# MATERIALS AND METHODS

# General materials and methods

## 3.1 Materials used and their source

### 3.1.1 Seeds and plant sample

| Plant | Species/ variety | Source of seed |
|---|---|---|
| Tomato | (*Lycopersicum esculentum* cv. Kashi Amrit ) | IIVR, Varanasi |
| - | *Nicotiana benthamiana* | CTRI, Rajamundry |
| Bitter gourd | *Momordica charantia* | Local |
| Chilli | *Capsicum annum* | Local |
| Cotton | *Gossypium hirsutum* | Local |
| French bean | *Phaseolus vulgaris* | Local |
| Okra | *Abelmoschus esculentus* | Local |
| Papaya | *Carica papaya* | Local |
| Raddish | *Raphnus sativus* | Local |

For agroinoculation experiment, plants were grown in sterilized soil, in 4''/10'' pots in an insect proof glass-house.

### 3.1.2 Clones and plant samples

Monomeric clones of *Tomato leaf curl Gujarat virus* (Chakraborty *et al.*, 2003a, 2003b) and *Tomato leaf curl New Delhi* (Padidam *et al.*, 1996) have been previously described. Clones of *Raddish leaf curl virus* (Singh *et al.*, 2007 a), Chilli leaf curl virus-Mograhat (Chakraborty *et al.*, unpublished) were also selected. All these clones were available in Molecular Viology Lab, School of Life Sciences, JNU.

### 3.1.3 Reagents, primers, DNA ladder, enzymes, antibiotics, kits, α-[$^{32}$P] (Radioactivity) filters and membranes

Trizma Base, EDTA, SDS, Bromophenol blue, Xylene cyanol FF, Antibiotics, Glycerol, X-Gal, IPTG, Agarose, Bovine Serum Albumin (BSA), Sodium Chloride, Tris-saturated Phenol, Sodium hydroxide, Bacto-tryptone, Yeast extracts and other chemicals were procured from Sigma, USA. Ethanol was purchased from Merck. Glacial acetic acid, Chloroform, Dextrose, HCl, $H_2SO_4$, Isoamylalcohol, Isopropanol, $KH_2PO_4$, $K_2HPO_4$, $MgCl_2$, $CaCl_2$, $NaH_2PO_4$, $Na_2HPO_4$, Sodium acetate and Potassium acetate were purchased locally. Ready to use 100bp and 1Kb, T 4DNA ligase, dNTPs, ATP, $MgCl_2$, Klenow fragment, Random hexamer primer, Taq

Polymerase and RNaseA were purchased from MBI Fermentas. Antartica Phosphatase was procured from New England Biolab. All the primers used in this study were synthesized from Sigma co. Restriction enzymes were obtained time to time from MBI Fermantas, Bangalore Genei and New England Biolab. α-[$^{32}$P] was obtained from BRIT, Mumbai (India). Elute™ Gel extraction kit, QIAprep$^{R}$ Miniprep and nucleotide removal kit were purchased from Qiagen, Germany. InsT/A Clone™ PCR Product Cloning kit was purchased from MBI Fermentas. For PCR product purification kit was purchased from Millipore. Membrane filters of 0.22 μm pore size was purchased from Millipore, USA. Nylon membrane for nucleic acid blotting is procured from Millipore, USA. Other filter papers were purchased from Whatman, USA.

## 3.2 General methods used for nucleic acid amplification, cloning and sequencing

### 3.2.1 Selection of *Escherichia coli (E.coli)* strains:

The *E. coli* strains used in the present study were streaked in LB agar plates containing Sodium nalidixate (15 μg/ml) for DH5α strain and without any antibiotic for DH10β strain.

### 3.2.2 Preparation of glycerol stocks of *E. coli* cells

Single isolated colony picked from LB agar plate was inoculated in 5 ml LB medium supplemented with suitable antibiotic, grown at 37°C, 220 rpm for 10-12 hrs. On the next day 25% glycerol stocks were prepared by adding 250 μl of sterile glycerol with 750 μl of grown *E. coli* cells with or without any plasmid DNA (as desired for different experiments) in a sterile eppendrof tube and gently vortexed to ensure the thorough mixing of glycerol stock. The culture was sealed by parafilm strip and transferred to -80°C for long term storage.

