Molecular Detection of Latent *Apple chlorotic leaf spot virus* in Elite Mother Plants of Apple

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Abstract *Apple chlorotic leaf spot virus* (ACLSV; family *Betaflexiviridae* genus *Trichovirus*) is one of the economically important latent virus infecting apple (*Malus × domestica* Borkh.). Reverse transcriptase polymerase chain reaction (RT-PCR) procedures were used to amplify coat protein gene of ACLSV. Among 5 primer sets used, two primer sets (1F1R and 1F2R) amplified fragments of expected size (432 bp). Products visible on agarose gel were produced using templates extracted from apple leaves. The results were further validated by sequencing fragment of 432 bp which was amplified from leaf of apple by using primer set 1F 1R. Comparisons with published sequences indicated that the isolate have very high 91 % identity values to the corresponding region of ACLSV isolate from apple. Selected primer pair (1F1R) was further used for screening 42 elite mother plants collected from apple growing areas of Himachal Pradesh, India, where in 17 were found free from ACLSV. Use of NAD5 gene in mitochondrial mRNA of the apple as an internal control, reduced the risk of false negative results that may occur with routine RT-PCR assays.

Keywords ACLSV · Apple · RT-PCR · *Malus* · Elite mother plants

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Apple is commercially the most important temperate fruit and is the fourth among the most widely produced fruits in the world after banana, orange and grape. China is the largest apple producing country in the world whereas India stands at 5th position with an area of 0.29 mHa, production 2.89 mt and productivity of 10 t/Ha. The relatively low productivity of apple in India is due to several biotic and abiotic factors. Biotic factors comprises of various fungal, bacterial and viral diseases. Among various viral diseases, ACLSV is widely distributed which was first reported in *Malus* spp. from the US by Mink and Shay in 1959 [4]. ACLSV is one of the important latent virus whose infection rates ranges up to 80–100 % in many commercial apple cultivars with yield losses of the order of 30–40 % [3, 12, 17]. In Himachal Pradesh (India) ACLSV appeared as a major virus on apple with disease incidence of 85–90 % [14].

ACLSV a type member of the genus *Trichovirus* [8] of *Betaflexiviridae* family, is a common virus of most fruit trees of *Rosaceae* family, which include apple, pear, quince, sweet and sour cherry, peach, plum and apricot [7, 13]. It is difficult to notice the infected plant in the field condition due to latent infection of ACLSV. Sometimes apparently healthy looking and high yielding (Elite mother) plants used as a source of bud stick may have latent virus infection. Hence detection of the virus in elite mother plants is a matter of great importance. Although, a natural spread under field condition has been detected, the natural mode of spread is unknown. Thus a program of disseminating virus-free material will help in controlling ACLSV infection. So, reliable detection of ACLSV is an important in the implementation of the program [10].

Biological indexing and ELISA are routinely used for detection of important viruses of apple. Both methods have their limitations, the major limitations of biological

Table 1 Details of ACLSV Primers derived from sequence AM409322.3 CP 17598 (NCBI)

S. no.	Code	Sequence	Polarity	Position of primer within ACLSV CP gene	Product size (bp)
1	1F	5' tcgcgaacatagcgatacag 3'	Sense	125–145	432
	1R	5' acgacattttcgccctcattc 3'	Antisense	556–536	
2	1F	5' tcgcgaacatagcgatacag 3'	Sense	125–145	433
	2R	5' gacgacattttcgccctcatt 3'	Antisense	557–537	
3	3F	5' tccgagcaaacagagttcct 3'	Sense	151–171	406
	1R	5' acgacattttcgccctcattc 3'	Antisense	556–536	
4	4F	5' ctccgagcaaacagagttcc 3'	Sense	150–170	407
	1R	5' acgacattttcgccctcattc 3'	Antisense	556–536	
5	3F	5' tccgagcaaacagagttcct 3'	Sense	151–171	407
	2R	5' gacgacattttcgccctcatt 3'	Antisense	557–537	

indexing are its application length (3 years), its cost in terms of glasshouse space and labour intensity and sometimes symptoms are difficult to interpret. ELISA often fails because of low virus titers or the inhibitory effects of plant polysaccharides or phenolic compounds. In addition, no internal controls are available to prevent false negative ELISA results. Therefore the development of reverse transcription (RT) coupled with PCR assay for detecting ACLSV [2, 5] provided rapidity and sensitivity to potentially overcome some of the problems mentioned above and it become an important method for the detection of low-titer, RNA-containing viruses [16].

