PRIMER DESIGNING AGAINST APPLE CHLOROTIC LEAF SPOT VIRUS INFECTING APPLES IN HIMACHAL PRADESH

Thesis

by

REENA KUMARI

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Dr. S.V. Bhardwaj
Professor & Head

Department of Biotechnology
College of Horticulture
Dr. Y. S. Parmar University of Horticulture
and Forestry, Nauni, Solan – 173 230 (H.P.)

CERTIFICATE- I

This is to certify that the thesis entitled, **"Primer designing against apple *chlorotic leaf spot virus infecting* apples in Himachal Pradesh "** submitted - in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY** to Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a record of bonafide research work carried out by **Ms. Reena Kumari (H-2006-17-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

31/12/08

Place : Nauni, Solan
Dated: 31st Dec 08

(S.V. Bhardwaj)
Chairperson
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis entitled, **"Primer designing against apple chlorotic leaf spot virus infecting apples in Himachal Pradesh "**, submitted by **Ms. Reena Kumari (H-2006-17-M)** to Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

16/1/09

(Dr. S.V. Bhardwaj)
Chairperson
Advisory Committee
Professor and Head

External Examiner

Members of Advisory Committee

16/01/09

Dr. Kamlesh Kanwar
Department of Biotechnology
Professor

16/1/09

Dr. P.D. Thakur
Department of Mycology and
Plant Pathology
Senior Scientist

16/1/09

Professor and Head
Department of Biotechnology

16/01/2009

Dean's Nominee

23.1.2009

Dean
College of Horticulture

CERTIFICATE-III

This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Primer designing against apple chlorotic leaf spot virus infecting apples in Himachal Pradesh"**, submitted to Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) by **Ms. Reena Kumari (H-2006-17-M)** in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY.**

21/1/09

Dr. S.V. Bhardwaj
Professor
Chairperson, Advisory Committee

21/1/09

Dr. S.V. Bhardwaj
Professor and Head
Department of Biotechnology
Dr Y S Parmar UHF, Nauni, Solan (HP)

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Needless to say errors and omissions are mine.

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Date: 31 Dec. 08

Reena
31/12/08
(Reena)

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LIST OF ABBREVIATIONS

ACLSV	-	Apple chlorotic leaf spot virus
BLAST	-	Basic local alignment search tool
bp	-	Base pair
cDNA	-	Complimentary Deoxyribonucleic acid
CP	-	Coat Protein
DAS – ELISA	-	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
DNA	-	Deoxyribonucleic acid
EMBL	-	European Molecular Biology Laboratory
et al.	-	Coworkers
IC-RT-PCR	-	Immunocapture Reverse Transcription Polymerase Chain Reaction
Kb	-	Kilobase pair
KDa	-	KiloDalton
µg	-	Microgram
µl	-	Micro litre
µm	-	Micromole
mM	-	Millimole
NCBI	-	National Centre for Biotechnology Information
ng	-	Nanogram
nm	-	Nanometer
ORF/s	-	Open Reading Frame/s
RT-PCR	-	Reverse Transcription Polymerase Chain Reaction
WWW	-	World Wide Web

INTRODUCTION

Chapter-1

INTRODUCTION

Apple belongs to the family Rosaceae, Sub family pomoideae and genus *Malus.* It is the most widely grown fruit and is cultivated throughout the temperate zone of both Northern and Southern hemispheres. Total production of apple throughout the world is 59, 683 thousand metric tonnes, and China is the largest producer of apple with the production of 23,000 thousand metric tonnes (Anonymous, 2003).

It is one of the most important temperate fruit crop of India with regard to average production, economic value and above all popularity (Kanwar, 1987). Here,it is mainly grown in three North Western Himalayan states viz, Himachal Pradesh, Jammu and Kashmir and Uttranchal, because of favourable agro climatic conditions. India's share in total world production is only 1.350 thousand metric tonnes.

It is the most dominating fruit crop of Himachal Pradesh constituting about 40 percent of total area and 76 percent of total production. Shimla, Kullu, Sirmour and Kinnaur are major apple growing belts, besides other temperate areas of the state. According to the statistical outline of the H. P. during 2006-07, annual production of apple was 2.68 lakh metric tonnes (Anonymous, 2007).

Commercial cultivation of apple in Himachal Pradesh started a few decades ago and the area under this crop is increasing exponentially. However, this increase in area is not commensurate with the production per unit area. There are many factors responsible for low productivity and one of the major constraints seems to be infection by different pathogens like bacteria, fungi and viruses. Amongst these viruses play most significant role because of their systemic nature. About 18 viruses have been reported to attack apples and these are known to cause major losses in terms of productivity and quality. Major viral diseases infecting apple are apples mosaic, apple rosette, apple chlorotic leaf spot,

apple stem pitting and apple green crinkle (Bhargava, 1957; Sharma *et al.*, 1979). Out of these, apple chlorotic leaf spot caused by apple chlorotic leaf spot virus belonging to flexiviridae family is the most commonly distributed virus (Adam *et al.*, 2004). Various strains of ACLSV have been reported all over the world. The virus causes chlorotic blotches (usually distriburted asymmetrically in young leaves), asymmetric leaf distortion and puckering. ACLSV mainly infects apple, peach, pear, plum, cherry and apricot. In addition, the virus also infects species in a few families, including chenopodiaceae and leguminoceae mechanically (Lister, 1970).

ACLSV was first reported in *Malus* species from USA (Mink and Shay, 1969), who described the virus as having flexuous filamentous particles 680-780 nm long and 12 nm in width, containing linear positive- sense ssRNA genome of 7, 549 to 7,555 nucleotides excluding a poly A- tail at 3' terminus where as the 5' terminus end of the RNA has a cap structure.

This virus has been detected by using techniques such as enzyme linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) (Herranz *et al.*, 2005; Menzel *et al.*, 2002). ELISA has been most commonly employed procedure used, because of its sensitivity, efficacy and economy. However, many viruses may be difficult to detect because of their low titer or uneven distribution in the plants through ELISA. Such difficulty is overcome by using molecular biology techniques like PCR, RT-PCR etcetera.

Now a days, computer analysis plays an important role in identification of genes in the given DNA sequence and also for finding similar DNA sequences in the databases. Using such techniques, we can also compute and design primers artificially. By using these primers in PCR, we can detect specific genes in a target sequence and use the amplified product for sequencing.

The ACLSV has been characterized by using available primers developed elsewhere. Random selection of primers followed by their application is not only time consuming but may be expensive and may not even be able to recognize particular strains. The ACLSV in Himachal Pradesh is a different strain of the

virus (Rana *et al.*, 2007) and so far its identification/characterization was being carried out ,using random primers because of the obvious strainal variances. Performance of such primers varies from place to place. There is, however, a possibility of picking up or designing specific primers for a strain to save time and money in addition to increase efficiency. It was therefore of interest, to design primers specific against the virus from Himachal Pradesh. The present studies thus have been planned with the following objectives in mind:

Objectives

- Identification of apple chlorotic leaf spot virus (ACLSV) using ELISA.
- Developing primers against apple chlorotic leaf spot virus CP gene using bioinfromatic tools.
- Testing efficacy of the developed primers using RT-PCR.

REVIEW OF LITERATURE

Chapter-2

REVIEW OF LITERATURE

Apple chlorotic leaf spot virus (ACLSV) is the most commonly distributed virus all around the world and it affects both yield and fruit quality of the crop. Cieslinska and Rutkowki in 2008 studied the effect of ACLSV on the yield and fruit quality of 'Sampion' and 'Golden Delicious' cultivars grafted on M 9 rootstocks. They observed that the quality parameters like weight, percentage of blush, total soluble solid , internal ethylene concentration of fruits and starch index of the virus free M 9 rootstocks was higher than from infected trees. The virus is classified as under :

2.1 CLASSIFICATION OF VIRUS

Family : Flexiviridae

Genus : Trichovirus

Species : Apple chlorotic leaf spot virus

Acronym: ACLSV

Synonyms: Pear ring pattern mosaic virus,
Apple latent virus type 1,
Plum pseudopox virus,
Quince stunt virus

Apple chlorotic leaf spot virus is classified into the closterovirus group, based mainly on its particle structure (Francki *et al.*, 1991).This group is composed of heterogeneous virus species with different particle length, transmission modes and tissue localization of the virus particles in infected material. The genome organization of ACLSV is quite different from that of the 3'- terminal genomic region of beet yellows virus (Agranovsky *et al* ., 1991) , the type member of the closterovirus group, indicating that ACLSV should be classified into a new virus group.

Martelli and his associates in 1994 discovered that a new genus trichovirus includes five viral species (two definitive and three tentative species) which have similar biological, morphological, physiochemical and ultrastructural properties. In definitive species, the genome was constructed by three ORFs. They also observed that the two tentative species viz: Grapevine virus A (GVA) and Grapevine virus B (GVB) may express small ORF extra to the above three ORFs.

2.2 STRUCTURE

ACLSV has very flexuous filamentous particles, approximately 680-780 nm long and 12 nm wide containing linear positive-sense , ssRNA with an M_r of $2.48x10^6$ and a single coat protein of 22kDa (Yoshikawa and Takahashi,1988). The particles require presence of divalent cation for the integrity of the quaternary structure (Lister and Hadidi, 1971).

2.3 PARTICLE COMPOSITION

2.3a NUCLEIC ACID: The virus is composed of linear positive sense ssRNA of M_r about 2.48 X 10^6 or 7549 to 7555 nucleotides excluding a poly A- tail about 5% of the RNA weight. The 5′ terminus of the RNA probably has a cap structure. Nucleotide base ratios for isolate P863 from *Prunus domestica* are: G23.8%; A31.5%; C17.7%; U27.0% (German *et al.*,1990; Sato *et al.*, 1993; Yoshikawa and Takahashi., 1988).

2.3b PROTEIN : The virus Particles contain a single polypeptide species of M_r 214-215 KDa (Lister *et al.*, 1973; German *et al.*, 1990; Sato *et al.*, 1993; Yoshikawa and Takahashi., 1988).

2.4 GENOME PROPERTIES

Complete nucleotide sequences of the single genomic RNA from four isolates have been determined. They are isolates P863 from *Prunus domestica*, P205 from apple, Bale from cherry and PBMI from plum (German *et al* .,1990; Sato *et al.*,1993 ;Yoshikawa and Takahashi., 1988). Identities of the nucleotide

sequences were 79.8% (P863/P205), 76.2% (P863/Ball), 81.5% (P863/PBMI), 76·5% (P205/ Ball) , 79·6% (P205/ PBMI), and 76·5% (BAL1/PBMI).

Complete nucleotide sequence of the genome of an apple isolate of apple chlorotic leaf spot virus (ACLSV-A) was 7552 nucleotides long excluding the poly (A) tail and it contains three ORFs in the positive strand. ORF 1 encoding proteins with M_r of 216.5KDa and it contains two motifs associated with the helicase (GxxxGxGKS) and the RNA polymerase (GxxxTxxxNT,GDD) at positions 1060-1067 and 1694-1728,respectively. The 50 KDa protein encoded by ORF 2 is localized on plasmodesmata in infected and transgenic plant leaves. However, ORF 3 has encoding protein with M_r of 21.4 KDa. Analysis of the dsRNA of ACLSV infected plants and in vitro translation of ACLSV RNA suggested that the 50 KDa protein and coat protein are expressed from 2·2 and 1·I kb subgenomic RNAs , respectively (Candresse *et al.*, 1996 ; German et al., 1992).

Al-Rwahnih *et al* in 2004 observed that most ACLSV coat protein isolates show variability in the N terminal portion and that the C-terminal is conserved. They compared ACLSV almond sequence with 64 other ACLSV-CP sequences through multiple alignment. Cieslinska and his associates in 2007 studied some molecular properties of apple chlorotic leaf spot virus isolates infecting fruit trees in orchards in Bulgaria. They performed sequence analysis of the coat protein (CP) and movement protein (MP) coding regions for isolates from peach, sweet cherry and apple trees, in order to determine sequence variability of ACLSV isolates. In 2007 Yaegashi *et al* suggested that amino acid alanine at position 40 and phenylalanine at position 75 or serine at 40 and tyrosine at 75 are important for incidence of ACLSV in host plant cells.

Complete nucleotide of an isolate of ACLSV from peach was sequenced by Marini and his associated in 2008. This is the first published sequence for the ACLSV infecting peach, and because of its history and the mode of transmission, is possibly the first complete genomic sequence for an isolate of the virus from China.

2.5 GEOGRAPHICAL DISTRIBUTION

ACLSV is probably the most widespread and distributed worldwide in fruit trees including apple, apricot, cherry, plum and peach. Geographical distribution of the virus had been reported in different countries viz; Australia, China , Eastern Asian region , North American region and New Zealand . Virus was first reported in Malus species from the U.S.A (Mink and Shay,1959 ; Lister *et al.*, 1965)

2.6 TRANSMISSION

The virus is readily transmitted by mechanical inoculation and by grafting. In 1968 Pfaeltzer was successful in transmitting ACLSV from apple to *Chenopodium quinoa* through sap inoculation. The symptoms produced on *C. quinoa* are local lesions followed by systemic infection.

2.7 SYMPTOMATOLOGY

Symptoms can be highly variable and depend on the virus strain and the host species or cultivar infected. The symptoms of ACLSV include line patterns, chlorotic rings, stem pitting, chlorosis and stunting and dark green mottle. (Bar joseph *et al.*, 1979; Yanase *et al.*,1975). In sensitive *Malus* cultivars, symptoms can include chlorotic leaf spots and/or ring and line patterns on foliage, asymmetric leaf distortion, premature leaf drop, stunting, terminal dieback, inner bark necrosis and xylem pitting, and local bark necrosis surrounding the inoculum buds (Nemeth *et al.*,1986; Mink 1989).

2.8 STRAINS

Desvigens and Boye (1988), Chairez and Lister (1973) and Yanase (1974) reported that many isolates of ACLSV from apple, peach, cherry and plum can be differentiated on basis of symptomatology in indicator plants. However, some isolates from peach and apple trees comprised of atleast two or three variants

that differ considerably from each other in nucleotide sequence (Candresse *et al.*,1995).

2.9 DETECTION

Apple chlorotic leaf spot virus is the most important virus infecting apples around the world and was first reported by (Lister *et al.*, 1965). There are numerous reports of ACLSV detection using enzyme based immunological techniques. Barba and Clark in 1986 used F_{ab} based indirect ELISA for the detection of strains of apple chlorotic leaf spot virus. This method was used to investigate feasibility of assaying ACLSV in fruit trees throughout the growing season and distribution of the virus in various parts of the trees. Anil kumar in 2000 used DAS and DAC – ELISA for the detection of ACLSV in apple. He used DAS and DAC- ELISA at maximum dilutions of 10^{-2},10^{-2}and10^{-3} of antigen, antibody and enzyme conjugate, respectively for reliable detection of this virus.

Generally alkaline phosphatase based DAS ELISA has been used widely. However, Bhardwaj *et al* in 1995 reported this virus from Himachal Pradesh, India using penicillinase based ELISA. They detected ACLSV from apple cultivars namely Golden delicious ,Red gold, Royal delicious , Starking delicious, Hardeman and Amb red by employing woody indicator R12740-7A under field conditions and penicillinase (PNC) based modified double antibody sandwich (DAS) elisa.

Kaur *et al.* (1995) also used penicillinase based indirect ELISA for detection ACLSV from four apple accessions viz. KIRI4A3, KIR33A39,KIRI3A27 and KIR44A16 maintained under glass house conditions. They also concluded that the differential symptoms in these four accessions may be due to either strainal difference in ACLSV or mixed infection with apple mosaic virus (ApMV).

Another method where the plates are first coated by protein A, ie Protein A coated enzyme linked immunosorbent assay (PAC ELISA) has also been

employed (Wu *et al.*, 1998) for detection of ACLSV and ASGV. However,this method involves more steps and thus is time consuming.

