MATERIALS AND METHODS
3.1 SURVEYS OF POME AND STONE FRUIT GROWING AREAS IN HP AND J&K FOR VIRUS INCIDENCE

3.1.1 Based on Visual Symptoms

Surveys were undertaken (2007-2009) in different locations of the major apple growing states (HP, J&K, Punjab, Uttarakhand) in India to identify the incidence of ACLSV in pome and stone fruit plantations (Fig 3.1).

Table 3.1: Various locations surveyed for different pome and stone fruit viruses.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Location</th>
<th>District</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Palampur</td>
<td>Kangra</td>
<td>HP</td>
</tr>
<tr>
<td>2.</td>
<td>Kaza</td>
<td>Lahul &amp; Spiti</td>
<td>J&amp;K</td>
</tr>
<tr>
<td>3.</td>
<td>Salooni</td>
<td>Chamba</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Tissa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Sangla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Kalpa</td>
<td>Kinnaur</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Kotkhai</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Annu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Kotgarh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Narkanda</td>
<td>Shimla</td>
<td>HP</td>
</tr>
<tr>
<td>11.</td>
<td>Nihari</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Sarahan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Theog</td>
<td>Mandi</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Dobi</td>
<td></td>
<td></td>
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<tr>
<td>15.</td>
<td>Thunag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Bajaura</td>
<td>Kullu</td>
<td>J&amp;K</td>
</tr>
<tr>
<td>17.</td>
<td>Seobagh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Manali</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Nauni</td>
<td>Solan</td>
<td>Punjab</td>
</tr>
<tr>
<td>20.</td>
<td>Renuka</td>
<td>Sirmour</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>Jammu</td>
<td>Jammu</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Phulwama</td>
<td>Phulwama</td>
<td></td>
</tr>
<tr>
<td>23.</td>
<td>Gandarbal</td>
<td>Gandarbal</td>
<td></td>
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<tr>
<td>24.</td>
<td>CITH</td>
<td>Srinagar</td>
<td></td>
</tr>
<tr>
<td>25.</td>
<td>Attari</td>
<td>Amritsar</td>
<td></td>
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<td>26.</td>
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</table>
Other pome (pear, quince) and stone (peach, plum, cherry, almond, apricot) fruits growing in these locations were also collected. Apart from these, samples from two nurseries at Palampur (HP) housing about 18 different cultivars and root stocks of apple were also indexed. Young leaves, flowers and twigs from plants showing typical symptoms of virus infection such as leaf deformation, curling, shot holes, necrotic spots, mosaic and mild chlorotic ring like symptoms were collected. Wild apricot (Chuli, Prunus armeniaca) and wild Himalayan cherry (Paja, P. cerasoides D. Don) were also sampled as these are wild relatives of Prunus spp. and are commonly used as rootstock for the stone fruit cultivars. Various pome and stone fruits were also surveyed from different locations of J&K and Punjab (Table 3.1).

3.1.2 Based on Enzyme Linked Immunosorbent Assay (ELISA)

Preliminary detection of the virus was done by ELISA using commercially available ELISA reagents as per manufacturer’s instructions. Symptomatic plants and randomly selected symptomless plants from each orchard were subjected to DAS-ELISA for the diagnosis of ACLSV. Other viruses viz. ApMV, ASGV, ASPV, PNRSV, PPV were also tested as ACLSV in nature occurs mostly in mix infection. Buds, flowers, young leaves, mature leaves and bark tissue were used for ELISA. DAS-ELISA was performed for all the mentioned viruses. The basic technique was the same as described by Clark and Adams (1977). DAS-ELISA uses antibodies which are bound to the surface of a micro plate to capture the antigens. The presence of antigen is detected using specific antibodies coupled with alkaline phosphatase. Finally the addition of the enzyme substrate p-nitrophenyl phosphate (pNPP) induces a yellow product, detectable at 405 nm, when the antigen is present.

3.1.2.1 Double Antibody Sandwich ELISA (DAS-ELISA)

(i) Microtitre plates (Tarsons, India) were coated with 100µl of coating antibodies diluted appropriately in coating buffer as per manufacturer’s instruction and plates were incubated for 4 hrs at 37°C in a humid box.

(ii) The plates were washed 3 times with PBS-T for 2min each. Antigen was prepared by macerating the virus-infected young leaf tissue (1g/10ml) in general extraction buffer. Several dilutions were made corresponding to 1X - 1/160X
Fig 3.1: District map of Himachal Pradesh showing areas surveyed for apple and other pome & stone fruits.
(iii) Dilution of the original antigen. Diluted antigen was pipetted (100µl) into the wells of the microtitre plate and incubated overnight at 4°C in a humid box to allow the trapping of antigen to the previously coated antibodies.

(iv) The plates were washed 3 times with PBS-T for 2min each.

(v) Conjugated antibodies were diluted appropriately in ECI buffer (as per manufacturer’s instruction) and loaded into the wells (100µl/well). The plates were then incubated at 37°C for 4hrs.

(vi) The plates were again washed with PBS-T for 3 times (for 2min) and 2 times (3min each). pNPP substrate solution prepared (1mg/ml) in PNP buffer was then loaded onto the well (100µl/well) and the plates were incubated at room temperature in dark till the colour developed.

(vii) After appropriate colour development (20-30min), the reaction was terminated by adding 50µl of 3 M NaOH to each well.

(viii) Positive and negative controls provided with the ELISA kit were also loaded on the same plate. Absorbance at 405 nm was measured with a Flow ELISA Microplate Reader Model 680 (BioRad, USA). The reaction was considered positive if absorbance was observed to be at least three times the background mean of healthy control.

Reagents:

**Coating buffer (0.05 M per litre):** 1.59g sodium carbonate and 2.93g sodium bicarbonate, pH 9.6.

**PBS Buffer:** 20mM sodium phosphate pH 7.4; 150 mM NaCl.

**PBS-T buffer:** 20 mM sodium phosphate pH 7.4; 150mM NaCl; 0.05% (v/v) Tween 20.

**Extraction buffer:** 1.3 g sodium sulfite (anhydrous); 20 g polyvinylpyrrolidone (PVP) (MW 24-40,000); 0.2g sodium azide; 2.0g powdered egg albumin grade II; 20.0g Tween-20 dissolved in 1000 ml 1X PBST and pH was adjusted to 7.4.

**ECI buffer:** 2.0g BSA; 20.0g PVP (M. wt. 24-40,000); 0.2g sodium azide dissolved in 1000ml 1X PBST and pH was adjusted to 7.4.

**PNP buffer:** 0.1g magnesium chloride; 0.2g sodium azide; 97ml diethanolamide dissolved in 800ml distilled water and volume was made to 1000ml and pH was adjusted to 9.8.
3.1.2.2 **Percent Disease Incidence of ACLSV**

The percent of ACLSV incidence on apple was calculated based on ELISA as:

\[
\text{Disease incidence (\%) = \frac{\text{Number of positive samples in ELISA}}{\text{Number samples tested}}} \times 100
\]

3.1.3 **Based on Hybridization**

3.1.3.1 **Preparation of Slot Blot**

Tissue blots were prepared by crushing sample in TNE buffer. Samples used were diluted to 200µl (100µl water + 100µl crushed sample) and denatured in water bath at 65°C for 10 minutes before loading. The apparatus was assembled with Zetaprobe membrane (Bio-Rad, USA) previously wetted with sterile water and 6X SSC for 2min each. The apparatus was connected to vacuum pump (<70mbar). To each slot 200µl of 10X SSC buffer was added and vacuum was applied till the liquid was completely absorbed but not dried. About 200µl of denatured sample was then added to each well. Vacuum was applied till the samples were absorbed completely. 200µl of 10X SSC was again applied and allowed to be completely absorbed. After this vacuum was released, apparatus was disassembled and the membrane was rinsed in 2X SSC. The membrane was dried by draining excess SSC onto blotting sheets and exposed to UV for 2min to bind the transferred nucleic acid to membrane. The membrane was wrapped in a cling wrap and stored at 4°C until hybridization.

3.1.3.2 **Probe Preparation**

Already sequenced pGEM-Teasy cloning vector containing the ACLSV CP (amplified using detection primers: Table 3.3, conditions: Table 3.4 and procedures detailed in section 3.2 to confirm the virus identity at molecular level) was digested with Eco RI restriction enzyme and the digested fragment was separated on 1% agarose gel. Viral DNA fragment was excised from the gel, eluted using AuPrep™ Gel™ gel extraction kit (Life Technologies Ltd., India) and then incubated in boiling water bath for 10 min for probe preparation. The reaction used was:

- Denatured DNA 200-500ng (~5µl)
- Random Primer 100ng (1µl)
- 10X Klenow buffer 3µl
- dNTP mix (-ATP)(3.3mM each) 4.5µl
- α\(^{32}\)-P dATP (10µCi/µl, specific activity 3x10\(^3\) Ci/mM) 10µCi (1µl)
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Klenow enzyme                                                                   5 units (1µl)

The volume was made 30µl with water and 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 7min. The contents were immediately transferred to ice before adding to the hybridization bottle.

Reagents:

dNTP mix (for α₃²-P dATP as the radioactive molecule, 100mM stock of dCTP, dTTP and dGTP): 3.3µl each in 100µl water i.e. 3.3mM

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- 100mM and EDTA (pH 8) - 10mM

3.1.3.3 Prehybridization/Hybridization

The blots were removed from cling wrap and placed in glass bottles. Prehybridization buffer was added and prehybridization at 42°C was carried out for 1hr in hybridization oven. Prehybridization/hybridization buffer consists of:

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

(Formamide was added to the buffer after autoclaving)

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

3.1.3.4 Washings of Blots

After hybridization solution was completely removed from the hybridization bottles containing the blot. The blots were then rinsed briefly with 2X SSC. Three wash buffers with progressive stringency were used for washings as follows:

<table>
<thead>
<tr>
<th>Wash Buffer</th>
<th>Wash Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Wash Buffer</td>
<td>2.0X SSC, 0.1% SDS (at 25°C) - two washings of 15min each</td>
</tr>
<tr>
<td>Second wash buffer</td>
<td>0.5X SSC, 0.1% SDS (at 25°C) - two washings of 15min each</td>
</tr>
</tbody>
</table>
Third Wash buffer          0.1X SSC, 0.1% SDS (at 65°C) - two washings of 30min each

After washing, the blots were placed on a Whatman 3MM filter paper to remove excess of liquid and wrapped in a cling wrap immediately, to prevent it from drying and for the purpose of autoradiography.

