2 and 1.5ml glycerol and aliquots of 200μl transferred to micro centrifuge tubes kept in ice.

6. The tubes were finally flash frozen in liquid nitrogen and transferred to -70°C ultra refrigerator for storage.

Transformation of competent E. coli.

Aliquots of competent cells as stated earlier were thawed on ice and mixed with the appropriate vector or the ligation mix and kept on ice for 30 min. The DNA-bacteria mixture was then transferred to a water bath preheated to 42°C and kept for 90 sec. One ml liquid LB was then added and incubated for 1h at 37°C. The culture (100μl) was then plated in each LB plate containing appropriate antibiotics and incubated at 37°C overnight.

Selection of transformed colonies

Blue white selection

Several cloning vectors like the frequently used pBLUESCRIPT harboring the β-galactosidase enzyme encoding lacZ gene, within which is placed the multiple cloning sequence (MCS), such that a series of unique restriction sites are engineered into it so that the plasmid can be restricted within the lacZ gene and the insert DNA recombined within the portion. Though neither the host, nor the plasmid is individually active, when transformed, the plasmid associates to form an
enzymatically active protein in a complementary manner, often referred to as α-complementation. Upon α-complementation, blue colonies are formed in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). When β-galactosidase is produced, it cleaves the colourless X-Gal complex and releases the blue indolyl derivative. On the other hand, plasmids having a DNA fragment inserted into the MCS causes a frame shift thus having no functional β-galactosidase activity, producing white colonies.

For each individual LBA plate, 40 µl of 2% X-Gal and 5 µl of IPTG (1M) was added and spread uniformly with a glass spreader. Transformed bacterial suspension (100 µl) was then spread uniformly on the plate and incubated at 37°C overnight. Next day, blue-white colonies were observed from which white colonies were selected for further confirmation of transformants.

**Colony PCR**

The PCR master mix comprising 1X PCR buffer, dNTPs, the required primer combination, Taq DNA polymerase and the requisite amount of HPLC water was dispensed in equal amounts in each PCR tube. White colonies were then individually transferred to the PCR tubes while marking and streaking in a master plate for subsequent recovery of the selected colonies. All operations were carried out in sterile conditions. The PCR tubes were then placed in a thermal cycler with a primary denaturation of 1 min 30 sec at 94°C, followed by 30 cycles of denaturation at 94°C, annealing at 58°C, extension at 72°C, and a final extension at 72°C for 7 min. The PCR product was subsequently run in an agarose gel for selection of the positively transformed colonies.
Material and Methods

Restriction analysis

Selected white colonies were inoculated in 10ml liquid LB and incubated overnight at 37°C. Next day, plasmid was isolated in a small scale and about 0.5μg DNA was taken for each marked clone for restriction analysis using the similar restriction enzymes used earlier for cloning. The mix comprising the restriction enzymes 1X SURECUT (Roche) buffer of the combination suitable for the enzymes considered, RNase A and appropriate amount of HPLC water was dispensed in equal volumes in each microcentrifuge tubes and the DNA added individually taking care that no contamination occurred. A minimum volume of 10μl was maintained for each clone. The tubes were eventually incubated at 37°C for 2h and whole of the product loaded into agarose gel adjacent to each non-restricted plasmid DNA for observation of the individual inserts.

Transformation of Agrobacterium

Preparation of competent cells

1. A single colony of Agrobacterium grown on LBA plates supplemented with Rifampicin (50mg L⁻¹) was transferred to 10ml liquid media with the similar concentration of Rifampicin and incubated at 30°C overnight, with shaking.

2. Next day, 1ml of the overnight culture was inoculated to 50ml liquid LB media and incubated under similar conditions for 6h to attain a final OD of 0.8-1.0 at 600nm. The bacteria were then pelleted upon centrifugation at 8,000 rpm for 10 min at 4°C.

3. The pellet was subsequently washed with 5 ml of 10:1 TRIS: EDTA (pH 7.5) and resuspended to form a uniform suspension.
4. The washed pellet was resuspended in fresh LB media and aliquots of 300µl transferred to microcentrifuge tubes placed on ice. The tubes were subsequently flash frozen in liquid nitrogen and stored at -70°C for subsequent use.

Transformation of competent cells

1. The competent cells were thawed on ice and 0.5µg of plasmid DNA was added. The bacteria-DNA mixture was incubated in ice for 5 min and then transferred to liquid nitrogen for 5 min.

2. From liquid nitrogen, the tube was quickly transferred to a water bath preset at 37°C for 5 min after which 1ml of liquid LB was added and incubated at 30°C on a shaker overnight.