Later in 2001, Corvo and Barros used a simple DAS-ELISA procedure for large scale detection of ACLSV and ASGV in apple trees. Numerous studies had shown that ACLSV detection should be done during a limited period in the growing season. To overcome this limitation they conducted a series of experiments using different apple tissues including winter bud sticks, flowers, leaves and growing shoots. This method was developed to be much faster and convenient procedure to detect ACLSV in apple tree tissues, as compared to standard procedure.

2.10 SOFTWARES FOR PRIMER DESIGNING

Use of softwares for primer designing seems, to be rather comparatively a new approach. There are many softwares available offline/online which meet the requirement for primer designing. These softwares are algorithm based and design primers suiting to specific requirements. In 1991 Hara used PRIMEGEN software for designing primers from multiple alignments. Later in 1994 Higgins and his coworkers obtained coat protein sequences from Genbank and used clustal W multiple sequence alignment software for designing virus specific primers. PRIMO software was used for designing forward and reverse primers and then the same were employed for large scale DNA sequencing Li *et al* in 1997.

Similarly another software PRIMESELECT was used for primer and probe designing by Plasterer in 1997. Singh and his coworkers in 1998 used PRIMER PREMIER program for designing degenerate primers from protein sequences.

Gibbs *et al* in 1998 developed GPRIME computer programme package to identify most homologous regions of aligned sequences. It was used to design primers that detect protex virus and tobamo virus in Australian orchird collections. In the same year Rozen and Skaletsky designed primers using primer

3 software. This software shows input parameters, excluded regions and targets to locate primers within the regions of interest.

A dual prime computer software was development in 2002 by Fernandes and Skiena. They used this software to identify short regions of high sequence similarity between the two genomes. This software, is designed at for finding reduced sets of primers sufficient to amplify all the genes in a genome .

Bystricka and his coworkers in 2005 used oligonucleotide based microarrays for detection of plant viruses. They presented a new approach towards detection of plant viruses using synthetic single standard oligomers (40nt) instead of PCR products as capture probes. All these softwares have specific qualities/parameters and their suitability/ utilities is a function of the sequence to be amplified and the parameters required.

2.11 EXTRACTION OF NUCLEIC ACIDS

Extraction of purified nucleic acids have been of concern since were reported. Newbury and Possingham in 1977 suggested that tissue from woody plants contain great amount of phenolic compounds and polysaccharides and these substances inhibit the activation of reverse transcriptase or Taq polymerase in RT-PCR. To overcome this problem Henson and French in 1993 used IPVP-40 or mercaptoethanol for extraction of nucleic acids. In 1999 Wu and his coworkers used sodium sulfite to inhibit polyphenolic oxides. Where as, Boom et al in 1990 used silica capture method for isolation of Total plant RNA from apple tissues. Now with the fast development in such techniques standard protocols are available, which can be applied as such or with slight modifications to purify specific nucleic acids.

2.12 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction is an extremely sensitive and specific technique (Saiki *et al.*,1988) for detection and identification of the plant pathogens. It is widely used for the diagnosis of plant diseases, allowing detection of very small amount of the disease agent in the infected plant and also

for the cloning of genomic fragments of the pathogen 'Henson and French,1993). RNA cointaining viruses require RT step to generate cDNA prior to PCR amplification (RT-PCR). Specificity of PCR is based on the use of oligonucleotide primers that are complementary to the regions flanking the target nucleic acid sequence to be amplified and the PCR usually requires purification of the target nucleic acid . Nickel *et al* in 1990 used silica capture method for total RNA extraction from infected leaves of *Chenopodium quinoa* and *Malus* species. This isolated RNA was used as template for RT-PCR with specific primers (Yoshikawa *et al.*, 1992 ; Mackenzie *et al.*, 1997). Hassan et al in 1995 used pentaplex RT-PCR for simultaneous detection of the pome fruits. Later in 2001 Fossaic and his coworkers used polyvalent nested RT-PCR for the detection of ACLSV. Later in same year Rott and Jelkmann used this method for detection of plant viruses.

Menzel *et al* in 2002 used multiplex RT-PCR assays for detection of four apple viruses with coamplification of plant mRNA as internal control. Four virus specific primer pairs and one primer pair specific for mitochondrial nad5 gene were used. This is the first report on the use of a specific internal RNA control from total nucleic acids. The multiplex RT- PCR assays described are reliable, rapid and sensitive methods for the detection of viruses.

Rana *et al* in 2007 used RNeasy plant mini kit (QIAGEN, Germany) for total RNA isolation from ACLSV infecting almond leaf tissue. They used primer pair (forward primer 5'-GATCAGAAGRMGRAGGAT-3' and reverse primer 5'-GTAGTAAAATATTTAAAAG-3' accession numbers, AM490253 and AM490254 respectively) to amplify coat protein gene and untranslated region from 3' end of the ACLSV genome. In the same year, while working on a similar disease in 17 different cultivars of apple collected from different regions of Himachal Pradesh and in 4 rootstocks which are extensively used throughout India, they found ACLSV to be present using direct antigen coating indirect ELISA. They further performed RT-PCR using degenerate primers for the cultivar Royal Delicious collected from Kinnaur area. This study confirms the presence of ACLSV in India.

Another modification of RT PCR is immunocapture reverse transcriptase polymerase chain reaction (IC-RT PCR) had also been used sometime for detection of plant viruses. Candresse *et al* in 1995 used polyvalent PCR based assay for the detection of apple chlorotic leaf spot virus and to achieve maximum sensitivity an immuno capture step was carried out directly in PCR tube before the reverse transcription and PCR reaction. To detect ACLSV, this assay proved very useful for the detection of this virus in a variety of plant material and from variety of woody hosts. The IC-PCR assay, was also used in conjunction with sequencing of the amplified fragment, to study the molecular variability of ACLSV.

Similarly Nemchinov *et al* in 1995 used specific primers for ACLSV for detection of the viral coat protein gene. These primers were utilized for amplification by RT-PCR and IC-RT-PCR of a 358 bp cDNA fragment extracted from ACLSV infected apple and peach from France.

In 1998, Pasquini and its coworkers characterized apple chlorotic leaf spot virus isolates from Italy. To determine the ACLSV isolates present, they performed western blotting, immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) and restricted fragment length polymorphism (RFLP). Later in 2004 Lebas and his coworkers also detected ACLSV using RT-PCR and IC-RT-PCR.

2.13 Development of primers for detection of viruses

As already mentioned developing specific primers for nucleic acid amplification is rather a new concept. Bateson and Rale in 1995 demonstrated that the sequence information obtained from cloned PCR product permitted the designing of specific PCR primers against particular virus/strain. Appropriate primer design determines RT-PCR specificity for one or more virus species, subgroups, strains or isolates. For detection and differentiation of virus species or strains, predominantly primers in conserved region of CP and viral RNA polymerase gene have been used (Singh, 1998).

The technique has been widely used to detect plant viruses in general and ACLSV in particular. Okuda and Hanada in 2001 used degenerate primers for detection of tospovirus species and Nickel *et al* in 2001 used two specific primers ASGV 6396 and ASGV 5641 for detection of apple stem growing virus. These primers were used to amplify 755 bp of ASGV coat protein. Similarly Lunello and his coworkers in 2005 used degenerate primers for detection of potyviruses in allium species.The technique has also found favour with scientists to detect Capillovirus and Trichovirus including ACLSV

Minafra and Hadidi in 1994 constructed the DNA specific primers specific for grapevine virus A, grapevine virus B and grapevine leafroll associated virus 111 based on nucleotide sequence of segment of each viral genome. Later Routh and his coworkers in 1998 also used single set of primers for detection of multiple serogroups of grapevine leaferoll- associated virus.

In 1996 Kinard and Scott used specific primer for detection of apple chlorotic leaf spot and apple stem growing viruses. Later in 2008 Rana and his coworkers designed AM408891-R primer for RT-PCR to detect and amplify genome of apple chlorotic leaf spot virus. Again in 2007 Rana and his coworkers designed AM408891-F primer to detect and amplify genome of apple chlorotic leaf spot, which were also used in 2008 for detection of ACLSV from almonds.

MATERIALS AND METHODS

Chapter-3

MATERIALS AND METHODS

The present investigations "Primer designing against apple chlorotic leaf spot virus infecting apples in Himachal Pradesh" were undertaken to detect coat protein gene of ACLSV and to test the specificity of primers under wet lab conditions. These studies were carried out in the Departments of biotechnology and Mycology & Plant pathology of Dr Yaswant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P).

To achieve these objectives the materials and methods used in the present investigations are described below under following headings.

3.1 Survey and collection of the plant material

3.2 Serological detection of virus

- 3.2.1 Source of antisera
- 3.2.2 Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)

3.3 Importing of sequences

- 3.3.1 Genbank at National Center for Biotechnology information. (NCBI)
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3.4 Primer designing using various softwares

- 3.4.1 Exome Horizon
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- 3.4.4 Gene Fisher
- 3.4.5 Primer Blast

3.5 Synthesis of primer

3.6 Isolation of nucleic acid (RNA)

- 3.6.1 Isolation of total plant RNA.

3.6.2 Purification of isolated RNA.

3.6.3 Agarose gel electrophoresis of RNA.

3.7 Detection of apple chlorotic leaf spot virus using RT-PCR.

3.7.1 Amplification of cDNA using ACLSV specific primers.

3.7.2 Agarose gel electrophoresis of PCR product.

3.7.3 Elution of DNA from agarose gel.

3.1 SURVEY AND COLLECTION OF PLANT

Extensive surveys were conducted in different apple growing areas of district Solan and Sirmour to ascertain identify the apple chlorotic leaf spot virus infected apple plants. Cultures of virus isolates were collected from naturally infected plants. Infected leaf samples expressing prominent symptoms of ACLSV were collected from the orchards of habban distt. Sirmour(H.P) and stored in liquid nitrogen . Other samples were taken from the fields Deptt of Mycology and Plant Pathology farms of Dr Yaswant Singh Parmar University of Horticulture and Forestry, Nauni, Solan(H.P).

3.2 SEROLOGY

Apple chlorotic leaf spot virus infected fresh or frozen leaves were put to serological indexing. The moisture of these leaves was maintained by keeping these in folds of moist filter paper or in liquid nitrogen cylinder. Double antibody sandwich form of enzyme linked immunosorbent assay was used as per the protocol of the suppliers of elisa reagents (M/S Agdia inc., Elkhart, Indiana,U.S.A), following Clark and Adams (1977). Planner for serological indexing of apple chlorotic leaf spot virus is presented in table 3.1.

Bioreba elisa reagents are optimized for use in the double antibody sandwich procedure (DAS- ELISA) using certified Nunc- immuno plates maxisorp 96 and operating with a working volume of 200ul per well. All the wells of microtitre plates were filled except extreme left, right ,top and bottom rows. According to the planner, wells were filled by diluting 1000x IgG in coating buffer (20µl in 20ml buffer) the plate was than covered tightly with aluminium foil, placed in a humid box and Incubated at 30°c for 4h. The coating

Table 3.1. Serological detection of apple chlorotic leaf spot virus

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		S_1	S_1	S_1	S_1	S_1	S_1					
C		S_2	S_2	S_2	S_2	S_2	S_2					
D												
E		C(+)	C(+)									
F		C(-)	C(-)									
G		B	B									
H												

S_1 = Plant sample from habban
S_2 = Plant sample from Pathology field
C(+) = Positive control
C(-) = Positive control
B = Buffer

antibody suspension was removed out of the plate wells by shaking it with a jerk. The wells were filled and emptied again with washing buffer. Washing was repeated for 3-4 times. Test sample (1gm) was then homogenize in an extraction buffer (2.5ml). All coated wells were filled with 200µl aliquots of the test sample, whereas, +ve and –ve control wells were filled with the solution provided for the purpose.The plates were incubated in humid box overnight at 4±1°C .The washing steps were repeated as mentioned above. Thereafter the wells, were filled by diluting conjugate antibodies in 1x conjugation buffer (20µl in 20ml buffer). The plate was again covered with aluminium foil and incubated in humid box for 4 hours at 37°C, followed by washing the plate as mentioned above . For visualizing the results, substrate Nitrophenyl phosphate(pNPP) tablet was dissolved at 1mg/ml in substrate buffer and each well was filled with 200µl of the substrate so prepared. The plates were again kept in humid box in an incubator at 37°C for 20 minutes or kept in dark by wraping the plate with carbon paper for 30 minutes at room temperature. The results were assessed by visually or colorimetric measurements. Flow chart diagram of the procedure is given in Fig. 3.1.

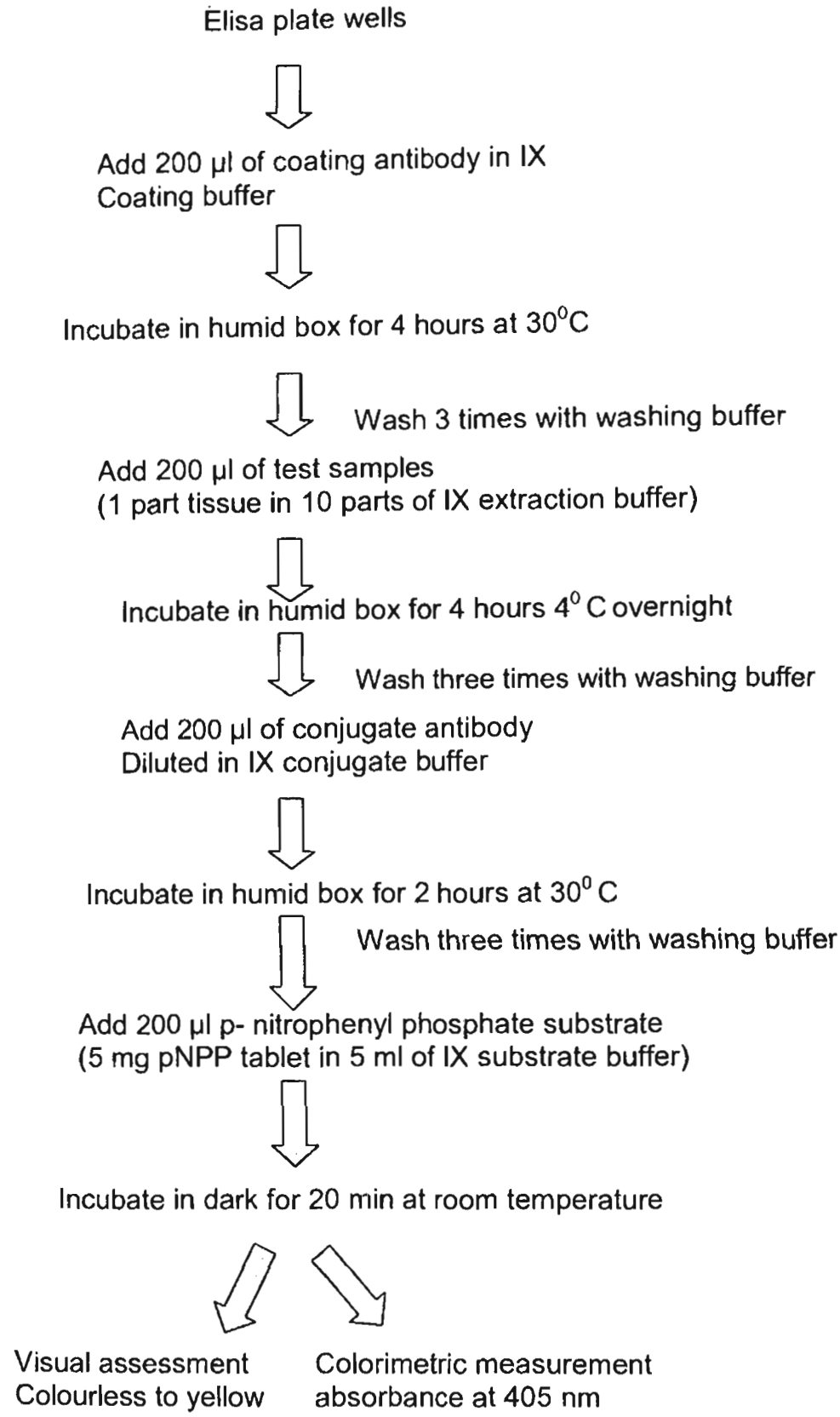


FIG. 3.1. Procedure of DAS ELISA for detection of ACLSV

3.3 IMPORTING OF SEQUENCES

The National center for biotechnology information (NCBI) and European molecular biology laboratory (EMBL) databases were searched for importing of sequences. Sequences of coat protein of apple chlorotic leaf spot virus were selected. Out of these, such sequences were taken which have highest possibility of showing conserved region of apple chlorotic leaf spot virus solan strains (the likely virus present in the state), so that primers, specific for this virus could be developed.