### 3.2.3 Preparation of competent *E. coli* cells

The competent cell for transformation was prepared by $CaCl_2$ method (Mendel and Higa, 1970). 1ml of overnight culture was inoculated with 50 ml of LB and allowed to grow at 37°C, 220 rpm. When an optical density OD 600 of 0.5 was reached, cells were chilled on ice for 15-20 min and centrifuged at 6000 rpm at 4°C for 5 min. The supernatant was discarded and cells were re-suspended in 30ml of ice-cold sterile

solution [(6 ml $CaCl_2$ 0.1M) + 24 ml $MgCl_2$ (0.1M)], mixed thoroughly. The tube containing mixture of cells and solution was incubated on ice for 30 min and contents were centrifuged at 6000 rpm for 5min at 4° C. Supernatant was discarded and 225µl of glycerol (100%) and 1275µl of 100mM $CaCl_2$ were added to the tube. The pellet was gently mixed and 100µl of competent cells were immediately aliquoted in eppendorf tubes and stored at -80°.

### 3.2.4 Transformation of competent *E. coli* strains

An aliquot of competent cell was thawed on ice for 5-10 min. 0.1-1 ng of plasmid DNA or 5 µl of ligation mixture were mixed with competent cells and kept on ice for 5 min. One hundred microlitres of competent cells were added to each DNA/ligation reaction and allowed to incubate on ice for 20 minutes. The cells were given heat shock at 42°C exactly for 90 seconds and immediately transferred to ice and left for 5 min. Eight hundred microlitres of LB medium was added to each tube and incubated at 37°C for 1 hour with shaking at 220 rpm. Two hundred microlitres of the transformation mixture was spread on a LB agar plate containing the appropriate antibiotic. Rest of the transformation mixture was pelletized at 8000rpm for 5 min, excess of media was discarded and cells were re-suspended in 100µl of media, which is used to spread one more antibiotic added LB agar plate. The plates were allowed to dry in the hood for 5 minutes and incubated overnight at 37°C.

### 3.2.5 Plasmid DNA extraction

Isolation of Plasmid DNA by alkaline lysis method (DNA minipreparation) as described by Birnboin & Dolly (1979)

*E. coli* cells from 1.5 ml of overnight grown culture with appropriate antibiotic were collected in eppendorf tubes and centrifuged at 13,000rpm for 2 mins. Supernatant was discarded, the pellet was re-suspended in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, sterilized by autoclaving and stored at 4°C). It was vortexed for 1 min at high speed to completely dissolve the pellet and 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added to the above. The solution was mixed well by inverting the tube. One hundred fifty microlitres of freshly prepared Solution III (For 2 ml: 1.2 ml of 5 M potassium acetate, 0.23 ml glacial acetic acid and 0.57 ml H2O, pre-chilled) was added to this tube, briefly mixed and kept on ice for 5 min. It was centrifuged at 12,000 rpm for 15

min at 4°C and the supernatant was saved. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed well by inverting the tube. Subsequently, tube was centrifuged at 13,000 rpm for 10 min at 4°C and the upper aqueous phase was collected. 2 volumes of Ethanol (or 0.8 volume of Isopropanol) were added. The tube was kept for 30 min at 4°C and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was drained out and the pellet was washed with 70% Ethanol. The pellet was dried and dissolved in 100 µl of sterile distilled water. For RNase treatment, RNase was added to a final concentration of 50 µg/ml and the tubes incubated at 37°C for 1h. The solution was then Phenol extracted and the plasmid were precipitated. The DNA pellet was resuspended in TE/sterile distilled water and the concentration of plasmid DNA was measured spectrophotometrically (Sambrook *et al.*, 1989, 2001).