3. The bacteria were pelleted on centrifugation at 5000 rpm for 5 min on the next day and the pellet was resuspended in 300µl liquid LB.

4. 100µl of the bacterial suspension was plated in LBA plates with Rifampicin and the appropriate selection antibiotic.

Selection of transformed Agrobacterium colonies

Colony PCR

A single colony was inoculated in 10ml culture in the presence of the respective antibiotic and grown at 30°C overnight with shaking. Next day, 1.5 ml of the culture was pelleted upon centrifugation at 10,000 rpm for 5 min. The supernatant was discarded and 1µl of the pellet added to 50µl PCR mix as described earlier, under similar PCR conditions in a thermal cycler. The PCR product was similarly run in an agarose gel for selection of the positively transformed colonies.
Material and Methods

Isolation of RNA

For the purpose of RNA isolation, utmost care was taken during handling using new tubes in all cases, baking glassware and the mortar-pestle so that no RNase contamination could occur. RNA was isolated using the QIAGEN RNAeasy minikit.

1. Tissue (500mg) was crushed to a fine powder in liquid nitrogen using a mortar and pestle and the powder directly transferred to a tube containing 5 ml RLT buffer (provided in the kit) and 50μl β-mercaptoethanol using a baked spatula. The suspension was mixed thoroughly and vortexed.

2. The solution was transferred to a QIAshredder (provided in the kit) in aliquots of 650μl, centrifuged and the flow through transferred to a fresh tube, taking care not to disturb the pellet. The flow through was adjusted to a volume of 1ml in each microcentrifuge tube and 500μl of dehydrated alcohol was added and mixed.

3. The resultant suspension was then transferred to the RNeasy minicolumn (provided in the kit) and centrifuged at 5000rpm for 30 sec. The flow through was discarded and 500μl of the buffer RW1 (provided in the kit) added directly to the matrix of the RNeasy minicolumn, centrifuged at 10,000rpm for 30sec and the flow through was discarded.

4. The buffer RPE (provided in the kit) was then added similarly, centrifuged at 10,000rpm for 30sec and the flow through discarded.

5. The column was then centrifuged blank at 10,000rpm for 2 min to ensure that no remnants of the previously used solutions were present and was finally air-dried.

6. The column was transferred to a fresh 1.5 ml collection tube and 35μl nuclease free HPLC water was added directly over the membrane, kept to stand for a minute and centrifuged at 10,000rpm for 1 min to elute the RNA. For effective
elution, the process was repeated once again and the RNA stored at -20°C for further use.

**RNA purification using RNase free DNase**

For effective purification of the total RNA from genomic DNA contamination, RQ1 (Promega) RNase free DNase was treated. To 35μl RNA appropriate amount of RQ1 10X reaction buffer was added along with 1 μl of the enzyme according to the manufacturer’s specification. The mixture was incubated at 37°C for 10 min, at the end of which an equal volume of phenol: chloroform was added, extracted and finally the RNA was precipitated using DEPC treated sodium acetate and dehydrated alcohol as described earlier. The RNA precipitate was finally washed with 70% ethanol (prepared with DEPC treated HPLC water), and finally dissolved in nuclease free water.

**Spectrophotometric estimation of nucleic acid**

The total DNA or RNA content was calculated according to the standard spectrophotometric estimates of 1 OD A$_{260}$ DNA corresponding to 50μg and 1 OD A$_{260}$ RNA corresponding to 40μg. If “x” OD was obtained upon loading 1μl nucleic acid, the total DNA content was calculated as 50x μg per μl DNA and 40x μg per μl RNA. If the total amount extracted was “y”, then the total DNA content was calculated as 50xy and the total RNA content as 40xy.
Material and Methods

3' RACE using anchored oligo dT primer

3' RACE from total RNA was performed using the anchored oligo dT primer 3'AP2dT and the MMLV reverse transcriptase. For the purpose, two solutions were formulated. Solution 1 comprising of 7.5µl total RNA, 2µl primer 3'AP2dT, and the appropriate amount of nuclease free water so that the final amounted to 20µl, was incubated at 70°C for 10 min and then transferred to 42°C for 5 min. The Solution 2 comprising of 1X RT buffer, 20mM DTT, 2mM dNTPs and appropriate amount of nuclease free water, such that the total volume amounted to 50µl was added. The Solution 2 was transferred to 42°C, and then 18µl Solution 2 was mixed with 20µl Solution 1 with 2µl RT and kept at 42°C for an hour. After the incubation period, the mix was incubated at 70°C for 15 min to inactivate the enzyme and then transferred to ice.

