MATERIALS AND METHODS

3.1 COLLECTION OF PLANT SAMPLES
Surveys were conducted in various parts of Kangra district, Himachal Pradesh, India from 2006-2009 and leaf samples of economically important crops and some weeds showing begomovirus-like symptoms were collected. Kangra district is situated in Western Himalayas between 31°2 to 32°5 N and 75° to 77°45 E, having varying altitude ranging from 427 to 6401m above mean sea level (amsl) and a sub-temperate climate in most of its regions (Imperial Gazetteer of India, v. 14, p. 380). Leaf curling, yellowing and mosaic symptoms typical of begomovirus infection were observed in *Lycopersicon esculentum* (tomato), *Capsicum annuum* (chilli), *Solanum tuberosum* (potato), *Phaseolus vulgaris* (common beans), *Zinnia elegans*, *Crassocephalum crepidioides* and *Ageratum conyzoides*, growing in green houses and open fields. The collected samples were stored at -80°C until use.

3.2 DETECTION OF BEGOMOVIRUSES BASED ON HYBRIDIZATION

3.2.1 Preparation of slot-blot
Samples were prepared for slot-blot by crushing them in TNE buffer. Crushed samples were diluted to 200µl (100µl water + 100µl crushed sample) and denatured in a boiling water bath for 5 min before loading. The slot-blot manifold (Amersham Biosciences, USA) was assembled with BrightStar® positively charged nylon membrane (Ambion, USA), previously wetted with sterile water. The manifold was connected to vacuum pump. To each well, 10x SSC buffer (200µl) was added and vacuum was applied till the buffer was completely absorbed but not dried. After that, 200µl each of the samples were loaded to the wells after their denaturation at 65°C for five min. Vacuum was applied till the samples were absorbed completely. Afterwards, 200µl of 10x SSC was added and allowed to completely pass through. Vacuum was released, apparatus was disassembled and the membrane was rinsed in 2x SSC. The membrane was air dried and exposed to UV light for 2 min in a UV crosslinker (Amersham Biosciences, USA) for binding of the transferred nucleic acids. The membrane was stored at 4°C until hybridization.
3.2.2 Probe preparation

Partial CP gene of ToLCNDV cloned in pGEM®-T Easy vector was digested with EcoRI restriction enzyme and electrophoresed in 1% agarose gel. Viral DNA fragment was excised from the gel, purified and then incubated in boiling water bath for 10 min, for probe preparation. The following reaction was used:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured DNA</td>
<td>200-500 ng</td>
</tr>
<tr>
<td>Random Primer</td>
<td>100 ng</td>
</tr>
<tr>
<td>10x Klenow buffer</td>
<td>3 µl</td>
</tr>
<tr>
<td>dNTP mix (-CTP) (3.3nM each)</td>
<td>4.5 µl</td>
</tr>
<tr>
<td>α-32P dCTP (10µCi/µl, specific activity 3x10³ Ci/mmole)</td>
<td>10 µCi</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>5 units</td>
</tr>
<tr>
<td>Final volume with ddH₂O</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 37°C for one hour. To this, equal volume of Buffer-A was added. The mixture was then denatured by incubating the tube in boiling water bath for 7 min. The contents were immediately transferred to ice before adding to the hybridization bottle.

dNTP mix: (for α32-P dCTP as the radioactive molecule, 100mM stock of dATP, dTTP and dGTP): 1+1+1+27µl water. 4.5µl of this mix was used for one reaction.

20x SSC buffer: 3M NaCl and 0.3 M Trisodium citrate

Buffer-A: 500mM Tris HCL (pH 7.5), 500mM NaCl, 5mM EDTA and 0.5% SDS.

TNE Buffer: Tris HCl- 20mM, NaCl- 10mM and EDTA- 10mM

3.2.3 Pre-hybridization/Hybridization

Pre-hybridization was carried out for 1 hr at 42°C in the pre-hybridization buffer. Pre-hybridization buffer consists of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>50% (v/v)</td>
</tr>
<tr>
<td>Na₂HPO₄ pH 7.2</td>
<td>120 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>250 mM</td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td>1 mM</td>
</tr>
<tr>
<td>SDS (w/v)</td>
<td>7%</td>
</tr>
</tbody>
</table>
Materials and Methods

After pre-hybridization, probe was added to fresh pre-hybridization solution and incubated at 42°C overnight (10-18 hrs).

3.2.4 Washings of Blots

After hybridization was complete the blots were removed from the hybridization solution, rinsed briefly in 2x SSC and placed in the first washing buffer followed by 2nd and third wash with progressive stringency as follows:

First Wash Buffer 2.0x SSC, 0.1% SDS (25°C) - two washings of 15 min each
Second wash buffer 0.5x SSC, 0.1% SDS (25°C) - two washings of 15 min each
Third Wash buffer 0.1x SSC 0.1% SDS (65°C) - two washings of 30 min each

After washing, the blots were placed on a Whatman filter paper to remove excess of liquid and wrapped in a saran wrap immediately, to prevent it from drying and for the purpose of autoradiography.

3.2.5 Autoradiography

The blots were exposed to X-ray films (X-OMAT, XK-5, and Kodak, India) by putting them between two intensifying screens (Kiran Hi-speed, India) for the autoradiography in a cassette at -80°C. The films were developed after sufficient exposure, depending on the intensity of the signal prior to autoradiography as determined by a portable radioactivity monitor.

3.3 DETECTION OF BEGOMOVIRUSES BASED ON PCR

Collected plant samples were also analyzed by PCR based diagnostics. First step in PCR diagnosis is extraction of total DNA from plant samples.

3.3.1 Extraction of total DNA

3.3.1.1 CTAB method

A slightly modified CTAB DNA isolation protocol (Permingeat et al., 1998) was followed (http://www.uni-stuttgart.de/bio/bioinst/molbio/RCA/protocols.html). CTAB is a cationic
detergent that binds to nucleic acids, denatures proteins and separates nucleic acids from proteins. Rapid freezing of the material using liquid nitrogen (N₂) stops oxidation and makes the tissue brittle. Incubation of disrupted samples at 60°C inactivates most cellular enzymes immediately. Tris prevents precipitation by acids and EDTA catches Mg²⁺ and other cations required for nuclease activity. NaCl prevents the precipitation of CTAB nucleic acid complexes. Dithiotreitol (DTT) is a reducing agent that prevents oxidation of phenolic compounds. Extractions with chloroform remove proteins and lipids, and isoamyl alcohol is added to improve phase separation. Nucleic acids remain in the aqueous phase which can be precipitated by isopropanol. Washing with 70% alcohol reduces the salt content in the pellet. The standardized protocol is as follows:

(i) The plant sample (50-100 mg) was ground in liquid N₂ in a pestle and mortar and the powder was taken in a 1.5 ml sterile tube

(ii) To the same tube, 500µl of CTAB buffer was added and the mixture was shaken for 1 h at 60°C

(iii) After that, 500µl of CI solution was added and shook for 5 min at room temperature

(iv) The mixture was spun down at maximum speed (14000 rpm) for 5 min at 4°C

(v) The aqueous phase was collected and transferred to a new tube

(vi) To the same tube, 0.8 volume of isopropanol was added

(vii) The reaction tube was inverted three-four times for precipitation

(viii) The pellet was spun down at maximum speed for 10 min at 4°C

(ix) The pellet was washed with 70% ethanol (EtOH)

(x) The mixture was spun down at maximum speed for 5 min at room temperature

(xi) EtOH was evaporated for 10 min at room temperature and the pellet was dissolved in 100µl of double distilled H₂O (ddH₂O) (stored at -20°C).

Notes: Interphase material should not come out with the aqueous phase.

Removed EtOH, but retained the water for easier solubility

**CTAB buffer:** 100mM Tris-HCl, pH 8.0, 20mM EDTA, 1.4M NaCl, 2% CTAB, 0.5M glucose, 100mM DTT

**CI solution:** Chloroform:isoamyl alcohol (24:1)
3.3.1.2 **Dellaporta’s method**

When total DNA was required in large amounts, extraction was performed by the method described by Dellaporta *et al.* (1983) as follows:

(i) Plant tissue (upto 5 g) was frozen in liquid N₂ and ground to a fine powder in a mortar/pestle.

(ii) The tissue powder was transferred to a centrifuge tube containing 50 ml extraction buffer supplemented with 500µl of β-mercaptoethanol. The tissue was suspended thoroughly by slow swirling.

(iii) This was followed by addition of 3.3 ml of 20% SDS, mixed properly and incubated at 65°C for 15 min.

(iv) The contents were cooled by incubating on ice for 15-20 min, followed by addition of 17ml of 5M potassium acetate, mixed thoroughly and incubated on ice for 20 min.

(v) The debris was pelleted down by centrifugation at 10,000 rpm for 20 min at 4°C and the supernatant was carefully decanted into a fresh tube avoiding any debris.

(vi) To the supernatant 33 ml of isopropanol was added, mixed and incubated at room temperature for 30 min for precipitation of nucleic acids.

(vii) The nucleic acids were pelleted down by centrifugation at 10,000 rpm for 20 min at room temperature.

(viii) Pellet of nucleic acids was dried by inverting the tube over paper towels and resuspended in TE₅₀.