### 3.2.6 Midi preparation for large scale plasmid DNA isolation:

A single colony of transformed *E. coli* cells was grown in 50 ml of LB media for overnight and supplemented with appropriate antibiotic. Culture was transferred to a 30ml centrifugation tube and centrifuged at 11000 rpm for 5min. Supernatant was removed and rest of the culture was also pelletized. It was then completely discarded from the pellet. The pellet was re-suspended in 1.5 ml of Solution I, vortexed for 1 min at high speed to completely dissolve the pellet. On Eighty microlitres of freshly prepared lysozyme (40mg/ml in 50mM TrisCl, pH 8.0) was added and incubated on ice for 8-10min. Three ml of freshly prepared Solution II was added to the above and mixed by inverting and kept on ice for 5min. After this 1.6 ml of chilled Solution III was added to this tube, briefly mixed and kept on ice for 15 min. It was centrifuged at 11,000 rpm for 15 min at 4°C and the supernatant was saved in a fresh tube. RNase A was added to the supernatant at a final concentration of 100µg/ml and incubated at 37°C for 30min. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and mixed well by inverting the tube. The tube was centrifuged at 11,000 rpm for 10 min at 4°C and the upper aqueous phase was saved. 0.8 volume of Isopropanol was added. The tube was kept for 30 min at 4°C and centrifuged at 11,000 rpm for 30 min at 4°C. The supernatant was drained out and the pellet was washed with 70% Ethanol. The pellet was dried and dissolved in 500 µl of sterile distilled water. The concentration of plasmid DNA was measured spectrophotometrically (Sambrook *et al.*, 1989, 2001).

### 3.2.7 Restriction digestion of plasmid DNA

The reaction mixture for restriction digestion for a particular enzyme(s) was determined according to the manufacturer's recommendation. DNA was subjected to restriction digestion with one enzyme (single digestion) or two enzymes (double digestion). Double digestion was performed in two different ways depending on the enzyme and the buffer compatibility. When only one component (salt concentration) of two-reaction buffer differed, the DNA was first cleaved with the restriction endonuclease that required lower salt concentration. Afterwards, the concentration of the reaction was adjusted to approximate the reaction conditions of the second restriction endonuclease. The other way of double digestion was to incubate the DNA with two required restriction endonucleases simultaneously depending on the buffer compatibility, according to the manufacturer's recommendation. If the enzymes and buffers were incompatible for the double digestion, the DNA was then first cleaved with one endonuclease, extracted with phenol:chloroform, and precipitated. It was then carried for the second restriction endonuclease digestion.

| Composition of restriction digestion reaction mixture | µl (final concentration) |
|---|---|
| Restriction Enzyme Buffer | 2µl from 10X stock (1X) |
| Template DNA | Plasmid-2 to 6 µl/Viral DNA- 10 to15µl (100ng DNA/µl) |
| BSA (if required) | 0.5µl (0.1mg/ml) |
| Restriction enzyme | 0.5µl to 1µl (1U/µg) |
| Sterile distilled water | To adjust the final volume 20µl |

The eppendorf tube was then incubated at the suitable incubation temperature (usually at 37 $^{0}$C) for 4 hrs to overnight, depending upon the nature of enzyme.

### 3.2.8 Dephosphorylation of linearized plasmid:

The linearized plasmid or partial clone in the case of tandem dimer preparation, singly digested with suitable enzyme was either purified by Gel extraction kit, or phenol:chloroform extracts (in case of non-heat inactivable enzyme), or enzyme inactivation were carried out simply by heat inactivation procedure at recommended temperature. This is now used as template for 5' dephophorylation.

| Composition of reaction mixture | |
|---|---|
| Reaction buffer | 1X |
| Template DNA | 500ng -1μg |
| Antartic Phosphatase (New England Biolab) | 1U/μg |
| Sterile distilled water | to adjust the volume up to 20μl |

The mixture was incubated at 37°C for 10-15 min and enzyme was heat inactivated at 65° C for 10min. Purified dephosphorylated plasmid was then used as vector in ligation mixture set up.

### 3.2.9 Ligation mixture set up for cloning DNA

Just before setting the ligation reaction, 1.0 μl of vector and 1.0 μl of insert were checked (if concentration is more than 50 ng dilution was made and loaded in the range of ~ 50-100 ng of each DNA) in a 1.0 % agarose gel in 1x TAE buffer to estimate the concentration of DNA. The ng equivalent of vector DNA and insert DNA for 100 fmole [1Mole ≡ 6.023 x $10^{23}$ molecules and for a 1000 bp (1.0 kb) DNA, 1000x 660 gms ≡ 1 mole] were calculated accordingly. 100 fmole of the vector and 100 fmole of the insert fragments were ligated in 1x $T_4$ DNA ligase buffer with 1.0 μl T4 DNA ligase (MBI, Fermentas) in a final reaction volume of 20 μl 16°C for 16-18 h as per the manufacturer's instruction (MBI,Fermentas). Only vector and $T_4$ DNA ligase in 1x $T_4$ DNA ligase buffer were used as negative control/background for ligation reaction. After completion of ligation the ligation reaction products we stored at -20°C, and used for transformation.

