ASSESSMENT OF VARIABILITY IN COAT PROTEIN GENE OF *Lily symptomless virus* INFECTING LILY AND TULIP

THESIS

By

RAMAWATAR

Submitted to

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IN

Partial fulfilment of the requirements for the degree

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AFFECTIONATELY DEDICATED TO MY REVERED PARENTS

who sacrificed their present to make my future

Dr. A.A. Zaidi
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CERTIFICATE – I

This is to certify that the thesis entitled, **"Assessment of variability in coat protein gene of *Lily symptomless virus* infecting lily and tulip"**, submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Mr. Ramawatar Nagar (Admission No. A-2003-30-06)** son of **Sh. Jagdish Narayan** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

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Chairman,
Advisory Committee

Place : Palampur
Dated: December 17, 2005

CERTIFICATE – II

This is to certify that the thesis entitled, **"Assessment of variability in coat protein gene of *Lily symptomless virus* infecting lily and tulip"**, submitted by **Mr. Ramawatar Nagar** son of **Sh. Jagdish Narayan** to Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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(Ramawatar Nagar)

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ABBREVIATIONS

%	Per cent
°C	Degree centigrade
µl	Microlitre
2ME	2-mercaptoethanol
A_{405}	Absorbance at 405 nm
APS	Ammonium per sulphate
BLAST	Basic local alignment search tool
bp	Base pairs
CAP	Cleaved amplified polymorphic
cccDNA	Closed circular DNA
cDNA	Complimentary DNA
cm	Centi meters
CP	Coat Protein
DAS-ELISA	Double antibody sandwich ELISA
DBIA	Dot Blot Immunoassay
dCTP	Deoxycytidine triphosphate
ddNTPs	Dideoxy nucleotides
DMSO	Dimethyl sulfoxide
DNA	Deoxy nucleic acid
dNTP	Deoxy nucleotide triphosphate
ds	Double stranded
DTT	Dithiothritol
dTTP	Deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMBL	European molecular biology laboratory
g	Gram
GC	Guanine + cytosine
GST	Glutathione s-transferase
h	Hour
HRP	Horseradish peroxidase
IC-RT-PCR	Immunocapture reverse transcription-polymerase chain reaction
IgG	Immuno globulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISEM	Immunosorbent electron microscopy
kb	Kilobase
kDa	Kilo Dalton
LA	Luria Agar
lb	Pounds
LB	Luria broth
M. Wt.	Molecular Weight
MAbs	Monoclonal antibody
MCS	Multiple cloning site
ml	Milliliter
mm	Milimeter

M-MLV	Moloney murine leukemia virus
MP	Movement protein
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnological informations
ng	Nanogram
nm	Nanometer
OD	Optical density
ORF	Open reading frame
P	Phosphorous
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffer saline tween
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	Picogram
P-NP	P-nitro phenyl phosphate
PVP	Polyvinylpyrrolidone
RCF	Relative Centrifugal field
RNA	Ribonucleic acid
rpm	Revolution per minute
RT	Reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
S	Svedberg unit
SDS	Sodium dodecyl sulphate
ss	Single stranded
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TAS-ELISA	Triple antibody sandwich enzyme linked immunosorbent assay
TBE	Tris-borate-EDTA
TBIA	Tissue Blot Immunoassay
TEM	Transmission electron microscopy
TEMED	N,N,N',N' tetramethyl ethylenediamine
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
UV	Ultravoilet
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YT	Yeast tryptone

Introduction

Chapter I

INTRODUCTION

Flowering plants originated during the Cretaceous Period, nearly 100 million years ago when Africa and South America were still connected to each other. Flower is the blossom of a plant; the reproductive structure of angiosperms. It stands for purity, beauty, tranquility, honesty and divinity of nature. Beautification of a place depends upon its floral diversity.

Lilium and tulip are amongst the most beautiful and popular ornamental bulbous flowers, grown widely for their brilliant colours. The appearance, beauty and colour of the bloom are exceptionally spectacular and fascinating. Hybrid *Lilium* (Oriental and Asiatic) are the most preferred among different types of lilies grown. Tulip and lily ranks 3rd and 4th after Gladiolus and Narcissus in the international flower trade (Beattie and White, 1993; Anonymous, 1996). They have aesthetic value and are often depicted as the symbol of purity and regality. William Blake rightly down the quote:

"The modest Rose puts a thorn,

The humble sheep a threatening horn.

While the Lily white shall in love delight,

nor a thorn a threat stains her beauty bright"

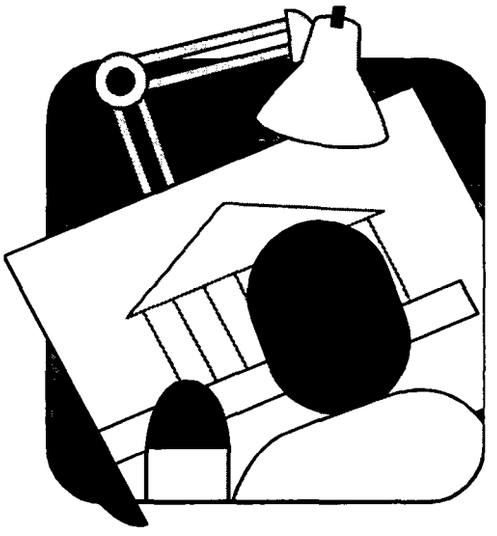
The genus *Lilium* belongs to family Liliaceae, includes more then 300 genera having 1, 20,000 species and characterized by features such as scaly bulbs, short lanceolate leaves and flowers either single or in loose racemes with six tepals. The fruit

of lily is capsule with many seeds. The name lily is used chiefly for plants of the genus *Lilium* and related species but is applied also to plants of other families, e.g., the water lily, the calla lily, and especially the numerous species of the Amaryllis family (often included in the Liliaceae) whose blossom closely resemble like true lilies in appearance. Because of the showy blossoms, characteristic of the family, many species are cultivated as ornamental. This is the chief economic value of the Liliaceae; over 160 genera of which have been represented in American trade. Most of the lilies are indigenous to the temperate region of Europe, Asia and America, while a few are native to the tropics (Beattie and White, 1993). Although floriculture is an age old profession, still India is in developing stage regarding their commercial cultivation. The importance of flower growing, from environment and economic point of view was not understood properly till seventies. However, in the last decade it has progressed to a global industry with commercial production of flower. Preferably lilies and tulips, should be grown under shade condition. However, lilies can also be grown well under protected condition provided that there is no damage of frost.

The main limiting factor in large scale cultivation of *Lilium* is its susceptibility to a number of pathogens including fungi, bacteria and viruses. Among these, diseases caused by viruses assume significant importance as they have direct effect on the quality and yield of cut flower, in addition to this, bulbs resulting in great economic losses (Lawson, 1981; Raju and Olseon, 1985). Various viruses that affect the lilies and tulip production are *Tulip breaking virus* (TBV), *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Lily mottle virus* (LMoV), *Turnip mosaic virus* (TMV) and *Strawberry latent ringspot virus* (SLRV). Viral disease causes a wide range

of symptoms that vary from a symptomless condition to severe leaf mottle and necrosis. Out of these viruses, LSV is an economically important virus having wider occurrence among the lily plants growing all over the world. LSV is a carlavirus having restricted host range to the Liliaceae family. Recent studies conducted on the status of viruses infecting *Lilium* in H.P. revealed that *Lily symptomless virus* is the commonly prevalent virus infecting lilies (Sharma, 2002; Bhaik, 2003), with an incidence about 65 per cent. The virus has been identified on the basis of symptomlogy, host range, biophysical properties, serology (ELISA) and electron microscopic studies (EM and ISEM). Mostly, lilies are propagated vegetatively so various results and reports from trials on the possibility of spreading *Lily symptomless virus* (LSV) by cutting flowers with a knife and by beheading plants with a machine has been found. Aphid is considered to be the most important insect vector responsible for the transmission of these viruses within plant population. Transmission through seed or dodder has not been reported for LSV. Studies have been carried out for the detection of the virus, cloning and sequencing of CP gene of various LSV isolates for understanding variability and higher level expression of CP gene in *E. coli* for the fulfillment of the objectives *viz.*

1. Amplification of the coat protein gene of the *Lily symptomless virus* from lilies and tulip.
2. Cloning, sequencing and understanding variability of the coat protein gene.
3. Higher level expression of the coat protein in *E. coli.*



Review of Literature

Chapter II

REVIEW OF LITERATURE

There are number of viruses known to infect lily and tulip which produce a wide range of symptoms. Most of these viruses have been reported from tulip and lily growing areas of the world (Lawson, 1981; Chang and Chung, 1987). The international trade of lily and tulip bulbs has increased the potential risk of introducing viruses/strains into further newer area (Cohen *et al.,* 1985). These viruses cause visible damages to floricultural produce and therefore, for plant health point of view they may pose serious problem for quality control. These viruses alone or in association with other viruses infect lily and tulip which increase the severity of symptoms.

Lilies have been reported to be susceptible to around twenty viruses under natural and glasshouse condition (Lee, 1992). However, Lawson and Hsu (1996) while describing lily disease and there control pointed out that there are ten viruses that infect lilies naturally. These include five aphid transmissible viruses *viz., Cucumber mosaic virus* (CMV) (Brierley, 1940), *Tomato aspermy virus* (TAV), *Lily symptomless virus* (LSV) (Brierley and Smith, 1944a, b; 1945). *Tulip breaking virus* (TBV) and *Lily mottle virus* (LMoV) (Derks *et al.,* 1982); four nematode transmitted viruses *viz., Arabis mosaic virus* (ArMV) (Mowat and Stefanac, 1974), *Strawberry latent ring spot virus* (SLRV) (Kulshrestha *et al.*, 2005), *Tobacco ring spot virus* (TRSV) (Travis and Brierley, 1957) and *Tobacco rattle virus* (TRV) (McWhorter and Allen, 1964). Among sap transmissible are *Tobacco mosaic virus* (TMV), *Lily virus x* (LVX), Lily mild virus, *Tomato ring spot* and *Narcissus mosaic viruses* (NMV) have also been reported (Bellardi *et al.,* 1988;

Stone, 1980; Memelink *et al.,* 1990; Lee *et al.,* 1996). The various viruses found infecting tulip are *Cucumber mosaic virus* (CMV), *Lily symptomless virus*, *Turnip mosaic* virus (TuMV), *Tulip breaking virus* (TBV) and *Strawberry latent ringspot virus* (SLRSV) (Cohen *et al.,* 1985).

Viruses infecting lily and tulip are mostly ssRNA viruses. The most common viral disease are caused by LSV either singly or in mixed infections with LMoV and CMV, whereas other viruses *viz., Strawberry latent ring spot virus , Arabis mosaic virus, Tobacco mosaic virus, Tomato aspermy virus, Lily virus x* and *Tobacco rattle virus* occur less frequently. Among them the most important virus is LSV. Under the present investigation, LSV, a Carlavirus was found to be widespread in *Lilium* and tulip grown commercially in H.P. Therefore, information regarding LSV has been reviewed in this chapter.

2.1 *Lily symptomless virus*

LSV was first reported in *Lilium* from Oregon in USA and described by Brierley & Smith (1944a) and Allen & McWhorter (1966). LSV belongs to Carlavirus group. Carlavirus name was derived from *Carnation latent virus* (CarLV) also having other name such as Lily curl strip virus (McWhorter and Allen, 1964), Lily virus (Allen, 1971) Lily yellow flat virus, Lily rosette virus, and Mormor mite (McWhorter, 1937). When this virus was first time reported in lily plants, some cultivars of lily did not show symptoms so the virus was named as *Lily symptom less virus.*

2.1.1 Geographical distribution

Geographically, LSV is reported to be worldwide in Origin. In Europe, Chavdarov and Denkova (1995) reported LSV from Bulgaria; Bellaridi and Bertaccini (1994) from Italy; Asjes *et al.* (1973) from Netherlands; Kaminska (1996) from Poland.

Mowat and Stefanac (1970, 1974) and Philips and Brunt (1986) from United Kingdom. The virus has been reported from Asia by Yang *et al.* (1993) from Taiwan; Hagita (1989) from Japan; Chang and Chung (1987) and Lee *et al.* (1996) from Korean Republic; Cohen *et al.* (1996) from Israel and Sharma (2002) and Bhaik *et al.* (2003) from India. From Australia, it was reported by Blake and Wilson (1996). In North America, LSV was reported by McWhorter (1937); Brierley and Smith (1944a) and Allen and McWhorter (1966).

2.1.2 Symptoms

The symptom of LSV appears on leaves. Leaves may show vein clearing or light brown spots on the lower side of the leaves may pass into yellow. purple or brown spots on the upper surface of the leaves, which lead to premature death of plant, is also seen (Derks and Hendricks, 1990). Many cultivars infected by LSV remain symptomless and plant often shows reduced growth with smaller flowers. Symptoms develop better at lower temperature (less then 15 ^{0}C), thus symptoms are more apparent in winter than in summer (Allen, 1972). While producing cut flowers in the green house, LSV symptoms became more evident, particularly under favourable light and growing conditions. However, LSV frequently occurs with *Cucumber mosaic virus* or *Lily mottle virus* in cultivated lily cultivars and complex infection results in severe leaf mosaic and dwarfing, which threatens the yield and commercial production of lily (Allen, 1972; Asjes, 2000; Derks & Asjes, 1975). The infected plants have low bulb yield and shorter vase life as cut flowers (Boontjes, 1983). The flowers produced in the axil of the lower stem soon turn yellow in infected plants (Blake and Wilson, 1996; Schouten *et al.*, 1997). Lily appears to gradually become more resistant to LSV after flowering. It is observed that there is 30%

reduction of flower number per plant in infected plant. Plant height of infected *Lilium* was shortened by half as compared with healthy *Lilium* (Kim *et al.*, 1998). Although this virus is prevalent in lilies, but there are few reports of its presence in tulip also. Symptoms are confined to tepals. Some cultivars develop fine venial streaks of intensified pigment (Allen, 1972; Derks and Asjes, 1975). The virus is transmitted to most of the progeny bulbs. Although interaction does not affect the number of progeny bulbs formed, but the bulb yield can be reduced by 7% (Derks and Asjes, 1975).

2.1.3 Disease caused by LSV

LSV causes curl-stripe and basal stripe in lily under some environmental conditions. It causes necrotic fleck disease in lilies when associated with CMV. Another virus, *Lily mottle virus*, often occurs in necrotic fleck-disease of *Lilium longiflorum* but apparently is not involved in the disease (Brierley & Smith, 1944a). It also causes brown ring in bulbs of lily mid-century hybrids and streak mottle on leaves of *L. speciosum* varieties when associated with TBV (Asjes *et al.*, 1973).

2.1.4 Economic Impact of LSV

LSV free *Lilum* plants have better quality flower with better colour, nice tint and large size than those of lily LSV infected plant (Vroomen, 1997).Hanks and Menhenett (1981) also observed that LSV free plants had more stem length and leaf spread than those of LSV infected plants and virus free plants produced more and large florets. Qualitatively the appearance of LSV infected lily and tulip in the field is mostly regarded as acceptable, apart from some premature senescence at the end of growing season. The overall incidence of LSV in most stocks of lilies was fatal in 1970s, but decreased considerably in 1980s and later (Asjes, 2000). However, there is lack of

concern due to the symptomless appearance of LSV infected lilies in the field, which indirectly block the efforts to attain the tolerance to zero for this virus. Moreover the most favourable growing conditions mask the development of clearly visible symptoms. The symptoms in LSV infected lilies and short vase life cause additional problem (Boontjes, 1978; Blake and Wilson, 1996; Schouten *et al.,* 1997). The lower leaves borne on the stem generally turn yellow fairly quickly and are shed earlier in LSV infected plants (Schouten *et al.,* 1997). Leaf and flower colour have also been considered to be duller in LSV infected plants.

A few quantitative data are available on the reduction in lily bulb yield caused by virus. Experimental data on the effect of LSV indicated around 20% reduction in different cultivars (Schouten *et al.,* 1997). In tulips, naturally infected plants have been found only in the Netherlands (Derks and Asjes, 1975) and (Allen, 1972). The incidence of infection within plant is usually less than 1% out breaks of high incidence seemed exceptional (Derks *et al.,* 1982).

2.1.5 Transmission

LSV is transmitted by aphids *viz., Myzus persicae, Macrosiplum euphorbiae, Aphis gossypii,* and *Aphis fabae* in a non-persistent manner (Mowat and Stefanac, 1974; Derks and Asjes, 1975). However, Brierley and Smith (1944a) reported persistent transmission by *Aphis gossypii.* The virus is sap transmissible and not seed borne in lilies (Brierley and Smith, 1944a; Allen, 1972).

In the Netherlands leaves of lily planted in the field in March appear above ground in April and persists till November. The non-persistent transmitted viruses including LSV spread by aphids flying from late April early October (Asjes, 1991). The

rapidity of spread of these viruses varies from cultivar to cultivar depending on the susceptibility of the cultivars. In some cultivars of lily, LSV may spread very rapidly (Asjes and Blom Barnhoorn, 2000).