Keeping in view of the sensitivity of the technique available, the importance of ACLSV and its latent/asymptomatic nature, the present investigation was carried out i.e. detection of ACLSV in elite mother plant by RT-PCR.

The samples of apple infected with ACLSV for RNA isolation were obtained from Division of Mycology and Plant Pathology, Dr Y.S Parmar University of Horticulture and Forestry, Nauni, Solan (HP). Elite mother plants collected from Shimla (Rohru) and Kullu districts of Himachal Pradesh (India) during the year 2010 by Dr Y.S Parmar University of Horticulture and Forestry, Nauni, Solan (HP). These plants were planted in the farm of IARI Shimla and leaf samples from each plant were collected for detection of ACLSV. All the lab based techniques were carried out in Division of Plant Protection, Central Potato Research Institute (CPRI) Shimla, HP (India).

Total RNA was isolated from leaf samples by using SpectrumTM Plant Total RNA kit (Sigma–Aldrich, USA) as per manufactures instructions. Isolated total RNA was used for c-DNA synthesis by using Revert AidTM of Fermentas Life Sciences as per manufactures instructions.

Coat protein gene of ACLSV was selected to design virus specific primers for RT-PCR amplification by using Primer 3 software. Sequence data of ACLSV (AM409322.3 CP 17598) was collected from the Gene-bank of the National

Center for Biotechnology Information (NCBI). The primer names, oligonucleotide sequences, and expected size of amplified products are shown in Table 1.

Performance of RT-PCR with an internal control can minimize the risk of obtaining false negative results [9, 15]. Although several plant internal control primers have been published [1, 11] none of them can distinguish DNA from RNA so that the complete removal of DNA is required before performing RT-PCR [9, 11]. To solve this problem, 9 designed the primers for amplifying apple mitochondrial NADH dehydrogenase subunit 5 (nad 5) gene i.e., sense: 5'-gatgcttcttggggcttctgtt-3' and antisense: 5'-ctccagtcacacattggcataa-3' with a product of 181 base pair. In this case the primers could only amplify the spliced mRNA derived cDNA but not the intron containing DNA. Therefore there is no need to eliminate contaminating DNA from plant extracts.

PCR was carried out in thin walled 1.25 ml tubes and volume was made up to 20 µl with sterile nano pure water in a GeneAmp PCR 9700 system (applied biosystems, USA). The reaction mixture of 20 µl containing 0.8 units of Red Taq DNA polymerase (Genei, Bangalore, India) 1.6 µl of 2 mM dNTP mix (Fermentas), 2.5 µl of 10× taq DNA Polymerase buffer (Genei, Bangalore, India), 0.5 µl of each downstream and upstream primers and 2 µl of cDNA. Amplification was carried out by following PCR conditions with five primer pairs along with an internal, water and healthy control. Denaturation was performed at 94 °C/30 s. annealing temperature of 57 °C/1 min. followed by extension at 72 °C/1 min. for 40 cycles along with a final elongation step at 72 °C for 10 min. About 10 µl of the reaction mixture from each tube was loaded onto 1 % agarose gel alongside 1 kb DNA ladder as molecular weight marker. Electrophoresis was done at 80 V. The buffer used was 1× TAE at 8.0 pH. The DNA bands in the gel were visualized on a UV-transilluminator and primer pair showing expected size without any multiple bands was selected.

After selecting a primer set, same PCR mix and PCR conditions were used on varying annealing temperatures i.e., 53, 55, 57, 59 and 61 °C. Once the primer set and annealing temperature was standardized, it was used for further studies.