### 3.2.10 PCR set up for plasmid and total DNA as template

The PCR is an *in vitro* procedure for synthesizing enzymatically, specific sequences of DNA. Two oligonucleotide primers that flank the DNA region of interest and hybridize to opposite strands of the DNA were utilised in this technique. A series of repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers using the thermostable Taq DNA polymerase accomplishes the DNA amplification. At the completion of the reaction, amplification products were analyzed by size fractionation using agarose gel electrophoresis and then purified with a MinElute™Gel extraction Kit (Qiagen, Germany) according to the

manufacturer's instructions. A condition under which a PCR was carried out varies depending mainly on the specific DNA sequence to be amplified and the oligonucleotide primers which were to be used for the amplification. Primers were designed such that the 5' and 3' ends of the genes/part of genome selected, to be amplified would be flanked by additional bases containing restriction sites necessary for subsequent cloning of the amplified product into plasmid vectors.

For different sets of primers used in this study, annealing temperature, primer extension with Taq polymerase, and cycles of amplification were varied as mentioned with respective description.

| Composition of PCR reaction mixture | |
|---|---|
| Reaction Buffer | 1X (2.5µl from 10X stock) |
| $MgCl_2$ | 0.5µl (25 mM) |
| Forward and Reverse primer | 0.1µl+0.1µl (from 100mM stock) |
| Template | plasmid DNA (20ng) or total DNA (1µg) |
| Taq polymerase | 1.5U |
| Sterile distilled water | to adjust the ready volume 25µl |

### 3.2.11 T/A cloning for amplified PCR product with poly-A tail

The PCR reaction mixture after the completion of PCR was immediately loaded on agarose gel to fractionate and amplified product i.e. band was cut out with the gel, purified with a MinElute$^{TM}$Gel extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. A ligation mixture was prepared according to the manufacturer's instrcuction. For this cloning INSTA cloning kit from MBI Fermentas, was used. The ligation mixture was transformed to competent *E.coli* cells. Blue white selection procedure was applied by spreading transformed cells in an X-gal, IPTG and ampicillin added LB agar plate. From their white colonies were selected and grown overnight in liquid culture media. Plasmid DNA was harvested and used for PCR to confirm the recombinant plasmids.

## 3.3 DNA extraction from leaf tissue and cloning of viral DNA

3.3.1 Modified Dellaporta (1983) method for total DNA isolation:

1. Preparation of tissue was carried out (1 leaf or upto 0.4 g) by grinding with liquid nitrogen using micropastles in 1.5 ml of microfuge tube. Seven hundred fifty microlitres of extraction buffer was added to frozen tissue and homogenized thoroughly and then stored at 4 ° C until preceded.

2. The microcentrifuge tube was then placed into 65 ° C (preset) water bath.

3. One hundred microletres of SDS(10%) was then added to the sample and inverted several times to mix properly.

4. Incubation was done for 10 min at 65 ° C in heat block and was allowed to cool to room temperature.

5. Next, 250 microlitre potassium acetate 5M pH (5.2) was added and inverted several times to mix properly.

6. Centrifugation was done for 10 min at 13500 rpm. The supernatant (900 microlitres) was taken in a two ml fresh microcentrifuge tube.

7. Equal volume of Chloroform-Isoamyl alcohol (24:1) was added to it and mixed well by inverting.

8. Centrifugation was again done at 13000 rpm for 10 minutes. The upper aqueous layer was taken in 1.5 ml of microcentrifuge tube.

9. 0.8 Volume of isopropanol was added to it and then microcentrifuge tube was kept for 30 min to overnight at -20 ° C.

10. Centrifugation was done at 13000 rpm for 30 minutes, at 4 ° C to get pellet. Supernatant was poured off and the pellet was washed with 70 % ethanol.