3.1.3.5 Autoradiography
Blots were exposed to X-ray film (Kodak, India) between two intensifying screens (Kiran Hi-speed X-ray film cassettes, 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.

3.1.5 Virus Transmission and Host Range
3.1.5.1 Mechanical Transmission
For the transmission of ACLSV, plant samples showing maximum and borderline ELISA values were selected. The samples were macerated using sterile pestle and mortar in 0.01M sodium phosphate buffer, pH 7 containing 2% PVP (M. wt. 8000) (Lister, 1970). Healthy seeds of diagnostically susceptible host species Chenopodium amaranticolor, C. quinoa, Phaseolus vulgaris and diagnostically insusceptible host species Nicotiana glutinosa, Cucumis sativus and few other hosts plants were sown in plastic trays filled with sterilized soil, sand and farm yard manure (FYM) mixture in 1:1:1, under insect proof glass house conditions. Nursery plants were further transplanted at 2 leaf stage in plastic pots to raise the test plants. The healthy and vigorously growing plants at 4-8 leaf stage were used in the inoculation experiments. A pinch of carborundum (800 mesh) powder was used as an abrasive to make injuries on leaves of test plants for easy entry of virus. Inoculation was done by gently rubbing the predusted leaves of test plants with forefinger dipped in the crushed plant extract. Inoculated leaves were rinsed with a gentle stream of distilled water after 1-2 min of inoculation. Inoculated plants were kept in the glass house for 2-8 weeks for symptom development. An optimum temperature of 25-26°C was maintained throughout the day in the glass house.
3.1.5.2 Host Range and Symptomatology
For this study, five plants of each species belonging to different families were used. Most of the test plants were inoculated at recommended leaf/growth stage. Inoculation was done mechanically as describe earlier. At least 5 plants of each species were kept as control. The experiment was repeated twice and observed for symptoms till 8 weeks after inoculation. Symptoms and severity along with incubation period was recorded. The plants were checked periodically by back inoculation of local lesion host (C. quinoa and P. vulgaris) to ascertain the reliability and purity of virus.

3.1.5.3 Preparation of Standard Inoculum
From the leaves of local lesion hosts several single lesions which were separated by appreciable distance were excised and macerated separately in a drop of above described buffer for ACLSV inoculation. This sap was termed as standard inoculum and was used for mechanical inoculations of young C. quinoa, P. vulgaris and C. amranticolor.

3.1.5.4 Maintenance of Virus Culture in-vivo
The viral culture of ACLSV used in this study was obtained from a diseased apple plant showing chlorosis, leaf deformation symptoms and also highest ELISA readings. To prepare the pure culture of the virus and to eliminate possible contamination from the other viruses, dilution of the sap were made and inoculated on the leaves of C. quinoa. From one of the inoculated plant, which showed symptoms, the culture was back inoculated using single lesion. The lesion was excised, macerated and inoculated onto young C. quinoa, the maintenance host.

3.1.5.5 Maintenance of Virus Pure Culture in-Vitro
To maintain pure culture of the virus in vitro tissue culture was used. ACLSV infected apple cultivar Gala from Uttrakhand was first of all tested for the presence of other viruses such as ApMV, ASPV, ASGV and PNRSV by ELISA and RT-PCR using virus specific primers (Table 3.3). The plant if found infected only by ACLSV would be used to maintain virus pure culture in vitro. Explants (apical/lateral meristems 0.2-1.0mm) were taken from infected apple plant and cultured on
Murashighe and Skoog (MS) medium (Table 3.2) (Murashighe and Skoog, 1962). Depending upon the solubility of the salts, separate stocks are made for Ammonium nitrate (*), Potassium nitrate (*), Vitamins (v), Macro nutrients (macro) and Micronutrients (micro). The explants were best multiplied on MS medium with 1mg/ml BAP, 0.1mg/ml NAA, 3%sucrose and 0.7% agar. The explants were routinely subcultured at 6 weeks interval to maintain the stock and for further use in molecular studies.

### Table 3.2 Components of MS Medium (Murashighe and Skoog, 1962)

<table>
<thead>
<tr>
<th>Components</th>
<th>Conc. (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>*NH₄NO₃</td>
<td>1650</td>
</tr>
<tr>
<td>*KNO₃</td>
<td>1900</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O (macro)</td>
<td>440</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O (macro)</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄ (macro)</td>
<td>170</td>
</tr>
<tr>
<td>H₃BO₃ (micro)</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO₄ 4H₂O (micro)</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄ (micro)</td>
<td>8.6</td>
</tr>
<tr>
<td>KI (micro)</td>
<td>0.83</td>
</tr>
<tr>
<td>Na₂MoO₄ 2H₂O (micro)</td>
<td>0.25</td>
</tr>
<tr>
<td>Cu SO₄ 5H₂O (micro)</td>
<td>0.025</td>
</tr>
<tr>
<td>Fe SO₄ 7H₂O (macro)</td>
<td>27.8</td>
</tr>
<tr>
<td>Na₂EDTA 2H₂O (macro)</td>
<td>37.3</td>
</tr>
<tr>
<td>Mesoinositol (v)</td>
<td>10.0</td>
</tr>
<tr>
<td>Pyridoxine HCl (v)</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl (v)</td>
<td>0.1</td>
</tr>
<tr>
<td>Nicotinic acid (v)</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine (v)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

are more sensitive than direct probing or serological techniques for detecting and characterizing plant pathogens (Hadidi et al., 1995). To further elucidate and confirm
the identity of virus, total RNA was extracted using two methods. Tissues from woody plants, especially when field- grown, can contain higher amounts of phenolic compounds and polysaccharides which are thought to be RT-PCR inhibitors (Nassuth et al., 2000; Singh et al., 2002). An extraction buffer (Foissac et al., 2001) with slight modification (no 100µl of 10% SDS added to clarified extract) for removal of the inhibitory activity from woody plant extracts was used to standardize both the RNA extraction protocols. For full genome characterization RNA was extracted from the samples showing highest ELISA readings (leaves of apple cultivars Gala Mast (in-vivo) and Gala (in-vitro) from Gandarbal (J&K) and Uttrakhand respectively and flowers of wild Himalayan cherry from Palampur (HP). RNA was also extracted from few other host samples to characterize and confirm the presence and diversity if any in ACLSV.

3.2.1 Total RNA isolation from Infected Tissue

3.2.1.1 By Guanidine Hydrochloride Method

(i) Plant tissue (100mg) was ground to fine powder in liquid nitrogen and 100µl of extraction buffer.

(ii) This mixture was homogenized with 10 volumes of homogenization buffer containing 150 mM β-mercaptoethanol.

(iii) Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by vortexing. The mixture was then centrifuged for 10min at 10,000 rpm at 4°C in a 30ml oak ridge tube to separate the phases.

(iv) The upper aqueous phase was transferred to a new oak ridge tube and equal volume of chloroform/isoamyl alcohol (24:1) was added followed by vortexing and centrifuged for 10min at 10,000 rpm at 4°C.

(v) The upper aqueous phase was transferred to a new corex tube, mix with 0.2 vol. of 1 M acetic acid and 0.7 vol. of chilled ethanol.

(vi) The mixture was then incubated at -70°C for at least 3 hrs.

(vii) RNA was pelleted by centrifugation for 10min at 10,000 rpm at 4°C.

(viii) The supernatant was discarded and 5ml of 3 M sodium acetate solution was added to the RNA pellet and vortex for 30 sec.

(ix) The tube was again centrifuged for 5min at 10,000 rpm at 4°C.

(x) The washing steps (viii – ix) were repeated twice.
(xi) The supernatant was discarded and the salt contamination in RNA pellet was removed by washing the RNA pellet with 5ml of cold 70% ethanol, followed by centrifugation for 5min at 10,000 rpm at 4°C.

(xii) RNA pellet was dried at room temperature and dissolved in 100µl 18.2MΩ millipore water.

If chlorophyll is visible in the RNA pellet, it is removed as follows:

(i) To the RNA solution 0.1 vol. of 3 M sodium acetate (pH 5.2) and 4 vol. of chilled absolute ethanol was added.

(ii) The mixture was incubated at -70°C for 2hrs. The tubes were then centrifuged for 10 min at 13,000 rpm at 4°C.

(iii) The pellet was washed twice with 70% ethanol and dried at room temperature.

(iv) The RNA pellet was dissolved in 100µl of 18.2 MΩ millipore water and stored in aliquots (10µl each) at -70°C for further use.

Total RNA was quantified by monitoring absorbance at 260 nm and its purity was checked by calculating the $A_{260}/A_{280}$ ratio using a UV spectrophotometer (Specord 200, Analytik Jena). To check the integrity of RNA, 5µl of total RNA (mixed with 2µl of gel loading dye and 1µl of 10 mg/ml ethidium bromide solution) was loaded on 1% denaturing agarose gel containing formaldehyde. The gel was run at 80 V for 2-3 hrs and was observed in UV transilluminator.

**Reagents:**

**Homogenization buffer (Guanidine hydrochloride buffer):** 100g guanidine hydrochloride was dissolved in 40ml 18.2MΩ millipore water. When dissolved, 5.2ml of 0.5M EDTA (pH 8.0) and 0.5075 g MES [2-(N-Morpholino) ethanesulfonic acid] was added and mixed to dissolve. Finally the volume was adjusted to 130ml.

**3 M Sodium acetate pH 5.2:** 123.05g of sodium acetate was dissolved in 400ml 18.2MΩ millipore water, pH was adjusted to 5.2 with glacial acetic acid and final volume was adjusted to 500ml.

**1 M Acetic acid (glacial):** 2.6ml of acetic acid was added to a 50ml sterile tube and the volume was adjusted to 45ml with 18.2MΩ millipore water.

**Diethyl pyrocarbonate treated water:** Diethyl pyrocarbonate (DEPC) was added to water at final concentration of 0.1% (1 ml/litre), mixed vigorously and incubated at 37°C overnight. The mixture was autoclaved at least twice to remove traces of DEPC.
Fomaldehyde gel running buffer (5X): 100mM MOPS, pH 7.0; 4mM Sodium acetate and 5mM EDTA, pH 8.0 [dissolved 20.6 g 3-(N-morpholino) propanesulfonic acid (MOPS) in 800ml DEPC treated 50mM sodium acetate. The pH was adjusted to 7.0 with 2 N NaOH. To this solution added 10ml DEPC treated 0.5M EDTA and volume was adjusted to 1 liter].

