Molecular Characterization and Diagnostic Development for *Apple chlorotic leaf spot virus*

SUMMARY

SUBMITTED TO THE FACULTY OF LIFE SCIENCES
GURU NANAK DEV UNIVERSITY, AMRITSAR
PUNJAB, INDIA
IN
PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
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2010
SUMMARY AND CONCLUSIONS

Work done in the present thesis entitled “Molecular characterization and diagnostic development for Apple chlorotic leaf spot virus” includes studies on incidence of the viruses infecting pome and stone fruits, virus transmission, host range, purification, molecular characterization, diversity analysis, heterologous over expression of viral coat protein (CP) in *E. coli* and development of nucleic acid and ELISA based diagnostic systems.

Horticultural crops play a unique role in India’s economy by supplementing the income of the rural people. These crops form a significant part of total agricultural produce and have become key drivers of economic development in many of the states in the country. In the past one decade, the change in cropping pattern indicates a shift towards the fruit and commercial crops. In Himachal Pradesh (HP) apple is the major fruit accounting for more than 40% of total area under fruits and about 88% of total fruit production from the state. Commercially, apple is the most important of all the fresh fruits grown in HP, Jammu & Kashmir (J&K) and Uttarakhand. Any losses occurring would affect the economy and livelihood of some growers for whom apple is the only cash crop. Some of the economically important major viruses and viroids known to cause diseases in apple and other pome and stone fruits are *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple scar skin viroid* (ASSVd).

During extensive surveys (2007-2009) of pome and stone fruit growing areas in HP and J&K, presence of typical virus like symptoms on these fruits indicated viral infection. Symptoms of leaf mosaic, severe chlorosis, deformation, curling etc were recorded in apple orchards. While plum, cherry, peach/nectarine, almond, cherry and apricots exhibited various symptoms like yellow flecking on leaves, mosaic, mottling, necrotic ring spots and shot holes. The symptoms were not persistent throughout the year, and disappeared late in the season making the plant seem healthy. Most of the orchards surveyed had mixed plantation of pome and stone fruits.

Preliminary analysis of samples by Enzyme Linked Immunosorbent Assay (ELISA) for ACLSV, ASPV, ASGV, ApMV and PNRSV confirmed presence of viral infections and
was found to be sensitive enough to detect viruses early in the season during flowering. Late in the season, detection of ACLSV and ApMV was ambiguous by ELISA while ASPV and ASGV were reliably detected by ELISA using even mature leaves and bark tissue. The ACLSV infection was generally latent, however characteristic leaf chlorosis, deformation and curling was observed in some cultivars (Golden Delicious and Gala Mast). A total of 290 apple plants were tested throughout the study and of these 90 were symptomatic while 200 were apparently healthy. More than half of the healthy samples were latently infected with one or more of the viruses. The overall percent disease incidence of ACLSV on apple in HP (80.8%) and J&K (32.6%) based on ELISA was ~ 75 %. Among other viruses, ASPV followed by ASGV were major viruses on apples. Further, ApMV was more common in cultivars from J&K in comparison to cultivars from HP. Due to mixed cultivation of fruit trees ACLSV was also detected in other pome (pear, quince) and stone fruits (plum, peach, apricot, almond, cherry). Some wild plants (wild Himalayan cherry, wild apricot) also tested ACLSV positive. Reverse transcription-polymerase chain reaction (RT-PCR) using virus specific primer pair and indigenously designed primer pair was done to confirm ELISA positive samples. All the cultivars which were earlier found to be positive for a particular virus by ELISA were also found positive by RT-PCR. This was for the first time that incidence of these viruses (ACLSV, ASGV and ApMV) was confirmed on various pome and stone fruits from India at serological and molecular level. Further sequenced ACLSV-CP was used as a probe for tissue blot radioactive hybridization so as to detect the presence of ACLSV in large number of pome and stone fruit samples. Host range studies (for six isolates India11, 13, 15, 16 [apple]; India27 [cherry] and India20 [peach]) showed that ACLSV triggered systemic infection on several hosts including Chenopodium quinoa, C. amaranticolor, Vigna sinensis (var. Chitlidana) and Phaseolus vulgaris. Hosts like Cucumis sativus, Gomphrena globosa, Dianthus barbatus, Lycopersicon esculentum, Nicotiana glutinosa, Cymopsis tetragonoloba and N. tabacum were symptomless for ACLSV. C. quinoa and C. amaranticolor were found to be the best maintenance and propagation hosts. However, N. megalosiphon and N. benthamiana showed mild mosaic and curling symptoms for isolates India11, apple and India27, cherry) respectively.
Virus purification was performed using established protocols with minor modification and crude virus extract (CVE) from the systemically infected *C. quinoa* plant inoculated with extracts of Gala Mast leaves showing chlorotic leaf spots symptoms from J&K, 10-15 days post inoculation, shoots/leaves from *in vitro* maintained pure culture (Gala from Uttarakhand) and young buds and flower petals (wild Himalayan cherry from HP) was obtained. The CVE contained partially purified virus particles. The virus was characterized through UV absorption spectrum, DAS-ELISA, molecular weight determination of coat protein, western blotting and electron microscopy (EM). CVE of ACLSV preparation showed absorption maxima Amax at 260 nm wavelength and absorption ratio $A_{260}/A_{280}$ were recorded to be 1.5. In DAS-ELISA the CVE of ACLSV reacted with upto 1:500 dilution of ACLSV specific indigenous antibodies. From the purified preparation, SDS-PAGE showed a protein band ~25 kDa, which was confirmed by western blotting with ACLSV specific antibodies. Flexuous filamentous particles of ~700nm were observed in the EM of the ACLSV CVE. End to end aggregation of particles was also seen.