3.4 PRIMER DESIGNING

With the discovery of polymerase chain reaction by Mullis *et al* in (1985), the detection /quantification of nucleic acids using this technology increased day by day. The procedure requires primers for detection of the nucleotides and then for further amplification of the target DNA. The primers to be used for these studies are traditionally selected randomly and then with repeated experimentation using different sets of primers, the best set is selected in wet lab experimentation. However, with bioinformatics tools we use different softwares for designing primers and these softwares narrow down the choice of primers to be used in such experiments. PCR involves denaturation, annealing and extension steps. All three steps are temperature sensitive and common choice of temperature is 94°C, 55°C, and 72°C respectively . Good primers are essential for successful reaction. The primers which are unique for the target sequence to be amplified should fulfill certain criteria given below:

1) **PRIMER LENGTH:** Specificity, temperature and time of annealing are partly dependent on primer length. This parameter is critical for successful polymerase chain reaction. For broad spectrum studies, optimum length of the primers for PCR is 18-30 bp (Wu *et al.,1991*). This length is enough for primers to bind easily to the template DNA at annealing temperatures.

2) **MELTING TEMPERATURE:** Tm is defined as the temperature at which one half of cDNA dissociates to form ssDNA. The optimal melting

temperature for Primers is in the range 52-58°C, which generally produce better results. Tm above 65°C should be avoided because of potential for secondary structures formation (Santalucia *et al.*, 1996).

3) **GC%**- It is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC content between 45-50% (Dieffenbach *et al.*, 1995). Higher GC% of the primer increases strength of annealing. GC content higher than this however, increases T_m resulting into formation of secondary structures and so is not recommended.

4) **SECONDARY STRUCTURES**- Secondary structures are formed by inter and interamolecular interactions and lead to poor and no product yield. (Kwok *et al.*, 1999). Secondary structures can be cross dimers ; self dimers and hairpins and their presence should be checked during primer designing.

All these aspects were taken care while designing primers under these studies. Different steps involved in designing primers using different softwares are given below:

3.4.1 PRIMER DESIGNING BY USING EXOM HORIZON

Exom horizon is bioinformatics softwares which was provided by M/S Mascon Global Limited as a trial version and was made available site: http://125.21.176.217/ehorizon2/login.htm

- Sequences of interest were searched in NCBI database and the format of sequence so selected opened in genbank format.
- Genbank format was changed to fasta by display button present on the web page.
- Exom horizon site was opened and gene sequence was pasted in the dialogue box.
- All the following parameters were filled

  Primer length -18-24

GC Content- 45-60

Tm- 52-60℃

- List of all the available primers along with the best pair of primer were displayed on screen.

3.4.2 PRIMER DESIGNING BY USING WEB PRIMER

This software is available free on site: genome-http://www2.stanford.edu/cgi-din/SGD/web-primer

- Nucleotide sequences of cp genes of ACLSV were imported from NCBI database.
- Their format was changed from Genbank to fasta and pasted on the dialog box of the software.
- All the parameters were filled as discussed in section (3.4.1).
- This software shows an option to choose best primer, which was clicked.
- List of designed primers was displayed on the screen.

3.4.3 PRIMER DESIGNING USING PRIMER3

- Primer3 software is used for designing specific primers. It is the most commonly used bioinformatic software for designing primers, which is freely available on site: (http://www.basic.nwu.edu/biotools/oligocale.html)
- Sequences of interest were imported and changed into fasta format
- All the required parameters were filled
- Five best pairs of primer were displayed on screen.
- The properties of these primers were checked using oligonucleotide properties calculator.

3.4.4 PRIMER DESIGNING USING GENEFISHER

Genefisher primer is used for designing degenerate primers.This software is freely available on site: http://bibiserv.techfak.uni-bielefeld.de/genefisher/.

DEGENERATE PRIMERS – Degeneracy in primer sequence should also be taken into consideration .Degenerate primers based on the amino acid

sequence of the conserved regions were also used to search for members of a gene family (Wilks et al., 1989). Computer programs have also been developed specifically for degenerate primer design (Chen and Zhn ,1997).

- Sequence of interest was pasted in dialog box in fasta format.
- Selected the PCR primer option and submitted.
- 5 best pairs of primers were displayed on the screen.
- Selected the best pair which fulfill all the desired parameters of primer designing.

3.4.5 PRIMER DESIGNING USING PRIMER BLAST

Primer blast software is used for designing specific primers. It is the most commonly used bioinformatic software for designing primer, which is freely available on site: (http://www.ncbi.nlm.nih.gov/)

- Sequence of interest was imported and changed into fasta format
- All the required parameters were filled
- Four best pairs of primers were displayed on screen.
- The properties of these primers were also cross checked using oligonucleotide properties calculator.

3.4.6 TESTING OF PRIMERS BY TOOL OLIGONUCLEOTIDE PROPERTIES CALCULATOR

Various parameters like length of primers, melting temperature, GC content, product size, secondary structures like hairpin loop formation, and self annealing determines the efficacy and success of experimentation in molecular biology. Primers are tested for above properties by the tool oligonucleotide properties calculator which is available on site: http://www.basic.northwestern.edu/biotools/oligocalc.html Selected oligonucleotide sequences (18-25) bp and pasted on the dialog box. Press calculate button. All the above parameters got displayed on screen.Another important parameter is secondary structures, which include self dimers, hairpin and cross dimers which were also checked by this softwares .For hairpin formation minimum basepairs

required are 4 and for self dimerisation minimum basepairs requiredare 5. So all the parameters required for designing best primers were calculated by this software.

3.5 SYNTHESIS OF DESIGNED PRIMERS

The designed primer were got synthesized from M/S Sigma Aldrich Chemicals Pvt. Ltd. Bangalore.

3.6 STERLISATION PROCEDURES

All the glass wares, tips, micro centrifuge tube and tip boxes, spatula, mortar and pestles were autoclaved at 1.2-1.5 kg/cm^2 (15psi to 22psi) above atmospheric pressure for 15 minutes. Double distilled water was also autoclaved and for RNA isolation and PCR, nuclease free water provided in the kit was used.

3.7 TESTING OF PRIMERS

3.7.1 ISOLATION OF TOTAL RNA:

Total RNA from ACLSV infected apple plant samples was isolated by using small commercially available RNA isolation kit (Bangalore genei). Procedure for RNA isolation is presented through flow chart in fig 3.2.

a) For RNA isolation, a lyasate was prepared by crushing the sample thoroughly in liquid nitrogen to make a fine powder.

b) Prewarmed buffer A+ phenolic mixture (80°c) in 1:4 ratio was added to the powdered tissue in a 30 ml sterile polypropylene tube and mixed for 5 minutes.

c) Equal volume of chloroform (4-7ml/gm) was added into the above solution obtained in step b. The mixture was incubated at room temperature for 30 minutes with intermittent mixing and centrifuged at a speed of 5000 rpm for 15 minutes. Upper phase was transferred into new tube for further use and lower phase was discarded.

d) 1/3 volume of precipitation solution (buffer b provided in the kit) was added to the solution obtained after step (c) and mixed well. The

resultant mixture was incubated at 4°C for 4 hours. RNA was precipitated by centrifuging the above mixture at 7000 rpm for 20 minutes and flow through was discarded.

e) Dissolved the precipitate obtained in above step (d) in 1-2 ml of wash buffer (buffer C provided in the kit) and centrifuged at 7000 rpm for 20 minutes. This step was repeated 2-3 times for effective precipitation of RNA and flow through was discarded .

f) Pellet was then washed twice with 70% ethanol and finally with 100% ethanol. Every time the solution was mixed well and centrifuged at 7000 rpm for 10 minutes and flow through was discarded.

g) After washing, air dried the pellet for 15-20 minutes and then dissolved in 150-200µl of nuclease free water.

h) Isolated RNA was then checked through agarose gel electrophoresis.

Flow chart of RNA isolation procedure is given in fig (3.2).

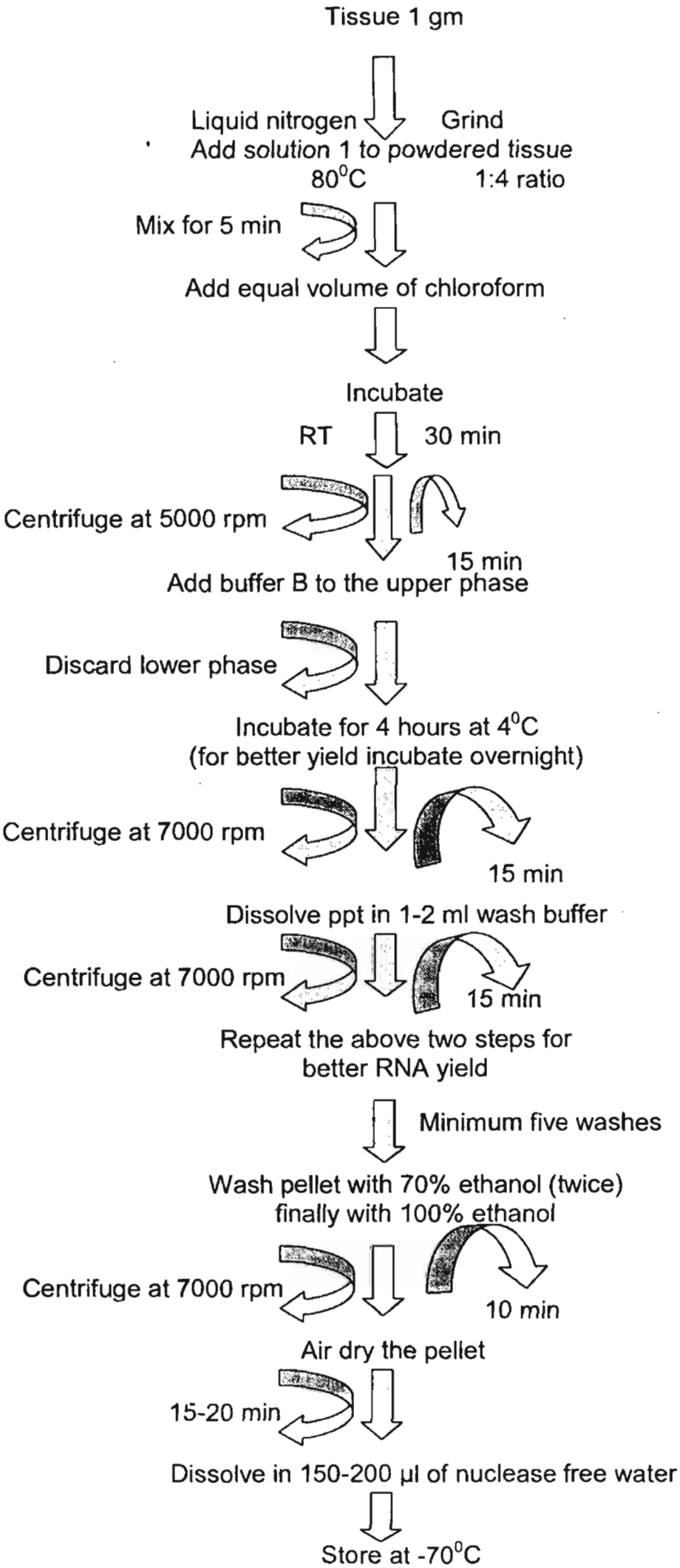


Fig 3.2 Procedure for isolation of Total RNA from Leaf sample

3.7.2 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was run for testing the presence of RNA isolated through RNA isolation kit. Solutions required for agarose gel were prepared as follows :

a) **Ethidium bromide dye**: Ethidium bromide solution was prepared by dissolving 10mg of ethidium bromide in 10 ml of sterile water and stirred on magnetic stirrer until the dye was completely dissolved. The solution was kept in dark bottle and stored at room temperature.

b) **50x TAE buffer:** 242 gm tris base and 57.1 ml of glacial acetic acid were mixed in a container. Then 100 ml of 0.5M EDTA was added (pH 8.0). Solution was stirred on magnetic stirrer until clear solution was obtained the buffer so prepared was stored at room temperature.

c) **6x loading dye:** 6x loading dye contains .25% bromophenol biue, .25% xylene cynol ff and 30% glycerol dissolved in water and kept at 4°c till use.

d) **Agarose gel (0.8 %):** 0.8% of agarose was poured in 100ml of distilled water having 2ml of 50x TAE buffer and boiled for 3-5 minutes. 8ul ethidium bromide was added when temperature reached 50-60^{0}C approximately. This solution was then poured into gel casting tray having already fitted comb. The mixture was then left for polymerization.

e) **Gel running procedure**: Isolated RNA was run in 0.8% agarose gel. Gel was placed in electrophoresis tank having 1x TAE buffer. Bromophenol blue dye (3.0µl) was mixed with 10µl of RNA sample on parafilm and loaded in wells and electrophoresis was run for 1hour at 70 volts.

f) **Viewing the gel and photography**: After completion of gel electrophoresis, gel was analyzed under UV transilluminator and photographs were taken with the help of gel documentation system .

3.8 DETECTION OF COAT PROTEIN OF APPLE CHLOROTIC LEAF SPOT VIRUS USING RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a molecular biology technique for amplifying a target nucleic acid by using a pair of specific

oligonucleotide primers. It is quite useful technique in the detection and diagnosis of viruses and other pathogens, specifically.

RT-PCR was performed with a specific primer pair that amplifies the coat protein gene of ACLSV. For synthesis of cDNA from RNA, a reaction mixture of 25µl was prepared . The reaction mixture contained RT buffer (5x), dNTP mix (100mM) reverse primer (200ng/µl), RNase inhibitor (25U/µl), RNA sample, reverse transcriptase (10U/µl) and nuclease free water. All the components were mixed through pipetting and then the RT mixture was incubated at 37°C for 1 hour after which same was incubated at 94°C for 1-2 min.

3.9 AMPLIFICATION OF cDNA USING ACLSV SPECIFIC PRIMERS

Further amplification of cDNA was done by preparing new reaction mixture containing PCR buffer (10x), dNTP mix (10mM), forward primer and reverse primer (200ng/µl), cDNA, Taq polymerase (5U/µl) and nuclease free water. PCR was carried out in thin walled 200µl tubes containing reaction mixture in a thermocycler (Applied Biosystem, USA). Denaturation was done at 94°C so that newly synthesized strand detached from the template and cooled; enabling the primer to hybridize at their respective positions, including positions on newly synthesized strands. Annealing temperature of the primers was different 56 and 57 respectively. Final extension was carried out at 72°C for 7 minutes. After completion of PCR, the amplified product was analyzed on 1.5% agarose gel at 75V with 100bp DNA ladder as a molecular weight standard. The gel was stained in ethidium bromide and visualized using an ultraviolet transilluminator and photographs were taken using gel documentation system (AlphaImager 22.0^{Tm}).