For further validation and conformation, the expected size amplicon was subjected to sequencing. For this the amplified DNA of desired size on the electrophoresis gel was eluted by mini elute gel extraction kit (Qiagen, Germany). Eluted DNA was subjected to cycle sequencing in the Thermal cycler for ACLSV. Twenty micro liter (20 µl) mix containing 3 µl Reaction mixture (Big dye® Applied Biosystem, USA), 2 µl 5× Sequencing buffer (Big dye® Applied Biosystem, UK), Selected primers 4 µl and Eluted DNA 8 µl, the PCR was performed at 96 °C/10 s, 50 °C/5 s, 60 °C/4 min up to 25 cycles. The cycle sequenced product was then subjected to purification in order to remove unused buffer, dNTPs etc. Then sequencing of purified product was performed by using 3,500 Genetic analyser (Applied biosystem-Hitachi). The sequence obtained was further analyzed by BLAST algorithm available at <http://www.ncbi.nlm.nih.gov>.

After standardization of RT-PCR conditions and further validation by sequence analysis, this protocol was used for the detection of ACLSV in elite mother plants collected from Shimla (Rohru) and Kullu districts of Himachal Pradesh (India).

Among five primer pairs (1F1R, 1F2R, 3F1R 4F1R and 3F2R), two primer pair i.e., 1F 1R and 1F 2R permitted amplification of c-DNA fragments containing ACLSV. The size of amplified product 432 bp agreed well with required size. Non specific bands were not observed in any experiment. Although with two pairs of primers specific amplification products were observed by RT-PCR, the primer pair 1F 1R was comparatively better. Therefore it was used for further studies.

The designed primer pair for the internal control amplified consistently a fragment of the expected size i.e. 181 bp (Fig. 1).

The optimal annealing temperature for selective primer i.e., 1F 1R was analyzed wherein, the varied optimal annealing temperatures ranging from 53, 55, 57, 59 and 61 °C was applied in PCR amplification by following standardize PCR conditions. An annealing temperature of 57 °C effectively amplified the specific fragment of ACLSV with 1F 1R. The reproducibility and reliability after standardizing primer pair with annealing temperature along with PCR conditions; it was further validated with an internal control (NAD 5 gene of Apple), water control, negative (healthy control) and positive control. After visualizing on a UV-transilluminator, internal control (as isolation control) and positive control gave a specific fragment of 181 and 432 bp respectively. There was no

band in negative and water control. After RT-PCR standardization and further sequencing, it gave a clear indication that the selective set of primer with standardized PCR conditions can be used for further detection and diagnosis of ACLSV in a given sample.

After validating the RT-PCR conditions the desired fragment was eluted from the gel and further processed for sequencing. After obtaining the sequence data it was subjected to BLAST which confirmed that the amplification obtained was of ACLSV cp gene.

The sequence obtained was further matched with the reported sequence from different geographical locations. The sequence exhibited identities of 91 % at the nucleotide levels, with apple ACLSV isolate from Brazil and Turkey. In addition to apple Indian isolates of ACLSV from different host such as almond, apricot and wild Himalayan cherry also shows 90–91 % identity.

After standardization and validation of the RT-PCR conditions the standardized protocol was used for molecular detection of ACLSV in 42 elite mother plants collected from Shimla (Rohru) and Kullu districts of Himachal Pradesh (India). Based on results obtained (Fig. 1) 25 plants have showed the presence of ACLSV (Table 2).

We report here the development of RT-PCR protocol for the detection of ACLSV in Apple. The coat protein gene appeared to be the most conserved region of the ACLSV encoding genes with identity levels ranging between 87 and 93.3 % [10]. Thus coat protein gene sequence was used for designing the primers. Among the five sets of primer, one primer set i.e. 1F1R was selected. This selected primer was further standardized by exposing it to different annealing temperature (53, 55, 57, 59 and 61 °C). Wherein the optimal annealing temperature of 57 °C was effectively amplified the specific fragment. By using the conditions described, we amplified a 432 bp fragment specific for coat protein region of ACLSV from leaves of apple tree. Similarly 5 were successful in amplifying fragments specific for ACLSV in apple by RT-PCR.