(ix) The suspended nucleic acids were successively extracted with equal volume of phenol (tris-saturated), phenol-chloroform and chloroform:isoamyl alcohol (24:1 mixture).

(x) After centrifugation at 10,000 rpm at room temperature for 10 min, the supernatant was collected, supplemented with 1/10th volume of 3M sodium acetate (pH 4.8) and precipitated by 2.5 volumes of 100% alcohol.

(xi) After incubation at -20°C overnight, the precipitated nucleic acids were recovered by centrifugation at 12,500 rpm for 20 min at 4°C.
**Extraction Buffer:** 100mM Tris HCl (pH 8), 50mM EDTA (pH 8) and 500mM NaCl. 1ml of β-mercaptoethanol was added per 100 ml of extraction buffer was added after autoclaving

**TE₅₀:** 50mM Tris HCl (pH 8) and 1mM EDTA (pH 8)

3.3.1.3 **RNaseA treatment of total nucleic acids**

The total nucleic acids were incubated with DNase and protease-free RNaseA (Fermentas, Maryland, USA) at 37°C for one hour as described below:

<table>
<thead>
<tr>
<th>Total nucleic acids</th>
<th>10µl (2-3µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNaseA (10 mg/ml stock)</td>
<td>0.5µl</td>
</tr>
</tbody>
</table>

After RNaseA treatment the nucleic acids were electrophoresed in 0.7% agarose gel after mixing with loading dye for analyzing their integrity and quality. Quantification of nucleic acids was performed by using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA)

3.3.1.4 **Total DNA isolation using a commercial kit**

For quick isolation of total DNA, AuPreP™ DNA easy Plant Mini Kit (Life Technologies, Delhi, India) was used as follows:

(i) The plant sample (100 mg) was ground in liquid N₂ to make fine powder and quickly transferred to a sterile 1.5 ml or 2 ml eppendorf tube.

(ii) To the tissue powder, 400 µl of PX1 Buffer and 4 µl of RNaseA stock solution (100 mg/ml) were added and vortexed vigorously. The mixture was incubated at 65°C for 10 min and mixed by inverting every 2 min during incubation.

(iii) To the lysate, 130 µl PX2 Buffer was added, and the mixture was vortexed and incubated on ice for 5 min.

(iv) A Shearing tube was placed onto a Collection tube and the lysate was added to it. It was centrifuged for 2 min at 14000 rpm. The flow-through lysate was transferred from the Collection tube to a new sterile tube.
(v) Volume of the flow-through lysate was determined and 0.5 volume of PX3 Buffer was added and mixed by pipetting. One volume of 98-100% ethanol was also added to the mixture and mixed by pipetting.

(vi) A DNA easy Plant Mini column was placed onto a Collection tube and 650 µl of the ethanol-added sample (including any precipitate) from the previous step was added to the column. It was centrifuged at 10,000 rpm for 1 min and the flow-through was discarded. This step was repeated for rest of the sample.

(vii) The column was washed twice with 0.7 ml WS Buffer by centrifuging for 30-60 sec and the flow-through was discarded.

(viii) It was centrifuged for another 2 min to remove any ethanol residue.

(ix) The column was transferred onto a new 1.5-ml tube. The collection tube and flow-through were discarded.

(x) To the same column, 100 µl of ddH₂O or TE buffer (preheated at 65°C) was added and kept for 5 min at room temperature. DNA was eluted by centrifuging for 2 min at 14000 rpm.

(xi) Eluted DNA was stored at 4°C for frequent use or -20°C for long-term storage.

### 3.3.2 PCR Amplification

For detection and characterization of begomovirus DNA components from the samples, PCR was carried out in a GeneAmp® PCR system 9700 (Applied Biosystems, California, USA) using degenerate primers for DNA-A, DNA-B and abutting primer pair for betasatellite (Table 3.1). The pattern of thermal cycling for each primer pair is also summarized (Table 3.2). A reaction mixture of 50µl consisted of:

- **Total DNA**: 5µl (200-500 ng)
- **10x Taq buffer A**: 5µl
- **10mM dNTP mix**: 2µl
- **Forward primer**: 1µl (200 ng)
- **Reverse primer**: 1µl (200 ng)
- **Taq DNA polymerase (Genei, Bangalore, India)**: 0.5µl (3 units/µl)
- **Added ddH₂O to final volume**: 50µl

**10x Taq buffer A**: 100 mM Tris (pH9.0), 500 mM KCl, 15 mM MgCl₂, 0.1% Gelatin
Materials and Methods

Table 3.1 Primers used for detection of different genomic components of begomoviruses

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-A</td>
<td>5'GCATCTGCAGGCCACATYGTCTTTCCNGT 3'</td>
<td>~1.3 kb</td>
<td>Rojas et al. (1993)</td>
</tr>
<tr>
<td>PAR1c496</td>
<td>5' AATACTGAGGGCTTTYCTRACATRGG 3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-A</td>
<td>5' GCCYATRTAYAGRAAGCCMAG 3'</td>
<td></td>
<td>Wyatt and Brown (1996)</td>
</tr>
<tr>
<td>AC1048</td>
<td>5' GGRRTDGARGCATGHGTACATG 3'</td>
<td>~0.57 kb</td>
<td></td>
</tr>
<tr>
<td>DNA-B</td>
<td>5' GCCYTTRAYAGRAAGCCMAG 3'</td>
<td>~0.57 kb</td>
<td></td>
</tr>
<tr>
<td>PBL1v2040</td>
<td>5' CTAGCTGCAGCATATTTACRWARWATGCCA 3'</td>
<td>~0.6 kb</td>
<td>Rojas et al. (1993)</td>
</tr>
<tr>
<td>PCrC1</td>
<td>5' GGTACCCCTCCAGGGGTACAC 3'</td>
<td>~1.4 kb</td>
<td>Briddon et al. (2002)</td>
</tr>
</tbody>
</table>


Table 3.2 Thermal cycling pattern for different primer pairs used in PCR

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Thermal cycling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL1v1978</td>
<td>Initial denaturation at 94°C for 3 min followed by 5 cycles consisting of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>PAR1c496</td>
<td></td>
</tr>
<tr>
<td>AV494</td>
<td>Initial denaturation at 94°C for 3 min followed by 35 cycles consisting of 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>AC1048</td>
<td></td>
</tr>
<tr>
<td>PBL1v2040</td>
<td>Initial denaturation at 94°C for 3 min followed by 5 cycles consisting of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>PCrC1</td>
<td></td>
</tr>
<tr>
<td>Beta01</td>
<td>Initial denaturation at 94°C for 3 min followed by 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min 30 sec and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>Beta02</td>
<td></td>
</tr>
</tbody>
</table>
3.3.3 Agarose gel electrophoresis

Gel electrophoresis was carried out using a submarine horizontal agarose slab gel as described by Sambrook et al. (1989). Appropriate amount of agarose (0.8-2.0%, as per requirement) was boiled in 1x TAE or 0.5x TBE buffer to dissolve completely. The mixture was cooled to 50°C and poured into an appropriate gel casting tray using a slot-forming comb. After solidification of the agarose, the comb was removed and the gel was placed in the gel tank for electrophoresis. PCR products (10µl) were properly mixed with 3µl of gel loading dye and loaded onto the wells of the gel. DNA ladder was also loaded to compare the size of the PCR product. Electrophoresis was carried out at a constant voltage of 5 V/cm. After electrophoresis, the gel was stained with ethidium bromide (EtBr; 1.0µg/ml) and visualized using a UV trans-illuminator and photographed using gel documentation system (Alpha Innotech Corp., CA, USA).

Sample loading dye (6X): 15% Ficoll 400; 0.25% bromophenol blue and xylene cyanol and stored at room temperature.

TBE (10X per liter): 108 g Tris base; 55 g boric acid and 9.3 g EDTA.

TAE (50X per liter): 242 g Tris base; 57.1 ml glacial acetic acid and 100 ml EDTA (0.5 M, pH 8.0).

3.4 MOLECULAR CHARACTERIZATION OF BEGOMOVIRUSES BASED ON PCR

For molecular characterization of begomoviruses, PCR amplified DNA was purified from 1% agarose gel using either GenElute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) or AuPrep™ GELX Gel Extraction Kit (Life Technologies) as per the manufacturer’s instructions and ligated to suitable vector.

3.4.1 Ligation of PCR amplicons to suitable vector

Either T-tailed pBluescriptII KS+ vector (Stratagene, La Jolla, CA, USA) or pGEM®-T easy vector (Promega, Madison, WI, USA) were used for ligation of PCR amplicons. Procedure of T-tailing was adapted from Marchuk et al. (1991) with following steps:

(i) pBluescriptII KS+ vector (10 µg) was digested with blunt end restriction enzyme SmaI (Fermentas) in a 50µl reaction mixture containing 5µl of 10x Tango™ buffer
(Fermentas) and 1 µl (10 units) of the enzyme. The mixture was then incubated at 30°C for 3-4 h.

(ii) Reaction mixture was heated to 65°C for 20 min to inactivate the enzyme followed by addition of 150 µl of sterile water to make the final volume up to 200 µl.

(iii) Extracted the reaction successively with equal volumes of phenol, phenol: chloroform and chloroform. After each extraction, aqueous phase was transferred to a new tube.