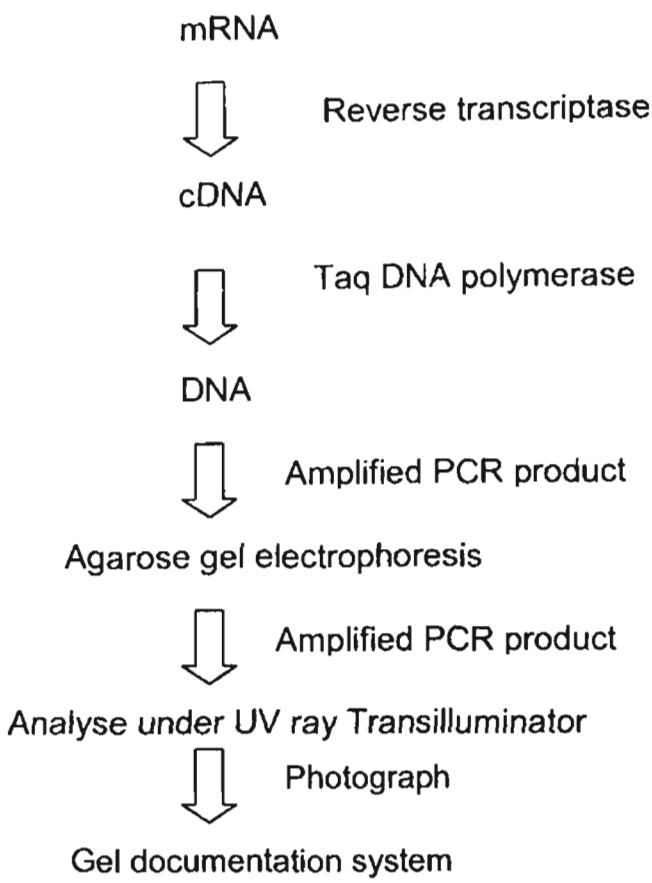


FIG. 3.3. Procedure of reverse transcription polymerase chain reaction

EXPERIMENTAL RESULTS

Chapter-4

EXPERIMENTAL RESULTS

Apple chlorotic leaf spot virus (ACLSV), the type species of the genus Trichovirus, is one of the most widely distributed plant viruses infecting pome and stone fruits. It affects both yield and fruit quality (Cieslinska and Rutkowski , 2008). It is difficult to detect infection of ACLSV on apples due to its latent symptoms and seasonal variations in detection. To overcome this difficulty specific primers were designed for detection of the virus. Three pairs of primers (Forward and Reverse) were selected out of total primers originally designed and were further tested under wet lab conditions for efficacy. The results of these experiments are being presented in the following paragraphs:

4.1 CULTURE IDENTIFICATION ON THE BASIS OF SYMPTOMS

For culture collection survey of apple orchards at Habban district Sirmour (H.P) was conducted. Plants were individually inspected and leaves, which showed prominent symptoms of ACLSV like chlorotic blotches (usually distributed asymmetrically in young leaves), asymmetric leaf distortion and puckering were drawn from the plants. ACLSV infected apple plants were also available in farms of Department of Mycology and Plant Pathology, Dr Y S Parmar University of Horticulture & Forestry, Nauni- Solan (Fig.4.1). Such infected plants were selected for further experimentations. The infected leaf samples were used for serological detection by DAS-ELISA and later on for RNA isolation to carry out molecular characterization of the virus.

Inference:

- Symptoms specific of the disease on the leaves of apple plants indicated presence of the virus (ACLSV).

4.2 DAS-ELISA FOR SEROLOGICAL INDEXING OF APPLE CHLOROTIC LEAF SPOT VIRUS

DAS ELISA was performed to confirm infection of the virus in the selected plants with the help of specific antisera against ACLSV. Under present investigations, leaves infected with ACLSV were brought to the laboratory. ELISA plate was first coated with 200 µl of coating antibodies and then filled with 200 µl of the test samples crushed in extraction buffer. After that 200 µl of conjugated antibodies and para nitrophenyl phosphate were added as mentioned in section (3.2), before recording of observation. For DAS ELISA the wells were coated as per planner (table 3.1). Visual assessment on the basis of colour is presented in (Figure 4.2).

Table 4.1 OD values of ACLSV in ELISA Plate

	1	2	3	4			
A							
B		1.114	1.118	1.220	1.222	1.224	1.220
C		1.197	1.198	1.338	1.355	1.458	1.430
D							
E		1.740	1.742				
F		.482	.485				
G		.363	.380				
H							

As it is evident from the results (Fig 4.2), sample B drawn from Nauni campus produced significantly darker yellow colour (C_2, C_3, C_4, C_5, C_6 & C_7) when compared to the – ve control (F_2 and F_3). However, the colour produced was lighter when compared to that produced by the +ve control (E_2 & E_4). The OD values of the sample were also in resonance with the visual observations (Table 4.1). The results confirmed presence of higher concentration of the virus in sample B.

Sample A was similarly evaluated visually as well as through ELISA plate reader. It was noticed that this sample (Fig 4.2; Table 4.1) also produced darker colour as compared to – ve control (fig 4.2). However, the intensity of colour was lighter than sample B (Fig 4.2) and the results were verified by

Fig. 4.1 Leaf of Apple showing symptoms of ACLSV infection (Var. Golden spur)

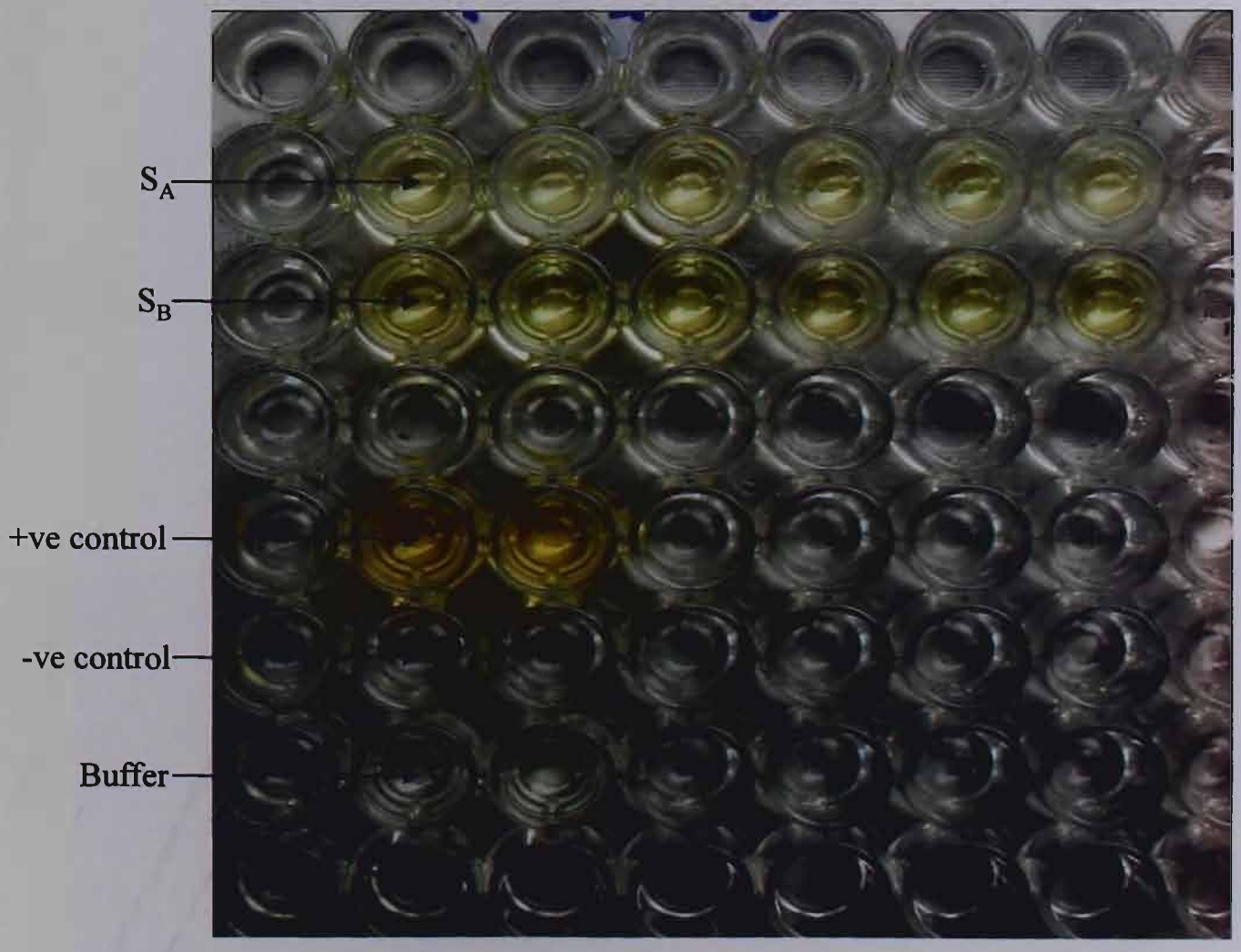


Fig. 4.2 Detection of ACLSV through DAS ELISA (SA - Sample A from Habban, **SB** - Sample B from Pathology farm of Nauni Campus **+ve** - Positive control, **-ve** - Negative control)

recorded OD values (table 4.1; F_2 and F_3) signifying presence of the test virus in sample A but in a lower concentration .

Inference .

- Both samples A and B have significant concentration of the virus.
- Sample B shows little higher concentration of the virus than that to sample A

4.3 PRIMER DESIGNING FOR DETECTION OF CP GENE OF ACLSV

Specific primers for detection of CP gene of ACLSV infecting apples for the H.P isolates were designed using online tools like Exome Horizon, Web Primer, Primer 3, Gene Fisher and Primer Blast against retrieved sequences of CP gene of ACLSV from NCBI (http://www.ncbi.nlm.nih.gov/) . Such retrieved sequences of the test virus are presented in table 4.2.

Table 4.2 Nucleotide sequences of coat protein gene of ACLSV retrieved from NCBI database

Accession number	Definition	Host	Place	Author
AM494507	Coat protein gene for ACLSV genomic RNA, Solan	Royal delicious apple	Solan	Rana *et al.*, 2007
AM498050	Coat protein gene for ACLSV genomic RNA, peach isolate from Solan	Peach	Solan	Rana *et al.*, 2007
AM494514	Coat protein gene for ACLSV genomic RNA, palampur	Apple	Palumpur	Rana *et al.*, 2007

4.3.1 Primers designed using Exome horizon

The trial version of EXOME horizon received from M/S Mascon Global limited was used to design primers. Trial version of the software was made available on site. http://125.21.176.217/ehorizon2/login.html.

- Nucleotide sequence of CP gene of ACLSV was downloaded from NCBI and Sequences were opened in Genbank format.
- Sequence of interest was pasted into dialog box in fasta format.

- All the Parameters were filled as mentioned in section (3.4.1).
- Five bests pairs of primers along with their properties were designed and are given in Table 4.3, 4.4 and 4.5)

Table 4.3. Designed Primers (5 pairs) against coat protein gene of ACLSV apple isolate from Solan (AM494507) using EXOME Horizon

	Primer	Length	Tm	GC	ANY	3'	Product length
Primer 1	Left Primer TCGCGAACATAGCGATACAG	20	60.00	50.00	6.0	1.0	158 bp
	Right Primer TCGGGTCCGAAGATGTAGTC	20	60.07	55.00	6.0	1.0	
Primer 2	Left Primer CAGACCCCTTCATGGAAGA	20	60.01	50.00	4.0	2.0	216 bp
	Right Primer TCGGGTCCGAAGATGTAGTC	20	60.07	55.00	6.0	1.0	
Primer 3	Left Primer TCTTCGGACCCGAACATAAG	20	60.07	50.00	8.0	2.0	240 bp
	Right Primer AAGACGCCGATTCATATTGG	20	59.99	45.00	4.0	3.0	
Primer 4	Left Primer GACCCCTTCATGGAAAGACA	20	59,90	50.00	4.0	0.0	213 bp
	Right Primer TCGGGTCCGAAGATGTAGTC	20	60.07	55.00	6.0	1.0	
Primer 5	Left Primer TCTTCGGACCGAACATAAG	20	60.07	50.00	8.0	2.0	238 bp
	Right Primer GACGCCGATTCATATTGGTT	20	59.79	45.00	4.0	0.0	

As is evident from data given in table 4.3 primers were designed against coat protein gene of ACLSV. All the five pairs of designed primer were 20 bp long, with melting temperature between 59-61 and GC content between 45-55. Any complementarity was between 4-8 and 3' complementarity was between 0-3. These primers were predicted to amplify a segment of approximately 240 bp.

Similarly this software was used to design primers against Solan sequence (AM 498050) and the results so obtained are presented in (Table 4.4).

Table 4.4 Designed Primers against coat protein gene of ACLSV peach isolate from Solan (AM498050) using EXOME Horizon

	Primer	Length	Tm	GC	ANY	3'	Product size
Primer 1	Left Primer CTGGATCTGGTGGTGGAAGT	20	59.96	55.00	4.0	1.00	230 bp
	Right Primer CGGCATGGTTGTAAAGAGGT	20	59.99	50.00	4.0	0.00	
Primer 2	Left Primer AGTTCCTGGATCTGGTGGTG	20	60.04	55.00	7.0	0.00	236 bp
	Right Primer CGGCATGGTTGTAAAGAAAT	20	60.06	50.00	4.0	0.00	
Primer 3	Left Primer CAGACCCCTTCATGGAAAGA	20	60.00	50.00	4.0	2.00	161 bp
	Right Primer GGTTGTATAACCCGATCACC	20	59.90	55.00	5.0	1.00	
Primer 4	Left Primer GAGAACGAGGCGAAACTGTC	20	59.96	55.00	3.0	3.00	159 bp
	Right Primer GGGTTCAAGAGTGGGATTCA	20	59.99	50.00	3.0	1.00	
Primer 5	Left Primer GAGAACAAGGCGAAACTGTC	20	60.00	55.00	3.0	3.00	160 bp
	Right Primer TAGGTTCAAGAGTGGGATTC	20	59.90	50.00	3.0	3.00	

As is shown in the table 4.4 all the primers designed against coat protein gene of ACLSV (AM498050) were 20 bp long. Tm and GC content of the primers were between 58-61 and 48-55% respectively. These primers were also predicted to amplify a product size upto 240 bp.

Table 4.5 Primer designed against coat protein gene of ACLSV apple isolate from Palampur (AM4980514) using exom horizon

	Primer	Length	Tm	GC	ANY	3'	Product size
Primer 1	Left Primer ACAACCTGAAGGAGGTGGTG	20	60.00	55.00	5.0	1.0	176 bp
	Right Primer CGGCATGGTTGTAAAGAGGT	20	59.90	50.00	4.0	0.0	
Primer 2	Left Primer CCAGAAAGTGATCGGGTCAT	20	59.93	50.00	5.0	3.0	106 bp
	Right Primer CGGCATGGTTGTAAAGAGGT	20	59.99	50.00	4.0	0.0	
Primer 3	Left Primer CAGACCCCTTCATGGAAAGA	20	60.01	50.00	4.0	2.0	155 bp
	Right Primer ATGACCCGATCACTTTCTGG	20	59.93	50.00	5.0	1.0	
Primer 4	Left Primer TCTAGCGGTGGAGGTGAAGT	20	59.87	55.00	4.0	1.0	226 bp
	Right Primer CGGCATGGTTGTAAAGAGGT	20	59.97	50.00	4.0	0.0	
Primer 5	Left Primer CTAGCGGTGGAGGTGAAGTC	20	59.87	60.00	4.0	2.0	225 bp
	Right Primer CGGCATGGTTGTAAAGAGGT	20	59.99	50.00	4.0	0.0	

Results for primers against ACLSV coat protein for apple isolate from palampur as designed using Exome Horizon are shown in (Table 4.5). Five pairs of best primers capable of amplifying a product of approximately 221 bp were designed through this software. Length of all primers was 20 bp, Tm between 59-60, GC between 50-55 respectively.