Primer pairs designed for the amplification of complete CP, MP and replicase were used for genome characterization and diversity studies. RNA for genome characterization was extracted by modified CTAB method from herbaceous maintenance host. For other samples commercial RNA extraction kit was used. It was revealed that ACLSV-CP was 584 bp (193 aa) long and MP was 1386 bp (460 aa) long as expected. About 27 ACLSV-CP isolates were characterized from various hosts and locations. Apart from this, deduced amino acid sequence of 3’end of ACLSV-MP (~111aa) overlapping with the CP was obtained by frame shift reading of 27 isolates. Replicase gene was amplified in two parts—first region was from 5’-28nucleotide (nt) to 1580nt -3’ and the second 420bp (from 5’-4502nt to 4922nt -3’). Apart from this a frame shift reading of MP and replicase overlapping region gave the extreme 3’end of replicase gene.

The first part of replicase consisted of 1572 bp [from 5’end 28th nt to 1580th nt -3’end containing complete methyl-trasferase domain], the second 420bp [from 5’-4502nd to 4922nd -3’ is region between helicase and polymerase domains] and third was overlapping region of 5’end MP [5675nt-5786nt] and replicase [region after the polymerase domain]. All the sequences obtained have been submitted to EMBL database.
Sequences from all the pome and stone fruit CPs from the present study (27) and those from earlier deposited sequences in GenBank were subjected to phylogenetic and multiple sequence analysis. The differences in phylogeny were evident from variations and co-variations in deduced amino acids (aa) by multiple sequence alignment of isolates from the various hosts. Sequence analysis of all the isolates from India and complete isolates from elsewhere show variability in sequences and 17 variations and co-variations in amino acid sequences. On basis of these variations supported by distinct cluster formation in the radiated phylogenetic tree constructed at amino acid level, two groups Group 1 and Group 2, were proposed. Recent work by Japanese group had reported similar cluster formation (namely P205 and B6 type) and noted that one cluster (P205) consisted of more infective isolates based on co-variation of five amino acids. Though mutational studies were not conducted in the present work, however symptoms observed on natural and herbaceous host plants for Group 1 (India11, 15, 16, 20, 27) and Group 2 (India13) isolates clearly indicated difference in infectivity. It was thus supporting that Group 1 members were P205 type and Group 2 as B6 types.