Extraction Buffer (Foissac et al., 2001)

- 6M Guanidine thiocyanate
- 0.2M Sodium acetate (pH 5.2)
- 25mM EDTA (pH 8.0)
- 1M Potassium acetate
- 2.5% Poly vinyl pyrrolidone (40,000 M.Wt.)
- 1% β mercaptoethanol

Guanidine thiocyanate was filter sterilized and added to autoclaved buffer along with β mercaptoethanol to make final volume to 100ml.

3.2.1.3 By Cetyltrimethylammonium Bromide (CTAB) Method

CTAB method of RNA extraction by Gasic et al. (2004) was standardized with slight modifications for RNA extraction from apple and other pome-stone fruits.

(i) Pre warmed 9.6ml of CTAB extraction buffer at 65°C and added 400µl β-mercaptoethanol to make volume of 10ml.

(ii) Plant tissue (1mg) was ground to fine powder in liquid nitrogen and added to 10ml of pre warmed CTAB extraction buffer.

(iii) The mixture was homogenized by vortexing for 2min and incubated at 65°C for 15min.

(iv) Equal volume (10ml) of chloroform/isoamylalcohol (24:1) was added and mixed by vortexing (2min). The mixture was transferred to autoclaved 50ml oakridge tubes and centrifuged at 10,000g for 15-20min at 4°C to separate the phases.

(v) The upper aqueous phase was transferred to a new oak ridge tube and re-extracted with equal volume of chloroform/isoamylalcohol (24:1) followed by vortexing and centrifugation at 10,000g for 15-20min at 4°C.

(vi) To the aqueous phase added ½ volume of 7.5M LiCl in each tube, mixed by inverting and stored overnight at 4°C.
(vii) The following day, tubes were centrifuged at 12,000g for 30min at 4°C. The supernatant was discarded by pipetting and the pellet was washed with chilled 70% ethanol (centrifuged at 12,000g for 5min at 4°C).

(viii) The RNA pellets were air dried at room temperature and dissolved in 100µl DEPC /
18.2MΩ water and stored at -80°C.

Total RNA was quantified by monitoring absorbance at 260,280,230 nm and its purity was checked by calculating the A_{230}/A_{260} and A_{260}/A_{280} ratio using a UV spectrophotometer (Specord 200, Analytik Jena). An A_{230}/A_{260} ratio higher than 2 indicates lack of polyphenol and polysaccharide contamination while, A_{260}/A_{280} ratio ranging from 1.91-2.02 indicates lack of protein contamination. To check the integrity of RNA, 5µl of total RNA (mixed with 2µl of gel loading dye and 1µl of 10 mg/ml ethidium bromide solution) was loaded on 1% denaturing agarose gel containing formaldehyde. The gel was run at 80 V for 2-3 hrs and was observed in UV transilluminator.

Reagents:

- **CTAB Extraction buffer:** 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble), 100mM Tris HCl (pH 8.0), 25mM EDTA, 2M NaCl, 0.5g/l spermidine (free acid) (HRS), 2% β-mercaptoethanol (added just before use). Extraction buffer autoclaved (without β-mercaptoethanol) and stored at room temperature.
- **Diethyl pyrocarbonate treated water:** Diethyl pyrocarbonate (DEPC) was added to water at final concentration of 0.1% (1 ml/litre), mixed vigorously and incubated at 37°C overnight. The mixture was autoclaved at least twice to remove traces of DEPC. Millipore water obtained at 18.2MΩ could also be used.
- **Others:** Chloroform-isoamylalcohol (Chl:Iaa) (24:1 [v/v]), 7.5M Lithium chloride (LiCl), 70% (v/v) Ethyl alcohol.

### 3.2.1.3 Using QIAGEN RNeasy Plant RNA Extraction Kit (Germany)

(i) Plant tissue (100mg) was ground to fine powder in liquid nitrogen and transferred to a 1.5ml centrifuge tube containing 100µl of extraction (Foissac et al., 2001).

(ii) RLT buffer was added to the plant extract in the extraction buffer and it was vortexed vigorously. Incubation of 3min at 55°C was given to disrupt the tissue.
(iii) Lysate was then transferred to QIAshredder spin column (lilac) placed in 2ml collection tube, and was centrifuged for 2min at full speed. Supernatant was carefully transferred to a new microfuge tube. This supernatant was used in the subsequent steps.

(iv) About 0.5 volume ethanol (96-100%) of was added to the cleared lysate and mixed by pipetting. Sample was then transferred to an RNeasy spin column (pink) placed in 2ml collection tube and was centrifuged for 15sec at 8000 x g. The flow through was discarded.

(v) 700μl of buffer RW1 was added to RNeasy spin column and was centrifuged for 15sec at 8000 x g to wash the spin column membrane. The flow through was discarded.

(vi) 500μl of Buffer RPE was added to the RNeasy spin column and was centrifuged for 15sec at 8000 x g to wash the spin column membrane. Flow through was discarded.

(vii) Again washing with buffer RPE was given to the RNeasy spin column for 2min at 8000xg. An empty spin was given for 1min at full speed.

(viii) Spin column were then placed in a new 1.5ml collection tube and 50μl of RNase free water was added directly to spin column membrane and was centrifuged for 1min at 8000 x g to elute the RNA.

**Reagents:**

Extraction buffer (Foissac et al., 2001) was made fresh and rest of the components (RLT, RPE, RW1 buffers, columns and collection tubes) were provided in the kit.

### 3.2.2 Primer Designing

Various primer pairs were designed for the amplification of different genes of ACLSV. For that, GeneBank available complete genome sequences of ACLSV reported from different parts of the world and from different hosts were downloaded from the NCBI data base (web site: [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). These sequences were then aligned with either MULTIALIN program (web site: http://prodes.toulouse.inra.fr/multialin/multialin.html) based on algorithm as reported by Corpet (1988) or by CLUSTALW program (web site: [http://www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)) based on the algorithm as described by Higgins et al. (1994). Based on the conserved regions identified at 5’ and 3’ regions of CP, MP and
replicase in the aligned sequences the primer pairs were designed keeping in mind the average GC content, sequence variations and the annealing temperature of the primer pairs. Degenerate primers for amplification of complete coat protein gene of ACLSV were designed. Other virus specific degenerate primers for genome characterization of Indian strain of ACLSV were also designed taking in account a little variability in earlier submitted sequences of the complete genomes of ACLSV. Primer designing softwares oligocal (Kibbe, 2007) was used to check the properties of the designed primers. The gene sequence of primer pairs were submitted to EMBL database. The designed primers were synthesized from Genei (India) and Sigma genosys (India).

3.2.2 First Strand cDNA Synthesis
Reverse transcription was performed in 0.2ml thin walled tubes using the total RNA as the template to obtain first strand cDNA. Detection primers available in literature were used for initial identification of the virus. The primers were also synthesized from Genei (India) and Sigma genosys (India). A 25µl reverse transcription (RT) reaction with 1-2µg of total RNA was mixed with 5µl of 5X RT buffer (reverse transcription buffer), 1.5µl of 40mM dNTP mix, 0.4 µg of downstream (reverse) primer, 20 units of RNase inhibitor and 200 units of M-MLV RT (USB Corp., USA) was set up. The reaction mixture was incubated at 37°C for 75 min and reverse transcriptase enzyme was inactivated by incubating the mixture at 70°C for 5 min. The longer cDNA fragments were generated using Protoscript® AMV Long Amp™ Taq RT-PCR Kit (USA). Total RNA (1-2µg) and antisense (reverse) primer (0.2-0.4 µg) were denatured at 70°C for 5min and immediately placed on ice. To this 10µl AMV Reaction Mix and 2µl of AMV Enzyme mix (RT) was added. Final volume was made upto 25µl. The reaction mixture was incubated at 42°C for 1hr and reverse transcriptase enzyme was inactivated by incubating the mixture at 80°C for 5 min as per manufacturer’s instructions.

3.2.3 PCR Amplification
PCR was also carried out in the automated Thermocycler 9700 (Applied Biosystems, USA) with 50 µl of total reaction mixture containing 7µl of cDNA product, 1µl (0.2µg) of
upstream (forward) primer, 1µl (0.2µg) of downstream (reverse) primer, 5µl of 10X Taq DNA polymerase assay buffer with 15mM MgCl₂, 3µl of 10mM dNTP mix and 0.5µl of (3 units/µl) of Taq DNA polymerase (Genei, India). For the amplification of longer fragments Protoscript AMV Long Amp Taq RT-PCR Kit (USA) was used with ACLSV specific primers as per manufacturer’s instructions. The patterns of thermal cycling for the
Table 3.3 Primers used for initial screening (detection) for various viruses and viroids

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer sequence in 5’ to 3’ orientation</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLSV</td>
<td>U TTCATGGAAAGACAGGGGCAA</td>
<td>~677bp</td>
<td>Menzel et al., 2002</td>
</tr>
<tr>
<td></td>
<td>D AAGTCTACAGGCTATTTATTATAAGTC TAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASPV</td>
<td>U ATGTCTGGAAACCTCATGCTGCAA</td>
<td>~370bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D TTGGATCAACTTTACTAAAAAAGCAT A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApMV</td>
<td>U CTAACAAATCTTCATCGATAAG</td>
<td>~700bp</td>
<td>Choi et al., 2003</td>
</tr>
<tr>
<td></td>
<td>D TCAACATGGTCTGCAAGTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNRSV</td>
<td>U AACTGCAGATGGTTTGCCGAATTTGCA A</td>
<td>~675bp</td>
<td>Kulshrestha, 2005</td>
</tr>
<tr>
<td></td>
<td>D GCTCTAGACTAGATCTCAAGCAGGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASGV</td>
<td>U CTG CAA GAC CGC GAC CAA GTT T</td>
<td>~755bp</td>
<td>Mackenzie et al., 1997</td>
</tr>
<tr>
<td></td>
<td>D ATG AGT TTG GAA GAC GTG CTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASSVd/</td>
<td>U TCGTCGTCAGCAAGGG</td>
<td>~267/261/252 bp</td>
<td>Faggioli and Ragozzino, 2002</td>
</tr>
<tr>
<td>PBCVd/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADFVd</td>
<td>D GAGCACCACAGGAACCTCACGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