11. Finally the pellet was dissolved in 50-100 microlitre of sterile double distilled water.

12. Concentration of DNA was quantified in spectrophotometer.

3.3.2 Total DNA extraction by CTAB method (adopted from Srivastava *et al.*, 1993)

1. 5gm of leaves was grinded in liquid nitrogen with mortar and pestle. Frozen powdered tissue was transferred into a 50ml capped centrifuge tube, 15ml extraction

buffer{1leaves (wt): 3extraction buffer (volume) was added and kept on ice (till all the samples were ready to proceed }.

2. Buffer was mixed by inverting; clumping of grinded tissue was avoided, and incubated at 65 ° C (preset) water bath for 1hr, mixed well at every 10-15 min.

3. Centrifugation was done at 1000rpm for 20min at 20°C, supernatant was transferred in a fresh centrifuge tube and extracted with equal volume of Chloroform:Isoamylalcohol (24:1).

4. Supernatant was taken in a open cap fresh tube and 1/10 volume of 3M Sodium acetate (pH 4.8), and 0.8 volume of chilled isopropanol was added and mixed by inverting.

5. Kept at -20°C deep freezer for 30min to overnight and centrifuged at 11000rpm for 30min at 4°C.

6. Pellet was washed with chilled 70% Ethanol for 15min, dried and dissolved in 500µl-1ml of sterile distilled water.

### 3.3.3 Viral DNA extraction (Alkaline lysis for concentrating supercoiled DNA from total DNA) (adopted from Birnboim and Dolly, 1979)

For isolating viral DNA, 500µl of total DNA was treated with RNase-A (100µg/ml) for 30min at 37°C. Now 1ml of solution II was added and mixed gently, kept at room temperature for 10min, followed by 15min incubation on ice (till the solution turns milky). Seven hundred and fifty microlitre of 3M sodium acetate (pH 4.8) was added and mixed gently, incubated on ice for 10 min and centrifuged at 11000rpm for 10min at 4°C. Supernatant was extracted with equal volume of Phenol: Chloroform: Isoamylalcohol. 0.8 volume of Isopropanol was added to the supernatant and mixed gently, kept at -20°C for 30min to overnight, followed by palletization at 11000rpm for 30min at 4°C. Chilled 70% Ethanol was used for washing the transparent DNA pallet for 10min. Pallet was dried and dissolved in 100µl of sterile distilled water.

### 3.3.4 Cloning of Viral DNA

Concentration of viral DNA isolated by above mentioned protocol was determined by spectrophotometer. 1µg of viral DNA is digested separately in ependorf tube with *Hind* III, *BamH* I, *Pst* I, *Kpn* I and *Xba* I restriction enzymes. 250ng of pUC18 vector was also digested with the same enzymes and given 5' dehosphorylation to avoid re-

ligation of plasmid. Enzymes were inactivated by heat from both the vector and digested viral DNA. Ligation was set up for each digested viral DNA with its respective linearized vector using a ratio of 750ng viral DNA and 250ng of vector. Ligation mixture was prepared by above described protocol and 1µl of ATP was also added. This was then incubated at 16°C for overnight and used to transform in *E. coli* cells. White blue selection was applied as discussed in T/A cloning procedure and recombinant plasmids were screened on agarose gel. Uncut recombinant plasmids showing mobility like 5.2 kb and approximately (determined by loading known plasmid construct; other monomeric clones as positive controls in gel) were selected and digested with respective restriction enzymes used for cloning. Presence of begomovirus in recombinant plasmids was confirmed by PCR using degenerate primer pairs (Wyatt & Brown, 1996). For cloning DNA-β, total DNA sample was subjected to amplification of satellite DNA- β molecule with universal beta primer (Briddon *et al.*, 2002). An approximately 1.3-kb fragment was amplified and ligated in the pTZ57R/T vector (INSTA cloning kit, MBI Fermentas, USA). Selected positive clones for DNA-A and DNA-β, were sent for sequencing.