On analyzing all the stone fruit isolates, with the exception of cherry isolates P1R9D9 (DQ329160) and C-2 (AY677106), all clustered in Group 2 suggesting co-evolution of ACLSV stone fruit isolates. However, the Indian stone fruit isolates did not show such co-evolution. Indian almond isolate (India24) was unique as it had asparatic acid (D) residue at 151 position instead of asparagines (N) when compared to rest of the isolates with the exception of an Indian apple isolate, India3. It is worth mentioning that these isolates were from the same orchard and in close proximity to each other. The isolates shared 93% sequence identity at amino acid level but different phylogeny (India3 - Group 1, India 24 - Group 2).

Among all peach isolates, India20 sequence was the only sequence having valine and phenylalanine at positions 59 and 75 respectively, similar to apple (India1, 3, 4), plum (India17, 18) and quince (India 23) isolates. It also indicated more variability at amino acid level with sequence identity of less than 87.1%. The India21 (peach) and India 19 (pear) isolates (100% identity) were obtained from different corners of the same orchard. ACLSV-CP isolates from plum (India 17, 18), quince (India 23), peach (India22) with various ACLSV apple isolates from different locations in HP clustered together.
Recombination analysis of all available complete CP sequences from India and elsewhere gave five potential recombination events (PREs). However, only event 1 (between apple isolates) seemed significant as it was detected by five of the recombination detection programs viz. MAXCHI (average P-value = 3.399 x10^-04), LARD (average P-value = 1.166 x10^-07), CHIMAERA (average P-value = 1.823x10^-02), 3SEQ (average P-value = 4.481x10^-06) and BOOTSCAN (average P-value = 2.877x10^-03) with identical breakpoints between nucleotides from 186-498 in India7 (major parent) and India12 (minor parent). The break points were also confirmed by drawing phylogenetic tree of 1-185,186-498 and 499-582 nt of all the Indian isolates. All the sequences involved, including the daughter isolate (India20, peach) fall in P-205 group which has Ser40 and Tyr75 indicative of higher infectivity. No recombination events were obtained in members of Group 2 (B6 type) which are the less infective group.

Sequence alignment of complete MP sequeneces showed some conservation in N-terminal portion except few changes, while C-terminal half showed a much higher variability, with only a few blocks of fully conserved residues. The Indian ACLSV-MP (FN673831, India15) isolate showed 61-86% similarity at amino acid level with other isolates from the world. The Indian isolate was found to be maximally similar to P-205 (apple) isolate (86%; D14996) and least to TaTao-5 (61%; peach, EU223295) isolate. The Indian MP isolate at 5’end has adjacent RNA-binding domains (A, B) and conserved ‘D’ motifs characteristic of ‘30K Super Family’ of movement proteins.

The 5’end (first 200aa) [8 world and 3 Indian ACLSV-MP isolates] was analyzed at sequence and phylogenetic level. The isolates show maximum variability in first 30 amino acid residues (~80-90nt) falling in the 3’end Replicase-5’end MP overlapping region. The Indian isolates [FN673831 (India15) and AM888397 (RC-Palampur apple isolate)] were unique from rest in having certain major aa changes in this overlapping region. Most of the amino acid changes were conserved except those in residues 20 and 22 in the RC-Palampur apple isolate (AM888397).

Apart from this deduced amino acid sequence of 3’end of ACLSV-MP (111aa) overlapping with the CP were obtained by frame shift reading of 27 Indian ACLSV-CP isolates. Sequence analysis using Clustal W revealed that all these Indian isolates among themselves shared identity ranging from 57-100% at amino acid basis indicating
maximum variability in the C terminal half. Phylogenetic relationship of the 3’ end of ACLSV-MP isolate shows separation into similar Group 1 and Group 2 clusters identified for ACLSV-CP isolates. Group 1 isolates were unique in having Glycine (hydrophobic) instead of Valine (polar) and Arginine (R, basic)/Glutamic acidic (E, acidic) at 395th and 438th amino acid (aa) positions respectively. However, partial ACLSV-MP (351-460 aa) isolates viz. MO-5, Balton-1, TaTao5, India27 and India15 (FN673831) show intermediate sequences identities to both the groups, These isolates cluster separately from the two groups also evident from Balton-1 isolate having Glycine as 438th aa residue like Group 1 isolates though it clusters seperately. All the Group 2 isolates show more variability in 351-377 aa as compared to Group 1 isolates.