U – Upstream Primer; D – Downstream Primer
Table 3.4 Thermal cycling pattern of Viruses/ Viroids tested

<table>
<thead>
<tr>
<th>Virus</th>
<th>Thermal Cycling Pattern</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACLSV</td>
<td>Initial denaturation for 2 min at 94°C and 30 cycles consisting of 94°C for 1 min, annealing temperature of 63°C for 1 min and elongation at 72°C for 1 min. Final elongation for 10 min at 72°C.</td>
<td>Menzel et al., 2002</td>
</tr>
<tr>
<td>ASPV</td>
<td>Initial denaturation for 2 min at 94°C and 30 cycles consisting of 94°C for 30 sec, annealing temperature of 61°C for 1 min and elongation at 72°C for 1 min. Final elongation for 10 min at 72°C.</td>
<td>Choi et al., 2003</td>
</tr>
<tr>
<td>ApMV</td>
<td>Initial denaturation for 2 min at 94°C and 30 cycles consisting of 94°C for 1 min, annealing temperature of 62°C for 1 min and elongation at 72°C for 1 min. Final elongation for 6 min at 72°C</td>
<td>Mackenzie et al., 1997</td>
</tr>
<tr>
<td>ASGV</td>
<td>Initial denaturation for 5 min at 94°C and 35 cycles consisting of 94°C for 1 min, annealing temperature of 57°C for 1 min and elongation at 72°C for 1 min. Final elongation for 10 min at 72°C</td>
<td>Kulshrestha, 2005</td>
</tr>
<tr>
<td>PNRSV</td>
<td>Initial denaturation for 1 min at 94°C and 30 cycles consisting of 94°C for 30 sec, annealing temperature of 60°C for 30 sec and elongation at 72°C for 30 sec. Final elongation for 1 min at 72°C</td>
<td>Faggioli and Ragozzino, 2002</td>
</tr>
<tr>
<td>ASSVd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
amplification of various viruses and viroids are summarized in Table 3.3. The PCR conditions for various isolates and genome parts was also standardized.

**Components:**

**M-MLV Reverse Transcription Buffer (1X):** 50mM Tris-HCl, pH8.3; 75mM KCl; 3mM MgCl₂ and 10 mM DTT.

**Taq DNA Polymerase buffer (1X):** 10 mM 3-[[tris(hydroxymethyl) methyl] amino] propane sulfonic acid (TAPS), pH 8.8; 15 mM MgCl₂; 50 mM KCl and 0.01% gelatin.

**Protoscript® AMV Long Amp™ Taq RT-PCR Kit, New England Biolabs (USA):**

The kit was supplied with AMV Enzyme mix (10X), AMV Reaction Mix (2X), LongAmp Taq 2X Master Mix.

### 3.2.4 Agarose Gel Electrophoresis

The gel electrophoresis was carried out in a submarine horizontal electrophoresis unit as described by Sambrook *et al.* (1989). Appropriate amount of agarose (0.8-1.0%, as per requirement) was dissolved in 1X TAE or 0.5X TBE buffer by boiling to dissolve completely. This agarose was cooled to 50°C and casted in an appropriate gel casting tray using a slot-forming comb. After the agarose had solidified, the comb was removed and the gel was placed in the gel tank containing electrophoresis buffer. PCR products (10µl) were properly mixed with 3µl of gel loading dye and loaded onto the wells of the gel. A known DNA ladder was also loaded to compare the size of the PCR product. Electrophoresis was carried out at a constant voltage of 50 V/cm.

After electrophoresis, the gel was stained with ethidium bromide (1.0µg/ml) and visualized on an UV Trans-illuminator. Gel Documentation system (Alpha Digi Doc system, USA) was used to photograph the gel.

**Reagents:**

**Sample loading dye (6X):** 15% Ficoll 400; 0.25% Bromophenol blue and Xylene cyanol

The dye was stored at room temperature.

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

**TAE (50X per liter):** 242g Tris base; 57.1ml glacial acetic acid and 100ml EDTA (0.5 M, pH 8.0).
3.2.5 Cloning of Amplified DNA Fragments

3.2.5.1 Isolation of PCR Amplified DNA Fragment from Agarose Gel
After visualization of the desired amplicons on gel the band was cut. DNA was eluted from agarose gel using the gel extraction kits from Sigma (India) and AuPrep (Life technology Ltd., India) as described by the manufacturer’s instructions. The eluted product was checked on gel and also quantified using a NanoDrop® ND 1000 (Nano Drop technologies Inc., USA).

3.2.5.2 Ligation of the PCR Amplified DNA Fragments into Suitable Cloning Vector
Ligation of gel eluted PCR amplified DNA was performed either in pGEM-T easy vector system (Promega, USA). Appropriate ratio of vector and insert (usually equimolar) was mixed and ligation was set up at 22°C for 1hr or 16°C (or 4°C) for overnight. The components were given in Table 3.5

Table 3.6 Various components of reaction mix for ligation

<table>
<thead>
<tr>
<th>Components</th>
<th>pGEM-Teasy vector system</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (vector + insert)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>Ligation Buffer</td>
<td>5.0 µl (from 2X stock)</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1.0 µl (3 Weiss units)</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>10µl</td>
</tr>
</tbody>
</table>

3.2.5.3 Preparation of Competent Cell
Competent cells can be prepared either freshly for each use or can be premade and stored at minus 70°C. For the transformation experiments DH5α, JM101, JM107 or XL1-Blue (tetR) strains of *Escherichia coli* (*E. coli*) were used. Single colony of the appropriate strain of the bacteria was streaked on to Luria agar (LA) plate with appropriate antibiotics and incubated at 37°C for 12-16hrs. These freshly grown colonies of *E. coli* cells were used for preparation of competent cells. A single colony was grown overnight at 37°C in Luria broth (LB) and a part of it was used to
inoculate larger volume of the medium. During the preparation of competent cells all the solutions, pipette tips were chilled and cells always kept on ice.

3.2.5.3.1 Competent Cells Which Can Be Stored At -70°C:
Luria broth (LB, 200ml) was inoculated with 1ml of the overnight culture of *E. coli* and incubated at 37°C on a rotary shaker till OD at 600 nm was around 0.5. Culture was cooled on ice immediately and cells were harvested by centrifugation at 6000 rpm for 5min at 4°C. Supernatant was removed carefully and traces of it were removed by inverting the centrifuge tube over paper towels. However, the tube was never removed from ice for a longer time. The pelleted bacterial cells were resuspended in 50-70 ml of ice-cold 0.1 M CaCl₂ and incubated on ice for 30min. Cells were recovered by centrifugation as above, and resuspended in 50-70 ml of ice-cold 0.1 M MgCl₂ and further incubated on ice for 30min. The cells were finally recovered by centrifugation and suspended in 10 ml of 0.1 M CaCl₂ containing 10% glycerol. Aliquots of 200μl each were prepared and stored at −70°C. For transformation, the cells were taken out from the deep freezer, thawed on ice for 15min and the DNA to be transformed was added to it.

3.2.5.3.2 Competent Cells Which Can Only Be Used Fresh Each Time:
Luria broth (50ml) was inoculated with 50μl of overnight grown culture of *E. coli DH5α* or XL1-Blue cells and was allowed to grow at 37°C on a rotary shaker till OD at 600 nm was around 0.5. Culture was cooled on ice and bacterial cells were pelleted down by centrifugation at 5000 rpm for 5min 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 15min. Cells were recovered by centrifugation as above. Cell pellet was then resuspended in 2ml of ice-cold 0.1 M CaCl₂ and aliquots of 200μl each were prepared to be used for a single transformation reaction. These cells could be stored on ice for not more than 12-16 hrs. This treatment results in increase of competence as well.

3.2.5.3.3 Ultra Competent Cells:
Ultra competent cells were also prepared for the transformation. Bacteria (*E. coli* DH 5 α overnight grown culture) was inoculated in 200 ml SOB medium and grown at
18°C. At OD close to 0.45, cells were split in to four 50 ml tubes and placed on ice for 10 minutes. After that a spin at 2500g for 10 minutes was given. Supernatant was discarded, and pellet was resuspended in 64 ml HTB (16ml each for each tube) and kept on ice for 10 minutes. Again spin was given as before, supernatant discarded, resuspended in 16 ml (each tube 4 ml) HTB and pooled in to one tube. Slowly added filter sterilized DMSO while gently swirling and aliquot of 100µl were made. These cells were also stored at -70ºC.

Reagents:

SOB: 20g bacto tryptone, 5g yeast extract, 0.5g NaCl (Dissolve in 900 ml water, added 10 ml 250mM KCl, adjusted pH to7 with NaOH (1N), autoclaved and added 5ml of 2M MgCl₂.

HTB: 10mM HEPES, 15mM CaCl₂.2 H₂O, 250mM KCl, dissolved in water, adjusted pH to 6.7 with KOH (1N), added 55mM MnCl₂, filter sterilized and stored at 4ºC.

3.2.5.4 Transformation of ligated product

Frozen competent cells were thawed on ice for 5-15min or 200 µl of freshly prepared cells were taken for transformation. Ligated product was mixed with competent cells and incubated on ice for 30min. The cells were given heat shock at 42°C for 90sec and immediately transferred to ice for at least 5min. To this, 800 µl of LB (without any antibiotic) was added and the tube was incubated at 37°C for 1hr in a rotary shaker set at 200 rpm. The cells were pelleted down (5000rpm/room temperature/5min) and then resuspended in 100-250 µl of LB. Transformed cells were plated on LB agar plate, supplemented with appropriate antibiotics (Ampicillin for pGEM-Teasy vector) and IPTG/X-gal in appropriate amounts for putatively selecting the recombinant clones. Cells were spread uniformly with autoclaved glass beads/disposable sterilized L-spreaders and the plates were incubated at 37°C for 12-16hrs till colonies are big enough for transfer. Colonies which turned blue were left out while the colonies which remained white (recombinant clones) were transferred on to a fresh plate containing appropriate antibiotics.

Reagents:
**Materials and Methods**

**X-gal/IPTG:** For each plate containing 20-25ml media, 40μl of X-gal (stock 20 mg/ml in dimethyl formamide (DMF) or Dimethyl sulphoxide (DMSO); light sensitive) and 10μl of IPTG (stock 20 mg/ml in water) were plated 15min before cell plating.