In all the reactions of RT-PCR a primer set amplifying apple mitochondrial NADH dehydrogenase subunit 5 (nad 5) gene was used as an internal control. Non DNase treated RNA extracts are often DNA contaminated [11], making the use of other internal control primers as RT-PCR control difficult for such extracts, because they do not distinguish between RNA and DNA. DNase treatment of the extracts will eliminate these problems, but each additional extraction step is time consuming, will raise costs and increases the risk of contaminations. In order to avoid this, a primer pair designed by [9] was used which allows the specific amplification of mRNA even in the presence of genomic DNA.

The specific/desired amplicon was eluted and further processed for sequencing. The sequence data after BLAST

Fig. 1 Screening of Elite mother plants for presence of ACLSV. Lane M 1 kb DNA ladder, Lane I Internal control, Lane W water control, Lane H Healthy control, Lane K₁–K₂₈ (samples from Kullu) to Lane R₁–R₁₄ (samples from Rohru) elite mother plant samples with primer set 1F1R

revealed that the amplicon belongs to ACLSV cp gene. After confirming that 1F 1R primer set able to amplify coat protein gene of ACLSV we screened the elite mother plant maintained in IARI RS Shimla (India). As these plants will be used as a source of bud stick for grafting and distribution to farmers in future, hence it is important to screen it for ACLSV. The collected elite mother plants were healthy in appearance, but based on results obtained we can conclude that among 42 different cultivars only 25 were found infected with tested virus (ACLSV). It proves asymptomatic nature of the virus and it suggest the urgent need for screening of all the mother plants prior to be used as source of scion for grafting or distribution to farmers. Results also imply that given primer set can be use to screen presence of ACLSV in different cultivars of apple. It shows wide distribution of this virus in farmer orchards. In similar study by [14] in Himachal Pradesh (India) ACLSV came across as a major virus on apple with disease

Table 2 Detection of ACLSV in elite mother plants

Cultivars	Code	ACLSV
Elite mother plants from Kullu		
Golden delicious	K ₁	–
Silver spur	K ₂	+
Red chief	K ₃	–
Oregon spur	K ₄	+
Well spur	K ₅	+
Starking delicious	K ₆	+
Starkrimson	K ₇	+
Tydemans early worsester	K ₈	+
Top red	K ₉	–
Red chief	K ₁₀	+
Vance delicious	K ₁₁	+
Golden spur	K ₁₂	+
Oregon spur	K ₁₃	–
Gloster	K ₁₄	+
Red delicious	K ₁₅	+
Starkrimson	K ₁₆	–
Vance delicious	K ₁₇	+
Top red	K ₁₈	+
Golden hornet	K ₁₉	+
Red fuji	K ₂₀	+
Silver spur	K ₂₁	+
Scarlet spur	K ₂₂	–
Red delicious	K ₂₃	–
Red flush	K ₂₄	–
Oregon spur	K ₂₅	+
Golden spur	K ₂₆	–
Well spur	K ₂₇	+
Red chief	K ₂₈	–
Elite mother plants from Rohroo		
Oregon spur	R ₁	–
Manchurian	R ₂	–
Silver spur	R ₃	+
Red chief	R ₄	+
Vance delicious	R ₅	+
Red flush	R ₆	–
Golden Hornet	R ₇	–
Red chief	R ₈	–
Top red	R ₉	+
Scarlet spur	R ₁₀	–
Well spur	R ₁₁	+

+ Positive with respect to ACLSV; – Negative with respect to ACLSV

incidence of 85–90 %. The infection was very widespread as ACLSV infection was confirmed in about 14 commonly grown apple cultivars from the 18 tested. [6] conducted survey in the month of June and September, 2010 in

Hatkoti, Rohru, Kuthara, Jubbal and Khadapathar areas of major apple producing Shimla district of Himachal Pradesh. A total of 250 samples were collected and analysed for the presence of ACLSV, where in the results indicated that a total of 117 samples were infected with *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV).

Based on this study we can conclude that there is a possibility of presence of avirulent strains of ACLSV providing cross protection to other pathogens as this are high yielding plants on farmer field which need to address in future. Also considering the performance of collected elite mother plants on farmer's field, virus free plant production is another researchable issue.

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