Inference

The sequence of CP gene of ACLSV is 794 bp long and because the primer designed by the software Exome horizon can amplify fragments length of 250 bp, the primers were not suitable for amplifying complete CP gene. Therefore, these primers were not selected for further studies.

4.3.2 Primers designed using web primer

CP gene sequences of ACLSV were downloaded from NCBI site and fasta format were used for designing primers. Primers were designed as explained in section (3.4.2). This software showed an option to select best primer pair and the result of such designing is presented in Table 4.6.

Table 4.6 Primers designed against three coat protein gene sequences of ACLSV (AM494507, AM498050, AM494514) using web primer

Sequence	Primer	Length	Tm	GC	ANY	End	Offset
AM494507	Forward Primer AGATCAGAAGGAGGAGGAT	20	49	50	12	6	2 bp
	Reverse Primer ATTTAAAAGTCTACAGGCT	20	40	30	12	4	784 bp
AM498050	Forward Primer TCAGAAGGAGAAGGATGG	19	51	52	8	8	5 bp
	Reverse Primer ATTTAAAAGTCTACAGGCT	20	40	30	12	4	776 bp
AM494514	Forward Primer CATCGCATTGCAATAAGATCA	21	53	38	20	6	6 bp
	Reverse Primer AAAAGTCTACAGGCTATTT	19	40	39	12	6	800 bp

As is shown in table 4.6, best primers were designed against the three sequences. The primer pair designed against the sequence AM494507 was predicted to amplify a segment of 783 nucleotides with forward and reverse

primer length of 20bp. Melting temperature and GC percent for the forward primer was 49^0C and 50% and that of reverse primer was 40^0C and 30%, respectively. However, primer pair designed against the sequence AM498050 showed the length of forward and reverse primers to be 19 & 20 bp respectively. Melting temperature and GC percent for the forward primer selected was 51^0C and 52% and that of reverse primer was 40^0C and 30% respectively. The primer pair designed against the sequence AM494514 was predicted to amplify a segment of 795 nucleotides. Melting temperature and GC content for the forward primer was 53^0C and 38% and reverse primer was 40^0C and 39% respectively.

Inference

Primers designed through this software have very low GC content of reverse primers in all the three cases and this ratio is less than optimal. The designed primers therefore can not be recommended for further studies and so were not used.

4.3.3 Primers designed using primer 3

Coat protein sequences of ACLSV were retrieved from NCBI, as in all other cases. Sequence of interest was opened in Genbank format and the format changed into fasta. Software primer 3 was opened through site: (http://www.basic.nwu.edu/biotools/primer 3.html). Five best pairs of primers were designed as explained in section 3.4.3 and the results so obtained are presented in (fig 4.7, 4.8 & 4.9). All the properties of primers were checked using oligonucleotide properties calculator tools. This software was opened through site (http://www.basic.nwu.edu/biotools/oligocalc.html).

This software was used to design primers against Solan sequence (AM494507) and the data so generated is presented in table 4.7.

As is evident from the data (Table 4.7), all the five pairs of primers designed were 20-22 bp long, had melting temperature between 58-61 and GC content between 40-50 and depicted for no secondary structure formation . These primer pairs were predicted to amplify segments of approximately 665 bp of nucleotides.

Table 4.7 Designed Primers against coat protein gene of ACLSV apple isolate from Solan (AM494507) using primer 3

	Primer	Length	Tm	GC (%)	Hairpin loop	3' compl	Product size
Primer 1	Left Primer CAGTTAAAGGTGGACGCAGA	20	58.92	50.00	None	None	662bp
	Right Primer TGGGTTCAAGAGTTTGACGTT	21	59.62	42.80	None	None	
Primer 2	Left Primer TCAGTTAAAGGTGGACGCAGA	20	58.92	50.00	None	None	665bp
	Right Primer CATGGGTTCAAAGAGTTTGACG	21	60.53	47.62	None	None	
Primer 3	Left Primer CAGTTAAAGGTGGACGCAGAT	21	59.25	47.62	None	None	662bp
	Right Primer TGGGTTCAAGAGTTTGACGTT	21	59.62	42.86	None	None	
Primer 4	Left Primer CAGTTAAAGGTGGACGCAGA	20	58.92	50.00	None	None	663bp
	Right Primer ATGGGTTCAAGAGTTTGACATT	22	59.91	40.91	None	None	
Primer 5	Left Primer TCAGTTAAAGGTGGACGCAGA	21	60.81	4.62	None	N None one	663bp
	RightPrimer TGGGTTCAAGAGTTTGACGTT	21	59.62	42.68	None		

Similarly, this software was again used for designing primers against solan sequence (AM 498050) and the results so obtained are represented in table 4.8.

Table 4.8 Designed primers against coat protein gene of ACLSV peach isolate from Solan (AM 498050) using Primer 3

	Primer	Length	Tm	GC (%)	Hairpin loop	3' compl	Product size
Primer 1	Left Primer GATCAGAAGGAGGAGGATGG	20	58.61	55.00	None	None	710 bp
	Right Primer TGGGTTCAAGAGTGGGATTC	20	59.90	50.00	None	None	
Primer 2	Left Primer GATCAGAAGGAGGAGGATGG	20	58.61	55.00	None	None	711 bp
	Right Primer ATGGGTTCAAGAGTGGGATTC	21	60.18	47.62	None	None	
Primer 3	Left Primer GATCAGAAGGAGGAGGATGG	20	58.61	55.00	None	None	715 bp
	Right Primer TTTCATGGGTTCAAGAGTGG	20	58.54	45.00	None	None	
Primer 4	Left Primer GATCAGAAGGAGGAGGATGG	20	58.61	55.00	None	None	716 bp
	Right Primer CTTTCATGGGTTCAAGAGTGG	21	59.58	47.62	None	None	
Primer 5	Left Primer GATCAGAAGGAGGATGG	20	58.61	55.00	None	None	711 bp
	Right Primer ATGGGTTCAAGAGTGGGATT	20	58.30	45.00	None	None	

Results presented in the table 4.8 predicted that the Left Primer GTCAGAAGGAGGAGGATGG was common in all the above designed primer pairs. Tnis primer was found well within the range of suitability in all properties without any tendency to form secondary structures like hairpin loop and 3′ complementarity. Length of this primer was 20 bp, Tm was 58.61 and GC % was 55.00. All the reverse primers as depicted in Table 4.7 had length between 20-21bp, Tm between 58 & 60 as depicted. These primers had GC % of 45-50. Also no hairpin formation and 3′ complementarity was depicted.

Similarly primers were designed against the coat protein gene of ACLSV(AM4940514) apple isolate from palampur using Primer 3 software and the results so obtained are presented in table 4.9.

Results from table 4.9 show that left primer AAGGTAGACGCAGATTTGAAGG was common in all five designed primers. The length of primer was 22 bp, Tm 60.1 and GC% 46. However, in case of reverse primer the GC content of the primers (1, 3, 4 & 5) was very low. As the second set of primers (table 4.8) fulfilled all the criteria of a good primer, it was selected to be used for wet lab experimentations.

Table 4.9 Designed Primers against CP gene of ACLSV infecting apple, palampur isolate (AM4940514) using primer 3

	Primer	Length	Tm	GC (%)	Hairpin loop	3′ comp	Product
Primer 1	Left AAGGTAGACGCAGATTTGAAGG	22	60.1	46	None	None	683 bp
	Right TCTTTATACTCTTTCATGGGTTCAA	25	60.9	32	None	None	
Primer 2	Left AAGGTAGACGCAGATTTGAAGG	22	60.1	46	None	None	702 bp
	Right CACTCCATTAATACCACGACTC	22	59.1	45	None	None	
Primer 3	Left AAGGTAGACGCAGATTTGAAGG	20	60.1	46	None	None	654 bp
	Right CTCTTTATACTCTTTCATGGGTTC	21	59.6	34.62	None	None	
Primer 4	Left AAGGTAGACGCAGATTTGAAGG	22	60.1	46	None	None	655 bp
	Right TCTTTATACTCTTTCATGGGTTCAA	24	57.0	33.33	None	None	
Primer 5	Left AAGGTAGACGCAGATTTGAAGG	22	60.1	46	None	None	653 bp
	Right CTCTTTATACTCTTTCATGGGTTCA	25	58.0	36%	None	None	

Inference:-

- Primers designed through this software fulfilled all the properties of good primers and amplifies desired CP gene product (600 to 800).
- Primer 2 (left Primer CAGTTAAAGGTGGACGCAGA; Right Primer TGGGTTCAAGAGTTTGACGTT) from Table 4.7 was used in further studies because this primer was found to amplify longest region (663 bp), depicted very small difference in Tm, and GC content and showed no tendency to form secondary structures.
- Primer 1 from Table 4.8 was also used in further studies because of comparable GC content of both Left and Right Primers as compared to other Primers.
- Primer 2 from Table 4.9 was used in wet lab experiments because of the comparable GC percentage of both primers (left and right).

4.3.4 Primers designed using Gene Fisher:

- This software also accepts sequences in fasta format, the sequences were similarly pasted into dialog box opening the software through site http://bibiserv.techfak.uni-bielefeld.de/genefisher/.
- Information regarding parameters was submitted.
- The results of Primer designing as obtained using this software are presented in Table 4.10, 4.11 and 4.12. In this case also properties of all the designed primers were checked using oligonucleotide properties calculator.

The data generated by this software against ACLSV solan sequence (AM494507) are presented in the table 4.10.

As it is evident from the results shown in table 4.10, primer 2 was chosen as the best primer (Left Primer TCAGTTAAAGGTGGACGCAGA; Right Primer TGGGTTCAAGAGTTTGACGTT). The primers are 20 bp long, had melting temperature of 60.8 and 59.2 respectively and GC% of 50 and 47 respectively. This primer was predicted to amplify largest region of 665 bp. However, Primer3 and Primer 5 were predicted to form hairpin loop structure.

Table 4.10. Designed Primers against coat protein gene of ACLSV (AM494507) *apple isolate from solan* using Gene Fisher software

	Primer	Length	Tm	GC%	Hairpin loop	3' comp	Product
Primer 1	Left Primer CAGTTAAAGGTGGACGCAGA	20	58.92	55.0	None	None	662 bp
	Right Primer TGGGTTCAAGAGTTTGACGTT	21	59.62	42.8	None	None	
Primer 2	Left Primer TCAGTTAAAGGTGGACGCAGA	21	60.81	50.0	None	None	665 bp
	Right Primer TGGGTTCAAGAGTTTGACGTT	21	59.62	47.62	None	None	
Primer 3	Left Primer CAGTTAAAGGTGGACGCAGATC	22	58.1	55.0	None	None	650 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56.2	41.3	Form	None	
Primer 4	Left Primer TTCAGTTAAAGGTGGACGCAGATC	24	59.6	42.1	None	None	646 bp
	Right Primer CATGGGTTCAAGAGTTTGAC	20	60.8	45.3	None	None	
Primer 5	Left Primer AAAGGTGGACGCAGATCTGAAAGC	24	58.2	45.1	None	None	645 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56	41.3	Form	None	

Similarly ACLSV apple isolate from palampur (AM494514) was also choosen and subjected to primer prediction using gene fisher. The software designed five primers against this strain as well and the properties of primers so designed were calculated using oligonucleotide properties calculator. The data so generated are presented in table 4.11

As it is evident from the results shown in (Table 4.11) Primer pair 1 (Left Primer GGATGGCAGCGGTTCTGAATCTAC; Right Primer CACTCCATTAATACCACGACTC) having length of 24 and 22 bp respectively, was predicted to amplify 690 bp of nucleotide. Primer pair 3 was found well with in the range of suitability in all properties without any tendency to form secondary structures and was predicted to amplify large product size of 702 bp.

However, the reverse primers of Primer 2, Primer 4 and Primer 5 showed Hairpin formation (5′ CTCTTTCATGGGTTCAAGAG 3′).

Table 4.11. Designed Primers against the coat protein of ACLSV (AM494514) apple isolate from Palampur using Gene Fisher

	Primer	Length	Tm	GC (%)	Hairpin loop	3′ comp	Product size
Primer 1	Left Primer GGATGGCAGCGGTTCTGAATCTAC	24	59	54	None	None	690 bp
	Right Primer CACTCCATTAATACCACGACTC	22	60	45	None	None	
Primer 2	Left Primer GGATGGCAGCGGTTGTGAATCTAC	24	59	54	None	None	684 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56	45	Present	None	
Primer 3	Left Primer AAGGTAGACGCAGATTTGAAGG	22	60	46	None	None	702 bp
	Right Primer CACTCCATTAATACCACGACTC	22	59	45	None	None	
Primer 4	Left Primer AAGGTAGACGCAGATTTGAAGG	22	60	46	None	None	680 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56	45	Present	None	
Primer 5	Left Primer AGGTAGACGCAGATTTGAAG	20	60	45	None	None	654 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56	45	Present	None	

Similarly the primers were designed against Solan sequence (AM498050). The results so obtained through this software were presented in table 4.12.

The details presented in table 4.12 show that primer pair 1 (Left Primer GATCAGAAGGAGGAGGATGG; Right Primer TGGGTTCAAGAGTGGGATTC) is the best primer because it accomplished all the criteria of a good primer. Whereas, the reverse primers of Primer 2, 3, 4 and 5 were predicted to form hairpin loop structure.

Table 4.12. Designed Primers against CP gene of ACLSV (AM498050) peach isolate from Solan using Gene Fisher

	Primer	Length	Tm	GC (%)	Hairpin	3' compl	Product size
Primer 1	Left Primer GATCAGAAGGAGGAGGATGG	20	58.61	55.00	None	None	710 bp
	Right Primer TGGGTTCAAGAGTGGGATTC	20	59.90	50.00	None	None	
Primer 2	LeftPrimer GAAGGAGGAGGATGGCAGCAGTTC	24	58.61	48.00	None	None	693 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56.18	45.00	None	Form	
Primer 3	Left Primer AGGAGGAGGATGGCAGCAGTTCTG	24	58.61	55.00	None	None	691 bp
	Right Primer CTCTTTCATGGGTTCAAGAG	20	56.18	45.00	None	Form	
Primer 4	Left Primer GAAGGAGGAGGATGGCAGCAGTTC	24	58.61	55.00	None	None	691 bp
	Right Primer CTCTTTCATGGGTTCAAGAGTG	22	58.36	45.00	None	Form	
Primer 5	Left Primer AGGAGGAGGATGGCAGCAGTTCTG	24	58.61	55.00	None	None	689 bp
	Right Primer CTCTTTCATGGGTTCAAGAGTG	22	58.36	45.00	None	From	

Inference :

- Primer 2 from table 4.10 was used in further studies because this primer was capable to fulfilled all the desired parameters of good primer.
- Primer 3 from table 4.11 (left Primer 5′ AAGGTAGACGCAGATTTGAAGG 3′; Right Primer 5′ CACTCCATTAATACCACGACTC 3′) was used in wet lab experiments because this primer fulfilled all the properties of a good primer.
- Primer 1 from table 4.12 was used in wet lab experiments because this primer accomplished all the criteria of a good primer and predicted to amplifies the largest region 710 bp.

4.3.5 Primer designing using Primer Blast

Primer Blast another freely available tool on site (http:// www.ncbi.nlm.nih.gov/) was also used to design primers against the selected coat

protein sequences of ACLSV. The results so obtained are presented in table 4.13, 4.14 and 4.15.

The data so generated by the software primer blast against ACLSV solan sequence acc no AM494507 (Table 4.13) clearly shows that the Primer 1 was capable of amplifying the nucleotide measuring 664 bp and length of forward and reverse primer was 20 and 21 respectively. Tm and GC percent of forward primer was 59.3^0C and 45% and reverse primer was 59.6^0C and 42.8% respectively. When we compare properties of Primer 2, Primer 3 and Primer 4 all had low GC content of reverse primer and that of forward primer. Primer 5 (Forward TCAGTTAAAGGTGGACGCAGA; Reverse CATGGGTTCAAGAGTTTGAGGTT) however, was predicted to amplify longest region of 665 bp. All the properties of this primer were suitable for wet lab experiments.