(iv) Finally 20 µl of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added to precipitate the digested plasmid DNA followed by incubation at -70°C for 30 min.

(v) Digested DNA was pelleted down by centrifugation at 14,000 rpm for 20 min, washed with chilled 75% ethanol and dried under vacuum in Savant SpeedVac Concentrator (Thermo Scientific) at room temperature.

(vi) The digested plasmid DNA was resuspended in 63 µl water making the final concentration ~130 ng/µl.

(vii) To the suspended DNA, 10 µl of 10x Taq DNA polymerase buffer (without MgCl₂), 20 µl of 10 mM dTTP, 6.0 µl of 25 mM MgCl₂ and 5 units of Taq DNA polymerase (Genei, Bangalore, India) were added. Incubated the reaction mixture for 3 h at 70°C.

(viii) The reaction mixture was extracted successively with equal volumes of phenol, phenol: chloroform and finally with chloroform. After each extraction, aqueous phase was transferred to a fresh tube.

(ix) Assuming ~75 µl recovery from the reaction, 75 µl of 2 M ammonium acetate and 150 µl isopropanol were added.

(x) Centrifuged at 14,000 rpm for 20 min at 4°C to pellet down the precipitated plasmid DNA. The pellet was washed twice with 75% ethanol, air dried, suspended in 30 µl of ddH₂O water and stored in aliquots of 5 µl each at -20°C.

Vector and insert DNAs were mixed in an appropriate ratio (usually equimolar), and ligation reaction was set up as described in table 3.3.
Table 3.3 Components of ligation reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>For pGEM®-T vector</th>
<th>For constructed vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (vector + insert)</td>
<td>4.0 µl</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase Buffer</td>
<td>5.0 µl (from 2x stock)</td>
<td>1.0 µl (from 10x stock)</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1.0 µl (3 Weiss units)</td>
<td>1.0 µl (5 Weiss units)</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>-</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>10.0 µl</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

The reaction was carried out either at 22°C for 1 h or 16°C (or 4°C) overnight. In case of constructed vector, T4 DNA ligase from Fermentas was used, whereas it is supplied as a kit component in pGEM®-T vector system.

3.4.2 Preparation of chemically competent *Escherichia coli*

It is difficult for normal *E. coli* cells to take up plasmid DNA from the medium. If they are treated with CaCl₂, the transformation efficiency can be significantly enhanced. Mainly DH5α or JM107 strain of *E. coli* was used for transformation experiments. Appropriate strain of the bacteria was revived from the stored cultures by streaking it on to a Luria Broth (LB) agar plate supplemented with appropriate antibiotics and incubating at 37°C for 12-16 h. A single colony from this plate was picked and cultured overnight in 5 ml of liquid LB medium at 37°C. A part of this culture was used to inoculate larger volumes of the medium for preparation of competent cells by either of the two methods described below.

3.4.2.1 Calcium method for a quicker preparation

LB medium (50 ml) was inoculated with 50µl of overnight grown *E. coli* culture and allowed to grow at 37°C on a rotary shaker till an OD around 0.5 was obtained at 600 nm. Culture was cooled on ice and bacterial cells were pelleted down by centrifugation at 5000 rpm for 5 min at 4°C. Supernatant was completely removed. The pellet was resuspended in 10 ml ice-cold 0.1 M CaCl₂ and incubated on ice for 15 min. Cells were recovered by centrifugation as above. Cell pellet was resuspended in 2 ml of ice-cold 0.1 M CaCl₂ and
aliquots of 200µl each were prepared to be used for a single transformation reaction. This is a quick method but the cells can only be stored for 12-16 h on ice.

3.4.2.2 Inoue’s method for a higher efficiency

The method was adapted from Inoue et al. (1990) and modified slightly. Competent cells prepared by this method are highly efficient and can be stored at -70°C. Overnight grown *E. coli* culture (50µl) was inoculated into 200 ml SOB medium and grown at 18°C on a rotary shaker till an OD around 0.5 was obtained at 600 nm. Cells were split into four centrifuge tubes (50 ml each) and placed on ice for 10 min. Cells were pelleted down by centrifuging at 2500 g for 10 min and supernatant was discarded. Cells were resuspended in 64 ml HTB buffer (16 ml in each tube) and kept on ice for another 10 min. Cells were pelleted again, resuspended in 16 ml (4 ml in each tube) HTB buffer and pooled into one tube. 3 ml of dimethyl sulfoxide (DMSO) was added slowly with gentle swirling. Aliquots of 100µl were made in 1.5 ml tubes, immediately frozen in liquid N₂ and stored at -80°C until use.

**SOB:** 20g tryptone, 5g yeast extract and 0.5g NaCl were dissolved in 900 ml water, added 10 ml 250mM KCl, adjust pH to 7.0 with NaOH, autoclaved and added 5 ml 2M MgCl₂.

**HTB Buffer:** 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ), 15 mM CaCl₂, 250 mM KCl, adjusted pH to 6.7 with KOH, added 55mM MnCl₂, filter sterilized and stored at 4°C.

3.4.3 Transformation of competent *E. coli* cells

Either frozen competent cells were thawed on ice for 10-15 min or 200µl of freshly prepared cells were taken for transformation. Ligated product was mixed with competent cells and incubated on ice for 30 min. The cells were given heat shock at 42°C for 90 sec and immediately transferred to ice for at least 5 min. To this, 800µl of LB (without any antibiotic) was added and the tube was incubated at 37°C for 1 h in a rotary shaker set at 200 rpm. The cells were pelleted down and resuspended in 100-250µl of LB. Transformed cells were plated on LB agar plate, supplemented with appropriate antibiotics. For selection of the recombinant plasmids by blue-white selection, IPTG and X-gal were used in appropriate amounts. Cells were spread uniformly with autoclaved glass beads or sterilized
glass spreader and the plates were incubated at 37°C for 12-16 h till the bacterial colonies grew big enough to transfer. Colonies that turned blue were left out while the colonies that remained white (recombinant clones) were transferred on to a fresh plate containing appropriate antibiotics.

**X-gal/IPTG:** For each plate containing 20-25 ml media, 40µl of X-gal (20 mg/ml stock in dimethyl formamide) and 10µl of IPTG (20 mg/ml stock in ddH₂O) were plated before cell plating.

**Concentration of the antibiotics:** Ampicillin-100 mg/ml dissolved in water; tetracycline-12.5 mg/ml dissolved in 50% aqueous alcohol.

### 3.4.4 Selection of recombinant clones

For confirmation of recombinant clones, plasmid DNA was isolated from the white colonies of the transformation experiment by either of the two methods described below. Isolated plasmid DNAs were digested with those restriction enzymes that have their sites at the flanking ends of the insert (in order to release the insert from the vector). Digested products were electrophoresed and analyzed in 1% agarose gel. Positive clones were selected for DNA sequencing.

#### 3.4.4.1 Alkaline lysis method of plasmid isolation

Alkaline lysis method (Birnboim and Doly, 1979) of plasmid isolation was adapted from Sambrook *et al.* (1989). A single bacterial colony was inoculated into 5ml LB medium (with appropriate antibiotics) and grown overnight at 37°C on a rotary shaker. From this overnight grown culture, 1.5 ml was pelleted down by centrifugation at 12,000 rpm in a microfuge for 1 min. Supernatant was discarded completely by using a micropipette. The pellet was resuspended in 100µl GTE and kept on ice. Two volumes (200µl) of alkaline SDS (freshly prepared) was added to the resuspended cells and mixed gently (avoiding vortexing) followed by incubation on ice for 5 min for lysis. To these lysed cells, 150µl of ice cold 3 M potassium acetate (pH 4.8) was added, mixed immediately and incubated on ice for 15 min. The tube was then centrifuged at 14,000 rpm in a microfuge for 10 min. The supernatant was transferred to a fresh tube and extracted with one volume of phenol and centrifuged at 14,000 rpm in a microfuge for 5 min. The DNA was then extracted with
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phenol: chloroform (1:1 v/v) and centrifuged as above for 5 min. Finally the DNA was extracted with 1 volume of chloroform: isoamyl alcohol (24:1 v/v) and centrifuged for 5 min. The DNA was finally precipitated with 2.5 volumes of ice cold 100% ethanol, incubated at -20°C for 1 h and centrifuged in a microfuge (12,500 rpm for 20 min at 4°C) to precipitate the DNA. Finally DNA pellet was washed with cold 80% ethanol, air dried and resuspended in 50-100µl of ddH₂O.

**GTE:** 50 mM glucose; 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA, pH 8.0.

**Alkaline SDS:** 0.2 N NaOH and 1% SDS (both mixed freshly from 10 N NaOH and 20% SDS stock, respectively).

**5 M Potassium acetate:** 49 g potassium acetate per 100 ml solution.

**3 M Potassium acetate, pH 4.8 (for 100 ml):** 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml sterile water.

### 3.4.4.2 Boiling miniprep method of plasmid isolation

The method by Holmes and Quingley, (1981) was adapted from Sambrook *et al.* (1989). Overnight grown culture (1.5 ml) was pelleted down in a microfuge by centrifugation at 12,000 rpm for 1 min. The supernatant was discarded and the pelleted cells were resuspended in 110µl of STET buffer. To the resuspended cells, freshly prepared lysozyme (in 10mM Tris HCl, pH 8.0) was added to a final concentration of 0.5 mg/ml. The tubes were kept at room temperature for 5 min for lysis of the cells followed by incubation in boiling water bath for 30-40 sec. The suspension was centrifuged at full speed (14,000 rpm) in a microfuge for 20 min. Pellet containing the cell debris was removed with the help of a sterile toothpick. DNA was precipitated by adding one volume of iso-propanol and mixing thoroughly. Precipitated DNA was collected immediately by centrifugation for 15 min at full speed (14,000 rpm). The supernatant was removed completely and the DNA pellet was air dried to remove traces of isopropanol. Pellet was dissolved in 50-100µl of ddH₂O.