2.1.6 Host Range

Host range of LSV is restricted to the Liliaceae family except of two reports where it has been reported from *Alstromeria* of Alstoroemeriaceae famiiy (Philips and Brunt, 1986) and spider lily of Amaryllidaceae family (Singh *et al.*, 2005a). However, the virus spreads effectively only among Liliaceae family plants like lily and tulip (Van Slogteren, 1971; Allen, 1972; Derks and Asjes, 1975; Dekker *et al.*, 1993) .Naturally infected tulips have been found in the Netherlands (Derks and Asjes, 1975). In 1960s, LSV occurred throughout all lily stocks because the lack of obvious deleterious effects caused little or no concern. In the 1970s this attitude changes as a consequence of the singular efforts of some growers (Asjes, 1976).

2.1.7 Particle properties

Sedimentation coefficient ($S_{20\,w}$) of LSV particle at infinite dilution is about 171-172S and A_{260}/A_{280} ratio is 1.20-1.43 (Civerolo *et al.*, 1968).

2.1.8 Particle structure

LSV particles are elongated, slightly flexuous rod, approximately 640 nm long and 17-18 nm in diameter. Particle centre is densely stained. For achieving the best contrast under electron microscopy virus particles were mounted in 2% sodium phosphotungstate or 1% uranyl acetate for electron microscopy. The electron microscopy is thus a useful aid in diagnosing LSV infection (Civerolo *et al.*, 1968; Lyons and Allen, 1969).

2.1.9 Genome

LSV nucleic acid constitutes 8.3% of total particle weight (Civerolo *et al.*, 1968) The genome of LSV is monopartite linear, positive sense (+), single standard RNA with genomic sizes of approximately 8.3 kb excluding poly A sequences. The 3' terminus has a poly (A) tail and the 5'- terminus sometimes has a methylated nucleotide cap, or a monophosphate (Cavileer *et al.,* 1994; Fuji, 2002; Zavriev, *et al.,* 1991). Carlavirus genomic RNA is encapsidated by a single type of coat protein (CP) with a Mr of 32 kDa. The triple gene block (TGB) consisting of 3 overlapping proteins of TGBp1, TGBp2 and TGBp3, known to function for cell-to-cell movement. Complete genomic nucleotide sequence and genome organization (Fig. 2.1) of LSV has been reported by Choi and Ryu (2003). The genome of the Korean strain of LSV (LSV-Kr), was 8394 nucleotides long and contain six open reading frames (ORFs) coding of proteins of Mr 220 kDa, 23 kDa, 12 kDa, 7 kDa, 32 kDa, and 16 kDa from 5' to 3' end respectively which is the typical genome structure of carlaviruses (Brunt *et al.,* 2000; Cavileer *et al.,* 1994; Fuji *et al.,* 2002; Hataya *et al.,* 2001; Mackenzie *et al.,* 1989; Zavriev *et al.,* 1991). This is the first report of the complete nucleotide sequence and genome structure of LSV.

2.1.10 Cytopathology

LSV found in all parts of the host plant; in cytoplasm (except possibly the meristematic region).No pinwheel or inclusion bodies have been observed from infected cells (Allen and Lyons, 1969). The virus like particle associated with the disease occurs in the cytoplasm (Civerolo *et al.*, 1968; Lyons & Allen, 1969)

2.1.11 Serology

Antiserum from a rabbit injected intravenously with clarified sap obtained from infected lilies had a titter of 1/1280 in tube precipitin tests (Van Slogteren and De Vos, 1966). A rabbit receiving three intravenous injections at weekly intervals followed

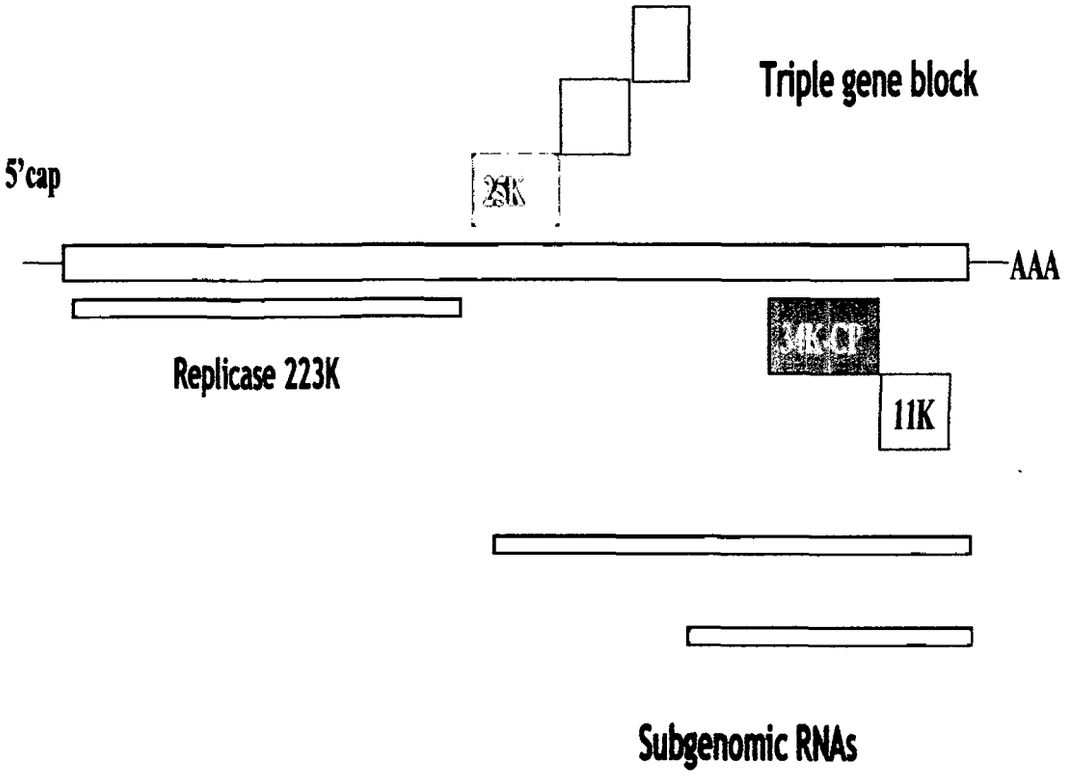


Fig. 2.1 Genome Organization of *Lily symptomless carlavirus*

Flexuous particle, size 660x18nm; Genome ssRNA and genome size 7.5-8kb

by two intramuscular injections of partially purified virus from lilies with curl-stripe symptoms developed a antiserum titer of 1/1024 (Allen, 1972). Tube or micro-precipitin tests, in which antisera form flocculent precipitates, are useful with clarified sap or partially purified virus.

2.1.12 Taxonomy and relationship

Properties and particle morphology place the virus in the Carlavirus group. The virus reacted positively with antisera prepared against Alstroemeria carlavirus, *Chrysanthemum virus B* (CVB), *Potato virus S & M* (PVS and PVM), *Carnation latent virus* (CLV) and *Passiflora latent virus* (PLV). Antiserum of *Lily symptomless virus* reacted positively with partially purified (about 10 times concentrated) suspensions of *Chrysanthemum B*, *Potato M* and *Potato S* viruses (Van Slogtren and De Vos, 1966).

2.1.13 Purification

LSV has been purified by various researchers. Civerolo *et al.* (1968) purified LSV by using 0.25 M potassium phosphate buffer, pH 7.5, containing 0.002 M $MgSO_4$, 0.1% thioglycollic acid, and 10.15 ml 1% bentonite solution per 100 ml of buffer solution. When centrifuged in sucrose density gradients, the virus gave a single light scattering band. (Allen, 1972) also purified LSV using 0.067 M phosphate buffer, pH (7.2) + 0.1 thioglycollic acid.

2.2 Detection of virus

As a result of advancement in biotechnology many new approaches for plant disease diagnosis and pathogen detection have been developed. These approaches are antibody based techniques, PCR based techniques and other techniques that form the

basis of modern plant disease diagnosis. Some of these techniques have been applied to plant disease diagnostic and pathogen detection at the particular level, whether in diagnostic clinics, in the hands of growers or others involve in crop management. Some techniques promise for future application in practical diagnostics or in studies of pathogen ecology or epidemiology. Viruses within a group have similar properties which are not shared by viruses in other group i.e. particle morphology, serological relationship and mode of transmission. A number of methods have been developed for detection and diagnosis of viral disease, the four methods most commonly used are:

- Bioassay
- Serology
- Electron microscopy
- RT-PCR

Bioassay is probably the most common approach. Electron microscopy is useful for the detection of a number of viruses, but the instrument is expensive and availability is limited. Although serological techniques have proved to be valuable diagnostic tool, their use in detecting broad spectrum of viruses is limited by the non-availability of antisera. RT-PCR is very accurate and sensitive techniques for virus diagnosis. In recent years, cytological techniques have been developed for the detection of virus-induced inclusions. There intracellular structures are characteristic for the viruses inducing them and have proved to be valuable agents in the diagnosis of plant virus disease (Christie *et al.*, 1995).

In case of LSV infection in lily and tulip, no inclusions are produced (Alper *et al.*, 1982). Derks and Abbink (1988) described distinctive symptoms caused by TBV and LSV in bulbs and in plants at different stages of growth from emergence to flowering, which made the diagnosis simpler.

2.2.1 ELISA

Immunoassay techniques can be characterized as quantitative analytical method applied for measuring biological important compounds or organism using antibodies as specific analytical reagents. These are based on unique reaction between antibodies and antigens, which elicit their production.

Immunological assay is the most important method for virus diagnosis these days. It offers great versatility in type of test and format used in specific serological test (Van Regenmortel, 1982). Micro plate method of ELISA has been introduced for diagnosis of variety of antigens (Voller *et al.*, 1976; Voller *et al.*, 1978a).

Heterogeneous assays, which are suitable for detecting macromolecules and plant or animal pathogens (Voller *et al.*, 1978b) of are various kinds. The most commonly used for detecting plant pathogens is double antibody sandwich ELISA (DAS-ELISA), which was first described in detail for plant viruses by Clark and Adams (1977). Since then various modification of this basic procedure, have been described.

Hagita (1989) detected LSV from bulb scales of *Lilium leichtlinci* var. maximowiczii plant by ELISA. The concentration of LSV appeared to be higher in the outer and inner bulb scales than in the middle ones.

Van Slogteren *et al.* (1980) detected LSV in leaves and bulb scales of lily plant with the immunodiffusion drop test and with ELISA. Derks *et al.* (1982) detected LSV in tulip by DAS-ELISA. They found that the particle concentration is considerably higher in tepals than in leaves.

2.2.2 Molecular techniques

2.2.2.1 Nucleic acid hybridization

Nucleic acid hybridization is a modern method of plant virus and viroid detection based on identification of specific molecule components of the causal agents in tested samples. The genetic material of the pathogen can be detected by nucleic acid hybridization. This technique was initially used in phytopathology for viroid detection (Owens and Diener, 1981). Considerable progress has been made in the nucleic acid hybridization, which seems to be a good alternative to ELISA technique, when virus-specific antisera are not available or pathogen specific protein is not produced is host plant. Combining PCR with molecular hybridization further increase the sensitivity of detection of plant pathogens (Vunsh *et al.,* 1990, 1991; Borja and Ponz, 1992).

Tissue print hybridization is also used extensively for detection of viruses. The most common procedure is the dot blot or slot-blot hybridization. Printing plant tissue directly to membrane was first reported by Cassab and Varner (1987) and subsequently the method has been modified to suit different plant species. This method has the added advantage of being able to localize virus within the plant (Mansky *et al.,* 1990; Chia *et al.,* 1995).

2.2.2.2 Polymerase chain reaction (PCR)

PCR is a method of *in vitro* amplification of template DNA sequence with very high specificity and fidelity using oligonucleotides, primer and *Taq* DNA polymerase which is a simple automated reaction (Saiki *et al.,* 1985; Mullis, 1990). Its current application are in the areas of disease diagnosis, detection of pathogens, detection of DNA in small samples, DNA comparisons, high efficiency cloning of genomic sequence and gene sequencing (Erlich, 1989; Erlich *et al.,* 1991). PCR has affected basic

molecular biological research, clinical research, forensics evolutionary studies and plant pathology. In 1990 Hadidi and Yang first reported the detection of viroids by RT-PCR amplification and also successfully utilized this technique for detection of RNA plant viruses from infected tissue and predicted the application potential of PCR technology in the field of plant pathology.

Wang *et al.* (2004) detected LSV by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Immunocapture (IC) RT-PCR method. The results showed that LSV was detected from dilutions equivalent to 2 and 200 ng of lily leaf materials by RT-PCR and IC-RT-PCR, respectively. RT-PCR and IC-RT-PCR were 1000- and 10-fold times more sensitive than DAS-ELISA. Nimi *et al.* (2003) also detected LSV in three lily cultivars by RT-PCR. Results were compared with those obtained in an enzyme-linked immunosorbent assay (ELISA). Use of RT-PCR has facilitated the production and cloning of full length cDNAs of plant RNA viruses and viroids. Takamatsu *et al.* (1994) cloned coat protein gene from naturally infected lily plants using the RT-PCR. The nucleotide sequence of the gene and deduced amino acid sequence of the protein are provided. It was suggested that this procedure makes it possible to omit complicated procedures and to complete DNA cloning of specific viral genes within a week.

2.3 Coat protein gene variability studies

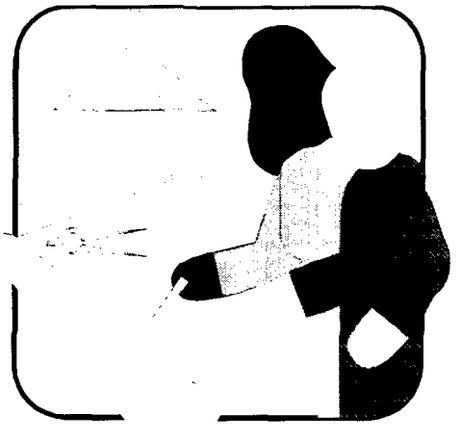
Coat protein of plant viruses determines virus antigenic property. It is also responsible for the virus-vector relationship and their mode of transmission. Variability in coat protein can lead to change in antigenic property of a virus which can also change virus-vector relationship. So the study of coat protein gene variability is very important. Severe case of addition and deletion can leads to evolution of new strain of virus. Coat protein gene variability can also be used for phylogenetic analysis.

Sharma *et al.* (2005) found nucleotide and amino acid similarity of coat protein gene of LSV of an Indian isolate (Accession no.AJ549932) to other LSV sequence available from GenBank. Nucleotide similarity was found to be 97-98% and amino acid homology 85-98%with other LSV sequence available from GenBank. Ahn *et al.* (1999) also compared two different Korean isolates of LSV, LSV-Ko and LSV-KII. The two Korean strain share 98.4% and 98.3% sequence identity at the nucleotide and amino acid levels, respectively. The CP gene of LSV-Ko showed 99.1% and 87.0% nucleotide sequence identity, and 99.0% and 96.6% amino acid sequence identity with those of the Netherlands and Japanese LSV strains, respectively. A pair-wise amino acid sequence comparison revealed a sequence similarity of 29.6% to 69.8% between LSV-Ko and other species of the Carlavirus. Choi and Ryu (2003) also compared complete nucleotide sequence of the genomic RNA of *Lily symptomless virus* to other Carlaviruses. In their study, they observed genetic heterogeneity in the ORF1 (replicase gene). A total of 221 of 5,847 nucleotides (nt) were heterogeneous in the ORF1 of replicase; 162 nt protein were silent and 59 nt resulted in amino acid changes. This heterogeneity indicates that the LSV-infecting lily plants contained a genetically heterogeneous population of LSV (quasi-species). Overall similarities to those of other Carlaviruses for the six ORFs of LSV were from 67.1% to 31.6% and from 87.3% to 13.7% at nucleotide and amino acid levels, respectively. The ORF1 replicase gene of LSV shares 40.9% to 56.8% and 48.9 to 58.6% identity with that of 5 other Carlaviruses at the amino acid and nucleotide levels, respectively. By their findings they observed that LSV was closest to *Blueberry scorch virus* (BlScV) in this ORF, among the other carlaviruses for which the sequence information available. The three triple gene blocks (ORF4), ORF5 (coat protein) and 3'-

proximal 16 kDa ORF6 gene were further analyzed, and phylogenetic trees for the coding region indicating the LSV was the most closely related to *Kalanchoe latent virus* and BlScV.

2.4 Coat protein expression studies

Ahn *et al,* (1999) studied the expression coat protein gene in *E. coli*. The 3'terminal region of the genomic RNAs of two Korean isolates of the LSV namely, LSV-Ko and LSV-KII, were cloned and their nucleotide sequences were determined. The nucleotide sequence analysis and protein analysis by Western blotting revealed that *E.coli* expressed a 32-kDa protein that is the viral coat protein (CP) for the LSV.