Table 4.13 Designed primer against coat protein gene of ACLSV apple isolate from Solan (AM494507) using Primer Blast

	Primer	Length	Tm	GC	Position	Product size
Primer 1	Forward TTCAGTTAAAGGTGGACGCA	20	59.3	45%	36	664 bp
	Reverse TGGGTTCAAGACTTTGACGTT	21	59.6	42.8%	699	
Primer 2	Forward GGTGGACGCAGATCTGAAAG	20	60.8	55%	46	654 bp
	Reverse TGGGTTCAAGACTTTGACGTT	21	59.6	42.8%	699	
Primer 3	Forward AGGTGGACGCAGATCTGAAA	20	60.8	50%	45	655 bp
	Reverse TGGGTTCAAGAGTTTGACGTT	21	59.6	42.8%	699	
Primer 4	Forward GTGGACGCAGATCTGAAAGC	20	60.9	55%	47	653 bp
	Reverse TGGGTTCAAGACTTTGACGTT	21	59.6	42.8%	699	
Primer 5	Forward TCAGTTAAAGGTGGACGCAGA	20	58.9	48%	37	665 bp
	Reverse CATGGGTTCAAGAGTTTGACGTT	23	60.5	48%	701	

Primer blast was again used to predict best primer against solan sequence (AM498050) and as in other case the properties of the primers so designed were calculated. The data so generated is presented in table 4.14.

The data presented in table (4 .14) demonstrated that all the five pairs of primers designed were 20-23bp long. Melting temperature of these primers were between 59-61 and GC% between 50-55% respectively.These primer pairs were capable for amplifying segments of approximately 711bp.

Table 4.14 Designed Primers against CP gene of ACLSV peach isolate from Solan Accession No (AM498050) using Primer blast

	Primer	Length	Tm	GC	Position	Product size
Primer1	Forward GGATGGCAGCAGTTCTGAAT	20	60.23	50%	15	698 bp
	Reverse CATGGGTTCAAGAGTGGGAT	20	59.78	50%	712	
Primer2	Forward ATCAGAAGGAGGAGGATGGC	20	60.6	55%	2	708bp
	Reverse GGGTTCAAGAGTGGGATTCA	20	59.9	50%	709	
Primer3	Forward ATCAGAAGGAGGAGGATGGC	20	60.6	55%	2	709 bp
	Reverse TGGGTTCAAGAGTGGGATTC	20	59.9	50%	710	
Primer4	Forward ATCAGAAGGAGGAGGATGG	20	60.6	55%	2	711bp
	Reverse CATGGGTTCAAGAGTGGGAT	20	59.78	50%	712	
Primer5	Forward GGATGGCAGCAGTTCTGAAT	20	60.23	50%	15	700bp
	Reverse TTCATGGGTTCAAGAGTGGG	20	60.9	50%	714	

Similarly this software was again used for designing primers against palampur sequence (AM494514) and the results so obtained are presented in table 4.15.

Result presented in table (4.15) depict that all the five pairs of primers designed were 21-25 bp long, had melting temperature between 58-60 and

product size between 683-702 bp. However, reverse primer of Primer 1, Primer 2, Primer 3 and Primer 5 has low GC content than optimum. Primer 3 was chosen for wet lab experiment. Length of forward and reverse primer was 21 and 22 respectively. Melting temperature was 60 and 50°C,GC content was 46 and 45%, respectively. This primer was predicted to amplify the fragment of 702 bp.

Table 4.15 Designed primers against coat protein gene of ACLSV apple isolate from palampur (AM494514) using Primer blast

	Primer	Length	T_m	GC (%)	Position	Product size
Primer1	Forward AAGGTAGACGCAGATTTGAAGG	22	59.47	45	44	683bp
	Reverse TCTTTATACTCTTTCATGGGTTCAA	25	59.81	32	726	
Primer2	Forward AAGGTAGACGCAGATTTGAAGG	22	59.47	45	44	684bp
	Reverse CTCTTTATACTCTTTCATGGGTTCAA	26	59.80	34	727	
Primer3	Forward AGGTAGACGCAGATTTGAAGG	21	59.41	46	44	702bp
	Reverse CACTCCATTAATACCACGACTC	22	59.21	45	746	
Primer4	Forward AGGTAGACGCAGATTTGAAGG	21	58.21	48	44	682bp
	Reverse TCTTTATACTCTTTCATGGGTTCAA	25	59.57	32	726	
Primer5	Forward AAGGTAGACGCAGATTTGAAGG	22	59.47	45	44	684bp
	Reverse CTCTTTATACTCTTTCATGGGTTCA	25	59.80	36	727	

Inference:-

- The results obtained through this software very similar when compared to those produced by Primer 3 and Gene Fisher.
- Primer 5 from table 4.13 was used in wet lab experiments because this primer fulfilled all the properties of a good primer.
- Primer 4 from table 4.14 fulfilled all the criteria of good primer and amplify largest fragment of cp gene of ACLSV. Thus used in wet lab experimentation.

- Primer 3 from table 4.15 was used in wet lab experiments because this primer fulfilled all the properties of a good primer.

Three best pairs of primers were selected from all the above designed primers through different softwares against CP gene sequences of ACLSV, Accession No (AM494514 and AM494507, AM498050). Three primer were selected on the basis of their properties and suitability for wet lab studies. Enlisted below are the primers used in further studies.

1) 5′ AAG GTA GAC GCA GAT TTG AAGG 3′ Forward
   5′ CAC TCC ATT AAT ACC ACG ACTC 3′ Reverse
   This primer was designed against CP gene sequence of ACLSV apple isolate from Palampur (AM494514)
2) 5′ TCA GTT AAA GGT GGA CGC AGA 3′ Forward
   5′ CATGGG TTC AAG AGT TTG ACG 3′ Reverse
   This primer was designed against CP gene sequence of ACLSV apple isolate from solan (AM494507)
3) 5′ GAT CAG AAG GAG GAG GATGG 3′ Forward
   5′ TGG GTT CAA GAG TGG GAT TC 3′ Reverse
   Primer designed against CP gene sequence of ACLSV peach isolate from Solan (AM494514).

4.4 Primer synthesis:

The best three primer pairs (section 4.3.5) were submitted to M/S Sigma Aidrich Chemicals Pvt. Ltd. Bangalore for synthesis. Necessary instructions regarding the primers were supplied by the manufactures. Table 4.16 shows sequences of the primers and other necessary parameters of the synthesized primers.

The synthesized primers were then used in wet lab experimentation to check their suitability. Stocks of these primers were prepared by resuspending these in sterile water. The same were then stored at -20^{0}C for further use.

Table 4.16. Synthesized primers and their properties

Name	Sequences (5'-3')	Length	Tm	GC (%)	MW	OD	Dimer	Hairpin
Primer 1	Left Primer AAGGTAGACGCAGATT TGAAGG	22	63.2°C	46%	6872	11.1	No	None
	Right Primer CACTCCATTAATACCAC GACTC	22	59.9°C	45%	6583	14.6	No	None
Primer 2	Left Primer TCAGTTAAAGGTGGAC GCAGA	21	63.6°C	50%	6519	7.1	No	None
	Right Primer CATGGGTTCAAGAGTTT GACA	21	63.3°C	48%	6501	13.6	No	None
Primer 3	Left Primer GATCAGAAGGAGGAGG ATGG	20	63.3°C	55%	6320	7.1	No	None
	Right Primer TGGGTTCAAGAGTGGG ATTC	20	63.8°C	50%	6228	15.2	No	

4.5 Testing of Primers using wet lab experiments

Total RNA was isolated from virus infected leaves of apple and RT PCR was performed for testing of the above designed primers.

4. 5.1 RNA Isolation

Results of serological indexing indicated presence of the virus in all the samples (Section 4.2). The concentration of the virus, however, was higher in the sample drawn from Nauni campus (Section 4.2). Therefore, plants showing prominent symptoms of ACLSV from Pathology farm of Dr Y S Parmar UHF, Nauni- Solan were selected as test plants for further studies. Fresh leaves from infected and healthy plants were collected and then used for RNA isolation using Bangalore genei RNA isolation kit as described in (section 3.6). Presence of RNA and its quality was checked by running it on 0.8% agarose gel under 75 V potential difference. The gel was first viewed by transilluminator under UV light. After confirmation of RNA bands; photographs were taken by gel documentation system. Photograph of the gel so obtained is presented in fig 4.3. As it is evident from the photograph the RNA from three samples S_1, S_2 and S_3 ; diseased plants C_{16} and C_{17} and healthy from entomology field respectively is

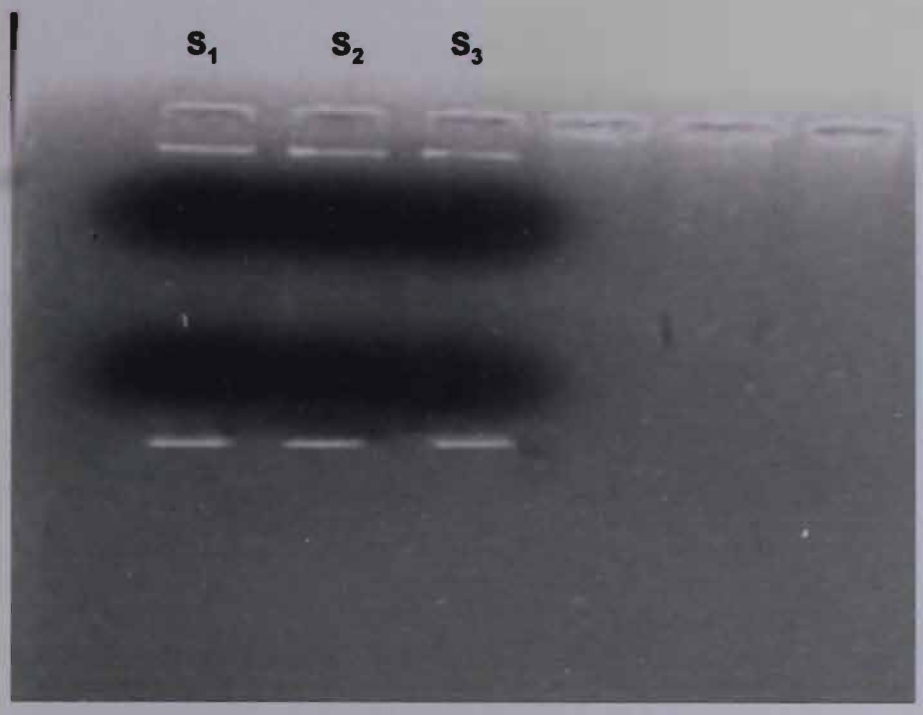


Fig. 4.3 Bands of isolated RNA (S1 and S2 : Diseased Samples; S3 : Healthy Sample)

Fig. 4.4 Product of RT-PCR in gel (M_1, M_2 and M_3- different reaction mixture, M – 100 bp DNA ladder)

clearly depicted in shape of bands in the three lanes. The lanes have bands at two positions i.e. at 23s and 16s respectively confirming presence of total RNA in the sample.

4. 5.2 RT-PCR for testing of Primers

RT –PCR was performed for testing the efficiency of the primers. Optimal primer sequence and appropriate primer concentration was essential for specificity and efficiency of RT-PCR. Experiments were performed with specific related primers. Enlisted below are the synthesized primers used in RT-PCR.

1. 5 'AAGGTAGACGCAGATTTGAAGG 3' (Upstream primer)
   5 ' CACTCCATTAATACCACGACTC 3' (Downstram Primer)
2. 5 'TCAGTTAAAGGTGGACGCAGA 3' (Upstream primer)
   5 ' CATGGGTTCAAGAGTTTGACG 3 ' (Downstram Primer)
3. 5 ' GATCAGAAGGAGGAGGATGG3' (Upstream primer)
   5 ' TGGGTTCAAGAGTGGGATTC3' (Downstram Primer)

Three different reaction mixtures and conditions were tried for RT-PCR studies as shown in fig 4.4. It is obvious from the photograph that reaction mixture M_1 M_2 showed lighter intensity band as compared to M_3. So out of these M_3 was chosen as best mixture (Appendix 1, 2) and was used for standardization and performing further experiments.

In RT-PCR step a reaction mixture of 25 µl was used for cDNA synthesis. It contained 9.5 µl of RNA , 0.6 µl of dNTP (100mM), 1 µl of RNase (25 U/µl) 1 µl mulv RT (10U/ µl), 1 µl of reverse primer (200 ng/µl), 5 µl of RT buffer and 14.8 µl of nuclease free water. Then reaction mixture was reverse transcribed by incubating at 37^0C for 1 hour and then at 94^0C for 2 min. This cDNA was then used in the next step. PCR was carried out in an automated thermocycler (applied biosystems, USA). The reaction mixture (50µl) used for PCR contained 5 µl Taq DNA A buffer (10X), 1.5 µl dNTP mix (10 mM) 1 µl (200 ng/µl) of reverse and forward primer, 1 µl taq DNA polymerase (5U/µl), 7 µl of cDNA and 32.8 µl of

sterile nuclease free water. Then different temperature conditions were employed for annealing of primers with template cDNA .

While working at different temperature for denaturation , annealing and extension it was observed in (table 4. 17) different results were obtained at different conditions.

Table 4.17. Different conditions for PCR

CONDITIONS	M_1	M_2	M_3
CONDITION 1	Band absent	Band absent	Band absent
CONDITION 2	Band absent	Band absent	Band absent
CONDITION 3	Band present	Band present	Band present

As it is depicted from the above table (4.17) conditions 1 and 2 presented in (Appendix 3) showed no bands whereas, condition 3 presented in fig 4.4 showed bands in all three reaction mixtures. This condition was standardized and used for performing further experiments.