**STET Buffer:** 8% sucrose; 0.5% Triton X-100; 50 mM Tris HCl (pH 8) and 50 mM EDTA (pH 8). Triton X-100 was added after autoclaving rest of the components in solution.
3.4.5 Purification of plasmid DNA for sequencing
Sequencing requires ultra clean DNA and therefore either the plasmid DNA prepared by alkaline lysis or boiling miniprep methods was further purified by precipitation with polyethylene glycol (PEG) 8000 or it was re-extracted using commercially available plasmid isolation kits.

3.4.5.1 Purification of plasmid DNA by PEG
The plasmid DNA from alkaline lysis or boiling miniprep methods was treated with RNaseA (as described before) and extracted twice with one volume of chloroform to remove RNase bound to DNA. To this DNA, 0.1 volume of 3 M sodium acetate (pH 4.8) and one volume of isopropanol was added followed by centrifugation at 14,000 rpm in a microfuge for 10 min. The pellet containing the DNA was washed with 80% alcohol, air dried and resuspended in 32µl of sterile water. To this DNA, 8µl of 4 M sodium chloride and 40µl of 13% PEG 8000 were added and incubated on ice for 20 min. The DNA was then pelleted down by centrifugation at 12,500 rpm in a microfuge for 20 min at 4°C. Pellet was washed with 80% alcohol twice, air dried and dissolved in 50µl sterile water.

3.4.5.2 Plasmid isolation using commercial kits
Plasmid was also isolated using either GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) or HiYield™ Plasmid Mini Kit (RBC, Taipei County, Taiwan) as per the manufacturer’s instructions.

3.4.6 Automated DNA sequencing
Sequencing of the PCR amplified (agarose gel purified) or cloned viral DNA was carried out by dideoxy chain termination method (Sanger et al., 1977) using ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems) and Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

3.4.6.1 Sequencing Reaction
Universal primers used for sequencing are listed below (Table 3.4). The sequencing reaction (5µl) was set up in 0.2 ml thin walled PCR tubes as follows:
**Materials and Methods**

<table>
<thead>
<tr>
<th>Material/Component</th>
<th>Volume/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR amplified or cloned plasmid DNA</td>
<td>2.5μl (200-500 ng)</td>
</tr>
<tr>
<td>5x Sequencing Buffer</td>
<td>1.0μl</td>
</tr>
<tr>
<td>Sequencing primer</td>
<td>0.5μl (2 pmol)</td>
</tr>
<tr>
<td>BigDye® Terminator v3.1 Ready Reaction Mix</td>
<td>1.0μl</td>
</tr>
</tbody>
</table>

**Table 3.4** Universal primers used for sequencing

<table>
<thead>
<tr>
<th>Vector</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescriptII KS+</td>
<td>M13 (-20) Forward</td>
<td>5’ GTAAAACGACGGCCAGT 3’</td>
</tr>
<tr>
<td></td>
<td>M13 (-26) Reverse</td>
<td>5’ CAGGAAACAGCTATGAC 3’</td>
</tr>
<tr>
<td>pGEM®-T Easy</td>
<td>T7 Forward</td>
<td>5’ TAATACGACTCACTATAGGG 3’</td>
</tr>
<tr>
<td></td>
<td>SP6 Reverse</td>
<td>5’ TATTTAGGTACACTATAG 3’</td>
</tr>
</tbody>
</table>

The PCR tubes containing the reaction mixture were placed in GeneAmp® PCR system 9700 (Applied Biosystems), which was programmed for 25 cycles on following conditions:

- Denaturation at 94°C: 10 sec
- Annealing at 50°C: 20 sec
- Amplification at 60°C: 4 min

### 3.4.6.2 Purification of the reaction mixture

At completion of the reaction, the mixture was purified either manually or using Montage SEQ<sub>96</sub> Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) as per manufacture’s instructions. In manual cleanup, the volume of the reaction mixture was raised to 100μl with ddH<sub>2</sub>O. To this, 10μl 3 M sodium acetate (pH 4.6) and 250μl of 100% ethanol were added followed by centrifugation at 14,000 rpm for 20 min. The pellet thus obtained was washed twice with 250μl of 75% ethanol and dried in air. The dried pellet was dissolved in 15μl of template suppression reagent (TSR; Applied Biosystems), denatured by heating for 4 min at 5°C and placed on ice before loading in the sequencer.
3.5 WHOLE GENOME CHARACTERIZATION OF BEGOMOVIRUSES

Whole genomes of the begomoviruses were amplified based on rolling circle replication mechanism using TempliPhi\textsuperscript{TM} DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA).

3.5.1 RCA reaction

In the first step of RCA, the sample is heated for two reasons; to destroy residual enzymes (nucleases, proteases etc.) and to denature dsDNA. During heating, the secondary structures of cccDNA are disrupted to a certain extent, which provides more templates for RCA. Random hexamer primers (supplied with the kit buffer) anneal to every ssDNA upon cooling to room temperature and serve as starter for subsequent polymerization. After addition of the enzyme (Φ-29 DNA polymerase), polymerization proceeds on the different substrates at room temperature. The enzyme displaces nascent strands and new hexamer primers anneal these strands, resulting in a huge network of DNA products. The standardized RCA protocol is described below:

(i) 5µl of TempliPhi Sample Buffer was transferred to an Eppendorf tube.
(ii) Sample DNA (isolated as described before) was transferred to the dispensed TempliPhi Sample Buffer. Amounts of sample DNA varied (20-200 ng) depending on the source of the starting material. However, total volume of the DNA never exceeded 1.5µl.
(iii) The sample was denatured by heating at 95°C for 3min and cooled down to room temperature or 4°C immediately.
(iv) In a separate tube, 5µl of TempliPhi Reaction Buffer was combined with 0.2µl of TempliPhi Enzyme Mix.
(v) 5µl of this mixture was transferred to the cooled, denatured sample (step 3).
(vi) The reaction mixture was incubated at 30°C for 4-18 h in a water bath.
(vii) The enzyme (Φ-29 DNA polymerase) was inactivated by heating the reaction mixture at 65°C for 10 min. The mixture was cooled down to 4°C.
3.5.2 RFLP of RCA amplified viral DNA components

Restriction fragment length polymorphisms are caused by variations in the DNA sequence. Its analysis involves fragmentation of genomic DNA by a restriction enzyme and gel electrophoresis for separation of the cleaved DNAs according to their length. Class II restriction enzymes which recognize four or six nucleotide-sequences were used for this purpose. Based on permutation theory, it is expected that a four base recognizing and a six base recognizing restriction endonuclease will have a restriction site in every 250 and 4100 bases respectively in a random sequence. As a begomovirus genomic component has 2500-3000 bases the actual frequency of restriction sites will vary considerably from virus to virus. Because a four base recognizing enzyme has higher probability of restriction sites in a begomovirus component, \( HpaII \) (recognizes \( C\ddownarrow CGG \)) was used to generate distinct restriction patterns for different viruses as described by Schubert et al. (2007). This method was quite useful in differentiating distinct viral species in different samples based on their polymorphic restriction patterns in a 2% agarose gel. The method also provided ideas about the genomic nature (monopartite/bipartite) of the virus under study. Restriction reactions were set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCA product</td>
<td>1.5µl</td>
</tr>
<tr>
<td>10x Reaction Buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>0.5µl  (5 Units)</td>
</tr>
<tr>
<td>Final volume with ddH(_2)O</td>
<td>20µl</td>
</tr>
</tbody>
</table>

The reaction mixtures were incubated at 37°C for 3-4 h and the enzyme was inactivated by incubation at 65°C for 10 min before loading directly to the gel.

3.5.3 Cloning RCA amplified complete viral DNA components

For cloning a complete viral DNA component into suitable vector, the primary requirement is identification of a restriction enzyme that has a single cut site in the entire component.

3.5.3.1 Preparation of the viral DNA for cloning

Aliquots (1µl each) of the RCA product were digested with several restriction enzymes (as described above) in separate reactions and electrophoresed in 1.5% agarose gel. Usually
those restriction enzymes were used that have a single cut site in multiple cloning site (MCS) of pBluescriptII KS+ and/or pUC19 vector. The enzyme that produced either ~2.8 kb (indicating complete DNA-A or DNA-B) or ~1.4 kb band (indicating alpha or betasatellite) was selected for cloning. More amount of the RCA product (4µl) was digested with that enzyme and electrophoresed in 1% agarose gel along with a DNA ladder. EtBr was not added directly to this gel because it reduces the cloning efficiency probably because of its property of intercalating into dsDNA. After completion of the electrophoresis, the gel-lane with DNA ladder was cut from the gel along with 1/4th portion of the lane with digested RCA product. The cut portion of the gel was stained with EtBr and assembled back into unstained gel on a UV trans-illuminator. The stained 1/4th portion of the desired band (to be cloned) illuminated under UV and the entire band was cut parallel to it. This DNA was purified from the gel using gel extraction kits (Sigma-Aldrich or Life Technologies) for cloning. This method avoided interference of EtBr and enhanced cloning efficiency.