Recombination analysis for complete MP sequences show one significant event between NC_001409 (major parent, plum) and FN673831 (minor parent, apple) leading to formation of daughter isolate AB326225 (MO-5, apple). The event was detected by four programmes with significant average P values. Recombination events were also detected by separately analyzing 5’ and 3’ ends of ACLSV-MP. The 5’end (overlapping replicase) indicated more significant recombination events as compared to 3’end overlapping CP. However, in the analysis of 3’end sequences the recombinant daughter India20 isolate was obtained by similar recombination (India7 major and India12 minor parenting) as reported for sequence analyses for CP isolates from almost the same region. This further supports that overlapping regions of ACLSV-MP are target areas for recombination.

Replicase was analyzed in three parts (5’end, middle portion and 3’end) for sequence variability, phylogeny differences and recombination evidences. The multiple alignment of first part (5’end sequences clearly indicated conserved methyl-trasferase domain (MET; 59-233aa). The TaTao-5 peach isolate was the most variable having maximum changes at amino acid level and sequence identity in the range of 76-78% only. The 3 Indian sequences among themselves shared 92-98% similarity at aa level while with others it ranged from 87% (Balton-1, French cherry isolate) to 96 % (P-205, Japanese apple isolate).

Analysis of 3’end sequences showed that FN673831 (India15 apple isolate) shared 74% identity at aa level with AM888396 (RD-Kinnaur apple isolate) and 88% to (RC-Palampur apple isolate). The RD-Kinnaur apple isolate shared maximum (100%) identity
to PBM-1 plum isolate from Germany while 88% with TaTao-5 peach isolate which is most variable from all the sequences. The middle portion of replicase (region between the helicase and polymerase domains) was mostly conserved and did not show significant recombination events. The Indian isolate shared sequence similarities ranged from 91 (TaTao-5 peach isolate) to 95 (Plum isolate). Three significant recombination events were detected in 5’ end portion of replicase giving recombinant Indian isolates while 3’ end replicase portion gave only one recombination that was significant.

For diagnostic development the ACLSV coat protein was over-expressed in *E. coli* BL 21 strain using pET32a vector and the conditions for optimal expression were standardized. Optimal expression was found after 3 hr incubation at 28°C with 1 mM IPTG. The expressed protein was purified and antibodies were raised by injecting the purified protein intramuscularly in rabbit. After 45 days immunization schedule, blood was collected and antibodies were purified from the serum. Antibodies were also tagged with alkaline phosphatase and were formulated in the form of a kit. The kit prepared in the present study was more sensitive for the detection of Indian strain of ACLSV in comparison to the standard kit. The indigenously developed antibodies, detection primer pairs (for CP and replicase) and sequenced CP genes were used to standardize various detection methods (ELISA, IC-RT-PCR and tissue blot hybridization).

**CONCLUSIONS:**

1. From the surveys of pome and stone fruit growing areas of HP and J&K, ACLSV came across as the most prevalent virus infecting most of the apple plantations of the country. ACLSV was confirmed at molecular level along with some other major apple viruses (ASGV and ApMV) and an apple viroid (ASSVd) for the first time in India. ACLSV mixed infection with other major apple fruit viruses viz. ASPV, ASGV and ApMV was common. ACLSV though not a major virus in stone fruits was detected in all of them (almond, apricot, cherry, peach, plum) and some wild relatives of Prunus spp.