Materials and Methods

Chapter III

MATERIALS AND METHODS

3.1 Sample collection

The lily and tulip samples were collected from different parts of Himachal Pradesh [Mandi, Solan, Chamba, Kangra] and J&K. Infected leaves were maintained in -80^{0}C and bulbs planted in quarantine condition in green house of Floriculture Division, Institute of Himalayan Bioresource Technology (IHBT, CSIR), Palampur, HP. These collected samples along with cultivars growing at IHBT Palampur were subjected for screening against LSV using the detection techniques *viz.* DAS-ELISA and nucleic acid based assays (RT-PCR and Northern hybridization). Further confirmation was carried out by sequencing of RT-PCR amplified products.

3.2 Detection of virus

3.2.1 Detection by Enzyme Linked Immunosorbent Assay (ELISA)

The plants were selected randomly from different cultivars and subjected to ELISA for the diagnosis of LSV. For the virus, DAS-ELISA was performed as described below. Diagnostic reagent for the detection of LSV were procured from Bio-Rad (USA). The basic techniques were same as described by Clark and Adams (1977).

Direct Antibody Sandwich ELISA (DAS-ELISA) protocol

1. Wells Plate (Nunc Immuno TM plates, Denmark) were coated with 100µl of polyclonal antibodies diluted appropriately (1:500 dilution) in coating buffer as per manufacture's instructions and plates were incubated overnight at 4°C in a humid box.

2. The plate was washed 5 times with PBS-T to remove unbound antibody.
3. Antigens were prepared by macerating infected tissue 1 g/10ml in extraction buffer. Several dilutions were made corresponding to 1X - 1/160X dilution of the original antigen. 100µl of the diluted antigen was pipetted into the wells of the microtitre plate as per the loading diagram and incubated for 2 hrs at 37°C in a humid box to allow coating of antigen in the wells.
4. Washing steps were repeated and appropriately diluted conjugate in ECI buffer (as per manufacturer's instruction) was filled into the wells (100 µl/ well). Plates were incubated for two hrs at 37°C in a humid box.
5. After washing the plates with PBS-T, the wells were filled with 100 µl solution of 1mg/ml p-nitro phenyl phosphate (PNP) prepared in PNP buffer (10% Diethanolamide solution) adjusted to pH 9.8 with HCl).
6. After appropriate colour development (15-20 min), the reaction was terminated by adding 50 µl of 3M NaOH to each well.
7. Positive and negative controls were also made on the same plate. Absorbance at 405 nm was measured for complete ELISA plate with a Flow ELISA micro plate reader (BioRed). The reaction was considered positive if absorbance was observed to be at least three times the background mean of healthy control.

Coating buffer (0.05M per liter): 1.59 gm sodium carbonate and 2.93 gm sodium bicarbonate.

PBST buffer: 20mM sodium phosphate pH 7.4; 150 mM NaCl and 0.05% (v/v) Tween 20.

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

ECI buffer: 2.0 g BSA, 20.0 g PVP 24-40,000 and 0.2 g sodium azide were dissolved in 1000 ml 1X PBST and pH was adjusted 7.4.

PNP buffer: 0.1 g magnesium chloride, 0.2 g sodium azide and 97 ml Diethanolamide were dissolved in 800 ml distilled water and the volume was made to 1000 ml and pH was adjusted to 9.8.

3.2.2 Nucleic acid based detection of *Lily symptomless virus* (LSV)

3.2.2.1 Detection of LSV by Northern hybridization (RNA-DNA hybridization)

Northern hybridization is an efficient means of detecting viruses based on RNA-DNA hybridization. To detect LSV, in lily cultivars, we used LSV CP gene (~875 bp), as radioactive labeled probe. The procedure followed is described below:

a) Preparation of the slot blot

Young leaf tissue (1 gm) of lily was frozen in liquid nitrogen in a mortar and pestle and ground to the fine powder. Fine powder (100 mg) was placed in an eppendorf and 300ml TNE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0) was added and mixed well. The tubes were centrifuged for 1 min at 12,000g. Supernatant (10µl) was diluted with 90µl of Millipore water and was denatured at 65°C for 10 min and immediately placed on ice before loading on to the slot blot apparatus containing the nitrocellulose membrane.

Nitrocellulose membrane was cut according to the size of the slot blot apparatus and pre washed in distilled water for 5 min. The membrane was equilibrated in 20x SSC buffer for 5 min. It was briefly air dried after equilibration and denatured samples were loaded into the slots of the apparatus and vacuum of 70 psi pressure was applied for 3-4 h. Then the membrane were placed in an UV cross linker for 2 min at 1,200 J energy to fix RNA on the membrane. A positive control probe for LSV was used while for negative control we used only TNE buffer.

b) LSV Probe Preparation

Denatured CP gene of LSV was labeled with P^{32} dATP isotope and was used as probes for the detection of LSV. The reaction mixture to form the probe contains as follows-

Material	Quantity
DNA	200-500 ng
Random hexamers	100 ng
10X Klenow buffer	3µl
dNTPs (-dATP) (10mM)	4.5µl
P^{32}dATP	1µl
Klenow enzyme(5U/ µl)	1 µl
Water	20.2µl
Total	30µl

It was mixed properly by pipetting and centrifugation followed by incubation for 1h at 37°C and kept on ice. The probes were kept at -20°C for further use. Just before use, the probes were denatured by incubating in boiling water for 8-10 min and kept on ice for further use.

c) Northern Hybridization using ^{32}P labeled DNA probes

Hybridization was carried out using Express Hyb solution (BD Biosciences, USA) as per the manufacture's instruction. Express hybridization solution was warmed at 68°C and stirred well to completely dissolve any precipitate. Nitrocellulose membrane (10x10 cm) was kept for pre-hybridization in 5-10ml of Express Hyb hybridization

solution with continuous shaking at 68°C for 30 min. The solution used for pre-hybridization was changed with fresh solution. To this equal volume of buffer A and the radioactively labeled DNA probes (denatured at 95°C and kept on ice for 8-10 min) were added. Taking care that no air bubble should be developed and the Express hybridization solution was evenly distributed over the entire blot. The tubes containing blot were incubated with continuous shaking at 68°C for 1 h. After hybridization was completed the blot was rinsed in wash solution-I at room temperature for 30-40 min with continuous agitation with few change of fresh wash solution-I. The blot was again kept in wash solution-II with continuous shaking at 50°C for 40 min with one change of fresh solution. The blot was removed with forceps and excess of wash solution was drained off. Immediately the blot was put on Whatmann filter paper (3mm) and wrapped with Saran wrap and exposed to X-ray film cassette. The X-ray film was with two intensifying screens and kept at -70°C for 1-2 days. Then it was developed and photographed in gel document system. (Alpha Digi Doc™).

3.2.2.2 Detection by RT-PCR

a) Primer Designing

Primer pairs were designed for the amplification of coat protein genes of LSV infecting lily and tulip. For that, nucleotide sequences of LSV coat protein available, in GenBank and reported from different parts of the world were downloaded from the NCBI database (web site: http://www.ncbi.nlm.nih.gov). These sequences were then aligned using either MULTIALIGN program (web site: http://prodes.toulouse.inra.fr/ multialin/multialin.html) based on algorithm as reported by Corpet (1988) or by CLUSTALW program (web site: http://www.ebi.ac.uk/clustalw) based on the algorithm

as described by Higgins *et al.* (1994). Based on the conserved regions identified at 5' and 3' regions of coat protein genes in the aligned sequences the primer pairs were designed keeping in mind the average GC content and the annealing temperature of the primer pairs. Restriction enzyme sites were also added at the 5' region of LSV CP upstream and downstream primers to facilitate the ligation into the appropriate vector. The gene sequences of primer pairs were submitted to EMBL database. The designed primers were synthesized from Microsynth (Switzerland).

Primer sequence of LSV coat protein gene:

Upward primer: 5' AAGG ATCCATGCAATCAAGACCAAGA 3'

Downward primer: 5'AAGAGCTCTCATCCATTAATTTGCCTA 3'

b) RT-PCR

Primarily the diagnostics was standardized and secondly the flux of viruses on lily and tulip were found out. Different lily and tulip cultivars were checked for the presence of LSV. LSV was detected in total RNA extracts from infected plants.

(i) Isolation of total RNA from plants

The total RNA was extracted from virus-infected tulip and lily plants using the two methods viz. RNA aqueous and RNA wiz ™ (Ambion, USA).

Total RNA isolation using RNA Wiz™ (Ambion, USA)

All steps were carried out at room temperature unless specified.

a) The infected leaves were crushed to fine powder in liquid nitrogen using baked mortar and pestle.

b) The tissue powder (100 mg) was immediately transferred to an eppendorf containing 500 µl of RNA Wiz solution and mixed properly.

c) The homogenate was incubated at room temperature for 5 min.

d) Chloroform (100 µl) was added to homogenate; tubes were covered with aluminum foil and incubated for 10 min.

The mixture was centrifuged at 10,000g for 15 min at 4°C. The mixture separated into 3 phases:

(i) Upper aqueous phase containing RNA

(ii) Inter phase containing DNA

(iii) Lower (organic) phase containing protein

f) Without disturbing interphase, the aqueous phase was carefully transferred to a new eppendorf and 0.5x starting volume of RNase free water was added and mixed well.

g) One starting volume of isopropanol was added, mixed well and mix was incubated for 10 min at room temperature. The mixture was centrifuged at 10,000g for 15 min at 4°C to pellet down RNA.

h) The supernatant was decanted and the pellet was washed with at least one starting volume of cold 75% ethanol by vortexing and centrifuged at 10,000g for 5 min at 4°C. Supernatant was discarded.

i) Pellet was air dried for about 10 min and RNA was resuspended in 50 µl of RNase free sterile water.

RNA aqueous Small Scale Phenol free Total RNA Isolation Kit:

1. About 50 mg leaves were homogenized to fine powder under liquid nitrogen.
2. The samples were added in 12 volumes of Lysis/Binding Solution.
3. The lysate was checked for viscosity so that it was not difficult to pipet.

4. The lysate was centrifuged 2-3 min at top speed in a microcentrifuged to remove debris.
5. Equal volume of 64% ethanol was added to the lysate and mixed gently.
6. RNAqueous filter cartridge was inserted into the supplied collection and elution tube.
7. 700 µl of lysate/ethanol mixture was applied to the filter at a time and centrifuged at RCF 10,000-15,000 X g for 1 min.
8. The Flow-through was discarded.
9. 700 µl Wash Solution #1 was applied to the RNAqueous filter cartridge and centrifuged for 1 min.
10. The flow-through was discarded.
11. 500 µl of wash solution #2/3 was added and the wash solution was drawn through filter as above. It was repeated once.
12. After discarding wash solution, centrifuge was continued for 30 seconds to remove the traces of wash solution.
13. The Filter Cartridge was put in fresh tube and 60 µl of Elution Solution preheated to ~95-100°C was applied to the center of the filter.
14. The equate was recovered by centrifugation for ~30 seconds at room temperature (RCF 10,000-15,000 X g) and stored in aliquots of 15 µl at -70°C till further used.

(ii) Checking of RNA

Agarose gel (0.1%) was prepared in 1X TAE buffer. RNA (5µl) were loaded with 1X RNA loading dye and electrophoresed at 80 V for 1-2 hr. After run, the gel was stained with ethidium bromide (0.5 mg/ml) and visualized in U.V. Transilluminator.

(iii) First Strand cDNA Synthesis

Synthesis of cDNA by RT-PCR:

Reverse transcription is a process by which cDNA is synthesized from RNA. This is carried out with the help of reverse transcriptase enzyme, which is RNA-dependent -DNA polymerase because it catalyzes the conversion of RNA into cDNA. In this process of reverse transcription, RNA serves as the template and thus cDNA is formed.

By use of RT-PCR we can amplify RNA sequence into DNA duplexes. At first a cDNA copy of RNA is produced using enzyme reverse transcriptase, this is then used for amplification.

A mixture for cDNA synthesis was prepared by adding the following reagents: --

Contents	Quantity
RNA	10.0 µl
First strand RT-buffer (5x)	5.0 µl
dNTP mix (40mM)	1.5 µl
Downstream primer (200ng/µl)	1.0 µl
Ribonuclease inhibitor (40u/µl)	0 .50 µl
Reverse transcriptase M-MLV (200U/µl)	1.0 µl
Water	6.0 µl
Total	**25.0 µl**

The mixture was thoroughly mixed and then centrifuged for a short run. This mixture was incubated for cDNA synthesis at 37°C for 1 hour 15 min. followed by incubation at 70 °C for 5 min. After completion of the reaction mixture containing cDNA was taken for PCR amplification.

(iv) Amplification of cDNA using PCR:

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. The PCR method was devised and named by Mullis and Faloona in 1987. A DNA polymerase uses two primers for a series of synthetic reactions that are catalyzed. The amplification reaction is performed in the PCR. Firstly, the double stranded DNA denatures at 94 ^{0}C, then annealing will occur at 52 ^{0}C and then, extension of the DNA chain take place at 72 ^{0}C with the help of *Taq* DNA polymerase to amplify the DNA at 72 ^{0}C. cDNAs of the two fragments of coat protein of *Lily symptomless virus* produced from RT step can be amplified by PCR which was prepared as follows: PCR was carried out in GenAmp PCR machine (Applied Biosystems, USA) with 50 µl of total reaction mixture containing 10 µl of cDNA product, 200 ng of each forward and reverse primer, 5 µl of 10x *Taq* DNA polymerase buffer with 10mM $MgCl_2$, 5 µl of 10mM dNTP, 1.5 units of *Taq* DNA polymerase (Bangalore, Genei) and rest water was added. It was then subjected to PCR amplification. The amplified products were resolved on the 1% agarose gel and analyzed in the presence of ethidium bromide.

Contents	Quality
Taq Polymerase buffer (10x)	5.0 µl
dNTP mix (10mM)	3.0 µl
Primer upstream (200ng/µl)	1.0 µl
Primer downstream (200ng/µl)	1.0 µl
Taq DNA polymerase enzyme (3U/µl)	1.0 µl
cDNA product	10.0 µl
Water RNase free	29.0 µl
Total	**50.0 µl**

The mixture was thoroughly mixed and incubated in PCR thermal cycler for the Following temperatures cycle:

Thermal cycling pattern

94 °C	3 min
94 °C	10 sec
52 °C	3 min
72 °C	1 min
Repeat	30 cycles
72 °C	10 min
4 °C	Till the samples are removed

After amplification all samples are to be stored at -20 °C till use.

MMLV Reverse Transcriptase buffer: 50mMTris-HCL (PH 8.3), 75mM KCl , 3mM $MgCl_2$.

***Taq* DNA polymerase buffer**: 10 mM TAPS (pH 8.8); 15 mM $MgCl_2$; 50 mM KCl and 0.01% gelatin.

(v) Agarose gel electrophoresis of the plasmid PCR product

The gel electrophoresis was carried out using a submarine horizontal agarose slab gel as described by Sambrook *et al.* (1989). After electrophoresis, the gel was stained with ethidium bromide (1.0μg/ml) and visualized using UV Trans-illuminator and photographed using Alpha DigiDoc system.

Preparation of the Agarose gel

1. The gel assembly was cleaned and dried.
2. 1% agarose was added in 40mL 1x TAE buffer.

3. The slurry was heated in the microwave and allowed to cool.
4. When the solution is around 50^0C, it was poured into the casting tray and was allowed to set for 15-20 min.
5. The comb was removed and gel along with casting tray was put in the electrophoresis tank

Loading of samples on the gel

1. 10µl of PCR sample mixed with 4µl of 6x gel loading dye was loaded in the well.
2. Gel was run at 8V/cm (DNA will migrate from cathode to anode).
3. After the dye had run considerable distance the gel was stained with EtBr (Ethidium bromide) (1µg/ml) and observed under U.V light.

If required destain the gel by further running the gel for about 10-20 minutes.

Reagents used

Loading dye

Contents	Quantity
Ficoll 400	15 %
Bromophenol blue	0.25 %
Xylene cyanol	0.25 %

TAE buffer (50x, per liter): --

Contents	Quantity
Tris base	242g
Glacial acetic acid	57.1ml
EDTA (0.5M,pH-8)	100ml

3.3 Cloning and Sequencing

3.3.1 Elution of the DNA band from the gel using GenElute™ gel extraction kit (Sigma)

The GenElute™ gel extraction kit is designed for the rapid purification of 50 bp to 10 kb linear DNA fragments and plasmids from standard or low melting agarose gels. The isolated DNA is suitable for a variety of downstream applications, such as automated DNA sequencing, PCR, restriction digestion, cloning and labeling.