**Concentration of the antibiotics used:** Ampicillin 100mg/ml dissolved in water and filter sterilized.

**3.2.5.5 Selection of Recombinant Clones**

After blue white selection of bacterial colonies, small scale preparation of plasmid DNA from 1-5 white recombinant *E. coli* clones was done to confirm the identity of recombinant clone by restriction digestion.

**3.2.5.5.1 Alkaline Lysis Method:**

In alkaline lysis method as detailed by Bimboim and Doly (1979) and Sambrook *et al.* (1989) five supposedly recombinant bacterial colonies were inoculated in five glass test tubes each containing 5ml sterile LB medium (with appropriate antibiotics) and grown overnight at 37°C. From this overnight grown culture 1.5-2ml was pelleted down by centrifugation at 14,000 rpm in a microfuge for 30sec. Supernatant was discarded completely by using a micropipette. The pellet was resuspended in 100μl GTE and kept on ice. Two volumes (200μl) of 0.2 N NaOH / 1% SDS (freshly prepared) was added to the resuspended cells and mixed gently (avoiding vortexing) followed by incubation on ice for 5min for lysis. To these lysed cells, 150μl of ice cold 3 M potassium acetate, pH 4.8 was added, mixed rapidly and incubated on ice for 15min. The tube was then centrifuged at 14,000 rpm in a microfuge for 10min. The supernatant was transferred to a fresh tube and extracted with one volume of phenol and centrifuged at 14,000rpm in a microfuge for 5min. The DNA was then extracted with phenol: chloroform (1:1 v/v) and centrifuged as above for 5min. Finally the DNA was extracted with 1 volume of chloroform: isoamyl alcohol (24:1 v/v) and centrifuged for 5min. The DNA was finally precipitated with 2.5 volumes of ice cold absolute alcohol, incubated at -20°C for 1hr and centrifuged in a microfuge (12,500 rpm for 20min at 4°C) to precipitate the DNA. Finally DNA pellet was washed with cold 80% alcohol and air dried. It was then resuspended in 50-100 μl sterile distilled water.
Materials and Methods

Reagents:

**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.

**3.2.5.5.2 Boiling Miniprep Method:**

For boiling miniprep method (Holmes and Quingley, 1981; Sambrook *et al.*, 1989) overnight grown cultures (1.5 ml) from five supposedly recombinant bacterial colonies was pelleted down in a microfuge by centrifugation at 14,000 rpm for 30sec. The supernatant was discarded and the pelleted cells were resuspended in 110 µl of STET buffer. To the resuspended cells, 10-12 µl of freshly prepared lysozyme (in 10mM Tris HCl, pH 8.0) was added to a final concentration of 0.5-1 mg/ml. The tubes were kept at room temperature for 5min for lysis of cells then incubated in boiling water bath for 30-50sec. The suspension was centrifuged at full speed (14,000 rpm) in a microfuge for 20min. Pellet containing the cell debris was removed with the help of a sterile toothpick. DNA in the supernatant was precipitated by adding one volume of iso-propanol, mixing thoroughly. Precipitated DNA was collected immediately by centrifuging for 20min at full speed (14,000 rpm). The supernatant was removed completely and the DNA pellet was air dried to remove traces of iso-propanol. DNA in the pellet was dissolved in 100-200 µl of sterile water and used as such for restriction digestion.

Reagents:

**STET Buffer:** 0.1M NaCl, 10 mM Tris HCl (pH 8) and 1mM EDTA (pH 8). Added 5% Triton X-100 after autoclaving rest of the components in solution.

**3.2.5.5.3 Digestion of plasmid DNA using restriction enzymes:**

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>10 units</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1 µg</td>
</tr>
<tr>
<td>water to</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
The above reaction mix was incubated at the temperature as recommended by the supplier of the enzyme for 3hrs. The digested DNA was then precipitated with 2.5 volumes of absolute ethanol and incubated at −20°C for overnight or at -80 for 1 hr. The DNA was collected by centrifugation at 12,500 rpm at 4°C for 20min in a microfuge, washed with 80% ethanol and suspended in 100µl of sterile water after vacuum drying. It was re-extracted successively with phenol-chloroform and chloroform and then re-precipitated with ethanol as above, before finally suspending it in 30µl of sterile water. This digestion was also sometimes directly loaded on to the agarose gel and analyzed for desired insert.

3.2.6 Purification of Plasmid DNA for Sequencing

Sequencing requires ultra clean DNA and therefore the DNA prepared by alkaline lysis was further purified by either by precipitation with PEG 8000 or by using commercially available GenElute Plasmid isolation kit (Sigma, India).

3.2.6.1 Purification of Plasmid DNA by Polyethylene Glycol

The isolated plasmid (from alkaline lysis or boiling prep) was mixed with DNase free RNase and incubated at 37°C for 1 hr. It was then extracted twice with one volume of chloroform to remove RNase bound to DNA. The DNA was then supplemented with 0.1 volume of 3 M sodium acetate (pH 4.8), mixed with one volume of iso-propanol and immediately centrifuged (14,000 rpm) in a microfuge for 10min. The pellet containing the DNA was washed with 80% alcohol, air dried and resuspended in 32µl of sterile water. To this, 8µl of 4 M sodium chloride and 40µl of 13% PEG 8000 were added and incubated on ice for 20min. The DNA was then pelleted down by centrifugation at 12,500 rpm in a microfuge for 20min at 4°C, washed with 80% alcohol twice, air dried and after dissolving in 50µl sterile water was used directly for sequencing.

3.2.6.1 Plasmid Isolation Using Kit

For high purity plasmid isolation, GenElute Plasmid isolation kit (Sigma, India) was used strictly as described by the manufacturer’s instruction.

3.2.7 Automated DNA Sequencing
Sequencing of the amplified and suitably cloned viral DNA was carried out using automated DNA sequencing system (ABI PRISM® 3130xl Genetic Analyzer, USA), using ABI prism Big Dye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) in accordance to Sanger’s dideoxy chain termination method (Sanger et al., 1977).
3.2.7.1 **Sequencing Reaction**

The reaction was set up according to the manufacturer’s instruction as follows:

- Terminator ready reaction mix 1.0 µl
- (containing Ampli Taq DNA Polymerase-FS)
- Sequencing Buffer (5X) 1 µl
- PCR amplified DNA fragment/vector 200-500 ng
- Primer T₇/SP₆ 1.66 pmol (~0.5 µl)

Volume of reaction mix was adjusted to 5 µl with H₂O. The thin walled PCR tubes (0.2 ml) containing the reaction mixes were placed in GeneAmp PCR system 9700 (Applied Biosystems, USA) and thermal cycling was carried out with initial denaturation of 1 min at 95°C followed by 30 cycles of:

- Denaturation 95°C 10 sec
- Annealing 50°C 40 sec
- Amplification 60°C 4 min

At the completion of above reaction, the volume of reaction mix was raised to 100 µl with water. To this 10µl sodium acetate (3M, pH 4.6) and 250µl ethanol were added and centrifuged at maximum speed for 20min. The pellet thus obtained was washed twice with 250µl of 70% ethanol and air dried. The pellet was dissolved in 15µl of template suppression reagent (TSR; Applied Biosystems, USA), thereafter it was denatured by heating for 4min at 95°C and placed on ice before loading in the sequencer. Another method to prepare sequencing PCR products for sequencing involved using Montage Seq Reaction Clean up Kit (Millipore, USA). About 25µl of injection solution was added to the PCR tubes and mixed by pippeting 20-25 times. This mix was loaded on to Millipore filter/wash plate and vacuum of about 15-20 mbar was applied for 5min to remove unused dNTPs and primers. A washing with 25µl injection solution was again given by pippeting as mentioned earlier on the filter plate and vacuum was re applied for 5min. Finally 25µl of injection solution was added to the plate, mixed by pippeting, sucked out from the filter plate and loaded to the 96 well optical sequencing plate/ capillary plate (Applied Biosystems, USA).
Components:
Injection solution and sequencing plates were supplied with the ABI prism Big Dye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA).

3.2.7.2 Gel Electrophoresis and Sequence Determination
Before loading the samples, the background fluorescence was measured and scan area was cleaned to get a uniform base line for all the sample lines. Then the samples were loaded and analyzed in automated sequencer.

3.3 PURIFICATION AND CHARACTERIZATION OF ACLSV FROM IN VITRO PURE CULTURE
3.3.1 Purification of ACLSV
ACLSV was purified as described by German-Retana et al. (1997).
(i) Young infected leaves of *C. quinoa*, tissue culture maintained ACLSV positive apple plantlets and flowers of Himalayan wild cherry showing severe symptoms were homogenized with three volumes of ice cold buffer B in a blender.
(ii) The homogenate was strained through a cheese cloth and clarified by adding bentonite suspension in steps. Initially 10mg bentonite per gram of leaves was added.
(iii) The homogenate was mixed and kept at 4°C for 10min and centrifuged at 1400 g for 5min at 4°C.
(iv) The supernatant was retained and bentonite at a concentration of 5mg/g of leaves was again mixed. Step (iii) was repeated till supernatant became straw yellow coloured and pellet grayish, each time using decreasing amount of bentonite.
(v) The virus was finally precipitated by adding PEG (M. wt. 6000) to 8 % volume of the clarified extract. The solution was gently stirred at 4°C till PEG was completely dissolved. The suspension was held for 1hr at 4°C and then centrifuged at 12,000g for 30min.
(vi) The pellet was then resuspended in maximum of 3ml of buffer C. Sucrose gradients 10-40% were prepared from two sucrose stocks and viral suspension was loaded directly onto six gradients (500μl/ gradient) and ultracentrifuged for 2.5hrs at 300,000g in a SW 41 rotor in Sorvall Ultra centrifuge (USA).
Gradient tubes were scanned in ultra violet light and UV absorbing region corresponding to the viral fraction was collected.

**Reagents:**

**Bentonite Suspension:** As per Lister and Hadidi (1971) protocol.

(i) 10g bentonite (Sigma, India) in 200ml buffer A was mixed in an electric blender.

(ii) Centrifuged for 3min at 600g and discarded the pellet. Centrifuged the supernatant at 5500g for 15min. Discarded supernatant, resuspended the pellet in 100ml buffer A.

(iii) The suspension was stored overnight at 4°C. Next day repeated step (ii) but the last pellet obtained was resuspended in 50ml buffer A. To confirm the concentration 1ml of the suspension was evaporated and bentonite obtained was weighed.