3.5.3.2 Preparation of the vector DNA for cloning

Either pBluescriptII KS+ or pUC19 vector DNA (1-2µg) was digested with the restriction enzyme selected for cloning followed by enzyme inactivation as per manufacturer’s instructions. The reaction mixture was purified using Wizard® SV Gel and PCR Clean-Up System (Promega). To remove the 5’ phosphate residues from the digested vector fragments, the purified DNA was treated with shrimp alkaline phosphatase (SAP) (USB, Ohio, USA) in a following reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested vector DNA</td>
<td>1 to 1.5µg</td>
</tr>
<tr>
<td>10x SAP reaction buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase</td>
<td>1µl (1 unit)</td>
</tr>
<tr>
<td>Final volume with ddH$_2$O</td>
<td>50µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 30 min followed by incubation at 65°C for 15 min to inactivate SAP. Removal of the 5’ phosphate residues prevents the digested vector to self-ligate and circularize thereby increasing the cloning efficiency many folds.
The SAP treated linearized vector DNA was electrophoresed in 1% agarose gel without EtBR and purified using gel extraction kits (as described above). Gel purification removes traces of undigested DNA (if any) that might produce unwanted colonies in the transformation experiment. Only 50-100 ng of the prepared vector was used in a ligation reaction and the remaining amount was stored at -20°C for future use.

3.5.3.3 Ligation reaction

Ligation reaction of viral and vector DNA was set up in an Eppendorf tube as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>20-50 ng</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>50-150 ng</td>
</tr>
<tr>
<td>10x T4 DNA Ligase Buffer (Fermentas)</td>
<td>2 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase (Fermentas)</td>
<td>1 µl (Weiss units)</td>
</tr>
<tr>
<td>Final volume with ddH₂O</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 16°C overnight in a water bath followed by storage at -20°C until transformation.

3.5.3.4 Transformation into E. coli and selection of recombinant clones

The ligated products were transformed into E. coli and recombinant clones were selected by blue/white screening (as described before). Plasmid DNA was isolated from the white colonies (as described before) and digested usually with PvuII restriction enzyme for confirmation of the recombinant clones. In pBluescriptII KS+ and pUC19 vectors, PvuII has a site at ~200 bp upstream/downstream of each end of the insert. Therefore, a self-ligated vector produces a band at ~400 bp after digestion with PvuII unlike the recombinant clones (in most cases). This strategy was followed because viral genomic components and the vectors being used have approximately similar sizes and are difficult to separate on agarose gels.
3.5.4 Sequencing of viral DNA components

Plasmid of recombinant clones was isolated using either GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) or HiYield™ Plasmid Mini Kit (RBC) as per the manufacturer’s instructions. End sequences of the cloned viral DNA components were obtained by sequencing the recombinant plasmids using vector specific M13 universal primers (as described before). Three clones from the same transformation experiment were sequenced for each component. As complete begomoviral components are ~2.8 kb in size, these could not be completely sequenced using vector specific primers only. Complete sequences were obtained by primer walking strategy either using self-designed specific primers or from 1st BASE Laboratories (Malaysia).

3.6 SEQUENCE ANALYSIS USING BIOINFORMATICS TOOLS

Database searches for obtained sequences were performed using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997) available at NCBI website (http://www.ncbi.nlm.nih.gov/blast). The program BLASTP was used to search the amino acid sequence database. Phylogenetic analysis was performed by neighbor-joining method selecting 1000 bootstrap replicates, using clustalW program (Thompson et al., 1994) version 1.83 available online (http://www.ddbj.nig.ac.jp) and trees were viewed and edited using TreeView software version 1.6.6 (Page, 1996). Multiple sequence alignments were (MSA) performed using MultAlin program available online (Corpet, 1988). MSA were also performed using ClustalW program (Thompson et al., 1994) included in MEGA5 software (Tamura et al., 2007), available at http://www.megasoftware.net. Phylogenetic analysis was inferred using neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) in MEGA5 software. Pairwise sequence alignment scores (percentage identities) were determined using ClustalW program version 1.83 available online (http://www.ddbj.nig.ac.jp). For recombination analysis, RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN and 3SEQ methods were used in the recombination detection program 3 (RDP3; Martin et al., 2010). Default settings were used throughout and the recombination events detected by more than three of the programs were considered significant. Various representative sequences of distinct
begomoviral species and alpha/betasatellites were used for phylogenetic and recombination analysis (described in results section). Online restriction maps of the sequences were constructed using either NEBcutter version 2.0 (Vincze et al., 2003) available at http://tools.neb.com/NEBcutter2/ or webcutter version 2.0 (http://users.unimi.it/~camelot/tools/cut2.html) software. Genome maps were constructed using BVTech Plasmid software (http://www.biovisualtech.com/).

3.7 TRANSMISSION OF BEGOMOVIRUSES
For characterizing genetic properties of the begomoviruses and fulfilling Koch’s postulates, their transmission was tried by the methods described below.

3.7.1 Mechanical transmission

3.7.1.1 Potting medium and test plants
Potting medium was prepared by mixing sand, soil and farmyard manure in 1:1:1 ratio. The mixture was sterilized by autoclaving for 30 min at 121°C and 15 lb per square inch. Seeds of the various test plants were sown in the autoclaved mixture taken in plastic pots. For germination of the seeds, the pots were placed under a transparent polythene sheet (for maintaining high humidity) at 30-35°C in an insect proof glass house. After germination, the seedlings were transferred to fresh pots and raised at a maintained temperature of 25-28°C and 14-16 h lighting conditions. Most of the test plants were inoculated at 4-8 leaf stage.

3.7.1.2 Sap transmission
Young leaves of the infected plants were macerated in inoculation buffer using sterile mortar/pestle and the slurry was squeezed through two folds of muslin cloth. The leaves of test plants were dusted with carborundum rubbed gently and the sap was applied using forefinger. Mock inoculations were performed with inoculation buffer alone.

**Inoculation buffer:** 100mM sodium phosphate buffer (pH 7.0), 0.1% sodium sulphite (w/v) and 5mM EDTA
3.7.1.3 Transmission using cloned viral DNA

Complete viral inserts were excised from the recombinant plasmids by restriction digestion and the DNA was applied to the leaves of test plant by rubbing the solution in the presence of carborundum. It was believed that the DNA will be re-ligated in the plant resulting in a systemic infection.

3.7.2 Construction of agro-infectious clones

For transmission of most of the begomoviruses it is necessary to construct dimers or partial dimers (also called bitmers) of the viral DNA components that harbour at least two origins of replication. These constructs can directly (without restriction) be rubbed onto plant surfaces, as the unit genome component will be released by replication and/or less efficiently by recombination. These constructs can be transferred to agrobacterial plasmids and delivered to plants by agro-infiltration for increased infectivity (Stenger et al., 1991). Two new begomovirus species infecting tomato and chilli plants were identified and molecularly characterized in the present study. Tomato infecting distinct begomovirus was named as ‘tomato leaf curl Palampur virus’ (ToLCPMV) whereas chilli infecting new species was named as ‘chilli leaf curl Palampur virus’ (ChiLCPaV) (described in detail in results section). Agro-infectious clones of these new species were prepared in binary vector pCAMBIA-1300 (Cambia Labs, Canberra, Australia) as described below.

3.7.2.1 Agro-infectious clones of tomato leaf curl Palampur virus

ToLCPMV was found to be a bipartite begomovirus, complete DNA-A and DNA-B of which were cloned into pUC19 vector, giving rise to recombinant plasmids pUCTLCPM-A and pUCTLCPM-B respectively (described in detail in results section). A partial tandem repeat (1.4 mer) of the DNA-A was constructed in two cloning steps. In the first step, an EcoRI/HindIII fragment of 1003 nucleotides (nt) was cut from the plasmid pUCTLCPM-A and cloned into EcoRI/HindIII sites of pCAMBIA-1300 to produce a recombinant plasmid pCAM-TLCEH-A. In the second step, a complete HindIII fragment was cut from the plasmid pUCTLCPM-A and cloned into HindIII site of pCAM-TLCEH-A to produce a recombinant plasmid pCAM-TLCIC-A. A partial tandem repeat (1.8 mer) of the DNA-B was also constructed in a two-step cloning procedure. In the first step, a 1018 nt long
Materials and Methods

A HindIII/PstI fragment was cut from the plasmid pUCTLCPM-B and cloned into pCAMBIA-1300 to produce a recombinant plasmid pCAM-TLCHP-B. In the second step a complete BamHI fragment was cut from the plasmid pUCTLCPM-B and cloned into BamHI site of the plasmid pCAM-TLCHP-B to produce a recombinant plasmid pCAM-TLCIC-B. Head to tail arrangement of the constructs was checked through restriction digestion analysis and end sequencing.