The procedure used was as follows

- Weigh empty eppendorf tubes.
- Sliced the electrophoresed PCR products or required length (~875) bands using sharp sterilized blades under UV illuminator.
- The sliced bands were put in the empty eppendorf tubes and weighed again.
- Gel solublization solution was added thrice the volume of the gel (weight we got after subtraction the weight of eppendorf with gel and the empty eppendorf) into the gel slice and kept on water bath at 60^0C for 10 minutes.
- In the mean time column was prepared. For preparation of binding column the GenElute Binding Column was placed into one of the provided 2 ml collection tubes. 500µl of the Column preparation solution was added to each binding column. Centrifuged for 1 min. Flow through liquid was discarded.
- Isopropanol equal to weight of the gel was added.
- 700µl of solublized gel solution mixture was added to the prepared column.
- Centrifuged for 1 minute after loading and flow-through liquid was discarded.
- 700µl of wash solution was added to the binding column. Centrifuged for 1 minute, the flow through liquid was discarded.
- The binding column was transferred to a fresh collection tube. 30-50µl of elution solution was added to the center of the membrane and centrifuged for 1 minute. The binding column was discarded and the DNA was collected in collection tube.

3.3.2 Ligation of foreign DNA fragment into the pGEM®-T easy (Promega, USA) vector

T4 DNA ligase

The pGEMT®-T Easy Vector System (Fig. 3.1) are convenient systems for the cloning of PCR products. The vectors consist of single 3' overhangs at the insertion site that greatly improves the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and provide a compatible overhang for PCR products generated by certain thermostable polymerases.

For the ligation the T4 DNA ligase enzyme is used. It catalysis the formation of the phosphodiester bond between juxtaposed 5'-phoshoryl and 3'-OH of DNA. It repairs single stranded nicks in the duplex DNA & will join both blunt ended & cohesive ended restriction fragments of duplex DNA.

The ligation reaction was set up as

Contents	Quantity
2 x ligation buffer	5.0 µl
50 ng pGEM-T-Easy-Vector	1.0 µl
Gel eluted PCR product	3.0 µl
T4 DNA ligase (3 u/ µl)	1.0 µl
Total	10.0 µl

The ligation reaction was kept at 4^0C for overnight.

3.3.3 Competent cell transformation

3.3.3.1 Preparation of competent cells

Competent cells can be prepared either freshly for each use or can be stored at -70^0C for future use. For the transformation experiments either DH5α (Amp^R) or XL1-Blue (tet^R) strains of *E. coli* were used. Single colony of the appropriate strain of the

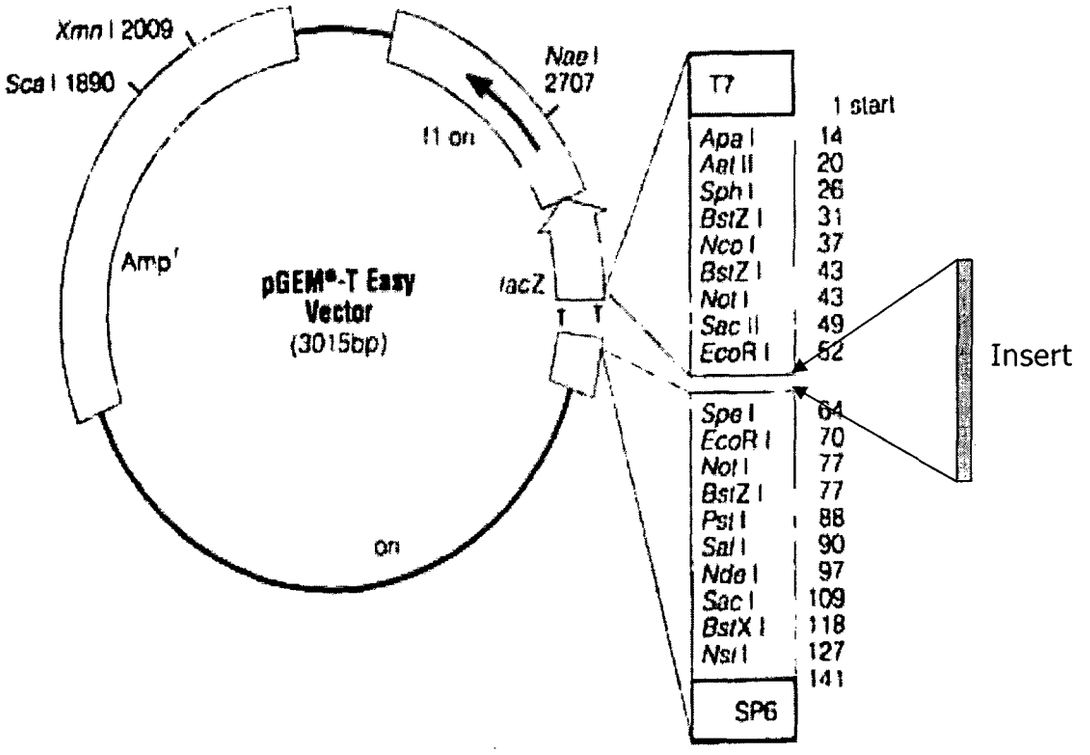


Fig. 3.1 **Diagrammatic representation of pGEM-T Easy vector (Promega, USA), showing restriction sites in multiple cloning site and a unique 3' T overhang which is compatible to 5' A overhang in PCR products. Inserts in this vector can be sequenced by T_7 and SP_6 primers.**

bacteria was streaked on to LB agar plate with appropriate antibiotics. This was incubated at 37^0C for 12-16 hrs. These freshly grown colonies of *E. coli* cells were used for preparation of competent cells or cultures were grown overnight at 37^0C and a part of it was used to inoculate larger volumes of the medium.

Competent cells which can be stored at –70^{0}C: 250ml LB was inoculated with 1ml of overnight culture of *E. coli* and grown at 37^0C on a shaker till OD at 600 nm was around 0.5. Culture was cooled on ice immediately and cells were harvested by centrifugation at 6000 rpm for 5 min at 4^0C in ss-34 rotor. Supernatant was removed carefully and traces of it were removed by inverting the centrifuge tube over paper towels. However, the tube was never removed from ice for long. The bacterial pellet was resuspended in 50-70ml of ice-cold 0.1M $CaCl_2$ and incubated on ice for 30 min. Cells were recovered by centrifugation as above, resuspended in 50-70ml of ice-cold 0.1M $MgCl_2$ and incubated on ice for 30 min. The cells were finally recovered and suspended in 10ml of 0.1M $CaCl_2$ containing 10% glycerol. Aliquots of 200µl each were prepared and stored at -70^0C. For transformation, the cells were removed from the freezer, thawed on ice and the DNA to be transformed was added to it.

Competent cells which can only be used fresh each time: 50ml LB was inoculated with 50µl overnight culture of *E. coli* DH5α or XL1-Blue cells and was allowed to grow at 37^0C on a shaker till OD at 600 nm was around 0.5. Culture was cooled on ice and cells were pelleted down by centrifugation at 5000 rpm for 5 min at 4^0C in ss-34 rotor. Supernatant was completely removed. The pellet was resuspended in 10ml ice-cold 0.1M $CaCl_2$ and incubated on ice for 15 min. Cells were recovered by centrifugation as above. Cell pellet was resuspended in 2ml of ice-cold 0.1M $CaCl_2$ and aliquots of 200µl each were prepared to be used for single transformation reaction. The cells can be stored on ice for not more than 12-16 hrs. This treatment results in increase of competence as well.

During the preparation of competent cells all the solutions were chilled, pipette tips cooled and cells always kept on ice.

3.3.3.2 Transformation of Competent cells (XL-Blue strain tet^R of *E.coli*)

Thawed the competent cells on ice after taking out from -80^{0}C (vol. 200 µl)

↓

Mixed 10 µl of the ligated product

↓

Kept on ice for 30 minutes

↓

Heat shock at 42^{0}C for 90 seconds

↓

Cooled on ice for 5 minutes

↓

800 µl of Luria broth was added

↓

Incubated at 37^{0}C for 1 hour with shaking
During that time LA plates were prepared

↓

Containing ampicillin (150 mg/ml) and
Tetracycline (12.5 mg/ml) 100 µl/ 100 ml of broth

↓

The Incubated culture was centrifuged and cells were pelleted down. 850 µl broth was discarded. Pellet was resuspended in remaining broth, and then plated on LA-amp + tetra plate.

↓

The above plates were incubated overnight at 37^{0}C. Next day plates were observed with transformed colonies.

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

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

3.3.3.3 Plasmid isolation from transformed cells

Transformed colony was picked up and inoculated on the LA-amp-tetra plates with the help of sterile toothpick and same was used for the inoculation of LB-amp-tetra tubes. These plates and tubes were grown overnight at 37^0C .

Principle

Bacterial plasmids DNA are widely used as cloning vehicles in recombinant research. Method denatures high molecular weight chromosomal DNA while covalently closed circular DNA (cccDNA) remains double stranded. There is a narrow range of pH (12-12.5) within which denaturation of the linear DNA occurs but cDNA does not.

Spin at 10,000 rpm for 2 min. Cells are lysed completely with NaOH and SDS. By choosing the ratio of cell suspension to NaOH solution carefully, a reproducible alkaline pH value is obtained without the need for monitoring the pH. Further, pH control is obtained by including glucose as a pH buffer. Chromosomal DNA, still in a very high molecular weight form is selectively denatured and when the lysate is neutralized by acidic NaOH (CH_3COONa), chromosomal DNA renatures and aggregates to form in soluble network. Simultaneously, high concentration of NaOAc causes precipitation of protein-SDS complexes and of high molecular weight RNA. Thus, the three major contaminants are co-precipitated and removed by a single centrifugation. Plasmid DNA and residual low molecular weight RNA are recovered from supernatant by ethanol precipitation.

Boiling miniprep method by Holmes and Quingely (1981)

1. 3 ml of the overnight grown culture was pelleted down in the centrifuge.
2. The supernatant was discarded and the pellet was resuspended in 110µl of STET.
3. Then 10.0µl of Lysozyme (10mg/ml) was added to the resuspended cells. The stock of Lysozyme was made in 10mM Tris pH 8.
4. The tubes were thoroughly mixed and incubated at room temperature for 5 minutes.
5. Then heat shock was given for 40 seconds in boiling water.
6. The suspension was centrifuged at full speed for 20-min at room temperature.
7. Pellet containing the cell debris was removed with the help of a sterile toothpick.
8. DNA in the supernatant was precipitated by adding one volume of iso-propanol (i.e. 120 µl).
9. Precipitated DNA was collected immediately by centrifugation for 20 min. at full speed.
10. The supernatant was completely removed and the pellet was dried and resuspended in 50µl nuclease free water.

STET buffer:

Contents	Quantity
Sucrose	8 %
Triton X-100	0.5 %
Tris HCl (pH 8)	50mM
EDTA (pH 8)	50mM

3.3.3.4 Identification of plasmid bacterial colonies that contain recombinant plasmids

Digestion of the plasmid DNA using *Eco*RI

pGemT® Easy Vectors contain multiple restriction sites within the multiple cloning regions. These restriction sites allow for the release of the insert by restriction digestion with a single restriction enzyme. pGemT® Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes *Eco* RI, *Bst* Z1, *Not* 1, *Pvu* II. The single enzyme can therefore be used for release of insert that is further proved by running the digested product on gel electrophoresis.

Digestion mixture:

Contents	Quantity
Plasmid DNA	10.0 µl
10x Buffer (Y^{+}Tango ^{TM}) (Fermentas)	10.0 µl(2x final concentration)
Eco RI (10u/µl) (Fermentas)	0.5 µl
Water	29.5 µl
Total	50.0 µl

Procedure:

- The digestion mixtures were thoroughly mixed and centrifuged for a short run.
- The digestion mixtures were incubated at 37°C for 3 hours.
- 2.5 volumes of cold ethanol was added in each mixture and incubated at -20°C for overnight.
- Next day the mixtures were centrifuged at maximum speed for 20 minutes at 4^0C .

- ❖ The supernatant was removed and the pellet was resuspended in 15µl RNase free water.
- ❖ The resuspended solution was mixed with 4µl of dye and loaded on 1% agarose gel.

3.3.3.5 Purification of plasmid DNA for sequence

Procedure

1. The bacterial colonies that contained recombinant plasmids were used to inoculate 5 ml of Luria- amp + tetra broth and incubated overnight at 37^0C in a shaking incubator.
2. After growth was obtained, the culture was stored in DMSO as well as in 50% glycerol.
3. The plasmid was isolated from the remaining culture as follows-
   a) 3ml overnight culture was pelleted down by centrifugation for 1 min. Supernatant was discarded. The cells were resuspended in 200 µl resuspension solution. Pipette up and down or vortex. 200 µl of lysis solution was added and inverted gently to mix. It was allowed to clear for 5 minutes.
   b) 350 µl of neutralization solution was added and inverted 4-5 times to mix. Centrifuged to pellet debris for 10 min at maximum speed i.e. 12,000 rpm.
   c) 500 µl column preparation solution was added to binding column in a collection tube and spun at 12,000 rpm for 1 min. The flow through was discarded
   d) Transferred cleared lysate into binding column.

e) Spun for 1 min and flow through was discarded.

f) 750 µl wash solution was added to the column. Spun for 1 min and flow through was discarded.

g) Spun for 1 min to dry the column.

h) Column was transferred to new collection tube.

i) 50 µl elution solution was added and spun for 1 min.

j) Plasmid DNA was collected.

3.3.3.6 DNA sequencing by the Sanger's dideoxy chain termination method (Sanger 1977)

This method makes use of the mechanism of DNA polymerase. DNA polymerase requires both a primer to which nucleotides are added, and a template strand to guide selection of each new nucleotide. The 3'-OH group of the primer reacts with the incoming deoxynucleoside triphosphate (dNTP), forming a new phosphodiester bond. The Sanger sequencing procedure uses di-deoxyribonucleoside triphosphate (ddNTP) analogs to interrupt DNA synthesis. When the dNTP is replaced by the ddNTP, strand elongation is halted after the analog is added because it lacks the 3'-OH group needed for the next step. The DNA to be sequenced is used as a template strand and a primer is annealed to it. By adding small amount of a single ddNTP, for example, ddCTP, to a normal reaction system, the synthesized strands will be prematurely terminated at locations where dc normally occurs, i.e., the position of dg on the template strand. Because there is much more dCTP, there is only a small change that the analog will be incorporated whenever a dC is to be added, but there is generally enough ddCTP, that each new strand has a high probability of acquiring one ddC at some point during synthesis. The result is a solution containing fragments representing each C residue in the sequence. The size of the fragments, separated by electrophoresis, reveals the location of

C residue in the sequence. The procedure is repeated separately for each of the four ddNTPs. And the sequence can be read directly from an autoradiogram of the gel. Because shorter DNA fragments migrate faster, the fragments near the bottom represent the nucleotide position closest to the primer (the 5'end) and the sequence is read from bottom to top. The sequence obtained is that of the strand complementary the strand being analysed.

Procedure

Sequencing extension reaction

Components	Quantity
Template DNA (100-500ng)	2-3 µl
Nuclease free water	4.0 µl
Reaction mixture	4.0 µl
Plasmid	1.0 µl
Primer	1.0 µl

For 1st cycle

94°C 30 Sec

50°C 40 Sec

60°C 4 min

Repeat 25 cycles

Washing to remove dye

- To the labeled product 90µl of Milli Q H_2O was added and transferred to 1.5ml tube. Now 10µl of 3M Sodium Acetate (pH 4.6) and 250µl of absolute alcohol was added. Mixed well by finger tipping.
- Spun at 10,000 rpm for 20 min at room temperature.

- The supernatant was quickly decanted off through pipetting and 250µl of 70% ethanol was added. The mix was gently inverted 2-3 times.
- Spun at 10,000 rpm for 20 min at RT.
- The washing step was repeated twice.
- The supernatant was discarded; pellet was air-dried and resuspended in 15µl Template Suppression Reagent (Applied Biosystems USA).

The samples were denatured at 94°C for 5 min and loaded to the automated DNA sequencer.

3.4 Sequence analysis

The sequence was aligned with corresponding sequences of other established LSV sequences from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.*, 1997). The program BLASTP was used to search the amino acid sequence database. Pairwise comparisons were performed by the ALIGN-2 program utilizing the DOTHELEX algorithm (Tatusova and Maiden, 1999). Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988). Phylogenetic tree was constructed with the help of ClustalW from the website http://www2.ebi.ac.uk/clustalw/ (Higgins *et al.*, 1994) and from the website www.ddbj.nig.ac.jp. Tree was subjected to bootstrap (using 1000 replicates) and viewed with the help of TreeView from the website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html (Page, 1996).

3.5 Cleaved amplified polymorphic (CAP) DNA analysis of coat protein gene of LSV

PCR amplification of LSV CP (50µl) was used as such for RFLP studies. This was used to confirm the amplification for CP. Four base pair cutters (restriction enzyme) were used which cut the DNA on specific sites. These enzymes (*Taq*I, *Hae*III) were chosen by restriction digestion analysis of available CP sequence of LSV in database using Webcutter (2.0) program.