**Buffer A:** 10mM Tris HCl pH 7.5 (autoclaved)

**Buffer B:** 10mM Tris HCl, 0.5mM MgCl₂, 0.2 % 3-3′ diaminodi-propyl amine (Sigma), USA). The pH was adjuted to 7.8 after addition of polyamines.

**Buffer C:** 10mM Tris HCl, 0.5mM MgCl₂ pH 7.8 (autoclaved)

10 % Sucrose: 10g Sucrose (Molecular Biology grade) in 100ml of buffer C.

40 % Sucrose: 40g Sucrose (Molecular Biology grade) in 100ml of buffer C. The sucrose solutions are used for gradient preparation, are aliquoted and stored frozen.

### 3.3.2 Characterization of the Virus

#### 3.3.2.1 UV Absorption Spectrum

The purified virus preparations obtained after purification were screened in Specord 200 Spectrophotometer (Analytik jena, Germany). Absorbance (A) of samples was recorded in UV range (200 to 300 nm; 254nm) and the values of A<sub>max</sub>, A<sub>min</sub> and A<sub>260/280</sub> were calculated to find out the quantity and purity of nucleoprotein particles. Spectral curves (absorbance vs. wavelength) were recorded using an automated recorder. 2.5 U of OD<sub>254</sub> corresponds to a viral concentration of 1mg/ml.

#### 3.3.2.2 Electron Microscopy

The Carbon-Formvar coated grid was overlayed with 1µl of 1:1000 times diluted commercial ACLSV antibodies. The grid was incubated at 37°C for 15min under moist conditions. The antibody dilution buffer was siphoned out from the grid using thin strips of filter paper leaving only the antibodies on the grid. The purified virus
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preparation (1µl) was mixed with equal volume of 2% uranyl acetate (fixative and negative stain) on a parafilm strip and transferred to the grid. The grid was again incubated at 37°C for 10-15min under moist conditions. The stain and additional water was removed from the grid using thin strips of filter paper. Three to five washings with sterile water were given to remove excess stain. The grid was incubated at room temperature under moist conditions for 5min. The grid was loaded onto the gun and into the electron microscope () for examination. Mean lengths and widths of the particles were determined using a procedure similar to that described by Monette and James (1990). Between 12 and 26 particles were measured to obtain the average size.

3.3.2.3 DAS-ELISA
To characterize the virus, the sap extracted from propagating host, different concentrations of partially purified preparation of virus was used for DAS-ELISA as described earlier.

3.3.2.3 Protein Analysis by Sodium Dodecyl Sulphate - Polyacrylamide Gel electrophoresis (SDS-PAGE)
Molecular weights of protein subunits were determined by SDS-PAGE following the method as described by Maizel (1971).

3.3.2.3.1 Preparation of Polyacrylamide Gel (PAGE):
Resolving gel (7.5-15%) and stacking gel (4-5%) were used to standardize the best separation. Above said amount of components were mixed for both resolving and stacking gel (excluding APS and TEMED) solutions separately in a properly covered flask and the solutions were degassed for 15-20min in vacuum desiccators. After degassing, APS and TEMED were mixed properly to the resolving gel solution and immediately poured onto the gel assembly, such that 2/3rd of the assembly should be filled, the solution was over layered with 500 µl of water saturated butanol to check aeration. It was then kept for 1-2 hr at room temperature for proper polymerization. After polymerization butanol was removed completely, APS and TEMED were then mixed to the stacking gel solution and over layered above the resolving gel. A comb of 10-13 wells was fitted in the assembly to make the wells such that 20-50µl of preparations can be loaded in the wells. The assembly was again kept for 1-2 hr at
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room temperature for proper polymerization. After the polymerization the gel assembly was placed in the running tank filled with 1X gel running buffer in both top and bottom chambers. Then the comb was removed from the stacking gel carefully and wells were washed with the running buffer properly. Compositions of resolving and stacking gel are given below:

**Reagents:**

**Acrylamide/Bisacrylamide stock:** Added 30.0 g acrylamide and 0.8 g bis acrylamide in 50 ml sterile water and stirred to dissolve completely. Made the final volume to 100 ml with water, filtered using Whatmann 3 MM and kept at 4°C in an amber coloured bottle.

**Resolving gel buffer:** 1.5 M Tris-HCl, pH 8.8 (dissolved 18.15 g Tris-base in 50 ml sterile water, adjusted the pH to 8.8 with 1 N HCl and made final volume to 100 ml). The buffer was autoclaved and stored at 4°C.

**Stacking gel buffer:** 0.5 M Tris-HCl, pH 6.8 (dissolved 6.0 g Tris-base in 50 ml sterile water, adjusted the pH to 6.8 with 1 N HCl and made final volume to 100 ml. Autoclaved and stored at 4°C.

**10 % SDS Stock solution:** Dissolved 10g sodium dodecyl sulphate in water with gentle stirring, made the final volume to 100 ml and keep it at room temperature.

**10% Ammonium persulphate (APS):** Dissolved 100 mg APS in 1 ml sterile water in foil covered collection tube (Prepared fresh and added to the gel solution just before pouring). **5X Electrode buffer (Gel running buffer):** Dissolved 15.0 g Tris-base, 72 g glycine and 5 g SDS in 800ml sterile water, adjusted the pH to 8.3 and made the final volume to 1000ml. Kept at room temperature.

**Resolving (Separating) gel compositions (ml)**

<table>
<thead>
<tr>
<th></th>
<th>7.5%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
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<tr>
<td>Water</td>
<td>14.54</td>
<td>12.05</td>
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<td>7.5</td>
<td>7.5</td>
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<td>7.5</td>
</tr>
<tr>
<td>10% SDS Stock</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Acrylamide/Bisacrylamide stock</td>
<td>6.15</td>
<td>10.0</td>
<td>12.0</td>
<td>15.0</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.150</td>
<td>0.150</td>
<td>0.150</td>
<td>0.150</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>Total solution</td>
<td>30 ml</td>
<td>30 ml</td>
<td>30 ml</td>
<td>30 ml</td>
</tr>
</tbody>
</table>
### Materials and Methods

#### Stacking (Spacer) Gel Composition (ml)

<table>
<thead>
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<th></th>
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<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>6.1</td>
<td>5.68</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>10% SDS Stock</td>
<td>0.100</td>
<td>0.100</td>
</tr>
<tr>
<td>Acrylamide stock</td>
<td>1.33</td>
<td>1.66</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.050</td>
<td>0.050</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>Total Solution</strong></td>
<td><strong>10 ml</strong></td>
<td><strong>10 ml</strong></td>
</tr>
</tbody>
</table>

3.3.2.3.2 **Protein Sample Preparation:**

Purified virus samples were dissolved in the sample buffer [1:4 (v/v)] and were incubated in boiling water bath for 5 min and immediately chilled on ice prior to loading on the gel.

**Reagents:**

**Sample buffer:** Mixed 1.0 ml of stacking gel buffer, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of 2-mercaptoethanol and 0.2 ml of Bromophenol blue (from 0.05% Bromophenol blue stock) in 4 ml of sterile water.

3.3.2.3.3 **Electrophoresis:**

The wells in the gel were rinsed thoroughly before loading the samples. The denatured proteins (10-15 µl) were loaded into the wells. Optimal concentration (3µl) of pre-stained protein markers (Fermentas, Lithuania) was also loaded into the wells. Electrophoresis was carried out in 1X electrode buffer at 25 mA in stacking gel and 35 mA in resolving gel until the bromophenol blue dye reached the bottom of the gel.

3.3.2.3.4 **Staining the Gel:**

3.3.2.3.4.1 **Coomassie Staining**

After completion of electrophoresis, gel was carefully removed from the glass plates and placed in 100 ml fixing solution (methanol:glacial acetic acid:water [50:10:40]) for 30 min with continuous gentle shaking. The Coomassie Brilliant Blue R-250 (0.12%) stain was prepared by adding 0.12 g Coomassie Brilliant Blue R-250 to 600 µl glacial acetic acid (mixed separately) to 100 ml of fixing solution. Staining was
carried out for 40 min to 1 hr with continuous gentle shaking. Gel was destained in solution containing methanol: glacial acetic acid: water (45:10:45) until the protein bands were clearly visible and the background was negligible.

3.3.2.3.4.2 Silver Staining

After the run was completed, gel was soaked in 50% methanol for 1 hr in a glass dish. The gel was then rinsed in deionised water for 10 minutes. Staining solution was prepared by adding solution A dropwise to Solution B with constant stirring in a covered flask. To this muddy coloured solution obtained by adding solutions A and B drop wise added concentrated ammonia till the solution becomes colourless. Then made the volume to 100 ml with deionised water (this silver stain was prepared immediately before use). The gel was stained in this solution for 15-30 min with gentle agitation on a gel rocker in dark. Then, gel was washed with deionized water for 10 min (twice) with gentle agitation. The gel was then soaked in developing solution until band appeared. After proper band development the gel was washed with deionised water and placed in 50% methanol to stop further reaction.

Reagents:

Solution A: 0.8 g silver nitrate in 4 ml distilled water.

Solution B: Mix 21 ml of 0.36% NaOH

Developing solution: Mix 2.5 ml of 1% citric acid with 0.25 ml of 37% formaldehyde and make the volume to 250 ml (freshly prepared).

3.3.2.4 Western Blotting

The western blots were prepared by transferring protein bands from SDS-polyacrylamide gel to Immun-Blot® PVDF membrane (BioRad, USA) in the T-70 Semi Dry Blot assembly (Amersham Biosciences, USA).

3.3.2.4.1 Preparation of Blot:

After electrophoresis, the gel was soaked in transfer buffer for 15 min at room temperature. PVDF (polyvinylidene difluoride, 0.2 micron) membrane was cut to the size of the gel and treated with 100% methanol for 5 min and then transferred to the transfer buffer (Towbin et al., 1979) for equilibration. After complete equilibration the blot could easily be submerged in any aqueous solution. The gel and membrane were sandwiched between the presoaked Whatman 3MM filter paper and pads.
provided with the apparatus. The sandwich was placed in blot assembly containing transfer buffer in such a way that membrane faced towards anode and the transfer was carried out at 400 mA at 4°C for 1-3 hr.

3.3.2.4.2 Probing of Western Blots with Antibodies:
Western blots were probed with monospecific polyclonal antibodies raised against the ACLSV as follows:

(i) After transfer of the protein on the PVDF membrane, it was washed in the transfer/TBS buffer and then incubated in the blocking solution for 2 hrs at room temperature with continuous shaking.