## 3.4 Agroinoculation protocol to study pathogenicity of cloned viral DNA on plants

### 3.4.1 Preparation of competent *Agrobacterium* cells

EHA105 *Agrobacterium* strain was used to grow 5ml culture media (LB media+ 1%sterelized glucose+ Rifampicin(30µgm/ml)) at 28°C for 36-48hrs. 2ml of grown culture was used to inoculate 50ml media in a 250ml conical flask. The culture was allowed to grow at 28°C, 220rpm until the OD reaches 0.6. Culture was chilled for 5min and 30ml of culture was transferred to a centrifuge tube. Culture was harvested at 4°C, 7000rpm for 5min. Supernatant discarded and pallet was resuspended in 10ml of ice-cold 0.15M NaCl, allowed for 15min incubation on ice. Again centrifuged at 7000rpm for 5min, pallet resuspended in 1ml of ice-cold 20mM $CaCl_2$ and 100µl of competent cells was aliquoted. Competent cells were kept in liquid $N_2$ for 5min, immediately stored at -80°C.

### 3.4.2 Preparation of Tandem dimeric constructs

From monomeric viral DNA clones the infectious clones were constructed in a binary vector. In all these cases (for DNA A, DNA B, and DNAβ) one restriction

enzyme was selected at which the viral DNA is cloned as monomer. Another restriction enzyme was selected in such a way that if doubly digested with both the enzyme, it would give a partial genome fragment with CR (common region of viral genome). This fragment containing CR, was first cloned in multiple cloning site (MCS) of pCAMBIA 2301. In this confirmed partial fragment containing construct then the full length genome was mobilized. The tandem orientation was then confirmed by appropriate restriction digestion of the genome.

### 3.4.3 Transformation of *Agrobacterium* cells

100µl of competent *Agrobacterium* (EHA 105) cells was thawed on ice and 1µg of plasmid DNA construct was added to it. After 30min incubation on ice tube was allowed to freeze in liquid $N_2$ for 1min. Frozen tubes were thawed in 37°C water bath for 1-2min, immediately kept on ice for 5-10min. 1ml of media (LB media+ 1% sterelized glucose+ Rifampicin(30µgm/ml)) was added and cells were allowed to recover at 28°C, 180rpm, for 3-5hrs. Cells were pelletized at 6000rpm for 5min and excess of supernatant was discarded. Pellet was resuspended in 100µl of media and spraded on plate (LB agar plate, 1%glucose, 30µg/ml Rifampicin, 50µg/ml Kanamycin). Plates were incubated at 28°C for 36-48hrs.

### 3.4.4 Selection of transformants

Transformants were identified based on growth on media supplemented with kanamycin (50µg/ml) and restriction digestion. Few colonies were selected and grown in 2ml of LB media with same antibiotic concentration for two days. Plasmids were harvested and digested with appropriate restriction enzymes to reconfirm the construct in *Agrobacterium*. These transformed *Agrobacterium* colonies were used for 50-100 ml culture. After 2days this culture was harvested in a centrifugation tube by pelletizing at 6000rpm for 5min. The pellet was washed twice by resuspending in sterile distilled water. After washing all the *Agrobacterium* construct pellets were dissolved in 500µl-1ml of sterile distilled water. Then OD was taken and equal no. of cells either used directly to inoculate on plants or different constructs is used to make combinations.

### 3.4.5 Agro culture and agro inoculation on plants

The *Agrobacterium* transformed cells in equimolar concentration were used to inoculate on plants. Five-leaf stage *N.benthamiana* plants (two week old seedlings,

one week after re-transplantation) and three-leaf stage tomato plants (two week old seedlings, one week after re-transplantation) were used for inoculation. For inoculating tomato, the first emerging leaf pricked with a sterile needle at the joint of petiole, and one drop of *Agrobacterium* culture was added. *N.benthamiana* plants are inoculated by pricking at the joint of third and second leaf from the apex and one drop of agroculture was added. All inoculated plants along with the plant inoculated with empty construct (mock) were allowed to grow at 28°C in glass house chamber maintaining 16 hrs day and 8 hrs night period.