2. In the genome characterization RT-PCR conditions for amplification of CP, MP and Replicase genes of ACLSV from *in vivo* maintained ACLSV apple (Gala Mast cultivar, India15) isolate were standardized.
3. Diversity assessment of ACLSV genes

**Coat Protein (CP)**

- In the diversity studies, CP from 27 isolates from different regions (HP, J&K, Uttarakhand, Punjab) and various hosts (apple, pear, quince, plum, peach, cherry, almond, apricot) were amplified. Other previously characterized isolates and Trichoviruses were used for comparative analysis. Indian isolates among themselves and with other isolates from the world shared 91-100% and 70-98% sequence identity at amino acid and nucleotide level respectively. The highest degree of variability was observed in the middle portion with 9 amino acid substitutions in contrast to the N-terminal and C-terminal ends which were maximally conserved with only 4 amino acid substitutions. In phylogenetic analysis no reasonable correlation between host species and/or geographic origin of the isolates was observed. Recombination analysis (RDP3 ver.2.6) done for all the available complete CP sequences indicated no significant evidence of recombination. However, one recombination event in CP among Indian isolates was detected.

- This is the first report of complete coat protein sequence variability study from India and also the first evidence of homologous recombination in ACLSV. The present analysis thus confirms the existence of differences in phylogeny among Indian isolates though they share high sequence identity (91-100% at amino acid level). The variability among Indian isolates was supported by host range studies and molecular data analysis (indicating variation in amino acids). Only one significant recombination was detected amongst Indian isolates. The lack of enough significant recombination among ACLSV-CP isolates (Indian and world) points toward coat protein gene being a not a much targeted sequence for virus recombination and subsequent evolution of the virus. It was thus reconfirmed as the most conserved region of the ACLSV genome.

- The closeness of maloidae / prunus isolates (in phylogenetic and recombination analysis) might reflect transmission routes that may be
prevalent in India. One important factor responsible for high sequence identities among pome and stone fruit isolates in India could be the mixed cultivation of these fruits. Vector (nematodes, bees), natural reasons (root graftings, pollen transmission) and unclean horticultural practices may also be the factors responsible.

**Movement Protein (MP)**
- Multiple sequence alignment of complete ACLSV-MP isolates from India and world revealed high divergence level that was unevenly distributed all along the 50K protein. The C terminal was most variable.

**Replicase**
- Three significant recombination events were detected in 5’end portion of replicase giving recombinant Indian isolates while 3’ end replicase portion gave only one recombination that was significant. Middle portion was conserved and gave no recombination. The region after the conserved MET domain was most variable.
- The variation between isolates was observed in three main regions: the hyper-variable segment downstream of the methyl-transferase domain, the C-terminal part of the 50K movement protein ORF, and the N-terminal part of the 28-22K ORF upstream of the CP coding region. The conclusions were supported by sequences variability data, evidence of phylogenetic differences and finally relatively by significant recombination events. These regions may be associated with the determination of the very varied biological properties of ACLSV, the variability observed may indicate less severe evolutionary constraints on these regions.

4. Diagnostic methods were standardized for the detection of ACLSV in various hosts.
   - Indigenous degenerate primer pair was designed from all the available full genomes of the virus in GenBank for amplification of complete coat protein gene. The RT-PCR conditions were standardized.
• Sequenced CP after enzyme or radiolabelling was used as a probe for large scale detection of ACLSV positive samples in tissue slot blot hybridization.
• ELISA based indigenous diagnostic kit was developed for the detection of ACLSV by expressing CP gene in the bacterial system. In the present study indigenous diagnostic kit was found to be more sensitive as compared to standard kit. Probably, it contained antibodies against the Indian isolate of ACLSV which made it more reliable for detecting Indian ACLSV isolates.
• Immuno capture RT-PCR using the indigenously raised ACLSV antibodies and designed primers was also standardized.

The various nucleic acid and ELISA based indigenous diagnostics developed could be useful in certification system for pome and stone fruit nursery material and in quarantines.
I Tanuja Rana certify that I have been registered in Guru Nanak Dev University, Amritsar for Ph.D. programme. I am submitting my thesis summary for the partial fulfillment of award of degree of Doctor of Philosophy.

yours sincerely

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Supervisor