(ii) The membrane was removed from blocking solution and transferred to 1X PBS solution containing the primary antibody (1:500 dilution) and 0.5% BSA. The membrane was incubated at room temperature for 2 hrs with continuous shaking.

(iii) The blots were washed thrice with washing buffer (PBST) for 5 min each.

(iv) Anti-rabbit Horseradish peroxidase (HRP) conjugated IgG (Roche, USA) was diluted 1:1000 times in ECI buffer and the blots were incubated in this solution for 2 hrs at room temperature with continuous shaking.

(v) The blots were washed again as described in step (iii) to remove excess of conjugated antibody.

(vi) Blots were then covered by cling film to completely disperse the substrate on the blot and incubate at room temperature for 5 min. The blots were then exposed to the x-ray film for 1 min and the film was finally developed.

Reagents:
**Tris-buffered saline (TBS):** 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, with 2% powder milk or 2% bovine serum albumin, and 10 μl Tween-20 (Sigma).

**Transfer buffer (Towbin buffer):** 25mM Tris-HCl (3.03 g/l), 192 M glycine (14.4g/l) and 20% methanol (v/v; 200ml/l). No acid and base was added to adjust pH. The buffer pH ranged from 8.1-8.5.

**Blocking solution:** 50 mM phosphate buffered saline, pH 7.0 containing 0.5% BSA.

3.3.2.5 Molecular Characterization

3.3.2.5.1 Extraction of Viral Nucleic Acid:
About 1 mg purified virus suspension (10 mg/ml) was taken in a volume of 100μl and incubated in the presence of proteinase K (200ng/μl) and 0.5 % SDS for 15min
at 50°C. The suspension was then extracted with equal volume of phenol, once with phenol: chloroform: isoamyl alcohol mix (25:24:1, v/v/v) and then with equal volume of chloroform: isoamyl alcohol (24:1, v/v). The aqueous phase was ethanol precipitated in presence of 0.1 volume of 3M sodium acetate, pH 5.3 and 2.5 volumes of 96 % ethanol at -20°C for atleast 1hr. Viral RNA was recovered by centrifugation at 15,000g for 20min at 4°C. The pellet was washed twice with 70% ethanol, followed by drying at room temperature and suspended in 10-30µl of DEPC treated water/TE. The suspended RNA was stored in aliquots of 10µl each at -70°C for further use.

**Reagents:**

**Proteinase K**: 20mg/ml in water (Sigma)

**TE**: 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0.

**Tris-saturated Phenol**: Extracted distilled phenol thrice with equal volume of 0.5 M Tris-HCl, pH 8.0 (pH of the supernatant should be 7.5-8.0). Add 0.1% 8-hydroxy quinoline. Overlay phenol with 0.1 M Tris-HCl, pH 8.0 and 0.1% 2-mercaptoethanol and used the lower layer as Tris-saturated phenol.

**20% SDS**: (w/v) 20g/ 100ml.

3.3.2.5.2 **RT-PCR, Cloning and Sequencing**:

RNA extracted from purified preparations of virus was used for RT-PCR reaction as described earlier for ACLSV using virus specific primers. The amplicons obtained were excised and eluted after gel checking. The eluted products were cloned and sequenced as mentioned earlier.

3.4 **ASSESSMENT OF DIVERSITY IN THE GENOME OF ACLSV INFECTING POME AND STONE FRUIT**

The sequences of CP, MP and replicase obtained from different stone and pome fruits were aligned with corresponding sequences of other established ACLSV sequences from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast (Altschul et al., 1997). Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988) for determining the sequence variability. Percent sequence identities among the isolates were obtained using Clustal W software.
(http://www.ebi.ac.uk/Tools/clustalw/) (Higgins et al., 1994). The phylogenetic trees were constructed using MEGA version 4 (Tamura et al., 2007) by neighbour joining method and 1000 bootstrap replicate with a cut off value of 65 % to determine the relationship of Indian isolates with other ACLSV isolates available from the world. Some phylogenetic trees were also constructed using 1000 bootstrap replicates with the help of Clustal W tool from DDBJ website (www.ddbj.nig.ac.jp). Phylogenetic trees were also constructed using Tree Explorer software 2.12 (Tamura, 199-1999). Four APCLSV (Apricot pseudo chlorotic leaf spot virus) partial CP isolates along with Peach mosaic virus (PeMV) and Cherry mottle leaf virus (CMLV) complete CP isolates were used as outgroup. All the sequences were also analyzed for putative recombination by Recombination Detection Program (RDP) ver. 3.26 or ver. 1.08 (Martin et al., 2005) in order to identify any recombination with other established Trichoviruses and subsequent evolution in the virus. RDP3 which identifies recombination breakpoints is based on various published recombination detection programs, including GENECONV, BOOTSCAN, MAXIMUM CHISQUARE, CHIMAERA and SISTER SCANNING.

3.5 DEVELOPMENT OF DIAGNOSTIC TOOL THROUGH HETEROLOGOUS EXPRESSION OF COAT PROTEIN

3.5.1 ELISA Based Diagnostics

3.5.1.1 Amplification, Cloning and Transformation of Cloned Fragment in E.coli

After getting the sequence of ACLSV CP from apple specific primers were designed on the basis of gene sequence obtained, containing restriction enzyme sites of Bam HI and Hind III for inframe cloning of the amplified CP gene in pET-32a expression vector as ACLSV CP gene does not contain the restriction sites of these enzymes and the sites of these enzymes are present in multiple cloning site (MCS) of vector pET-32a. ACLSV CP was amplified using these designed primers from ACLSV CP already cloned in pGEM-T easy vector as template in a PCR at similar thermal cycling conditions as mentioned in Table 3.5. The amplified fragment was separated on 1.0% agarose gel, specific band excised from the agarose gel as previously described. Eluted PCR product and pET-32a vector were double digested with Hind III and Bam HI, digested fragments were run on gel and specific digested fragment were eluted from the gel and suspended in 15-20 µl of water or TE. The gel eluted double digested pET-32a(+) vector and modified ACLSV-CP were ligated in-frame,
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according to manufacturer instructions using T4 DNA ligase (2 units) (Fermentas, Lithuania), at 16°C for 14-16 hrs and transformed by heat shock into *E. coli* BL21 competent cells prepared according to Hanahan (1983). After transformation, recombinant clones were screened both with restriction digestion (with *Bam* HI and *Hind* III) and with PCR in order to check the recombinant colonies having desired insert. Plasmid was isolated from several recombinant clones and all were sequenced in an automated sequencer in order to identify the orientation and frame of insert. After getting the sequence, one clone was selected containing ACLSV CP in the correct orientation and correct frame that was used for expression studies.

### 3.5.1.2 Standardization of Optimal Expression Conditions

The clone containing ACLSV CP in the correct orientation and frame was selected and used for expression studies. The selected colony was restreaked on LB agar plate, single colony was picked and cultured in 5 ml of LB supplemented with ampicillin and grown overnight at 37°C at 200 rpm in an incubator shaker. Yeast trypton (YT) broth (HiMedia, India) 250 ml supplemented with ampicillin was then inoculated with 1 ml of overnight grown culture. Different IPTG concentrations (0.5, 1, 1.5 mM), temperature combinations (28, 30, 37°C) and time intervals (1, 2, 3, 4 hr) were used to standardize optimal expression so that expressed protein should be present in maximum concentration.

**Reagents:**

**IPTG (0.5 M):** Dissolve 0.119 g IPTG (Isopropyl-β-D-galactopyranoside) in 1 ml of sterile water and mixed properly to dissolve. Filter sterilized the solution and stored in aliquots at -20°C.
3.5.1.3 **Checking of Expression Level**
The bacterial pellet taken from the above step was re-suspended in 800 µl of 1X PBS and sonicated till it became clear. This was then centrifuged at 12,500 rpm for 10 min at 4°C, pellet and supernatant were collected in separate tubes. Pellet was again suspended in 800 µl 1X PBS. Supernatant and resuspended pellet (5-10 µl) from each of the tubes was run on the gel (12% SDS-PAGE) with sample loading buffer and gel was silver stained.

3.5.1.4 **Purification of Protein**
The recombinant protein was extracted using Bug Buster HT Protein Extraction Reagent (Novagen, USA) as per manufacturer’s instruction. The 6x His tagged protein was purified using precharged His-Bind column under native condition (Novagen, USA) in accordance to the manufacturer’s directions. The purified protein was analyzed again on 12% SDS-PAGE for integrity and purity. Concentration of the purified protein was determined by UV absorption and by Bradford method (Bradford, 1976).

3.5.1.5 **Raising of Antisera**
Purified protein obtained after purification from *E. coli* was used as antigen for immunization of rabbit to raise the hyperimmune sera against ACLSV coat protein. Immunizations were performed by priming 2 Angora female rabbits, approximately 5 months old, four times at weekly intervals. Aliquots of purified ACLSV-CP/His fusion protein (0.6mg) were emulsified with complete Freund’s adjuvant (FCA) (Genei, India) (1:1 v/v) and injected subcutaneously into the hind legs. After 5 weeks, a booster injection emulsified with incomplete FA (FIA) (0.6mg protein) was given and rabbits were bled beginning 1 week later at weekly intervals for 7 weeks. The animals were bled from the marginal ear vein. The blood was collected in a glass tube and allowed to clot at room temperature for an hour. Subsequently the glass tube containing the clotted blood was kept at 4°C overnight. The serum was collected by using pasture pipette and centrifuged at 5000 rpm for 10 min at 2-6°C. The supernatant was collected and stored at 4°C after adding sodium azide to a concentration of 0.02% (w/v). To collect more serum, booster injections were given 5, 12, 16 and 22 weeks after an initial injection. Specificity of antibodies was checked by DAS-ELISA (as previously described) using known positive and
negative samples. For reference and serological testing, commercial ACLSV ELISA kit (Agdia, USA) was used.

3.5.1.6  **Purification of Antibody (Separation of IgG From Whole Serum)**

3.5.1.6.1  **By Ammonium Sulphate Precipitation Method**

(i) Distilled water (9 ml) was added to 1 ml of crude serum. Slowly drop wise 10 ml of neutralized saturated ammonium sulphate (Sigma) was added with continuous stirring.

(ii) After stirring, it was kept at room temperature for about 1 hr. The resulting solution should appear viscous and cloudy because of precipitation of antibodies i.e. IgG.