Gene specific amplification from 3' RACE product

For amplification of specific gene products, the complete gene sequences were acquired as specific Genbank sequences from NCBI (National Center for Bioinformatics). Primers were subsequently designed.

For effective amplification of the specific gene sequences, two rounds of PCR were performed. The first round comprising the 3'AP2dT corresponding 3'AP2 primer and the gene specific forward primer, followed by the second round amplification using product of the first round as template and gene specific primers. For both rounds of PCR, high fidelity Deep Vent DNA polymerase (New England Biolabs, NEB) was used.
Table 4: Primer sequences used for amplification of the promoter sequences:

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>PROMOTER</th>
<th>ACCESSION</th>
<th>SEQUENCE (5'-3')</th>
</tr>
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<td>PINF</td>
<td>PINII</td>
<td>X04118</td>
<td>AGT CAA GCT TAC TCT TGG ATT TTG TAT G</td>
</tr>
<tr>
<td>PINR</td>
<td>PINII</td>
<td>X04118</td>
<td>ATG CGG ATC CGA TGG ATA ATT AAT TAG TAC</td>
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<tr>
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<td>LAPAI</td>
<td>AF162854</td>
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<tr>
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<td>LAPAI</td>
<td>AF162854</td>
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Table 4a. Vectors used.

<table>
<thead>
<tr>
<th>VECTOR</th>
<th>REFERENCE</th>
</tr>
</thead>
</table>
Construction of vectors with jasmonic acid inducible promoter(s) as chimeric GUS constructs

Isolation of PINII and LAPal

Genomic DNA was isolated from Potato (Solanum tuberosum L.) and tomato (Lycopersicon esculentum Mill. cv. Moneymaker) using the CTAB method as described earlier (Murray and Thompson 1980). Template DNA (500ng) was amplified with gene specific primers (Table 4), using 1U Taq DNA Polymerase (NEB), in a 50μl PCR mixture. PCR conditions were as follows: initial denaturation of 4 min at 94 °C followed by 35 cycles consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of 7 min at 72°C in a Peltier effect Thermal Cycler PTC100 (MJ Research). The PINII product (~800bp) and LAPal product (~300bp) was observed as single bands upon agarose gel electrophoresis and considered for subsequent restriction analysis for the purpose of directional cloning in the vector pBlueScript (SK+).

Cloning of PCR products into pBluescript(SK+)

Restriction analysis

The PCR products were extracted with an equal volume of phenol:chloroform and subsequently precipitated with two volumes of ethanol in the presence of 3M Sodium Acetate solution. The products were then washed with 70% ethanol and dissolved in sterile HPLC water. The appropriate restriction buffer was then added and the enzymes HindIII and BamHI added in required amount depending upon the enzyme unit as supplied and incubated at 37°C for 2h. The products were finally eluted from gel using LMP agarose followed by phenolisation. The vector pBS(SK+) was
similarly restricted using the same enzymes, phenolised and precipitated similarly for the purpose of ligation.

**Ligation**

The PINII and LAPaI products were ligated into pBluescript using T₄ DNA ligase as described earlier.

**Transformation**

The ligated product was subsequently transformed into competent *E. coli* strain DH5α and plated in presence of Ampicillin, X-Gal and IPTG for screening using Blue-white selection. The resultant white colonies were grown in liquid culture and the plasmid DNA isolated in small scale, treated with RNase and the products confirmed for positive transformation using HindIII/BamHI mediated restriction analysis. The clones with positive inserts were selected for the subsequent steps.

*Cloning as chimeric GUS constructs in the vector pBI221*

The vector pBI221 was considered for the subsequent cloning process that harbors the GUS gene under the CaMV35S (*Cauliflower Mosaic Virus*) promoter and the NOS (Nopaline Synthase) terminator. For the purpose of chimeric GUS constructs with the promoters of interest, the CaMV35S promoter was replaced with the promoters of LAPaI and PINII respectively. For the said purpose, the vector was digested with HindIII/BamHI to remove the 35S promoter, the vector backbone was eluted from the gel and the previously digested HindIII/BamHI inserts were ligated with T₄ DNA ligase, maintaining all previously discussed interim procedures. The resultant product was used for transformation into *E. coli* strain DH5α. The positive transformants were
selected using blue-white selection and subsequent plasmid isolation followed by restriction with *HindIII/BamHI*. Size of the inserts was confirmed to be exactly similar to the previously isolated PCR products.