Reaction mixture for CAP was prepared differently for different restriction enzymes in microfuge tube on ice as follows:

1) *Hae* III:

Reaction mix components	Quantity
Hae III (8u/µl)	0.5 µl
Buffer M(10X)	5.0 µl
DNA	20 µl
Water	19.5 µl
Total	50µl

2) *Taq* I:

Reaction mix components	Quantity
Taq I (10u/µl)	0.5 µl
Taq Buffer (10X)	5.0 µl
DNA	20 µl
Water	19.5 µl
Total	50µl

Temperature profile for enzymes used: -

Restriction enzymes	Temp.
Hae III (8u/µl)	37°C
Taq I (10u/µl)	65°C

Mixture was incubated at suitable temperature for 2-3 h.

b) Absolute alcohol (2.5 volumes) was added to mixture and incubated at – 20°C for overnight or at –80°C for 30-60 min for precipitation.

c) After incubation it was centrifuged at 14,000g for 20 min at 4°C.

The pellet was washed twice with 75% alcohol and air dried. The pellet was dissolved in 15 µl of H_2O and stored at –20°C for further use or directly loaded on the agarose gel.

Agarose gel (0.8-1%) was prepared and 15 µl of DNA mixed with 4 µl of gel loading dye (1x) was loaded. Electrophoresis was carried out at 80V for 2-3 h in TAE buffer. The gel was stained in ethidium bromide (0.5µg/ml) for 2 min and visualized in UV transiluminator.

3.6 Expression of LSV cp in *E .coli*

3.6.1 Amplification, Cloning and Transformation of Cloned fragment in *E. coli*

After getting the sequence of LSV CP from lily, specific primers were designed on the basis of gene sequence obtained, containing restriction enzyme sites for inframe cloning of the amplified CP gene in pET32 (+) (Fig. 3.2) expression vector as LSV CP gene does not contain the restriction sites of these enzymes and the sites of these enzymes are present in multiple cloning site (MCS) of the expression vector.

CP was amplified using designed primers (expression) from LSV CP already cloned in pGEM-T easy vector as template in a PCR reaction using Advantage 2 HF PCR system (BD Biosciences USA). The reaction composition and conditions were same as described earlier except the annealing temperature which is 53°C for this case. The Amplified fragment was separated on 1.0% agarose gel; specific band was excised from the gel and eluted from the agarose gel as previously described. Eluted PCR product and

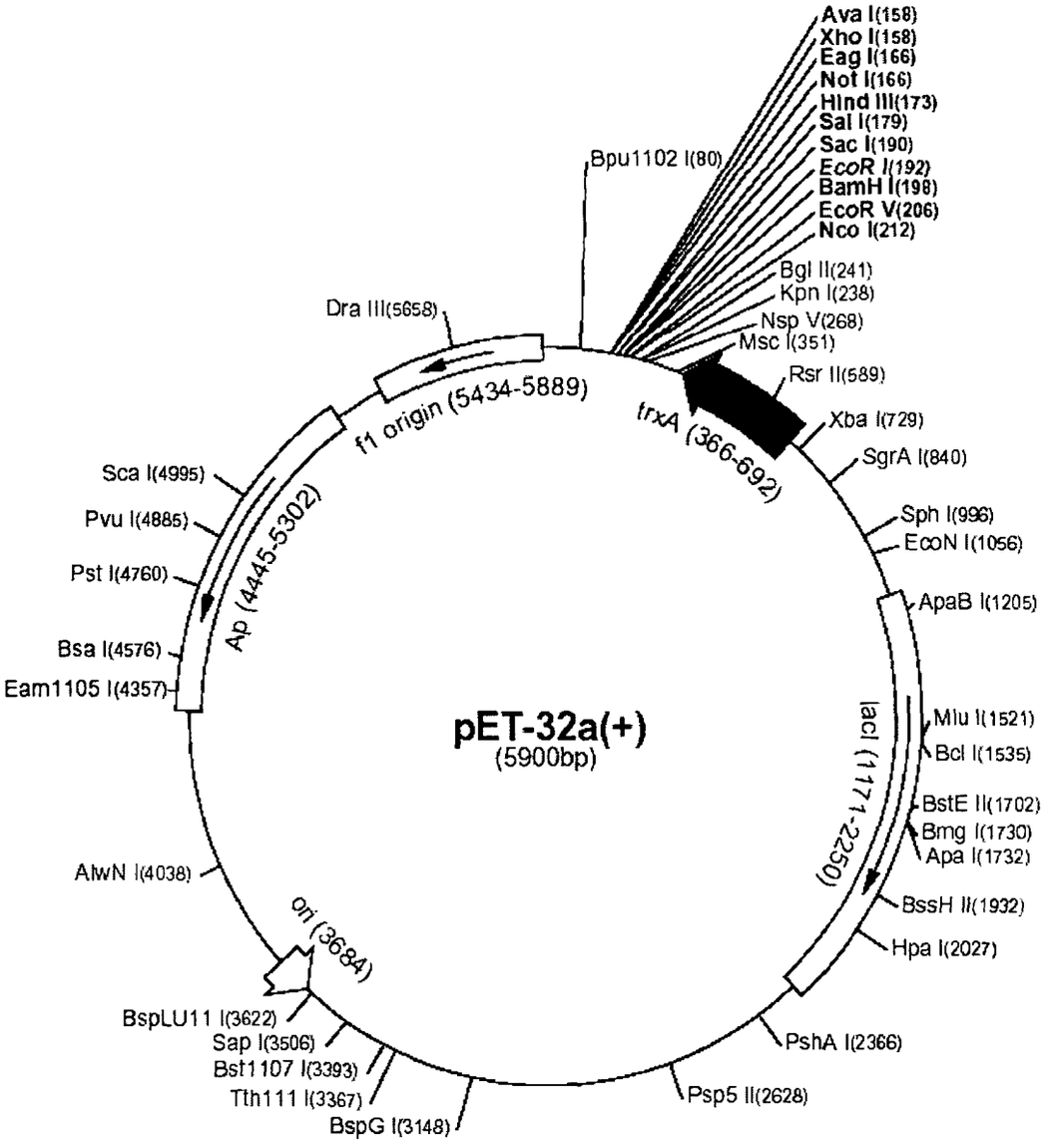


Fig. 3.2 Diagrammatic representation of pET32 (+) vector (Promega, USA), showing restriction sites in multiple cloning sites, reporter gene(Amp), and origin of replication (ori site).

pET32 (+) vector were digested with *Eco* RI and *Hind* III, digested fragments were run on gel and specific digested fragment were eluted from the gel and suspended in 30-40 µl of water or TE. The gel eluted digested PCR fragment and the vector (pET32 (+)) were ligated (as described earlier) and competent *E. coli* (BL21) cells were transformed with the ligated product. After transformation, recombinant clones were screened both with restriction digestion (with *Eco* RI and *Hind* III) and with colony PCR in order to check the recombinant colonies having desired insert. Plasmid was isolated from several recombinant clones and all were sequenced in an automated sequencer in order to identify the orientation and frame of insert. After getting the sequence one clone was selected that contain LSV CP in the correct orientation and in correct frame and that clone was used for expression studies.

3.6.2 Standardization of optimal expression conditions for LSV CP

The selected colony was restreaked on LB agar plate and single colony was picked in 5 ml of LB supplemented with ampicillin and grown overnight at 37°C at 200 rpm in an incubator shaker. YT broth (250 ml)(supplemented with ampicillin) was then inoculated with 1 ml of overnight grown culture and kept for incubation at 37°C at 200 rpm till the OD_{600} reaches 0.5. After that, 500 µl of IPTG was added to it and again incubated in a shaker. 5 ml of culture was pelleted after different incubation time intervals (1 h, 2 h, 3 h and 4 h) and the pellet was kept for checking of expression level. Different IPTG concentrations (ranging from 0.5 mM to 1.5 mM) and different temperature combinations (ranging from 20-37°C) were used to standardize the condition (IPTG concentration and temperature) so that the expression level was optimum and much of the expressed protein should be present as soluble fraction.

3.6.3 IPTG Checking of Expression Level

Bacterial pellet taken from the above step was suspended in 800 µl of 1x PBS and sonicated till it becomes clear. It was then centrifuged at 12,500 rpm for 10 min at 4°C. Pellet and supernatant were collected in separate tubes. Pellet was again resuspended in 800 µl 1x PBS. 5-10 µl from each of the tubes were run on the gel (12% SDS-PAGE) and gel was silver stained.

3.6.4 SDS-PAGE of expressed coat protein

Molecular weights of coat protein were determined by SDS-PAGE following the method as described by Maizel (1971).

3.6.4.1 Preparation of polyacrylamide gel (PAGE)

Composition of stacking gel and resolving gel are given below:

(i) Resolving gel/Separating Gel /lower gel Composition

	7.5%	**10%**	**12%**	**15%**
Distilled Water	19.4 ml	16.05 ml	13.4 ml	9.4 ml
10% SDS (w/v) stock	400 µl	400 µl	400 µl	400 µl
Tris-HCl (1.5 M; pH 8.8)	10 ml	10 ml	10 ml	10 ml
Acrylamide/Bis-acrylamide(30% stock)	10 ml	13.33 ml	16 ml	20 ml
10% Ammonium persulphate	200 µl	200 µl	200 µl	200 µl
TEMED	20 µl	20 µl	20 µl	20 µl
Total	**40 ml**	**40 ml**	**40 ml**	**40 ml**

2x resolving gel buffer: 0.75M Tris HCl (pH 8.8) and 0.2% SDS.

(ii) Stacking gel (5%), upper gel

Distilled Water	6.1 ml
10% SDS (w/v) stock	2.5 ml
Tris-HCl (1.5 M; pH 8.8)	100 µl
Acrylamide/Bis-acrylamide(30% stock)	1.3 ml
10% Ammonium persulphate	50 µl
TEMED	10 µl
Total	**5.00ml**

4x stacking gel buffer: 0.5M Tris HCl (pH 6.8) and 0.4% SDS.

* Ammomium persulphate (APS) was prepared fresh (10%) and added to the gel solution just before pouring.

7.5-15% resolving gel and 4-5% stacking gel was used to standardize the best separation. Above said amount of components were mixed for both resolving and stacking gel (excluding APS and TEMED) solutions separately in a properly covered flask and the solutions were degassed for 20 min in a vacuum desiccator before poring to the gel assembly. After degassing, APS and TEMED were mixed properly to the resolving gel solution and immediately poured to the assembly, such that 2/3 rd of the assembly should be filled, the solution was over layered with 500 µl of water saturated butanol to check aeration. It was then kept for 1-2 h at room temperature for proper polymerization. After polymerization butanol was removed completely, APS and TEMED were mixed to the stacking gel solution and over layered above the resolving gel. A comb of 10-13 wells was fitted in the assembly to make the wells so that 20-50 µl of preparations can be loaded in the wells. The assembly was kept for 1-2 h at room temperature for proper polymerization. After the polymerization the gel assembly was

kept in the running tank filled with 1x gel running buffer in both top and bottom chambers. Then the comb was removed from the stacking gel carefully and washed with the running buffer properly.

Acrylamide/Bisacrylamide stock: Add 30.0 gm Acrylamide and 0.8 gm Bis Acrylamide in 50 ml sterile water and stir to dissolve completely. Make the final volume to 100 ml with water and keep it at 4°C in an amber colored bottle.

Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8 (Dissolve 18.15 gm Tris-base in 50 ml sterile water and adjust the pH to 8.8 with 1 N HCl and make final volume to 100 ml) and keep it at 4°C.

Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8 (Dissolve 6.0 gm Tris-base in 50 ml sterile water and adjust the pH to 6.8 with 1 N HCl and make final volume to 100 ml) and keep it at 4°C.

10 % SDS Stock solution: Dissolve 10 gm Sodium dodecyl sulphate in water with gentle stirring and make the final volume to 100 ml and keep it at room temperature.

10% Ammonium persulphate (APS): Dissolve 100 mg APS in 1 ml sterile water (Prepared fresh and added to the gel solution just before pouring)

5x Electrode buffer (Gel running buffer): Dissolve 15.0 gm Tris-base, 72 gm glycine and 5 gm SDS in 800 ml sterile water and adjust the pH to 8.3 and make the final volume to 1000 ml and keep the solution at room temperature.

Sample preparation: Pellet was resuspended in denaturing buffer (1x loading dye). The samples were denatured in boiling water for 3-5 minutes and immediately chilled on ice, prior to loading on the gel.

Denaturing buffer (5x loading dye): 250mM Tris HCl (pH 6.5); 10% SDS, 1% β-mercaptoethanol, 0.625% bromophenol blue, 0.625% xylene cyanol and 50% glycerol. 1% β-mercaptoethanol was added every time in the end while denaturing protein suspension.

3.6.4.2 Electrophoresis

The wells in the gel were rinsed thoroughly before loading the samples and pre-run was carried out for 15 min at 25mA. Then the denatured protein samples were loaded into the wells. Optimal concentration of protein markers (Pharmacia) were also solubilized in denaturing buffer and loaded into the wells. Electrophoresis was carried out in 1x electrode buffer at 25 mA in stacking gel and 35 mA in resolving gel until the bromophenol blue dye reached the bottom of the gel.

1x electrode buffer: 25mM Tris HCl (pH 8.8); 192mM glycine and 0.1% SDS.

Coommassie blue R-250: 0.2% (w/v) in a mixture of 10% methanol and 7% acetic acid.

3.6.4.3 Staining of protein gel

Coomassie staining

After completion of electrophoresis, gel was carefully removed from the glass plates and stained with Coomassie Brilliant Blue R-250 (0.2%). Staining was carried out for 1 h to overnight with continuous gentle shaking. Gel was de-stained in solution containing 16.5% Methanol and 7% Acetic acid until the protein bands were clearly visible and the background was negligible.

Silver staining

After the run was complete gel was soaked in 50% methanol for 1 h in a glass dish. Staining solution was prepared by adding solution A dropwise to Solution B with constant stirring and then making volume to 100 ml with deionised water (this silver stain

should be prepared immediately before use). The gel was stained in this solution for 15 min with gentle agitation on a gel rocker in dark. Then, gel was washed with deionized water for 5 min with gentle agitation. The gel was then soaked in developing solution until band appears. After proper band development the gel was washed with deionised water and placed in 50% methanol to stop further reaction.

Solution A: 0.8 gm Silver nitrate in 4 ml distilled water.

Solution B: Mix 21 ml of 0.36% NaOH with 1.4 ml of freshly prepared 14.8 M Ammonium hydroxide.

Developing solution: Mix 2.5 ml of 1% Citric acid with 0.25 ml of 37% Formaldehyde and make the volume to 500 ml (freshly prepared).

Results

Chapter IV

RESULTS

4.1 Natural symptoms

Virus infected plants of lily and tulip in the experimental field of Floriculture Division (IHBT, Palampur) and other samples collected from different locations of Himachal Pradesh *viz.* Chamba, Solan, Kangra, Mandi and J&K found to exhibit symptoms like mosaic, mottling and light brown spot on back side of leaves, flecking of leaves, reduced plant size, distorted and twisted growth, colour breaking of flower, and smaller flower size (Plate 4.1 and 4.2).

4.2 Detection of LSV

4.2.1 Serological based detection of LSV

LSV is strongly immunogenic and as a result serological methods can be applied reliably. In the present study, DAS-ELISA was used for their detection in lily and tulip plants.

Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

Virus infected leaf samples of *L. longiflorum* (Ester lily), *L. tigrinum* (Tiger lily), *Hymenocallis spp.* (Spider lily), *Zentedeschia spp.*(Cala lily) and 21 cultivars of Oriental and Asiatic hybrid were subjected to ELISA, of which *Lilium longiflorum*, *L. tigrinum* and *Hymenocallis spp.* (Spider lily) and 7 cultivars of Asiatic and Oriental hybrid lilies were found positive (Table 4.1). The incidence of virus was around 33.3%. The absorbance value for tested samples was near positive control or 4-5 times the negative control was considered positive for the virus. The absorbance values for positive and negative controls were found to be 2.505 and 0.158, respectively.