(iii) Solution was centrifuged at 9,000 g for 15 min and precipitate was washed with 2 ml of half-strength PBS. Washing step was repeated three times to remove the traces of ammonium sulphate.

(iv) Finally precipitate was dissolved in 1 ml of half-strength PBS.

(v) O.D. was measured at a wavelength of 280 nm.

(vi) The antibodies were diluted in a way that final concentration became 1 mg/ml (O.D. reading 1.4 = 1 mg/ml).

(vii) Aliquots of 1 ml along with 0.02% w/v sodium azide were stored at -20°C for further use.

**Reagents:**

**PBS (100 ml):** Na$_2$HPO$_4$.12H$_2$O (5.8 g); NaH$_2$PO$_4$.2H$_2$O (1.0 g); NaCl (8.76 g).

3.5.1.6.2  **By Affinity Chromatography using Protein A Agarose columns (GeneiTm IgG Purification Kit, India)**

The IgG was extracted from the antiserum using Protein A antibody purification kit (Genei, India) as per the manufacturer’s instructions. IgG samples were stored frozen at -20°C in 0.5 ml aliquots. Antibody concentration was estimated based on the specific extinction coefficient A$_{280}$, 1 cm = 1.4 (= 1.0 mg/ml).

(i) Column Preparation: Top cap and then bottom cap of the protein A columns were removed sequentially. The storage buffer was poured out.

(ii) Protein A column was equilibrated by applying 10 bed volumes of 1X equilibration buffer and allowed to drain through the column.
(iii) Sample Preparation: Diluted crude serum (for 1ml column 10ml of 1X equilibration buffer and for 2ml column 20ml of 1X equilibration buffer) to ensure that proper ionic strength and pH were maintained for binding (Samples were centrifuged for 5min to separate particulate matter and preventing clogging of columns).

(iv) Sample Purification: Applied 2-4ml of diluted serum onto equilibrated 2ml Protein A columns. Flow through was collected for analysis of binding efficiency.

(v) The column was then washed with 25 bed volumes of 1X equilibration buffer or until the absorbance of eluate at 280nm is less than 0.03.

(vi) Elution: Enough tubes were set up to collect up to 5 bed volumes of eluate, each containing 25µl of neutralizing buffer.

(vii) To elute the antibody, initial 0.5ml buffer was drained out and 1ml fraction was collected by adding 1X elution buffer to the top of the disc of the column. Each time eluate was collected in a fresh tube.

(viii) The absorbance of eluate in each tube was measured at 280nm and IgG fractions with high absorbance ($A_{280} \geq 0.4$) were pooled.

(ix) Absorbance of the pooled fraction at 280nm was measured. Estimated total IgG was calculated as-

\[ (A_{280 \text{ nm}}/ 1.4) \times \text{ Dilution Factor} \]

(Where 1.4 is extinction coefficient of IgG)

(x) Purified antibody was analysed by loading on SDS-PAGE or dialyzed against buffer of interest (preferably 1X PBS).

(xi) Column Regeneration: The column was regenerated for re use by washing with 5 bed volumes of elution buffer. The column was washed with 5 bed volumes of 1X storage buffer and could be stored at 4°C till further use.

**Reagents:**

10X equilibration buffer, neutralization buffer, elution buffer and Protein A agarose columns all provided in the kit.

**3.5.1.7 Preparation of Antibody-Enzyme Conjugate (Using Alkaline Phosphatase)**

A part of antibodies were tagged with alkaline phosphatase by the method of Avrameas (1969).
(i) Alkaline phosphatase (Sigma, USA) (1ml) was dissolved in 2ml of purified ACLSV antibodies.

(ii) Fresh gluteraldehyde (25% stock) was added to the solution in a way to make the final concentration 0.05% and mixed well.

(iii) It was incubated at room temperature for 4 hr. A faint brown colour was developed.

(iv) After incubation it was centrifuged at 9,000 g for 20 min.

(v) The precipitate was washed twice with half strength PBS and finally dissolved in 2 ml of half strength PBS.

(vi) Bovine serum albumin (BSA) to 5 mg/ml and sodium azide to 0.02% w/v were dissolved in it to enhance its shelf life. It was stored at 4°C till further use.

3.5.1.8 Evaluation of Alkaline Phosphatase Conjugate

Activity of conjugate was checked by DAS-ELISA as described earlier using known positive and negative samples.

3.5.1.9 Field Screening to Check the Activity of the Diagnostic Kit

To check the reliability of kit, different apple cultivars, *Prunus* spp. and inoculated host plants were checked by DAS-ELISA (as previously described) using the developed antibodies and enzyme conjugate. At the same time same samples were also checked using standard commercial kit (Agdia, USA). Sensitivity and specificity of purified IgG against the ACLSV-CP were evaluated by western blotting of fusion proteins using indigenously raised antiserum and Direct Tissue Blot Immuno Assay (DTBIA).

For DTBIA, plant extracts made for ELISA was also plotted onto nitrocellulose membranes (Biorad, USA). After the membrane was imprinted with the tissue samples and semi dried, it was placed in a solution of 1% BSA in PBS and incubated for 1hr at 25°C, or overnight at 4-6°C to block any remaining protein binding sites. The membrane was then plotted with indigenous ACLSV antibodies and a washing with PBS(T) was given for 2-5min. Biotinylated secondary antibodies coupled with streptavidin-HRP (Roche, USA) were applied and again the membrane was allowed to semi dry at room temperature. Finally, the membrane was dipped in 3,3'-Diaminobenzidine (DAB) 1% solution till colour developed (1-3min). These reacted
to produce a brown staining wherever primary and secondary antibodies were attached.

**Reagents:**

1% DAB Peroxidase Substrate Solution (20X)-Blue: Added 0.1g of DAB (3,3’-diamino benzidine or DAB-tetrahydrochloride (Sigma, USA) in 10 ml distilled water. Added 10N HCl 3-5 drops till solution turns light brown in color. Stirred till DAB completely dissolved, aliquoted and stored at –20 °C.

1% Nickel Ammonium Sulfate (20X): Added 0.1g of nickel ammonium sulfate in 10 ml distilled water. Stirred till dissolved. Stored at 4 °C or aliquoted and stored at –20 °C.

1% Cobalt Chloride (20X): Added 0.1g of Cobalt Chloride in 10 ml distilled water. Stirred till dissolved. Stored at 4 °C or aliquoted and stored at –20 °C.

0.3% H$_2$O$_2$ (20X): Added 100ul of 30% H$_2$O$_2$ in 10 ml distilled water and mixed well. Stored at 4 °C or aliquoted and stored at –20 °C.

**Final Dilution:** 0.05% DAB, 0.05% Cobalt Chloride, 0.05% Nickel Ammonium Sulfate and 0.015% H$_2$O$_2$ in PBS, pH 7.2 (pH < 7.0 would reduce staining intensity while, pH >7.6 will cause background staining)

3.5.1.10 **Keeping Quality of the Diagnostic Kit**

To check its keeping quality, DAS-ELISA was conducted using known positive and negative samples during 2008-2009. A part of antibodies and conjugate were kept in refrigerator (4-10°C) whereas other part was kept at room temperature (20-35°C) and were used for the study.

3.5.2 **Development of PCR based Diagnostics**

3.5.2.1 **Standardization RT-PCR for molecular detection**

Degenerate primer pair (AM490253, AM490254) specific for amplification of complete ACLSV CP was designed from all the available full genomes of ACLSV (apple, plum, cherry) to have maximum detection ability. This primer pair was selected as ACLSV detection primers due to maximum conservation of CP gene in ACLSV. PCR conditions were standardized w.r.t. various cultivars, hosts and DNA polymerases.
3.5.2.2 Nucleic Acid / Hybridization Based Diagnostics

Sequenced coat protein genes obtained after molecular detection were used as probes (radio labeled/ enzyme labeled) for detection of ACLSV from plant extracts (mature trees, nursery plants and tissue culture raised plants) according to the procedure mentioned in section 3.1.3 by slot blot hybridization as mentioned earlier.

3.5.2.3 Standardization of Immuno Capture-RT-PCR based Diagnostics

To increase the reliability and sensitivity of the diagnostics developed (nucleic acid based and antibody based) both the approaches were combined. Various ACLSV positive, border line and negative samples were tested. Immuno-capture RT-PCR was standardized with indigenous antibodies and degenerate primers designed for complete coat protein amplification. The procedure is based on the method by Gillaspie et al. (2000) and is as follow:

(i) The PCR tubes (0.2 ml) were soaked in 0.1N HCl and 4M NaOH at room temp for 15min were given. The PCR tubes were rinsed three times with PBST wash buffer (section 3.1.2.1) after each treatment.

(ii) Final soak in 95% ethanol for 15min was given and the PCR tubes were air dried. All this was to condition the PCR tube surface for firmly binding the antibodies.

(iii) Several dilutions of the indigenously raised antibodies were made (1:100, 1:200, 1:300, 1:500, 1:1000). About 200µl of each dilution was loaded onto pretreated PCR tubes and incubated at 4°C overnight and 37°C for 4hrs.

(iv) Next morning three washings with PBST was given and 200µl extract of plant samples crushed in extraction buffer (3.1.2.1) was loaded in the PCR tubes. These were incubated at 4°C overnight and 37°C for 4hrs.

(v) The PCR tubes were rinsed three times with PBST wash buffer.

(vi) About 10µl of 18.2 millipore water was added to the rinsed and air dried PCR tube. The water was mixed thoroughly in the tube and finally incubated at 63°C for 10min.

(vii) This 10µl was used to set up a RT reaction. Firstly 1µl of down primer (complete CP- 7552) was added to this 10µl, denatured at 70°C for 5min and immediately placed on ice.

(viii) Added all the components mentioned below to make final volume to 15µl
Materials and Methods

5X RT Buffer 1.5 µl
40mM dNTPs 1 µl
RT enzyme (USB Corp., USA) 0.5 µl
water 1 µl

(ix) The reaction mixture was incubated at 37°C for 75min and reverse transcriptase enzyme was inactivated by incubating the mixture at 70°C for 5 min.

(x) PCR conditions were standardized for a 50µl reaction in the same PCR tube using primer pair designed for complete coat protein amplification.

(xi) The PCR product was checked on 1% agarose by ethidium staining. The desired band from one of the positive sample was proceeded for cloning and sequencing.