**Cloning of chimeric promoter GUS constructs in the binary vector pBIN19**

Though the promoter-GUS constructs were successfully cloned in the vector pBI221, subsequent cloning into a binary vector was necessary for proper *Agrobacterium* mediated transformation. For the said purpose, the binary vector pBIN19 was considered, which possessed the appropriate multiple cloning site. The vector pBIN19 as well as the promoter-GUS constructs in the vector pBI221, was digested with *HindIII/EcoRI* for effective isolation of the GUS gene under the control of the promoters of interest and the NOS terminator. The digested product upon electrophoresis did not resolve completely in 1% agarose gel, since the truncated product and the vector backbone was similar in size i.e., about 2.5 kb. For a better resolution, the digested product was electrophoresed in a lower volume of 1.2% agarose gel. The vector backbone and the inserts were visible, which however could not be eluted, since the amount loaded for proper resolution was not sufficient for effectively eluting from the gel, which could be used for subsequent purification and ligation. A direct ligation of the digested products was thus performed. The resultant products were transformed in DH5α and positive transformants selected by blue-white selection. The selected white colonies were further confirmed as the clones of interest by colony PCR since chances of pBI221 ligation with the pBIN19 vector was equally possible taking into consideration the procedure of direct ligation. Plasmid DNA of the positive clones was isolated and restriction analysis with multiple enzymes was performed to confirm the fidelity of the cassette. Large-scale isolation
of plasmid DNA from the resultant positive clones was performed and the DNA stored at -20°C for future use, as well as used for transformation into Agrobacterium tumefaciens strain LBA4404.

Transformation in Agrobacterium

Previously prepared competent cells were transformed with the positive clones of the pBIN19 chimeric GUS-promoter constructs. The positively transformed colonies were selected by colony PCR, which were finally stored as glycerol stabs for subsequent plant transformation.

Cloning of antimicrobial protein encoding genes

Of the various identified antimicrobial protein encoding genes, the Arabidopsis leaf thionin, THI2.1 and defensin PDF1.2 were isolated from Arabidopsis thaliana var. UK-4 and Col-0 respectively. Since the genes were identified to be highly inducible under various conditions, induction of the genes was considered individually for RT-PCR based cloning strategy. The Petunia hybrida defensin PhDI and the radish defensin RsAFP2 identified from Raphanus sativus was also considered, though the strategy employed for theses genes were not similar.

Cloning of THI2.1 and PDF1.2

Isolation of THI2.1 and PDF1.2

The Arabidopsis leaf thionin, THI2.1 was reported to be developmentally regulated and highly inducible upon treatment with methyl jasmonate and heavy metals like silver nitrate (Epple et al. 1997). Based on this consideration, 3-week-old Arabidopsis thaliana var. UK-4 was infiltrated with 1000μM silver nitrate, 8h prior to RNA
Table 5: Primer sequences used for amplification of the genes sequences THI2.1 (Accession L41244), PDF1.2 (Accession A68653), RsAFP2 (Accession U18556), PhDI (Accession AF507975).

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>GENE</th>
<th>SEQUENCE (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>3AP2DT</td>
<td>3' RACE ANCHOR</td>
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<tr>
<td>3AP2</td>
<td>PRIMER</td>
<td>CTGAGGATCCGTCGACTAGTAC</td>
</tr>
<tr>
<td>THI2.1F</td>
<td>THI 2.1</td>
<td>GTC AGG ATC CAT GAA AGG AAG AAT TTT GA</td>
</tr>
<tr>
<td>THI2.1R</td>
<td>THI 2.1</td>
<td>CAT GGA GCT CTT ACA ACA GTT TAG GCG GCC CAG</td>
</tr>
<tr>
<td>PDF1.2F</td>
<td>PDF 1.2</td>
<td>CTG AGG ATC CAT GGC TAA GTG TGC TTC CAT C</td>
</tr>
<tr>
<td>PDF1.2R</td>
<td>PDF 1.2</td>
<td>GAT CGA GCT CTT AAC ATG GGA CGT AAG AAG TAC</td>
</tr>
<tr>
<td>RsAFP2</td>
<td>RsAFP2</td>
<td>GAC TGG ATC CAC ATA TAC ATT AAA AAG TAG</td>
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<td>RsAFP2</td>
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<tr>
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<td>PhD1</td>
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</tr>
<tr>
<td>PhiDR</td>
<td>PhDI</td>
<td>GAT CGA GCT CCT ACA CCA TCA TAT CTG CCT CAA G</td>
</tr>
</tbody>
</table>
Material and Methods

Extraction. For the purpose of effective induction of the defensin PDF1.2, 2-week-old Arabidopsis thaliana var. Col-0 was infected with Alternaria brassicicola MUCL20297 48h prior to RNA extraction. Since systemic induction was reported to occur within 48h, directly inoculated leaves were avoided. Total RNA from both the varieties was then isolated using the RNeasy plant minikit as described earlier. To reduce chances of contamination of genomic DNA, the product was treated with RQ1 RNase free DNase.