3.7.2.2 **Agro-infectious clones of chilli leaf curl Palampur virus**

ChilliLCPaV was found to be a monopartite begomovirus associated with a betasatellite (described in detail in results section). Complete viral genome and the betasatellite component was cloned into pBluescriptII KS+ giving rise to recombinant plasmids pKSChiLC-A and pKSChiLC-β respectively. A partial tandem repeat (1.9 mer) of the viral genome was constructed in two cloning steps. In the first step, an EcoRI/KpnI fragment of 2669 nucleotides (nt) was cut from the plasmid pKSChiLC-A and cloned into EcoRI/KpnI sites of binary vector pCAMBIA-1300 to produce a recombinant plasmid pCAM-ChiEK-A. In the second step, a complete EcoRI fragment was cut from the plasmid pKSChiLC-A and cloned into EcoRI site of pCAM-ChiEK-A to produce a recombinant plasmid pCAM-ChiIC-A. A partial tandem repeat (1.7 mer) of the betasatellite was also constructed in a two-step cloning procedure. In the first step, a 1018 nt long EcoRI/BamHI fragment was cut from the plasmid pKSChiLC-β and cloned into pCAMBIA-1300 to produce a recombinant plasmid pCAM-ChiEB-β. In the second step a complete BamHI fragment was cut from the plasmid pKSChiLC-β and cloned into BamHI site of the plasmid pCAM-ChiEB-β to produce a recombinant plasmid pCAM-ChiIC-β. Head to tail arrangement of the constructs was checked through restriction digestion analysis and end sequencing.

3.7.3 **Agrobacterium transformation and agroinfection**

*Agrobacterium tumefaciens* was transformed either by tri-parental mating or by freeze-thaw transformation of chemically competent cells.
3.7.3.1 Tri-parental mating procedure

As the name suggests, the procedure involves three strains, finally resulting in the transformation of *Agrobacterium* with the desired recombinant plasmid. Donor *E. coli* DH5α cells carrying recombinant pCAMBIA plasmids were grown overnight at 37°C in LB supplemented with kanamycin (50µg/ml). A single colony of freshly streaked *A. tumefaciens* strain LBA4404 was grown for 16-22 hours at 28°C in 5 ml of YEP medium containing rifampicin (25µg/ml) and streptomycin (100µg/ml). *E. coli* DH5α cells carrying pRK2013 helper plasmid were grown overnight in LB containing kanamycin (50µg/ml). The overnight grown cultures (1.5 ml each) were centrifuged at 13,000 rpm for 1 min. The supernatant was discarded and each pellet was washed 2-3 times with sterile water by dispensing and re-pelleting the cells in the water (to remove traces of antibiotics). Finally each pellet was dispensed in 500µl of YEP medium and all three cultures were mixed in 1:1:1 ratio. The mixture was spotted on YEPA plates (without any antibiotics) and incubated at 28°C for 2 days allowing conjugation. After 2 days, the spotted culture was scraped with the help of a sterile blade, dispensed in 1 ml of YEP medium (without any antibiotics) and spotted on YEPA plates containing streptomycin (100µg/ml), rifampicin (25µg/ml) and kanamycin (50µg/ml). The plates were incubated at 28°C for 3-4 days after which the transformed *Agrobacterium* colonies appeared on the plate.

**YEP medium (Composition per liter):** 10 g yeast extract, 10 g peptone, 5 g NaCl. pH of the medium should be 7.

**YEPA medium:** 1.5 g agar and rest of the components were same as YEP medium

3.7.3.2 Preparation of chemically competent cells of *Agrobacterium* LBA4404

The method was adapted from Xu and Li, (2008) and competent cells were prepared as described below:

(i) Single colony of freshly streaked *Agrobacterium* LBA4404 strain was grown overnight in 5 ml of YEP medium without any antibiotic at 28°C.

(ii) To 50 ml of YEP medium, 2 ml of the overnight culture was added.

(iii) Cells were allowed to grow until and OD of 0.5 was obtained at 600 nm.
Materials and Methods

(iv) Cells were incubated on ice for 10 min.
(v) Cells were pelleted down by centrifugation at 3000 g for 5 min at 4°C.
(vi) The pellet was dispensed in 1 ml CaCl$_2$ (20 mM chilled CaCl$_2$).
(vii) Cells were pelleted down again as above.
(viii) The pellet was finally dispensed in 1 ml CaCl$_2$ (20 mM chilled CaCl$_2$).
(ix) Aliquots of 100µl of the cells were made in 1.5 ml Eppendorf tubes and transferred immediately to liquid N$_2$ for quick freezing.
(x) Stored at -80°C until use.

3.7.3.3 Transformation of Agrobacterium competent cells by freeze-thaw method
The competent cells were transformed with the recombinant pCAMBIA plasmids as follows:

(i) Competent cells were thawed on ice for 20 min.
(ii) Plasmid DNA (1µg) was mixed with the competent cells.
(iii) Tubes were immediately put in liquid N$_2$ for 5 min for freezing.
(iv) After that the tubes were immediately put at 37°C for 25 min (heat shock).
(v) After the incubation, 1 ml of YEP medium (without any antibiotic) was added to the tubes.
(vi) Transformed cells were allowed to grow for 3 hrs at 28°C with continuous shaking (250 rpm) in a rotary shaker.
(vii) Cells were pelleted down by centrifugation at 12,000 rpm for 2 min.
(viii) Supernatant was discarded but approximately 150µl supernatant was retained and cells were dispensed in it.
(ix) Cells were spread on YEPA plates containing streptomycin (100µg/ml), rifampicin (25µg/ml) and kanamycin (50µg/ml).
(x) The plates were incubated at 28°C for 3-4 days.
3.7.3.4 Selection of transformed Agrobacterium by colony PCR

Colony PCR was performed to select the transformed Agrobacterium colonies using specific primers for different components. A single Agrobacterium colony was picked and placed in 50 µl of sterile water. Boiled for 2 min and immediately placed on ice. PCR reaction was set up as follows:

- Boiled mixture
- 10x Taq buffer A
- 10 mM dNTP mix
- Forward primer
- Reverse primer
- Taq DNA polymerase (Genei, Bangalore, India)
- Final volume with ddH₂O to

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled mixture</td>
<td>10 µl</td>
</tr>
<tr>
<td>10x Taq buffer A</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5 µl (100 ng)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5 µl (100 ng)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.25 µl (3 units/µl)</td>
</tr>
<tr>
<td>Final volume with ddH₂O to</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

After completion, the reaction was directly electrophoresed in 1% agarose gel to look for desired amplification.

3.7.3.5 Agro-infiltration of infectious clones

Agro-infiltration was performed by a method described by Zhou et al. (2003). Transformed Agrobacterium cultures were grown at 28°C for 48 hours (till OD₆₀₀ =1) in 10 ml of YEP medium. The cultures (0.2-0.5 ml per plant) were injected into stems and petioles of the test plants at 4-6 leaf stage using 2 ml syringe and a fine needle. For inoculation of two types of clones into single plant, their cultures were mixed in 1:1 ratio before inoculation. At least five test plants were inoculated with each type of the inoculum. After agroinoculation, the plants were kept in an insect-free chamber at a constant temperature (25-28°C) under 14-16 h lighting. Plants were photographed 25 dpi.

3.7.4 Detection of viral DNA components in agro-inoculated plants

Viral components were detected from the systemically infected leaves of agro-inoculated plants by southern blotting and direct sequencing of RCA products.
3.7.4.1 Southern blotting and hybridization

For detecting viral DNA components, total nucleic acids were isolated from systemically infected leaf tissue of agro-inoculated plants by Dellaporta’s method and treated with RNase A (as described before). The DNA (~10µg) was electrophoresed in 0.7% agarose gel and transferred to the positively charged nylon membrane by capillary transfer method. After completion of electrophoresis, the DNA in the gel was denatured by placing the gel in a plastic tray containing the denaturation solution with slow shaking on a gel rocker for 30 min. After denaturation, the gel was soaked in neutralizing solution for 30 min with slow shaking. The two chambers of the electrophoresis tank were filled with transfer solution (10x SSC). A wick of Whatman® 3MM paper was soaked in 10x SSC and placed over the gel platform of the tank with its ends submerged in the solution. Three more sheets of Whatman® 3MM paper (slightly bigger than the gel) were soaked in 10x SSC and placed over the wick. The gel was carefully inverted and placed over the sheets avoiding any air bubbles between the gel and Whatman® papers. Nylon membrane was cut to the gel size, wetted in sterile water and placed over the gel avoiding any air bubbles. Three more sheets of Whatman® 3MM paper were cut to gel size, soaked in 10x SSC and placed over the membrane. A stack of gel-sized dry blotting papers (8-10 cm height) was placed over the 3MM papers and approximately 500 g weight was placed on top of the stack. The transfer was allowed to take place for 14-16 h.

After completion of the transfer, the blotting paper stack was removed, the membrane was marked so as to indicate the orientation and rinsed in 2x SSC briefly. The buffer was removed by placing the membrane between the folds of a dry 3MM sheet but the membrane was not completely dried. The membrane was wrapped in a cling film and placed under UV light in a UV cross-linker for 2 min for cross-linking the DNA to the membrane. The blot was then stored at 4°C until hybridization. Radioactive DNA probe (specific to DNA component to be detected) preparation, hybridization and autoradiography were performed as described before (section 3.2).