Plate 4.1 Color breaking pattern on flower and mosaic pattern on leaves of tulip

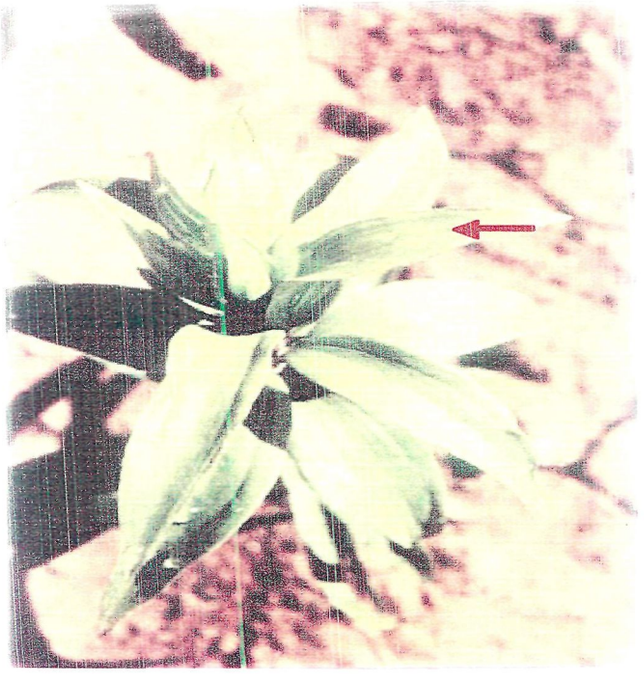


Plate 4.2 Mosaic pattern on leaves of Asiatic and Oriental hybrids lilies

Table: 4.1. Detection of LSV by DAS-ELISA in different species and cultivars of lily

S. No.	Cultivars	Result	S. No.	Cultivars	Result
Asiatic hybrid lilies					
1	Ucaris	-	9	Cavi	-
2	Nerone	-	10	Alaska	+
3	London	+	11	Prato	-
4	Romano	+	12	Monobracia	-
5	America	-	13	Nova Cento	+
6	Brunello	-	14	Tascana	-
7	Nova Cento (TCPs)	-	15	Pollyanna	+
8	Adelina	-			
Oriental hybrid lilies					
16	Expression	-	19	White Mero Star	+
17	Galilei	+	20	Star Gazer Max	-
18	Woodriff's	-	21	Mediterrannee	-
Other lilies					
22	*Zentedeschia spp* (Calla lily)	-	24	*Hymenocallis spp.* (Spider lily)	+
23	*L. tigrinum* (Tiger lily)	+	25	*L. longiflorum* (Ester lily)	+

4.2.2 Nucleic acid based detection of LSV

4.2.2.1 Northern hybridization for detection of LSV

For detection of LSV in lily cultivars by northern hybridization, ^{32}P labeled probe was used. Total RNA isolated as described earlier from various lily cultivars collected from different parts of the Himachal Pradesh, were blotted on nitrocellulose membrane and probed. After autoradiography dark colour bands were observed on X-ray film in LSV infected plants of lily. The results are shown in Table 4.2. Out of *L. longiflorum* (Ester lily) and 14 cultivars of Asiatic and Oriental hybrids, 6 were positive of which one was *L. longiflorum* and five were cultivars of Asiatic and Oriental hybrids, namely Corida, Brunello, Nova Cento, London, Cavi and positive control Tiger-lily RNA (Fig. 4.1) indicating 40% incidence.

Table 4.2 Results of Northern Hybridization with *L. longiflorum* and 14 cultivars of Asiatic and Oriental hybrid lilies

S. No.	Samples	Reaction	S. No.	Samples	Reaction
1	Corida (AH*)	+	9	London (AH)	+
2	*L. longiflorum* (Ester lily)	+	10	Cavi (AH)	+
3	Brunello (AH)	+	11	Expression (OH)	-
4	White Mero Star (OH**)	-	12	Nerone (AH)	-
5	Mediterrannee (OH)	-	13	Adelina (AH)	-
6	Nova Cento (AH)	+	14	Prato (AH)	-
7	Romano (AH)	-	15	Pollyanna (AH)	-
8	Star Gazer Max (OH)	-	16	Tiger-lily RNA (positive control)	+

AH* = Asiatic Hybrid lily; OH** = Oriental Hybrid lily

Figure 4.1 Northern hybridization used for the detection of LSV in lily samples using radiolabeled CP gene probe. Out of 16 samples tested 6 were found positive:

a). The positive samples were Corida (AH) (No.2), *L. Longiflorum* (Ester lily) (No.3), Brunello (AH) (No.4), Nova Cento (AH) (No. 10), London (AH) (No.13), Cavi (AH) (14) shows the presence of LSV.

b). No. 1, 5-9, 11-12 and 15-16 were found negative for LSV.

c). No. 17-23 are blank numbers.

d). No. 24 is a positive control.

4.2.2.2 Detection by RT-PCR

Total RNA was isolated from leaves of lily and tulip and checked on 1% agarose gel (Fig. 4.2). CP gene of LSV was amplified using total RNA isolated from lily and tulip using LSV CP gene specific primers (LSV-U and LSV-D). An amplicon of ~875 bp was observed as expected on 1% agarose gel in LSV infected sample (Fig. 4.3).

The desired PCR product i.e. DNA of LSV CP gene (~ 875bp) was eluted and its concentration was determined and ligated in pGEM-T Easy vector (Promega, USA). The *E. coli* XL-Blue (tet^R) competent cells were transformed with those ligated vector. Clones were selected on LB agar plates supplemented with ampicillin + tetracycline. From overnight grown culture plasmid was isolated and subjected to restriction digestion with *Eco*R1 to check and confirm the presence of ligated CP genes in plasmids. Fig. 4.4 a shows photograph of the gel showing *Eco*R1 digested recombinant plasmid DNA. It gave fragment of approximately 875 bp in case of LSV CP gene in colony No. 1 and 3. Clones were also checked with colony PCR for the presence of desired fragment. Gel photograph of colony PCR revealed the amplification of same size (~875 bp) (Fig. 4.4b) in 5 out of 5 clones checked, showing that the transformed colonies (5 in number) contain LSV CP gene.

4.3 Cleaved amplified polymorphic (CAP) DNA analysis for checking variability in coat protein gene of LSV

To check the variability of LSV in lily and tulip cultivars grown in H.P. and J&K, cleaved amplified polymorphic DNA analysis was done. CP gene of four different LSV isolates, two tulip isolates from Palampur and Srinagar and two lily isolates from Palampur were amplified and cloned. These clones were then further used for CAP analysis. Fig. 4.5 shows restriction digestion pattern with two 4 bp (frequent cutter)

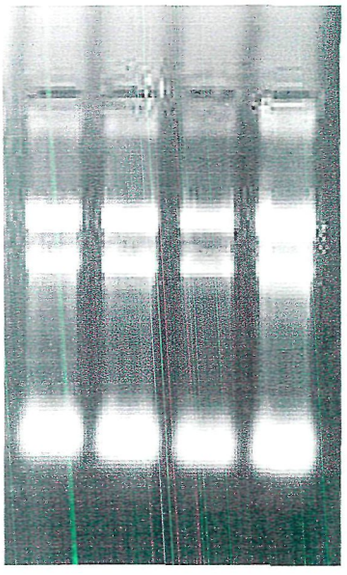


Figure 4.2 Total RNA isolation of lily plant showing three different RNA bands for different RNA.

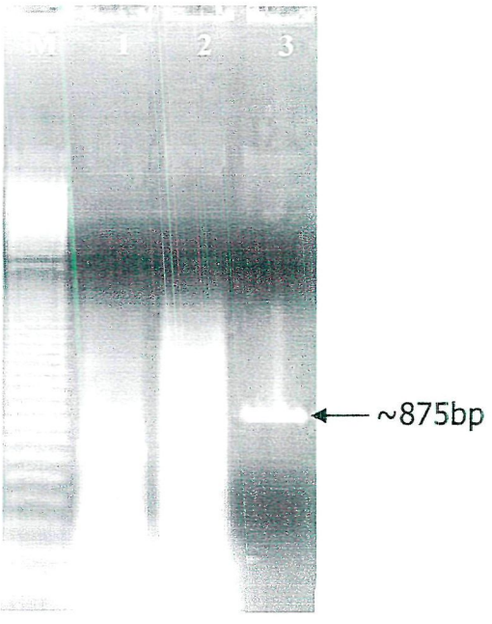


Figure 4.3 Agarose gel electrophoresis (1%) showing reverse transcription-polymerase chain reaction (RT-PCR) using LSV CP specific primer pair. Lane M: DNA marker (100 base pair ladder). Lane 1 and 2: showing no result. Lane 3: Showing amplified products of ~875 bp from infected leaf samples. The approximately 875 bp band in lane 3 was indicative of LSV infection.

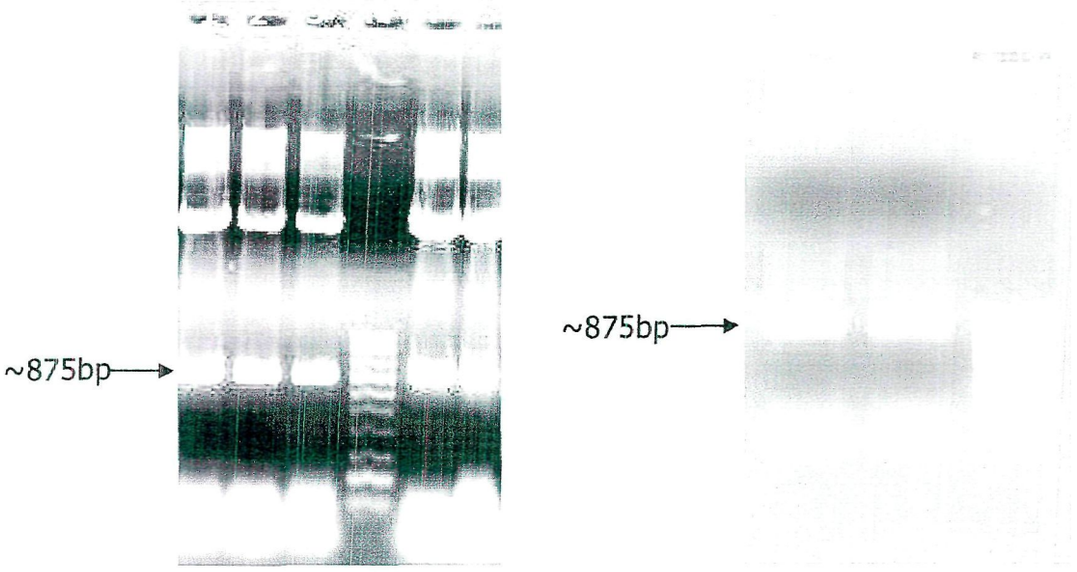
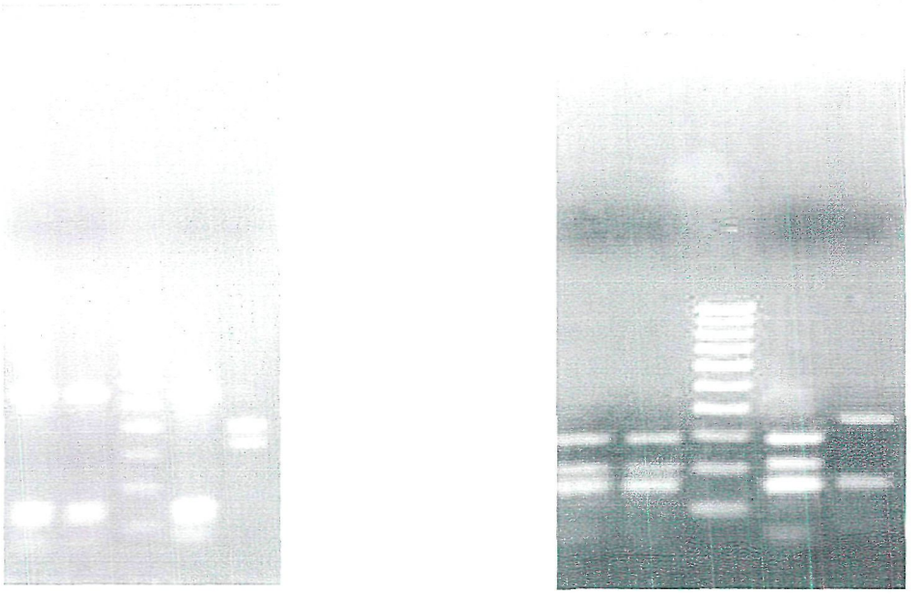


Fig. 4.4 (A) Agarose gel electrophoresis (1.0%) showing restriction digestion product of clones of LSV to check the identity of clones. Lane 1-5: Clones contain the LSV insert of Kangra isolate. Lane M: 100 bp DNA marker (λ DNA).

Fig. 4.4 (B) Agarose gel electrophoresis (1.0%) showing colony PCR of clones of LSV to check the identity of clones. Lane M: 100 bp DNA marker (λ DNA). The approximately 875 bp band in lane 1-2 after colony PCR reaction confirms presence of LSV insert in clones.



(A)Restriction digestion by *Hae* III (B) Restriction digestion by *Taq* I

Fig. 4.5 Agarose gel electrophoresis (1.0%) showing cleaved amplified polymorphic (CAP) analysis of cloned LSV isolates using A) *Hae*III, B)*Taq*I (all 4 base pair cutter). Lane 1: Oriental hybrid lily from Palampur, Lane 2: Tulip from Palampur, Lane 3: Tulip from Srinagar, Lane 4: *Lilium longiflorum* and Lane M: 100 bp DNA marker.

cutters (*Hae*III, *Taq*I). Restriction pattern with these enzymes was able to differentiate various isolates of LSV depending upon their digestion pattern. Digestion with *Taq*I and *Hae*III enzyme was giving maximum variability.

4.4 Sequencing

The cDNA clones of LSV CP genes of different isolates of lily and tulip were sequenced using T7 and SP6 primer in automated sequencer (ABI prism 310). The sequenced data (Fig. 4.6-4.9) were submitted to the GenBank and accessions numbers were obtained. The complete sequence of CP gene of different LSV isolates were submitted to GenBank with accession number AJ831416 for Asiatic hybrid lily isolate from Solan, AJ831415 for Asiatic hybrid lily isolate from Chamba, AJ831417 for Asiatic hybrid lily isolate from Kangra, AM087402 for tulip isolate from Srinagar, AM087401 for tulip isolate from Palampur and AM087400 for Oriental hybrid lily isolate from Palampur, AJ781318 for tiger lily from Palampur.

4.5 Sequence analysis

The nucleotide sequence and deduced amino acid of the coat protein gene was compared between the seven LSV isolates reported from India and with the 14 isolates of the virus reported from other countries and other parts of India (Table 4.3). A total of 21 isolates were used for generating multialignment using MUITALIN programme. Alignment of the nucleic acid and deduced amino acid sequence shown in Fig. 4.10 and 4.11 respectively. As seen in the figure, various isolates showed the presence of unique residues in the rows of alignment. Among these rows of alignment the first row (Acc. No. AM087402) tulip from Srinagar and second last row (Acc. No. AJ81318) tiger lily from Palampur which are two of seven Indian isolates showed a stretch of 11 and 21 amino

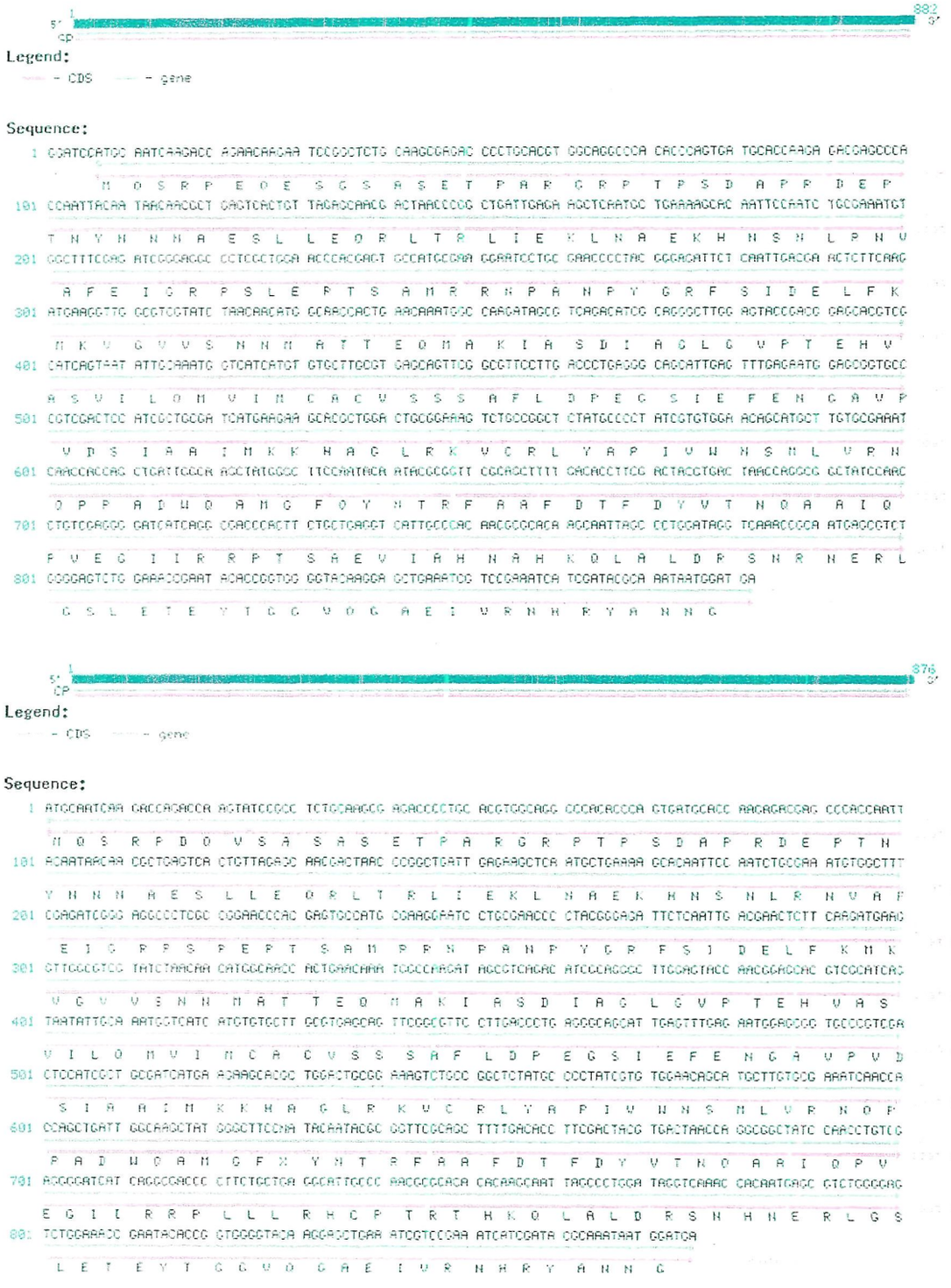


Figure 4.6 The nucleotide and deduced amino acid sequences of *Lily symptomless virus* coat protein gene of two different isolates (Accession no. AM087402 and Accession no. AJ831415).