### 3.4.6 Symptom study on plants

All the agroinoculated plants were studied daily for symptom development and compared with mock to record the symptom appearance. Score was given for symptoms as per the criteria decided given below. (Respective score was given if one or more of the symptoms from that category were appearing)

| Score | Symptom description |
|---|---|
| Symptom-0 (0) | No symptom |
| Symptom-1 (+) | Mild chlorosis and little deformation in new leaves, systemic leaf has little crumpling on surface |
| Symptom-2 (++) | Curling of leaf, chlorosis and/or mosaic |
| Symptom-3 (+++) | Curling of leaf, chlorosis, vein clearing and/ or yellowing, growth of plant is reduced |
| Symptom-4 (++++) | Curling of the newly emerging leaves and shoot apex growth affected, new systemic leaves are arising curled and/ rolled |
| Symptom-5 (+++++) | Severe mosaic, curling/ crumpling of whole of the shoot apex, growth stunted, no new leaf |

## 3.5 Collection of agroinoculated leaf samples, viral DNA detection and viral DNA accumulation study

### 3.5.1 Collection of sample and DNA isolation

Field samples were collected as described at respective places with the experiments (Chapter 4). From agroinoculated glass house plants samples were collected on different dpi depending on the experiment set ups and either proceeded immediately

for total DNA isolation or stored at -80°C and isolated later, by the methods described above.

### 3.5.2 Southern hybridization

1. Running agarose gel and transfer to membrane: Equal amount (depending upon the experiment and plant samples) of total DNA was loaded on 0.9% agarose gel and fractionated by applying voltage not more than 50V to allow smooth and clear separation of viral DNA bands. The transfer of high molecular weight DNA from agarose gel to nylon membrane was achieved by capillary method as described by Sambrook *et al.* 1989. Depurination is usually practiced during the transfer of high molecular weight DNA. Again the gel was soaked in 200ml of depurination solution (0.25 N HCl) for 15 min with gentle rocking. This acid depurination step causes the partial hydrolysis of the DNA before alkali denaturation and helps in the transfer of DNA. After the depurination step, the gel was washed briefly in deionized water and the DNA in agarose gel was denatured by soaking the gel in 250ml of denaturing solution (0.4 N NaOH, 0.6 M NaCl) for 45 min with gentle agitation. The gel was rinsed in deionized water twice. The gel was bathed in 250ml neutralizing solution (1 M Tris.Cl, pH 7.4, 1.5 M NaCl) for 30 min once with constant agitation. Again the gel was rinsed with water and the gel was bathed in neutralizing solution for 10 min. While the gel was in neutralizing solution, a glass plate was placed over a 10X SSC buffer containing tank/deep tray and adequate volume of 10X SSC [(20X SSC (3 M NaCl, 0.3 M NaCitrate, pH 7.0)] was added. A filter paper (larger than size of the gel, should cover maximum area of glass plate) was placed in such a way so that it two sides were completely dipped in tank buffer and above then filter paper was saturated with 10 X SSC. After neutralization, the gel was placed on the top of the filter paper in an inverted position so that the under side was now uppermost and also no air bubbles remained trapped under the gel. Parafilm was kept over areas of filter paper not covered by gel to avoid direct contact of filter paper and membrane. Pre-soaked nylon membrane of the same size of the gel was layered on the gel so that no air bubble remained trapped under the membrane. 2-3 pieces of pre-soaked 3 mm whatmann filter papers were kept over the membrane. Blotting papers (paper towels) were placed on the filter paper and with a flat plate load on the top. The DNA was kept for transfer, overnight (Sambrook *et al.*, 1989).

Next day, the blot was carefully removed with the gel and the marker positions were carefully marked. It was then UV cross-linked at 15Kcalories for 1min. The membrane was pressing between two 3 mm whatmann filters, wrapped properly and stored.

2. Probe labelling: Random probe labelling method was used (Feinberg & Vogelstein, 1983), reaction mixture was prepared as follows; Random hexamer primer- 0.5µl(0.2µg/µl), DNA template- 4µl(30ng), sterile distilled water- 17µl, were mixed in a 1.5ml eppendorf tube and boiled at 100°C for 5min, snap-chilled on ice for 3-4min, now added Klenow buffer- 3µl(10X), Klenow fragment- 1µl(5U/µl), α-[$^{32}$P]- 3µl, and the reaction was incubated at 37°C for 1hrs. After the random labelling step was completed, free nucleotides were removed from labelled DNA by nucleotide removal kit (Qiagen, Germany) following manufacturer's instruction. The labelled DNA was denatured by boiling at 100°C for 10 min and snap-chilled on ice for 5 min, and then added in the running pre hybridizaton solution and allowed to hybridize.