**Denaturing Solution:** 1M NaCl and 0.5M NaOH

**Neutralizing Solution:** 1.5M Tris HCl (pH 8) and 3M NaCl

**SSC (20x):** 3M NaCl and 0.3M trisodium citrate
3.7.4.2 Direct sequencing of RCA products
RCA was performed from the total DNA isolated from systemically infected leaves of agro-inoculated plants. To confirm the identity of viral components, RCA products were directly sequenced using primers specific to the DNA component to be detected (described in results section).

3.8 CHNS ANALYSIS OF TOMATO LEAF CURL PALAMPUR VIRUS INFECTED AND HEALTHY TOMATO
Carbon, hydrogen, nitrogen and sulphur (CHNS) content in healthy and virus infected plant leaves, stem and roots were quantified using CHNS analyzer (Elementar, vario micro). The fresh samples (agroinoculated and healthy) were kept in an oven at 45°C until weight loss became constant. Dry plant tissues were crushed in a mortar and pestle to a very fine powder. About 10-15 mg of each plant samples were mixed with tungsten oxide (3-4 mg) as a catalyst and packed in a very thin tin board. Similar procedure was repeated for all the plant samples. The packed sample boats were kept in sample holder of instrument for the analysis of CHNS. On placing the sample boats in holder, instrument was started and proper entry of parameters like weight of blank, standard and samples were done in a computer attached with the instrument. Sulfanilamide was used as a standard, which has a fixed percentage amount of C (41.8), N (16.25), hydrogen (4.65) and sulfur (18.62). After putting the weight of all these parameters, instrument was started and left for the analysis. A statistical factor was applied to deduct the blank from each plant sample.

3.9 RNA SILENCING SUPPRESSORS ENCODED BY TOMATO LEAF CURL PALAMPUR VIRUS
For identification of RNA silencing suppressors encoded by tomato leaf curl Palampur virus, Agrobacterium co-infiltration and reversal of silencing assays (described in section 2.5.4) were performed as described below.

3.9.1 Vector construction
For Agrobacterium based transient expression, each ORF encoded by DNA-A and DNA-B components of tomato leaf curl Palampur virus was cloned into the binary vector
Materials and Methods

pCAMBIA-1302, driven by *Cauliflower mosaic virus* (CaMV) 35S promoter. Primers carrying *Nco*I and *Spe*I restriction sites were designed (Table 3.5) to PCR amplify and clone each ORF (except ORF BV1) of the virus into respective sites of pCAMBIA-1302 vector. It was made sure that these ORFs do not contain any *Nco*I or *Spe*I recognition site. ORF BV1 contained an *Nco*I site; therefore *Xho*I site was introduced into its primers (Table 3.5) for its cloning into respective sites of pCAMBIA-1300. PCR conditions were standardized (described in results section) and all ORFs were PCR amplified from plasmids pUCTLCPM-A and pUCTLCPM-B. PCR products were purified from 1% agarose gels and cloned into pGEM®-T Easy vector (as described before). Recombinant pGEM®-T plasmids (carrying viral genes) were digested with *NcoI/SpeI* (*XhoI* for BV1), electrophoresed in 1% agarose gel and viral fragments were purified from the gel. The fragments were cloned (as described before) into *NcoI/Spel* digested and agarose gel purified pCAMBIA-1302 vector. ORF BV1 was cloned into *XhoI* sites of pCAMBIA-1300. Specific primers for pCAMBIA-1302 were designed (Table 3.6) to sequence the cloned viral ORFs and confirm their in-frame cloning.

3.9.2 Agro-infiltration of tissues

For agro-infiltration, all binary plasmids were transferred to *A. tumefaciens* (strain LBA4404) by freeze-thaw transformation method (as described before). Transformed colonies were selected by colony PCR (as described before) using gene specific primers (Table 3.5). Empty pCAMBIA-1302 vector was also transferred to *Agrobacterium* and used as a negative control as well as an inducer of RNA silencing against GFP gene in a co-infiltration assay.
### Table 3.5 List of primers designed to clone ORFs of ToLCPMV into pCAMBIA vectors

<table>
<thead>
<tr>
<th>ORF</th>
<th>Primer Sequence</th>
<th>ORF size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV2</td>
<td>5’ CCATGGATATGTGGGATCCATTATTGCA 3’</td>
<td>365 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTTCAGCCCCTGGGACGTC 3’</td>
<td></td>
</tr>
<tr>
<td>AV1</td>
<td>5’ CCATGGGTAAGCGTCCACGAGA 3’</td>
<td>770 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTTAATTTTACTGGGATCAT 3’</td>
<td></td>
</tr>
<tr>
<td>AC1</td>
<td>5’ CCATGGCTCCGCCAACTCGTTT 3’</td>
<td>1094 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTCAACTCTCTCTCCTGGGAT 3’</td>
<td></td>
</tr>
<tr>
<td>AC2</td>
<td>5’ CCATGGATATGCATTATTACACGAC 3’</td>
<td>410 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTTAATGCTTTTGGTGGTTTGA 3’</td>
<td></td>
</tr>
<tr>
<td>AC3</td>
<td>5’ CCATGGCTTCCGCAATCCATCCAT 3’</td>
<td>411 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTTAATGCTTTTGGTGGTTTGA 3’</td>
<td></td>
</tr>
<tr>
<td>AC4</td>
<td>5’ CCATGGGTCTCCGCATATCCAT 3’</td>
<td>176 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTCAACTCTCTCTCCTGGGAT 3’</td>
<td></td>
</tr>
<tr>
<td>BC1</td>
<td>5’ CCATGGGTATGCTCAAATTGAAATGATAG 3’</td>
<td>807 bp</td>
</tr>
<tr>
<td></td>
<td>5’ ACTAGTTTAATGCTTTTGGTGGGAGT 3’</td>
<td></td>
</tr>
<tr>
<td>BV1</td>
<td>5’ CTCGAGATGGCTTTTCTCTCTCTCTTA 3’</td>
<td>846 bp</td>
</tr>
<tr>
<td></td>
<td>5’ CTCGAGTCAACTATATAATTAAGAA 3’</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.6 Primers designed to sequence cloned ORFs in pCAMBIA-1302

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCAM-1302F</td>
<td>Forward 5’ ATTGATGTGATATCCTCCACT 3’</td>
</tr>
<tr>
<td>pCAM-1302R</td>
<td>Reverse 5’ TAAGTTTTCCGTATGGTGCA 3’</td>
</tr>
</tbody>
</table>
3.9.2.1 Preparation of Agrobacterium cultures

Agro-infiltration protocol was adapted from Karjee et al. (2008). Single Agrobacterium colonies harboring pCAMBIA-1302 vector with or without the viral ORFs were inoculated into 5 ml of YEM medium (as primary culture) and grown overnight at 30ºC with a shaking at 200 rpm. About 2% of this inoculum was then used to inoculate 50 ml of the YEM broth and grown at 30ºC, 200 rpm, until the OD at 600 nm reached 0.5. The cultures were centrifuged at 5,000 rpm for 5 min in centrifuge tubes and bacterial pellets were dispensed in 20 ml of infiltration solution. These cultures were kept at room temperature for 3-4 h before infiltration.

Infiltration solution: 10 mM MgCl₂, 10 mM MES pH 5.6 and 150µM acetosyringone.

YEM medium (contents per litre): 0.4 g yeast extract, 10 g mannitol, 0.1 g NaCl, 0.2 g MgSO₄·7H₂O, 0.5 g K₂HPO₄. pH of the medium was set to 7.0. 15 g agar was added for making YEM agar plates.

3.9.2.2 Agrobacterium co-infiltration assay

The approach was based on the assay described for identification of RNA silencing suppressor encoded by tomato yellow leaf curl virus-Israel (Zrachya et al., 2007) as non-transgenic Nicotiana plants were used in the experiment. For co-expression of GFP, Agrobacterium culture carrying empty pCAMBIA-1302 (inducer of GFP silencing) vector was mixed with cultures carrying cloned viral ORFs (1:1 v/v ratio) in separate tubes. This homogeneous culture suspension was taken in a 5 ml needleless syringe and infiltrated into the young leaves of 5-6 week old, greenhouse grown N. tabacum cv. Xanthi. Infiltration was performed by generating a vacuum with the help of a finger on the dorsal side of the leaf and mouth of the syringe on the ventral side. As the vacuum was created, the plunger of the syringe was pushed to transfer the culture into the leaf through the stomata. Each agro-infiltrated leaf was labeled for the construct used for infiltration. A plant was infiltrated with only one of the ORFs for best results. Suppressor of RNA silencing encoded by Mungbean yellow mosaic India virus (MYMIV; AC2 gene) cloned into binary vector pBI121 was kindly provided by Dr. Sunil K. Mukherjee (International Centre for Genetic Engineering and Biotechnology, New Delhi, India), which was used as a positive control.
*Agrobacterium* culture carrying empty pCAMBIA-1302 vector was used as a negative control.