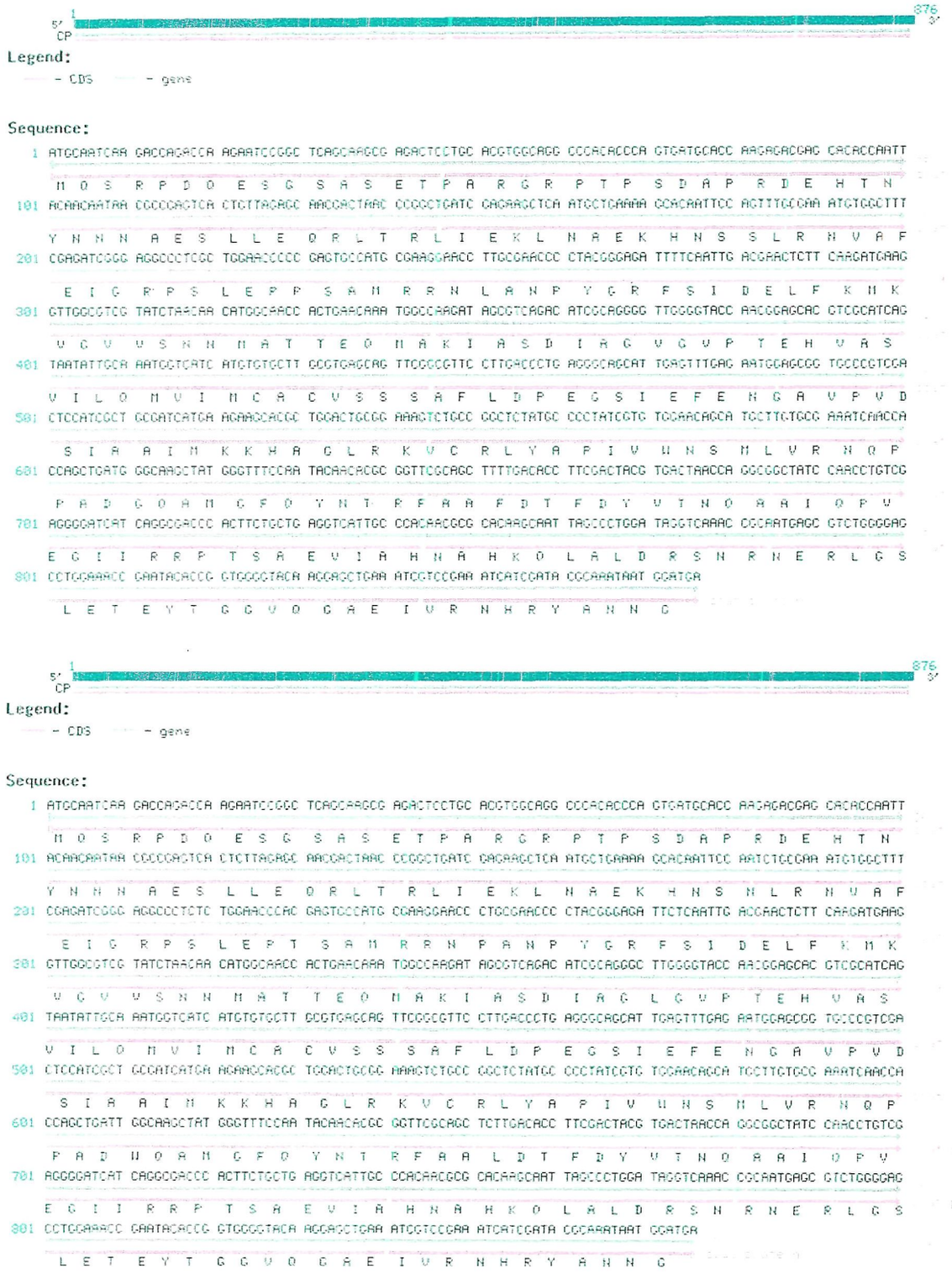


Figure 4.7 The nucleotide and deduced amino acid sequences of *Lily symptomless virus* coat protein gene of two different isolates (Accession no. AJ831416 and Accession no. AJ831417).

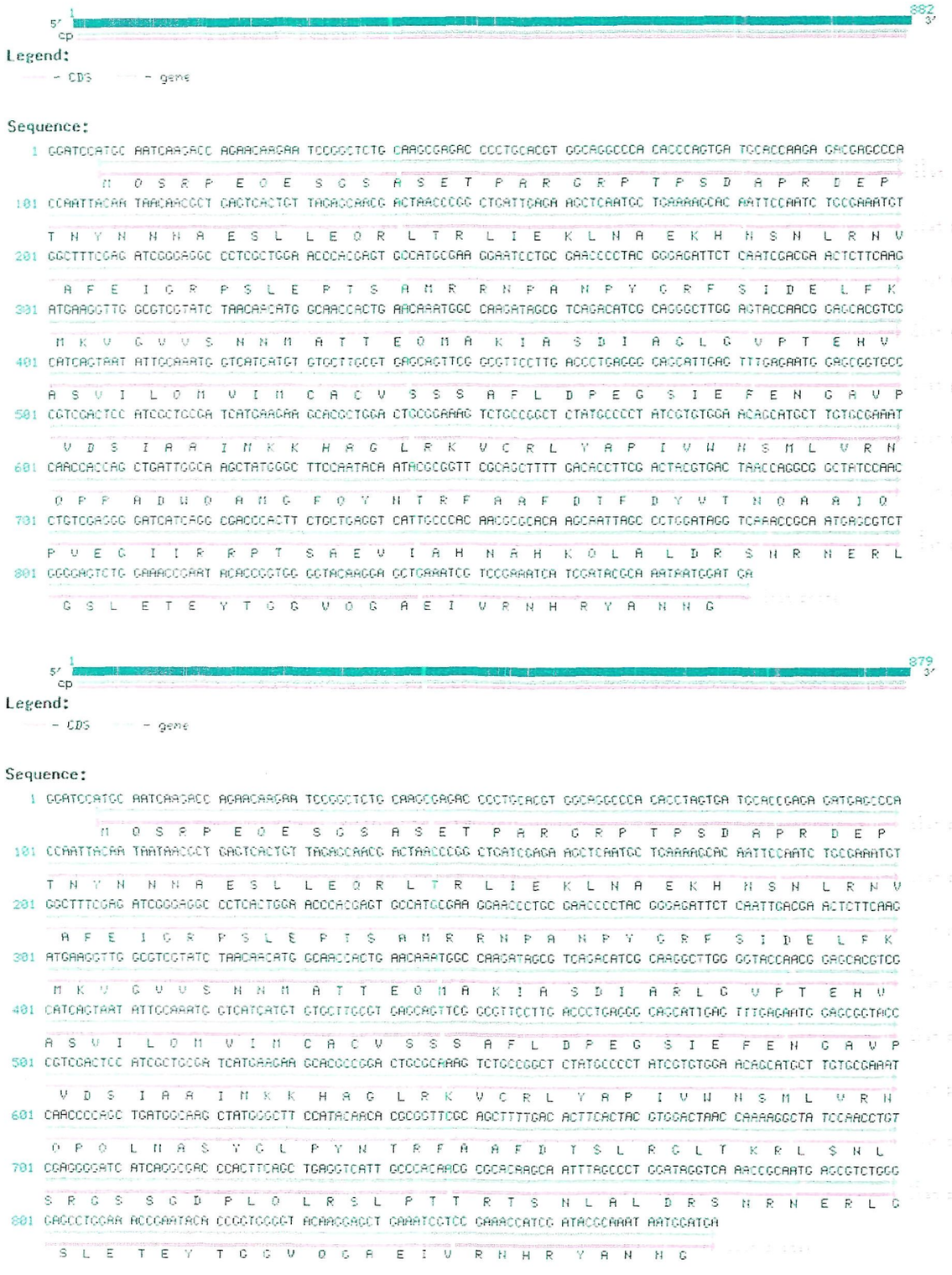


Figure 4.8 The nucleotide and deduced amino acid sequences of *Lily symptomless virus* coat protein gene of two different isolates (Accession no. AM087400 and Accession no. AJ781318).

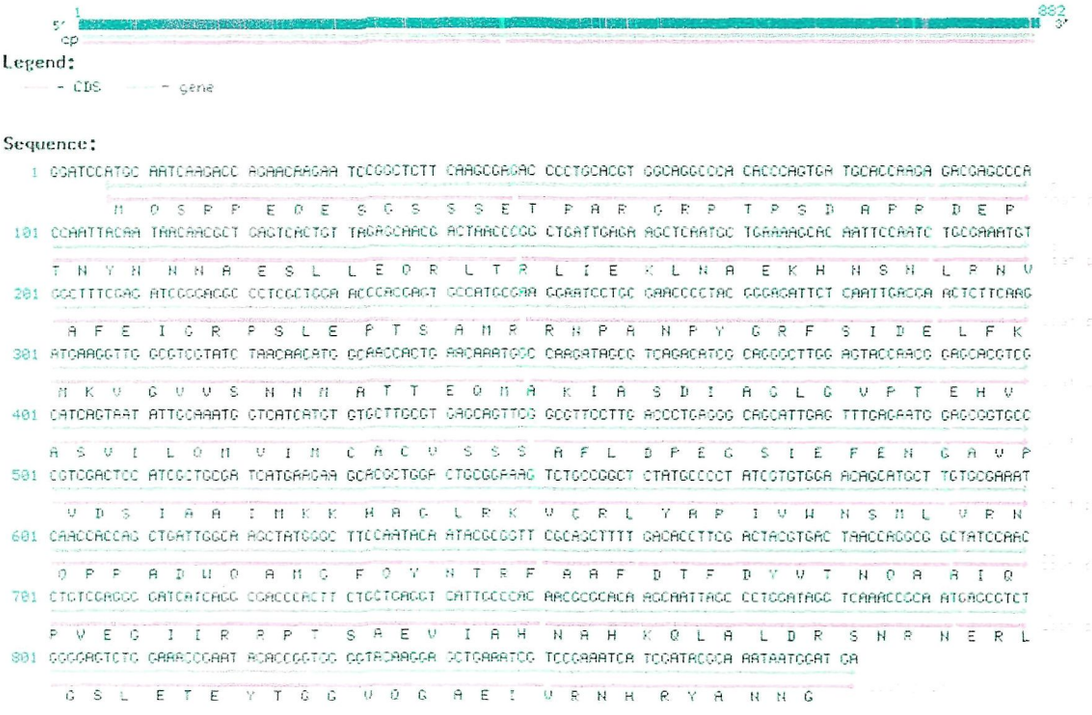


Figure 4.9 **The nucleotide and deduced amino acid sequences of *Lily symptomless virus* complete coat protein gene isolate (Accession no. AM087401).**

Table: 4.3 **Different isolates of LSV reported from India and other countries used for multiple alignments**

S.No.	Accession No.	Country	Plant
1.	**AM087402**	India	Tulip
2.	**AM087401**	India	Lily (Oriental Hybrid lily)
3.	**AM087400**	India	Tulip
4.	**AJ831415**	India	Lily (Asiatic Hybrid lily)
5.	**AJ831416**	India	Lily (Asiatic Hybrid lily)
6.	**AJ831417**	India	Lily (Asiatic Hybrid lily)
7.	**AJ781318**	India	Lily (Tiger lily)
8.	AJ748320	India	*Lilium spp*
9.	X15343	Netherland	*Lilium spp*
10.	AJ780923	India	Spider lily
11.	AJ564638	China	*Lilium spp*
12.	AJ564641	China	*Lilium spp*
13.	AF103784	South Korea	*Lilium spp*
14.	U43905	South Korea	*Lilium spp*
15.	AJ748277	India	*Lilium spp*
16.	AJ516059	South Korea	*Lilium spp*
17.	AJ564640	China	*Lilium spp*
18.	AF015286	USA	*Lilium spp*
19.	AY326460	China	*Lilium spp*
20.	AJ585052	India	*Lilium spp*
21.	AJ564639	UK	*Lilium spp*