Table 4.18. Standardized temperature conditions for Primer 1, 2 and 3

Primers	**Primer 1**		**Primer 2**		**Primer 3**	
Steps	**Temperature**	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
Initial Denaturation	94^0C	5 min	94^0C	5 min	94^0C	5 min
Denaturation	94^0C	30 sec	94^0C	30 sec	94^0C	30 sec
Annealing	56^0C	30 sec	57^0C	30 sec	57^0C	30 sec
Extension	72^0C	30 sec	72^0C	30 sec	72^0C	30 sec

From above table it is evident that the temperature & time optimized for PCR using Primer 2 and Primer 3 was the same however, in case of primer 1 annealing temperature was 56^0C. The final extension was done at 72^0C for 7 min and then incubation was done at 4^0C. The results of the PCR amplified product was confirmed by running agarose gel electrophoresis and the results of agarose gel electrophoresis are presented in the following paragraphs:

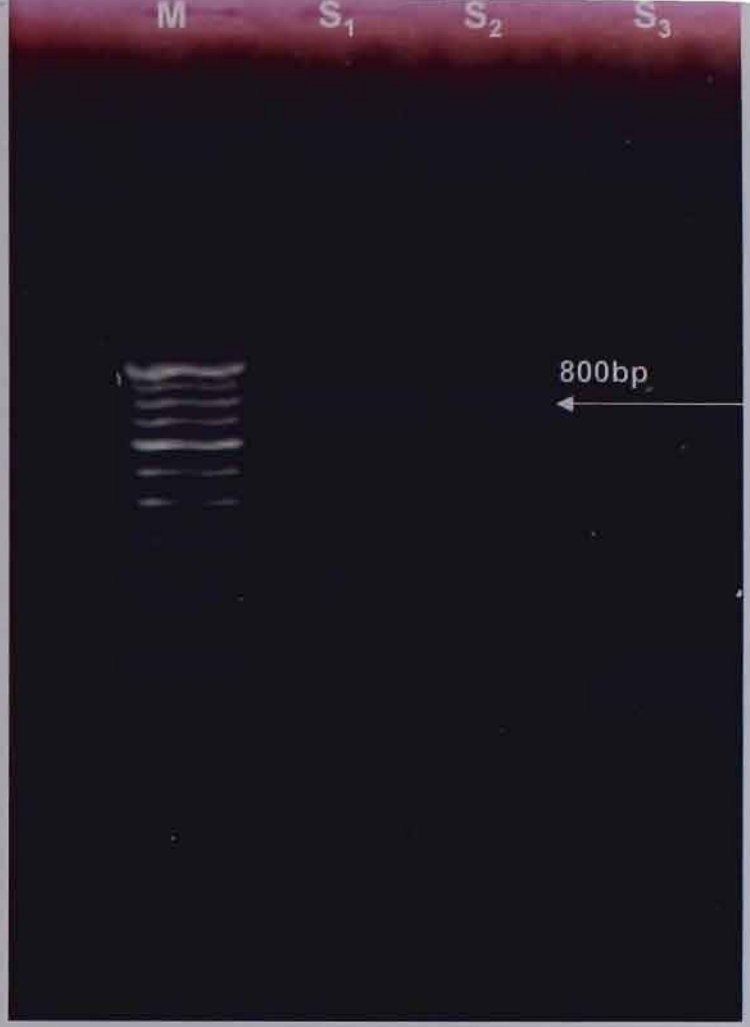


Fig 4.5 Amplification of CP gene of ACLSV through RT PCR using primer-1 (M -100bp Ladder; S_1 and S_2 diseased samples; S_3 healthy sample)

Fig 4.6 Amplification of CP gene of ACLSV through RT PCR using primer-2 (M -100bp Ladder; S_1 and S_2 diseased samples; S_3 healthy sample)

Fig. 4.5 shows amplification of CP gene of ACLSV by using Primer 1 (Forward 5' AAGGTAGACGCAGATTTGAAGG 3'; Reverse 5'CACTCCATTAATACCACGACTC 3') which was designed against CP gene of ACLSV genome RNA Palampur isolate. Gel showed that in lane 1 100 bp marker was loaded. S_1 and S_2 were the ACLSV infected samples loaded in lane 2 and lane 3. However in lane 4 healthy sample (S_3) was loaded. After loading of the samples in gel was run for 1 hour and 30 minutes at 80 v and then viewed under gel documentation system. The photograph of gel showed that samples S_1 and S_2 had amplification near 800 bp, however, there is no band in lane 4 where, healthy sample was loaded. From the photograph this was thus confirmed that this primer amplified only the CP gene of ACLSV.

The result with Primer 2 (forward 5' TCAGTTAAAGGTGGACGCAGA 3'; Reverse 5' CATGGGTTCAAGAGTTTGACG 3') designed against cp gene of ACLSV, apple isolate from Solan is presented in fig 4.6. Again lane 1 of the Gel was loaded with 100bp DNA ladder. Lane 2 and 3 were loaded with S_1 and S_2 diseased and lane 4 was loaded with healthy sample. Fig 4.6 shows amplification at 600 bp in ACLSV infected sample S_1 and S_2 however, no band was observed in case of S_3.

The result of primer 3 presented in Fig 4.7 shows amplification of coat protein gene of ACLSV using primer 3 (forward 5' GATCAGAAGGAGGAGGATGG 3'; Reverse 5' TGGGTTCAAGAGTGGGATTC 3') designed against cp gene of ACLSV infecting peach, isolate Solan. In lane1 100 bp DNA ladder was loaded ,whereas, S_1 and S_2 diseased samples were loaded in lane 2 and lane 3, S_3 the healthy sample was loaded in lane 4 as usual. Photograph of gel (fig 4.7) shows amplification near 450 bp in all the samples. From these results it is confirmed that the primer gave non specific amplification and it may have amplified plant DNA because the band was present in diseased as well as in healthy samples.

Inference

- Primer 1 showed specific amplification of 800 bp (Fig 4.5). The band was present only in diseased samples, however, the amplified product was larger as compared to expected (702 bp).
- Primer 2 showed specific amplification at 600 bp (fig 4.6) and the band was present in diseased sample only.
- Primer 3 (Fig 4.7) showed non specific amplification as the band was present in all samples the (S_1, S_2 and S_3).

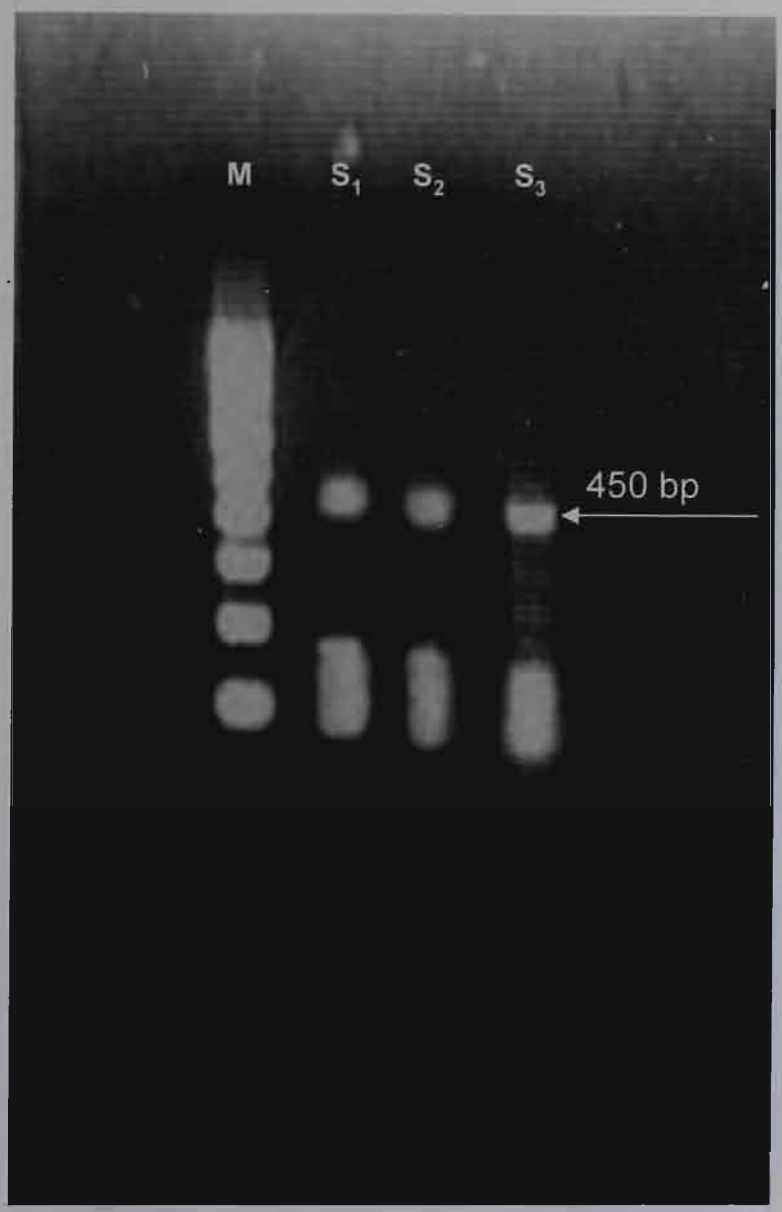


Fig 4.7 Amplification of CP gene of ACLSV through RT PCR using primer-3 (M -100bp Ladder; S_1 and S_2 diseased samples; S_3 healthy sample)

DISCUSSION

Chapter-5

DISCUSSION

Apple is the most important temperate fruit crop of India with regard to average production and economic value (Kanwar, 1987). It is mainly grown in India in three North Western Himalayan states viz; Himachal Pradesh, Jammu and Kashmir and Uttranchal. According to the statistical outline of the H. P during 2006-07, annual production of apple was reduced to about 2.68 lakh metric tonnes during 2006 from 4.59 lakh metric tonnes in 2003 (Anonymus, 2007). The major constraint for this is susceptibility to a number of pathogens like bacteria, fungi and viruses. Amongst them viruses play most significant role, because of their systemic nature. About 18 viruses have been reported to attack apple, out of these, apple chlorotic leaf spot virus is the most commonly distributed virus (Cieslinska and Rutkowski 2008). This virus is a member of trichovirus genus (Marteli *et al.*, 1994) and Flexiviridae family (Adams *et al.*, 2004). Traditionally viruses were detected by ELISA. Now RT-PCR for the detection of plant RNA viruses has led to improved assay sensitivity. Most critical parameter for successful PCR amplification is designing of primers and so the present investigations were aimed to design specific primers against CP gene of ACLSV using different softwares. The results so obtained (chapter 4) are being discussed in the following paragraphs:

Selection of virus culture

Viral cultures under present investigations were selected using visual symptoms. ACLSV has been reported to produce characteristic symptoms like chlorotic spots, asymmetric leaf distortion, puckering and inner bark necrosis (Yanase *et al.*, 1979). Local lesions were produced on mechanically inoculation, which are characteristic of apple chlorotic leaf spot virus. Production of local lesions on *Chenopodium amranti*color is in confirmation to the similar results obtained by Ptaeltzer (1968) and Cropley (1963) .

Serological Indexing

Leaves from infected plants from both locations were subjected to indexing. For detection of viruses, DAS ELISA was selected as a convenient procedure (Clarks and Adam, 1977; Derks *et al.*, 1990). DAS ELISA confirmed presence of ACLSV in all the samples collected from both of these locations. Samples collected from the Pathological farm of Dr Y S Parmar UHF, Nauni, Solan (H.P) showed higher concentration, whereas, lower concentration of the virus was observed in samples from Habban distt Sirmour (H.P). There are numerous reports on detection of ACLSV in infected plant samples through DAS ELISA (Detienne *et al.*, 1981; Wu Y Q *et al.*, 1998; Anil Kumar., 2000) and the present results are line with these reports. Recently a new host (*Prunus celasoides* D. Don) infected by ACLSV was tested using ELISA by Rana and his associates (2008) at Palampur (Himachal Pradesh). Concentration of a virus increases with time, after infection has set in. In case of fruit crops especially temperate fruits, the viruses are not evenly distributed in the plants and different concentrations in different samples have been reported (Anil Kumar, 2000).

Softwares for Primer designing

Bioinformatics has become an essential tool not only for basic research but also for applied research in biotechnology and biomedical sciences. Use of softwares in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Softwares are now available online or otherwise, to study genomics/proteomics of known sequences of nucleic acids/proteins. These software packages may be used for complete DNA and protein analysis, secondary structure predictions and primer designing etc. Some scientists have also developed algorithms and computer programs for various purposes including primer designing (Rychlik and Rhoades, 1989; Lowe *et al.*, 1990; Lucas *et al.*, 1991; O' Hara and Venezia, 1991; Tamura *et al.*, 1991; Makarova *et al.*, 1992; Gorelenko *et al.*, 2001).

Different softwares like Web Primer, Exome-Horizon, Gene Fisher 2, Primer 3 and Primer Blast were used for designing PCR primers for HP isolate of ACLSV. Three best pairs of primers were predicted using Primer 3, Gene Fisher and Primer Blast. Primers designed by Exome Horizon and Web Primer were not selected for Wet lab experimentation because of short product size and low Tm and GC content of the primers. A particular software out of the presently available softwares can not be used to designing primers against all the sequences. In fact it seems that a software is a function of nucleotide sequence to be amplified and the properties required and so has to be selected out of the available ones for such designing, like Malhotra *et al* in 2007 used Web Primer for designing specific primers against lily symptomless carlavirus. The selected primer pairs were scanned for the presence of complementary sites within themselves and complementary sites were observed in the predicted primer pairs.

Optimal design of DNA primers aids the diagnosis of plant viruses. In general, the melting temperature T_m of primer should be calculated (Lockhart *et al.*, 1998) and the secondary structures of oligonucleotides must be considered for avoiding dimmer and hairpin loops (Wang and Seed., 2003). The main parameters which have to be taken care are:

Primer Length: The length of forward and reverse primer are important factor for proper amplification of nucleic acid sequence. This parameter is critical for successful PCR (Wu *et al.*, 1991). Under present investigations the length of predicted primers were between 19-21 bp and good amplification of the viral coat protein was observed under wet lab experiments. The results therefore, are in consonance with studies carried out by Wu *et al.*, 1991.

Melting temperature: The primers designed under present investigations have melting temperature between 55-60^{0}C for both forward and reverse primers and the RT PCR results show good amplification with this temperature range. While scanning through literature it was observed that generally the Tm for primers in the range of 52-58^{0}C produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65^{0}C have been

recommended to be avoided because of potential for secondary annealing (Santalucia *et al.*, 1996).

GC content: GC content is another important characteristic of DNA and provides information about the strength of annealing. Primers with a GC content between 45 to 60 percent are highly suitable for good polymerisation (Dieffenbach *et al.*, 1995). Most of the predicted primer pairs have GC ratio within this acceptable range and in wet lab these primers shows specific amplification .

3'end Sequences: 3' terminal position in PCR primers is another essential consideration for the control of mispriming (Kwok *et al.*, 1990). It has been reported that a primer should be "Stickier" on their 5' end as compared to their 3' ends because it has been reported that high G/C content at 3' end of primers results in mispriming. Predicted forward and reverse primers in present studies have been seen to have less 3' sticky end and no such mispriming had been reported in wet lab experiments. So could prove to be good primers.

Secondary structure: Primers should not contain complementarity within themselves. That is, they should not form hairpins. Breslauer *et al* (1986) reported that complementary sites in a primer fold back on it and result in unproductive priming. There is rather no point of discussion on this issue as it is a common sense that complementarity will arise into folding back and so forming hairpins. As the present primers designed had no complementarity and so no hairpins formation. Further good amplification was expected which was noticed in wet lab experimentations.

Under present investigations the three best primer pairs selected which fulfilled all the criteria of good primers as mentioned above. The three best pairs of primer selected for wet lab experimentations are given below:

1.	AAGGTAGACGCAGATTTGAAGG	Left Primer
	CACTCCATTAATACCACGACTC	Right Primer

2.	TCAGTTAAAGGTGGACGCAGA	Left Primer
	CATGGGTTCAAGAGTTTGACG	Right Primer
3.	GATCAGAAGGAGGAGGATGG	Left Primer
	TGGGTTCAAGAGTGGGATTC	Right Primer

No such palindromic sequences were observed in all the predicted primers under present investigations and so could be used for PCR studies.

RT-PCR: Primer pairs selected on the basis of above parameters are also to be supported by wet lab experiments to put these into use. Under present investigations a reverse transcriptase polymerase chain reaction assay was performed. RNA was isolated using commercially available Total RNA isolation kit (Bangalore genei) and the results of RNA isolation in agarose gel electrophoresis showed presence of 23s and 16s RNA. There were numerous reports on RNA isolation through different kits (Malhotra *et al .., 2007* ; Rana *et al.*, 2008, and Sharma *et al* ., in 2008). RT-PCR was performed using three ACLSV specific primers for the CP gene of the HP isolates under specific programe (Section 4.5.2). Primer 1 showed a band near 800 bp in both the ACLSV infected samples showing amplification of desired product. Similarly second primer pair 2 showed amplification near 600 bp in both the diseased samples and primer pair 3 showed amplification at 450 bp in all the three (diseased as well as healthy) samples, which showed that primer 3 gave non specific amplification. There have been many reports of simple and rapid techniques to detect plant viruses using RT-PCR. Lately in 2008, detection of ACLSV using RT-PCR was carried out in almonds by Rana and his coworkers. The amplified product of 800 bp through Primer pair 1 is in consonance with the finding of Rana *et al* 2008, who reported approximately similar size (800 bp) for 3' terminal region of almond. In case of primer 2, which shows a band near 600 bp is supported by the findings of Menzel *et al.*, 2002 and Cieslinska *et al.*, 2007 who reported approximately (677 bp) for 3' terminal region of apple isolates. However, Kinard and Scott in 1996 reported approximately (551 bp) for 3' terminal region of apple isolate of ACLSV.