### 3.9.2.3 Reversal of silencing assay

A GFP silent transgenic line of *N. tabacum* cv. Xanthi (Karjee *et al.*, 2008) was kindly provided by Dr. Sunil K. Mukherjee (International Centre for Genetic Engineering and Biotechnology, New Delhi, India). *Agrobacterium* cultures carrying cloned viral ORFs were infiltrated (as described for co-infiltration assay) into the young leaves of greenhouse grown GFP silent transgenic *N. tabacum* cv. Xanthi plants. MYMIV AC2 gene cloned in pBI121 and empty pCAMBIA-1302 vector were used as positive and negative controls respectively.

### 3.9.2.4 Analysis of the inoculated leaves

The inoculated leaves were plucked from the plant for fluorescence and biochemical analysis at 2 dpi, 5 dpi, 7 dpi and 10 dpi (Karjee *et al.*, 2008). This period was required for the optimum expression and suppression activity of the viral ORFs. The leaves were visualized under UV light for fluorescence and photographed. After photography, the leaves were immediately stored at -80°C until further use.

### 3.9.3 Analysis of GFP mRNA

To confirm the results obtained by visual observation of GFP fluorescence under UV light, GFP mRNA levels were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Agro-infiltrated portions of the leaves were cut with the help of a sterile blade and the tissue was used to isolate total RNA using TRI Reagent® (Sigma-Aldrich) as described below.

#### 3.9.3.1 RNA isolation

(i) Leaf tissue (100-150 mg) was ground to fine powder in liquid N2 using mortar/pestle and dispensed in 1 ml of TRI reagent (in 1.5 ml Eppendorf tubes). The samples were incubated at room temperature for 5 min.
(ii) To this mixture, 200µl of chloroform was added, vortexed vigorously for 15 sec and incubated in dark at room temperature for 10 min.

(iii) The resulting mixture was centrifuged at 13,000 rpm for 15 min at 4°C and aqueous phase was transferred to a fresh tube.

(iv) To the aqueous phase, 500µl of isopropanol was added, allowed to stand for 5 min at room temperature and centrifuged at 13,000 rpm for 10 min at 4°C.

(v) Supernatant was discarded and RNA pellet was dissolved in 1 ml of 80% ethanol.

(vi) Centrifuged at 10,000 rpm for 5 min at 4°C.

(vii) Supernatant was decanted using a paper towel. The pellet was air-dried for 10 min and dissolved in 50µl of nuclease-free sterile water by incubating at room temperature for 10 min.

(viii) Aliquots of 10µl were stored at -80°C until use.

(ix) RNA (2µl) was electrophoresed in 1% agarose gel to assess the quality of isolated RNA. RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific).

3.9.3.2 DNaseI treatment of isolated RNA

Isolated RNA was treated with RNase-free DNaseI (Ambion) to remove traces of DNA (if any).

(i) RNA was thawed on ice and about 2µg of it was transferred to an autoclaved 0.2 ml PCR tube.

(ii) To the RNA, 2µl of 10x DNaseI buffer (supplied with DNaseI) and 1µl of DNase I (2 Units/µl) were added.

(iii) Adjusted the volume to 20µl with RNase-free water and incubated at 37°C for 30 min in a thermocycler.

(iv) After incubation, 2µl of 25mM EDTA (pH 8.0) was added to the reaction mixture.

(v) DNaseI was inactivated by incubating the reaction mixture at 65°C for 10 min. The reaction mixture was directly used for reverse transcription.
3.9.3.3 RT-PCR

Reverse transcription (RT) was performed in 0.2 ml thin walled PCR tubes using DNaseI treated total RNA as the template. Specific primers were designed and synthesized for ACTIN (internal control) and GFP (Table 3.7). For RT reaction, 1 μg of total RNA was mixed with 10 μl of 5x RT buffer (USB), 2.5μl of 40 mM dNTP mix, 0.4μg of downstream (reverse) primer, 20 units of RNase Inhibitor (USB) and 200 units of M-MLV RT (USB) to a final volume of 50μl. The reaction mixture was incubated at 37°C for 75 min and the enzyme was inactivated by incubating the mixture at 70°C for 5 min in an automated thermocycler. PCR was performed using 10μl of the RT product in a 50μl reaction mixture (as described before) and thermal cycling pattern for each primer pair is described below (Table 3.8).

Table 3.7 List of primers designed to amplify GFP and ACTIN genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Forward</td>
<td>5’ GGAGTTGTCCCAATTCTTGTT 3’</td>
<td>~600 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ GTCTCTCTTTTCGTTGGGATC 3’</td>
<td></td>
</tr>
<tr>
<td>ACTIN</td>
<td>Forward</td>
<td>5’ CTGAGGGAAGCCAAGATAGA 3’</td>
<td>~300 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’ CTGAGGGAAGCCAAGATAGA 3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8 Thermal cycling pattern of PCR for GFP and ACTIN genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Thermal cycling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Initial denaturation at 94°C for 3 min followed by 30 cycles consisting of 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>ACTIN</td>
<td>Initial denaturation at 94°C for 3 min followed by 30 cycles consisting of 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 10 min</td>
</tr>
</tbody>
</table>
3.9.3.4 Specificity of amplified products
Amplified PCR products were electrophoresed in 1% agarose gel and purified using a gel extraction kit (as described before). Purified products were directly sequenced using specific primers (Table 3.7). Around 50 ng of purified PCR product was used and sequencing was performed as described before.

3.10 SUB-CELLULAR LOCALIZATION OF IDENTIFIED SUPPRESSORS

3.10.1 Plasmid construction
For revealing sub-cellular localization of the identified suppressor proteins (AC4 and AV2) of ToLCPMV, the ORFs were cloned at 5’ end of modified GFP gene (mGFP5) available in pCAMBIA-1302 driven by CaMV 35S promoter for expression of AC4:GFP and AV2:GFP fusion proteins. The ORFs were amplified by PCR using the specifically designed primers carrying NcoI and SpeI sites without any stop codon (Table 3.9). The ORFs were PCR amplified (Table 3.10) and cloned into pGEM®-T Easy vector first and sub-cloned into the NcoI and SpeI sites of pCAMBIA-1302 (Fig. 3.1). In-frame cloning of the ORFs was confirmed by sequencing of the recombinant clones using vector specific primers (Table 3.6). GFP expressing pCAMBIA-1302 vector was used as control.

Table 3.9 List of primers used for PCR amplification of the ORFs for fusing with mGFP5 gene in pCAMBIA-1302

<table>
<thead>
<tr>
<th>ORF</th>
<th>Primer Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV2</td>
<td>Up  5’ CCATGGATATGTGGGATCCATTATTGCA 3’</td>
<td>362 bp</td>
</tr>
<tr>
<td></td>
<td>Down 5’ ACTAGTCAGCCCTGGGCACGTC 3’</td>
<td></td>
</tr>
<tr>
<td>AC4</td>
<td>Up  5’ CCATGGGTCTCCGGCATATCCAT 3’</td>
<td>173 bp</td>
</tr>
<tr>
<td></td>
<td>Down 5’ ACTAGTAAACGTCTCCGTCTTTG 3’</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

Table 3.10 Thermal cycling pattern for PCR amplification of the ORFs for fusing with mGFP5 gene in pCAMBIA-1302

<table>
<thead>
<tr>
<th>Target</th>
<th>Thermal cycling pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV2</td>
<td>Initial denaturation at 94°C for 3 min followed by 30 cycles consisting of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 10 min</td>
</tr>
<tr>
<td>AC4</td>
<td>Initial denaturation at 94°C for 3 min followed by 30 cycles consisting of 94°C for 30 sec, 60°C for 30 sec, 72°C for 25 sec and final extension at 72°C for 10 min</td>
</tr>
</tbody>
</table>

Fig. 3.1 Schematic representation of the constructs made for sub-cellular localization studies of the identified suppressors
3.10.2 Biolistic assay and GFP visualization

Epidermal strips of onion (*Allium cepa*) were placed on Murashige and Skoog media supplemented with agar in Petri plates and bombarded with each of the above plasmids as described previously (Selth *et al.*, 2005). Gold Particles (1.6µm, Bio-Rad) were coated with the plasmids by placing 2 mg of gold particles (per six shots) in 100µl of absolute ethanol. The particles were vortexed for 2 min, centrifuged for 10 sec in a microcentrifuge and washed twice with sterile water. Gold particles were resuspended in 25 µl of 40% (v/v) glycerol. While gently vortexing, 4 µl of plasmid solutions (500 ng/µl), 10 µl of cold 0.1M spermidine, and 25 µl of 2.5 M CaCl₂ were added drop-wise and the resulting mixture was incubated on ice for 10 min. The particles were pelleted down by brief centrifugation, washed with 70% (v/v) ethanol, and resuspended in 65µl ice-cold absolute ethanol. Then 10µl aliquots were placed onto sterile marcocarriers and allowed to dry at room temperature. Gold particles were bombarded using Biolistic® PDS-1000/He particle delivery system (BioRad Laboratories, Hercules, CA, USA) and 1100 psi rupture discs (BioRad). Following bombardments the Petri plates were covered with aluminum foil and kept in dark for 48 hours. Localization of the fusion protein was ascertained using AX10 Imager.M1 (Zeiss, Germany) and GFP filter set (excitation 460–500 nm, emission 515–550 nm).