3. Pre hybridization and hybridization of DNA bound membrane: Prehybridization was done at 65°C for 4-6hrs using Pre-hybridization buffer in a hybridizing oven. The blots were hybridized with denatured α-[$^{32}$P]-dCTP-labelled DNA probe at $10^6$cpm/ml, by adding it in the Pre-hybridization buffer. Hybridization was continued for 12-16 hours at 65°C.

4. Washing of blots: Membranes were washed sequentially as follows: 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; 0.2X SSC, 0.1% SDS for 10 min each at 65° C until the non-specific counts had substantially reduced.

5. Scanning and Phosphomoimager analysis: Membranes were air-dried and exposed to imaging plate. The image was read by Phosphoimager (Fuji film FLA-5000, Japan) using Image Quant software. The membranes were also developed by autoradiography where they were exposed to an X-ray film and kept at $-80^0$C. The X-ray film was developed after the required exposure time.

## 3.6 Sequencing and computational analysis

### 3.6.1 ORF and their amino acid sequence analysis

DNA sequencing of full-length DNA-A and betasatellite clones were carried out using automated DNA sequencer commercially using primers specific to cloning vectors and viral sequences. The primary sequence results were checked with NCBI VecScreen for removing vector sequence from sequence. The sequence results were verified for the presence of all begomovirus specific ORFs (using NCBI ORFfinder) and conserved nonanucletide sequence. For analysing the sequence result, NCBI (www.ncbi.nlm.nih.gov) blast search was followed by sequence analysis on EBI (www.ebi.ac.uk); percentage identity/similarity analysis for the sequence with species that showed maximum identity in blast search. Amino acid sequence of all ORFs and CR region of different species were aligned using clustalW on EBI, to study the conserved regions and changes between them.

### 3.6.2 Phylogenetic relationship study

The full-length genome of all begomovirus species selected were aligned using ClustalW (www.ebi.ac.uk), followed by dendrogram generated through Maximum Composite Likelihood (MCL) method for estimating evolutionary distances between all pairs of sequences simultaneously, using Mega program version 4.0 ((http://www.megasoftware.net/features.html, Tamura *et al.*, 2007). Genetic distances were calculated with the Kimura-2 parameter model (Kimura, 1980) with a transition/transversion ratio of 2.0, and the reliability of the trees was determined by bootstrap analysis with 1,000 pseudoreplicate data sets. All the ORFs nucleotide sequence, amino acid sequence, intergenic region of DNA-A molecules were compared with that of other ToLCV species from India as well as outside India and other begomovirus species infecting different crops. Similarly βC1 ORF and complete nucleotide sequence of DNA-β were compared with satellitebeta species associated with ToLCV and other begomoviruses species.

### 3.6.3 Recombination and bootscan analysis

To unmask probability of natural recombination for the origin of DNA A and DNA β molecules associated with Tomato leaf curl disease in India and to identify recombinant sequences and recombination breakpoints, all the viral sequences (cloned in this study and related sequence available at Gene Bank) were analysed. This study was accomplished using boot scanning and similarity plot analysis programs of Simplot program version 3.2

(http://sray.med.som.jhmi.edu/SCRoftware/Simplot), with a sliding window of 200 nucleotides moving in 20-nucleotide steps. (Lole *et al.*, 1999; Bouslama, *et al.*, 2007; Blawid, *et al.*, 2008). Recombination analysis was also repeated with the recombination detection program (Martin and Rybicki, 2000) version RDP3beta27. Defaults RDP settings were used throughout with a P-value cut-off= 0.05 and the standard Bonferroni correction. In general recombination detection sequences were considered circular, consensus daughters were found and sequences were polished. In case where considered parental sequences were having more sequence identity in themselves than the daughter sequence, close relative scan was carried out during manual bootscan in RDP. For bootscan analysis 200b replicates with 95% cut-off percentage was taken and for GENECONV analysis g-scale parameter was set to 1. After X-over analysis under RDP, each and every detected recombination event was checked with other programs separately with P-value cut-off= 0.05, in each case. For RDP both internal reference and external reference options were checked alternately and for small dataset only internal reference was considered with 70%-100% sequence identity.