As discussed earlier the primers have properties; length, 20 nucleotides; GC content above 45% and Tm between 50^0C-60^0C. These properties fall within the most suitable range as suggested by Wu et al., 1991; Rychlik *et al.*, 1990; Dieffenbach *et al.*, 1995 and Breslauer *et al.*, 1986. The primers designed by earlier workers were large in size as compared to primers (20bp) designed and used during present studies. Thus these primers are more economic and cost effective. Results of RT-PCR confirmed that the primers designed against ACLSV could amplify the 3' terminal region of ACLSV genome and it also confirms that the properties as suggested are ideal for good quality primers.

It is also confirmed that for designing primers against present CP gene of ACLSV isolate the softwares Primer 3, Gene Fisher, Primer Blast are most suited where as Exome Horizon designs primers which are not capable of amplifying full length CP gene. Similarly primers designed by Web Primer also have low T_m and GC content so, can not be used for designing primers against CP gene of ACLSV isolates.

SUMMARY AND CONCLUSION

Chapter-6

SUMMARY AND CONCLUSION

Under present investigations, Primer designing against apple chlorotic leaf virus infecting apple was carried out. During investigations the selected virus infected plants were serologically tested and total RNA was isolated from infected and healthy leaves. Primers specific for CP gene of ACLSV were designed, using different bioinformatics tools. The best pair of primers were then selected on the basis of several parameters including length of primer, GC content, Tm and their tendency to form secondary structures. RT-PCR conditions were standardized for testing the efficacy of above selected primer pairs. The results so obtained are summarized below:

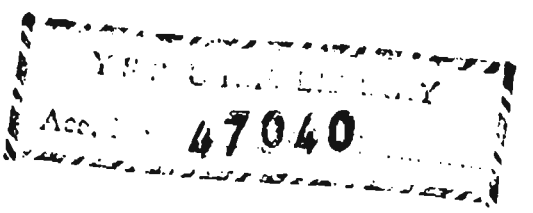
- Virus infecting apple plants were showing symptoms like chlorotic bloches, asymmetric leaf distortion and puckering. Apple plants showing such symptoms were collected from pathology farm of Dr Y S Parmar UHF, Nauni Solan and Habban distt Sirmour (H.P).
- Serological detection of the virus infected samples using DAS-ELISA showed high concentration of ACLSV samples collected from Pathology farm of Dr Y S Parmar UHF, Nauni- Solan.
- Three sequences of coat protein gene of ACLSV (AM494507, AM 498050 and AM 494514) were downloaded from NCBI.
- Primers specific for CP gene of ACLSV were designed against three sequences using different softwares ie Exome Horizon, Web Primer, Primer 3, Gene Fisher, Primer Blast. Oligonucleotide property calculator software was used for checking properties and secondary structure formation of designed primers.
- 5 pairs of primers were designed by Exome Horizon against each CP gene sequences. Primers designed by this software were rejected because the CP gene sequence of ACLSV was approximately 794bp and the product amplifies through these primers was predicted to be 250 bp.

- Web primer showed an option to choose the best primer, however, primers designed through this software were not recommended for wet lab studies due to low T_m and GC content of reverse primers.
- Primers designed through Primer 3, Gene Fisher and Primer Blast was selected for wet lab studies, because primers designed through these softwares fulfilled all the required parameters of good primers.
- Three best pairs of primers were selected from all the picked primers on the basis of parameters like length, T_m, GC% and tendency to form secondary structures.
- Primer pair 1 (Forward AAGTAGACGCAGATTTGAAGG; Reverse CACTCCATTAATACCACGACTC) was designed against CP gene of ACLSV apple isolate from Palampur.
- Primer pair 2 (Forward TCAGTTAAAGGTGGACGCAGA; Reverse ATGGGTTCAAGAGTTTGACA) was designed against CP gene of ACLSV apple isolate from Solan.
- Primer pair 3 (Forward GATCAGAAGGAGGAGGATGG; Reverse TGGGTTCAAGAGTGGGATTC) was designed against CP gene of ACLSV peach isolate from Solan.
- The efficacy of the designed primers was tested using RT-PCR. The RT reaction mixture was (5µl of RT buffer, 9.5µl of RNA, .6 µl of dNTP (100 mM), 1 µl of RNase (25U/ µl), 1 µl mulv RT (10U/µl), 1 µl of reverse primer (200 ng/ µl) and 14.8 µl of nuclease free water) and PCR reaction mixture was (5 µl Taq A buffer (10x), 1.5 µl dNTP mix (10mM), 1 µl (200 ng/ µl) of reverse and forward primer, 1 µl Taq DNA polymerase (5U/ µl), 7 µl of cDNA and 32.8 µl of sterile nuclease free water. For achieving best amplification RT steps were conducted at (37^0C for 1 hr; 94^0C for 2 min) and for polymerase chain reaction the denaturation, annealing and extension temperature for primer 1 was (94^0C for 30 sec, 56^0C for 30 sec, 72^0C for 7 minutes), and for Primer 2 and Primer 3 were (94^0C for 30 sec, 57^0 C for 30 sec, 72^0 C for 7 minutes) respectively.

- Primer 1 amplified 800 bp CP gene of ACLSV. Probably including UTR region of 3' terminal end of ACLSV genome.
- Primer 2 showed 600 bp amplification in both diseased samples.
- Primer 3 showed non specific amplification as the band at 450 bp was present in diseased as well as healthy sample.

Conclusion

Designing of specific primers added an advantage for detection of particular strain and isolate. Under present investigations Primer 1 and Primer 2 proved suitable for amplification of coat protein gene of ACLSV. The length of these primers was short as compared to primers used earlier. Thus these primers were less expensive ie more economic.



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ABSTRACT

Dr. Y. S. Parmar University of Horticulture and Forestry,
Nauni, Solan (H.P.)
Department of Biotechnology

Title of Thesis	:	Primer designing against apple chlorotic leaf spot virus infecting apples in Himachal Pradesh
Name of the student	:	Reena Kumari
Admission No.	:	H-2006-17-M
Year of award of degree	:	2008
Major Advisor	:	Dr S. V. Bhardwaj
Major Field	:	Biotechnology
Minor Field	:	Mycology & Plant Pathology
Degree Awarded	:	M.Sc. in Biotechnology
Year of Award of Degree	:	2008
No. of pages in thesis	:	69+V
No. of words in abstract	:	253

Abstract

Apple chlorotic leaf spot virus (ACLSV) belongs to genus trichovirus and family flexiviridae is most commonly widely distributed plant virus. It causes great looses to both yield and fruit quality of apple production world wide. Pure cultures of virus were collected from Habban (orchard) district Sirmour H.P. and Pathology farm of Dr Y S Parmar University of Horticulture and Forestry Nauni, Solan H.P. After assuring the presence of virus by serological testing, primers were designed using different software viz: Exome Horizon, Web Primer, Primer 3, Gene Fisher and Primer Blast and the properties of designed primers were checked by using oligonucleotide property calculator. Three best primer pairs (Forward/Reverse) were selected on the basis of important parameters like primer length, T_m, GC% and formation of secondary structures which are necessary for proper working of primers. Efficacy of these primers was tested using RT-PCR. Results of RT-PCR showed that Primer pair 1 (Forward 5' AAGGTAGACGCAFGATTTGAAGG 3'; Reverse 5' CACTCCATTAATACCACGACTC 3') showed amplification at 800 bp and Primer 2 (Forward primer 5' TCAGTTAAAGGTGGACGCAGA 3' Reverse primer 5' CATGGGTTCAAGAGTTTGACG 3') showed amplification at 600 bp. Both primer pair amplified the coat protein gene of ACLSV. However, primer 3 (forward primer 5' GATCAGAAGGAGGAGGATGG 3' Reverse Primer 5' TGGGTTCAAGAGTGGGATTC 3') showed non specific amplification at 450 bp as band was present in both healthy and diseased sample. So out of these three primers Primer 1 and 2 are recommended to use for detection of CP gene of ACLSV. These primers were small in size and thus cost effective .

Signature of Major Advisor

Reena 21/01/09
Signature of the student

Countersigned

Professor & Head
Department of Biotechnology
Dr Y S Parmar University of Horticulture and Forestry,
Nauni, Solan-173230 (H.P.)

APPENDICES

APPENDIX-I

Composition of RT-PCR mixture for cDNA synthesis

MIXTURE A

RT buffer (5x)	5 µl
dNTP mix (100 mM)	.6 µl
Primer (200 ng/ µl)	1 µl
RNase inhibitor (25U/ml)	1 µl
RNA sample	6 µl
Reverse transcriptase (10U/ µl)	1 µl
Water	10.4 µl
Total	**25**

RT-PCR conditions

37^0C	1 hr
94^0C	2 min

MIXTURE B

RT buffer (5x)	5 µl
dNTP mix (100 mM)	.6 µl
Primer (200 ng/ µl)	1 µl
RNase inhibitor (25U/ µl)	1 µl
RNA sample	9.5 µl
Reverse transcriptase (10U/ µl)	1 µl
Water	6.9 µl
Total	**25**

APPENDIX-II

Composition for PCR mixture

MIXTURE 1

Buffer (10 x)	5 µl
dNTP mix (100 mM)	1.2 µl
Forward Primer(200 ng/ µl)	1 µl
Reverse Primer(200 ng/ µl)	1 µl
CDNA sample	5 µl
Tag DNA polymerase (5U/ µl)	.5 µl
Water	36. 3
Total	**50**

MIXTURE 2

Buffer (10 x)	5 µl
dNTP mix (100 mM)	1.2 µl
Forward Primer(200 ng/ µl)	1 µl
Reverse Primer(200 ng/ µl)	1 µl
CDNA sample	6 µl
Tag DNA polymerase (5U/ µl)	1 µl
Water	34.8
Total	**50**

MIXTURE 3

Buffer (10 x)	5 µl
dNTP mix (10 mM)	1.5µl
Forward Primer(200 ng/ µl)	1 µl
Reverse Primer(200 ng/ µl)	1 µl
CDNA sample	7 µl
Tag DNA polymerase (5U/ µl)	1 µl
Water	33.5
Total	**50**

APPENDIX-III

Conditions for PCR

Condition 1

94°C	5 min
94°C	10 sec (denaturation)
57°C	3 min (annealing)
72°C	1 min (extension)
72°C	7 min
4°C	infinity

40 cycles

Condition 2

94°C	5 min
94°C	10 sec (denaturation)
57°C	1 min (annealing)
72°C	1 min (extension)
72°C	7 min
4°C	infinity

40 cycles

Condition 3

94°C	5 min
94°C	30 sec (denaturation)
57°C	30 sec (annealing)
72°C	30 sec (extension)
72°C	7 min
4°C	infinity

40 cycles

APPENDIX-IV

Sequence ¹.

LOCUS AM498050 793 bp RNA linear VRL 16-SEP-2007
DEFINITION Apple chlorotic leaf spot virus cp gene for coat protein, genomic RNA, peach isolate from Solan.
ACCESSION AM498050
VERSION AM498050.1 GI:157310300
KEYWORDS coat protein; cp gene.
SOURCE Apple chlorotic leaf spot virus
ORGANISM Apple chlorotic leaf spot virus
Viruses; ssRNA positive-strand viruses, no DNA stage; Flexiviridae; Trichovirus.

REFERENCE 1

AUTHORS Rana, T., Chandel, V., Hallan, V., Handa, A., Thakur, P. D. and Zaidi, A. A.

TITLE Molecular characterization of peach isolate of Apple chlorotic leaf spot virus from India

JOURNAL Unpublished
REFERENCE 2 (bases 1 to 793)
AUTHORS Rana, T.
TITLE Direct Submission

JOURNAL Submitted (07-MAR-2007) Rana T., Floriculture Division, IHBT, CSIR, Institute of Himalayan Bioresource Technology (CSIR), Palampur, Himachal Pradesh, 176061, INDIA

FEATURES Location/Qualifiers
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/translation "MAAVLNLQLKVDADLKAFLAAEGRPLHGKTGAILEQMLESI
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ORIGIN

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Sequence 2.

LOCUS AM494514 793 bp RNA linear VRL 16-SEP-2007
DEFINITION Apple chlorotic leaf spot virus cp gene for coat protein, genomic RNA, isolate Palampur.
ACCESSION AM494514
VERSION AM494514.1 GI:157310286
KEYWORDS coat protein; cp gene.
SOURCE Apple chlorotic leaf spot virus
ORGANISM Apple chlorotic leaf spot virus

Viruses; ssRNA positive-strand viruses, no DNA stage; Flexiviridae; Trichovirus.

REFERENCE 1
AUTHORS Rana,T., Chandel,V., Hallan,V., Handa,A., Thakur,P.D. and Zaidi, A. A.
TITLE Molecular characterization of ACLSV from India
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 793)
AUTHORS Rana,T.
TITLE Direct Submission
JOURNAL Submitted (26-FEB-2007) Rana T., Floriculture Division, IHBT, Institute of Himalayan Bioresource Technology (CSIR), Palampur, Himachal Pradesh 176 061, INDIA
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1 gatcagaagg aggaggatgg cagcggttct gaatctacaa ttaaaggtag acgcagattt
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Sequence 3.

LOCUS AM494507 784 bp RNA linear VRL 16-SEP-2007
DEFINITION Apple chlorotic leaf spot virus cp gene for coat protein, isolate Solan.
ACCESSION AM494507
VERSION AM494507.1 GI:157310272
KEYWORDS coat protein; cp gene.
SOURCE Apple chlorotic leaf spot virus
ORGANISM Apple chlorotic leaf spot virus
Viruses; ssRNA positive-strand viruses, no DNA stage; Flexiviridae; Trichovirus.

REFERENCE 1

AUTHORS Rana,T., Chandel,V., Hallan,V., Handa,A., Thakur,P.D. and Zaidi, A.A.
TITLE Molecular characterization of ACLSV from India
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 784)
AUTHORS Rana,T.
TITLE Direct Submission
JOURNAL Submitted (26-FEB-2007) Rana T., Floriculture Division, IHBT, Institute of Himalayan Bioresource Technology (CSIR), Palampur, Himachal Pradesh 176 061, INDIA
FEATURES Location/Qualifiers
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ORIGIN

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121 acagacactg gaggccatct tcgcgaacat agcgatacag ggcacctcgg agcaaacgga
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781 ctac
//

APPENDIX-V

Fasta format of sequences

>gi|157310300|emb|AM498050.1| Apple chlorotic leaf spot virus cp gene for coat protein, genomic RNA, peach isolate from Solan
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>gi|157310272|emb|AM494507.1| Apple chlorotic leaf spot virus cp gene for coat protein, isolate Solan

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AAATATTTTACTAC

CURRICULUM VITAE

Name	:	**Reena Kumari**
Father's Name	:	Sh. Roop Lal
Date of Birth	:	29.08.1985
Sex	:	Female
Marital Status	:	Unmarried
Nationality	:	Indian

Educational Qualifications:

Certificate/Degree	Board/University	Year	Division
10+2	CBSE	2002	First
B.Sc Medical	HPU	2005	First
B. Ed	HPU	2006	First

Whether sponsored by some state/ Central Govt./Univ./SAARC : No

Scholarship/stipend/Fellowship, any other financial assistance received during the study period. : University Stipend

Publications:

Kumari Reena, Bhardwaj S. V. Katoch Monisha, Thakur P. D and Kanwar K. 2008. Primer designing against apple chlorotic leaf spot virus infecting apples in Himachal Pradesh. Presented at National Symposium of Indian Virological Society, VIROCON at PGIMER, Chandigarh. Abst-29.

Katoch Monisha, Bhardwaj S. V, Kumari Reena. 2008. Comparative genome analysis of cucumber mosaic virus using Bioinformatic tools. Presented at National Symposium of Indian Virological Society, VIROCON at PGIMER, Chandigarh. Abst-30.

Reena
21/01/09
(Reena Kumari)