3’ RACE (Rapidly Amplified cDNA Ends) and amplification of gene specific product

The purified RNA was used for 3’RACE using the anchored 3’AP2dT primer. The subsequent rounds of PCR, using the 3’ RACE product as template, were used for amplification of the required gene by nested PCR. To ensure positive amplification of lowly expressed transcripts, a step-up PCR was attempted at the first round using the dT3’AP2 corresponding primer, 3’AP2 and the gene specific forward primer THI2.1F for THI2.1 and PDF1.2F for PDF1.2 (Table 5). The PCR cycle consisted of a primary denaturation of 2.30 min at 94°C followed by 10 cycles of 30 sec at 94°C, 45 sec at 50°C and a one min extension at 72°C, followed by 25 cycles of denaturation at 94°C, followed by 15 sec annealing and extension at 72°C for one min. The resultant product was used as template for the subsequent PCR using gene specific forward and reverse primers (THI2.1F&R for THI2.1 and PDF1.2F&R for PDF1.2) (Table 5). The same cloning strategy was employed for both THI2.1 and PDF1.2 considered individually. To ensure fidelity of the clones, Deep Vent DNA Polymerase was used in both rounds of PCR, which possessed high proofreading activity and reduced chances of mutation.
Cloning in pBluescript

The PCR products were phenolised and precipitated with three volumes ethanol and 3M Sodium acetate solution. The purified product was subsequently digested with *BamHI* and *SacI* along with the vector pBluescript. The vector was then purified by phenolisation while the gene specific products were eluted from the gel using the QIAquick gel extraction kit. Both the vector and the gene specific products were ligated with T₄ DNA ligase and transformed into *E. coli* strain DH5α. The transformed clones were selected using blue-white selection. The white colonies were isolated and colony PCR was performed for both *THI2.1* and *PDF1.2* clones. Plasmid DNA of the positive colonies was finally extracted and subjected to restriction digestion with *BamHI/SacI* for further confirmation.

Isolation and cloning of the *Petunia hybrida* defensin PhD1.

The full-length cDNA clone of the *Petunia hybrida* defensin was obtained as a generous gift from Dr. Fillipa Bruglieri. Since no suitable restriction site flanking the gene sequence was identified, gene specific primers were designed with the appropriate restriction sites at the 5' end for subsequent cloning.

The 311bp PhD1 fragment was amplified using Deep Vent DNA polymerase and PhD1 gene specific primers PhDF and PhDR (Table 5), with an initial denaturation of 4 min at 94 °C followed by 35 cycles consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final extension of 7 min at 72°C. The PCR product was purified by phenolisation and subjected to restriction digestion with *BamHI/SacI*. The product was run in 1.2% agarose gel and finally eluted from the gel using the QIAquick gel extraction kit. The *BamHI/SacI* restricted vector pBluescript was ligated with the eluted product and transformed in *E. coli* strain DH5α. The
transformed colonies were selected using blue-white selection. The white colonies were further confirmed to be positively transformed by plasmid isolation followed by restriction digestion of the plasmid DNA using \textit{BamHI/SacI}.

\textit{Isolation and cloning of the Radish defensin RsAFP2.}

Primers were designed across the flanking regions of the 98bp intron (RsF and RsR), along with the gene specific primers (RsAFPF and RsAFPR)(Table 5), with the aim of intron removal and PCR mediated ligation. A combination of PCR amplifications was attempted at from the genomic DNA with the purpose of removing the intron. The first set of amplification RsF1 with the gene specific forward primer RsAFPF and the intron flanking reverse primer RsR was followed with the second set RsF2 with the gene specific reverse primer RsAFPR and the intron flanking forward primer RsF. All amplifications were performed using high fidelity \textit{Deep Vent} DNA polymerase with an initial denaturation of 4 min at 94°C followed by 35 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 7 min at 72°C. Both the products RsF1 and RsF2 were eluted individually from the gel using the QIAquick gel extraction kit. Equal amount of both the products were then considered for PCR mediated ligation using the gene specific primers RsAFPF and RsAFPR with a final concentration of 4mM MgCl$_2$. The product was reamplified using the same PCR conditions and purified by phenolising. The product was subsequently digested with restriction enzymes \textit{BamHI/SacI} and ligated with the similarly restricted vector pBLUESCRIPT, using \textit{T$_4$ DNA ligase}. The vector was subsequently transformed and confirmed.