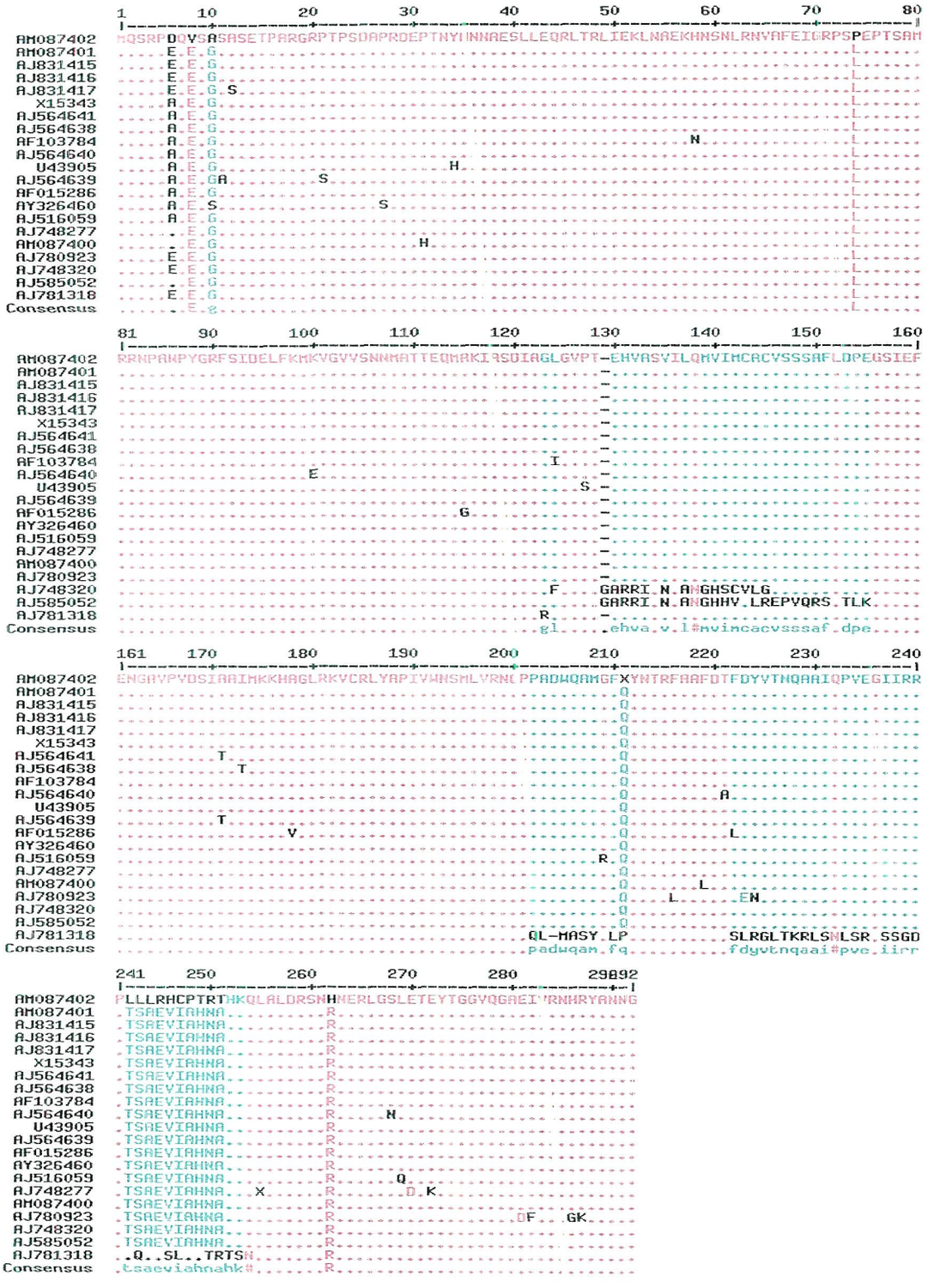
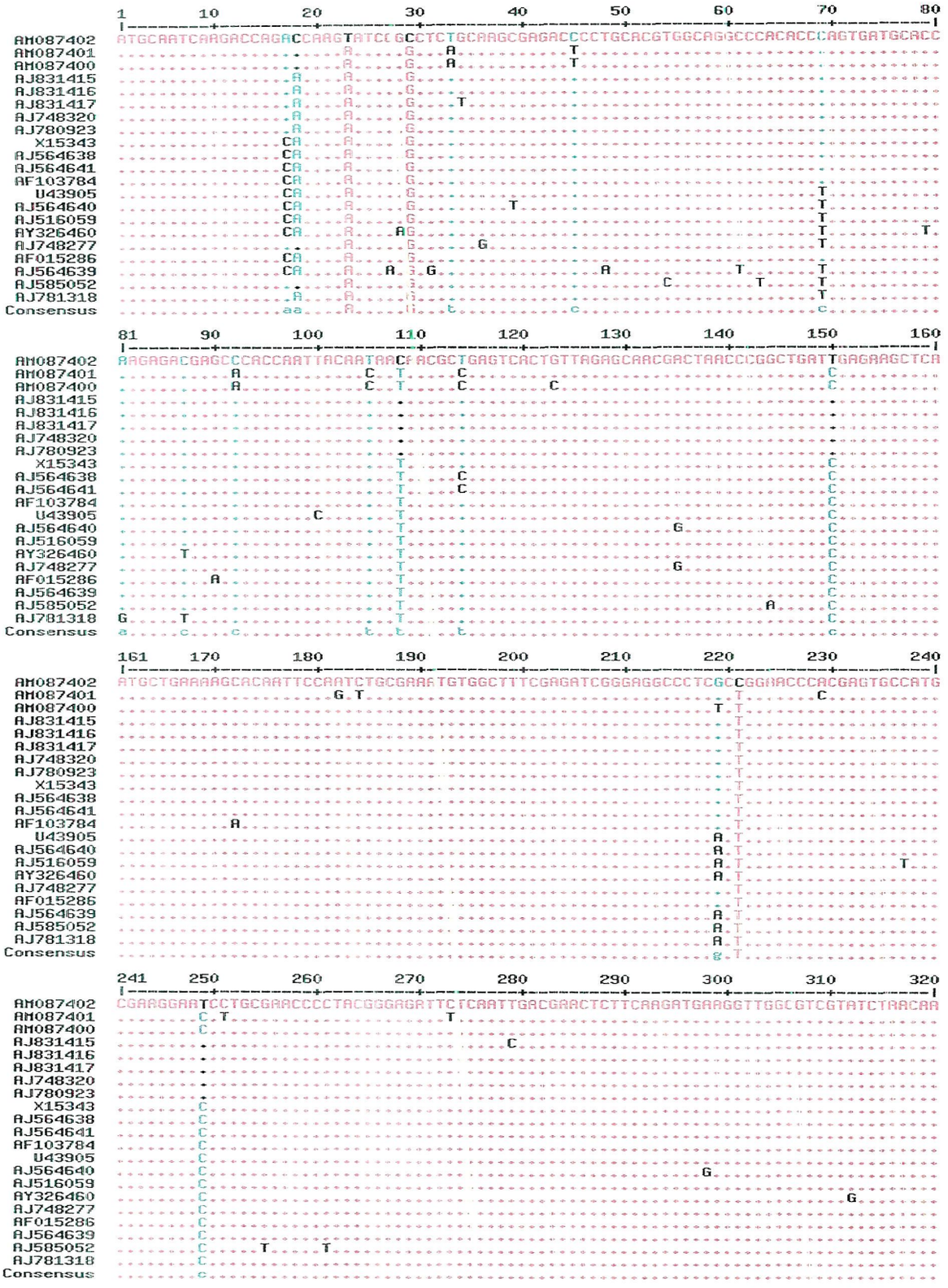


Figure 4.10. Multiple sequence alignment of seven Indian isolates of LSV CP with 14 different LSV isolates reported from other countries and hosts using Multalin V. 1.2.3. Dots in alignment denote the homologous amino acids.


1 10 20 30 40 50 60 70 80
AM087402 ATGCAATCAAGACCAGACCAAGTATCCGCCTCTGCAAGCGAGACCCCTGCACGTGGCAGGCCCACACCCAGTGATGCACC
AM087401
AM087400
AJ831415
AJ831416
AJ831417
AJ748320
AJ780923
X15343
AJ564638
AJ564641
AF103784
U43905
AJ564640
AJ516059
AY326460
AJ748277
AF015286
AJ564639
AJ585052
AJ781318
Consensus
81 90 100 110 120 130 140 150 160
AM087402 AAGAGACGAGCCCACCAATTACAATAACCAACGCTGAGTCACTGTTAGAGCAACGACTAACCCGGCTGATTGAGAAGCTCA
AM087401
AM087400
AJ831415
AJ831416
AJ831417
AJ748320
AJ780923
X15343
AJ564638
AJ564641
AF103784
U43905
AJ564640
AJ516059
AY326460
AJ748277
AF015286
AJ564639
AJ585052
AJ781318
Consensus
161 170 180 190 200 210 220 230 240
AM087402 ATGCTGAAAAGCACAATTCCAATCTGCGAAATGTGGCTTTCGAGATCGGGAGGCCCTCGCCGGAACCCACGAGTGCCATG
AM087401
AM087400
AJ831415
AJ831416
AJ831417
AJ748320
AJ780923
X15343
AJ564638
AJ564641
AF103784
U43905
AJ564640
AJ516059
AY326460
AJ748277
AF015286
AJ564639
AJ585052
AJ781318
Consensus
241 250 260 270 280 290 300 310 320
AM087402 CGAAGGAATCCTGCGAACCCCTACGGGAGATTCTCAATTGACGAACTCTTCAAGATGAAGGTTGGCGTCGTATCTAACAA
AM087401
AM087400
AJ831415
AJ831416
AJ831417
AJ748320
AJ780923
X15343
AJ564638
AJ564641
AF103784
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AJ748277
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AJ781318
Consensus

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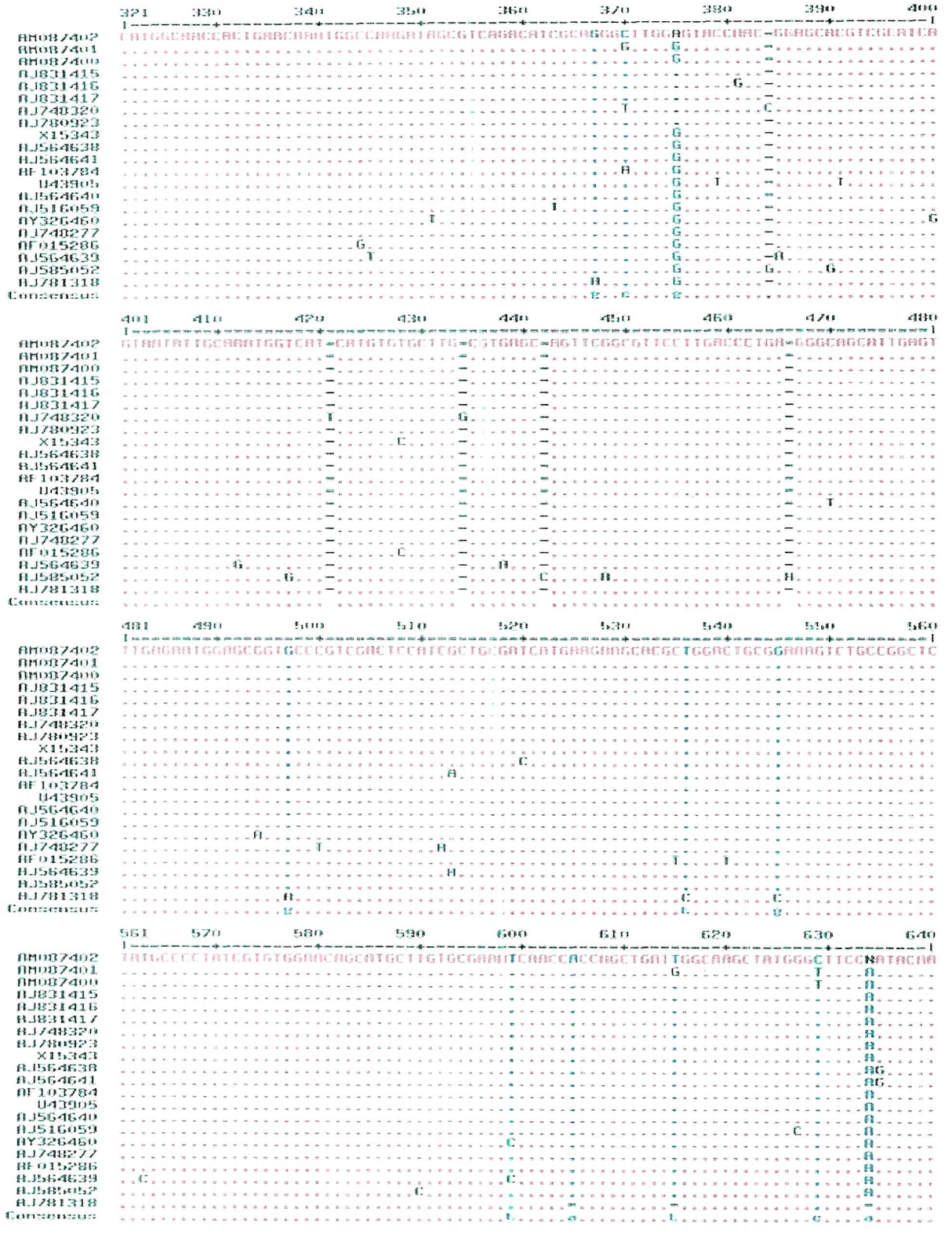

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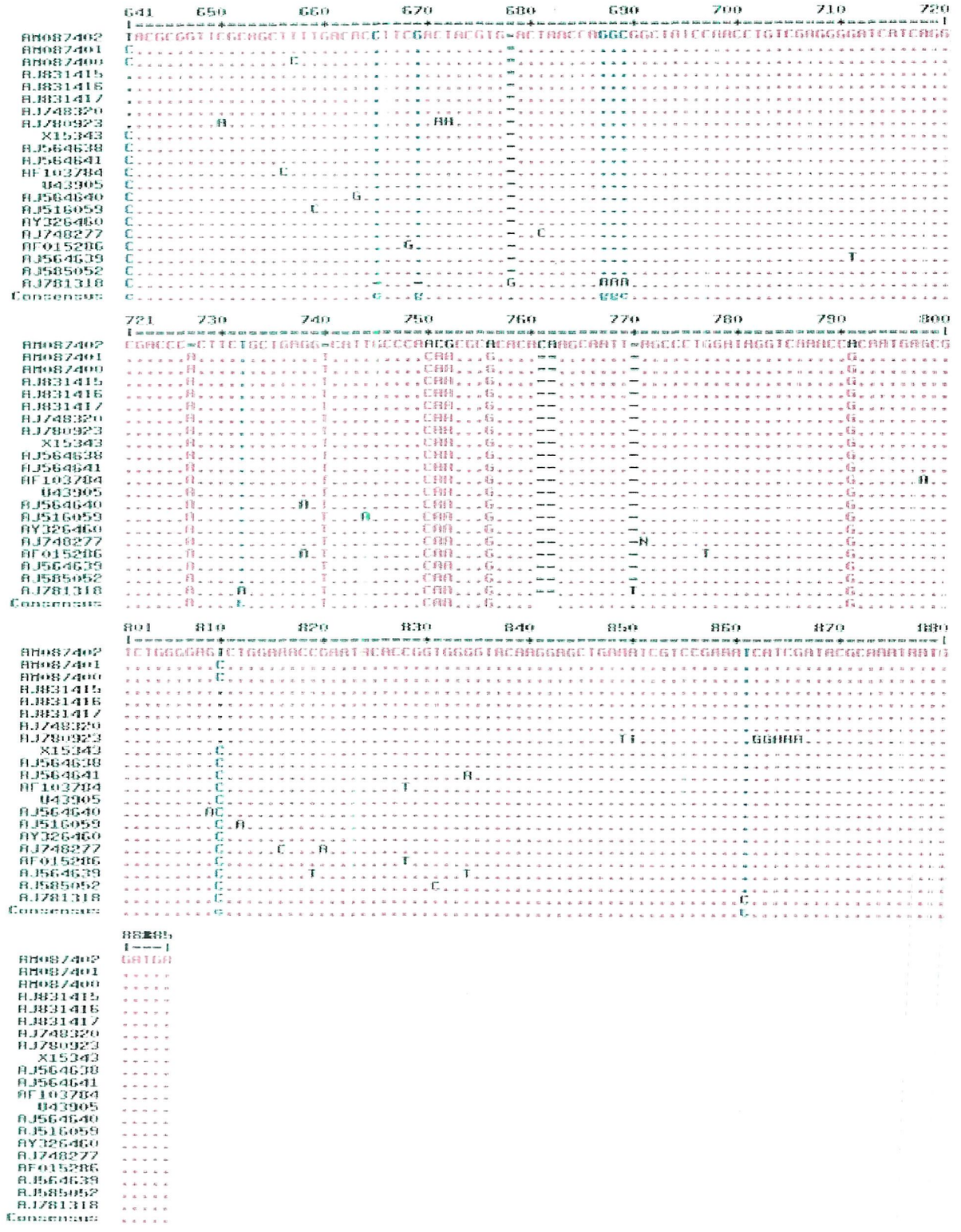


Figure 4.11. Multiple sequence alignment of seven Indian isolate of LSV CP with 14 different LSV isolates reported from other countries and hosts using Multalin V. 1.2.3. Dots in alignment denote the homologous nucleotides acids.

acid long variable region respectively (Fig. 4.10). The tiger lily isolate showed unique residues toward the C-terminal of the protein; residues at position 210-206, 220-238 and 240-235 were variable and unique to this isolate and tulip isolate showed unique residues at their C- terminal region of protein at position 241-251. Other regions of the protein for these isolates were similar.

A total of twenty one LSV isolates were chosen (Table 4.3) for study the percentage nucleotide and amino acid similarities. The Indian tiger lily isolates of LSV showed least homology 85-99% at amino acid and 74-85% at nucleotide level with all other isolates of LSV reported from other country and other part of India. At amino acid level two Indian lily isolates (AJ 831415 and AJ 831416) of LSV showed highest homology 100% with tulip isolate of LSV reported from Srinagar (AM087401) and the least homology 92% with isolate of LSV reported from India (AJ585052). The other isolates showed 88-99% homology among themselves at nucleotide level and 92-99% homology at amino acid level (Table 4.4).

4.6 Phylogenetic tree analysis

All the sequences used for phylogenetic analysis were analyzed for phylogenetic tree by TREEVIEW program therein (Page, 2005). The isolates infecting tiger lily (Acc. No AJ 781318) and tulip (Acc. No AM 087402) showed significant deviation from all other isolates (Fig. 4.12).

4.7 Expression of LSV CP in *E.coli*

4.7.1 Amplification, Cloning and Transformation of Cloned fragment in *E. coli*

On the basis of LSV CP sequence, sequence specific expression primers were designed containing the appropriate restriction enzyme sites. Using the above primer pair LSV CP was amplified using the positive clones of pGEMT®-T Easy Vector containing LSV insert and run on the gel. Gel showed the amplification product of ~875 bp specific

Table: 4.4 **Amino acid and nucleotide homology of Indian isolates to other isolates of the world. (Above diagonal is nucleotide homology and below diagonal is amino acid homology)**

Lily symptomless virus	AM08740	AM08740	AM08740	AJ831415	AJ831416	AJ8314-17	AJ78131	AJ748320	X15343	AJ780923	AJ564638	AJ564641	AF10378	U43905	AJ748277	AJ516059	AJ564640	AF01528	AY32646	AJ585052	AJ564639
AM087402	x	96	97	98	98	98	96	98	97	97	97	97	97	97	97	96	96	96	96	96	96
AM087401	94	x	98	97	97	97	96	97	98	96	98	98	97	97	97	97	97	97	97	97	96
AM087400	94	98	x	98	98	98	97	98	98	97	98	98	98	98	97	97	97	97	97	97	97
AJ831415	94	100	98	x	99	99	97	99	98	98	98	98	98	98	98	98	98	98	97	97	97
AJ831416	94	100	98	100	x	99	97	99	98	98	98	98	98	98	98	98	98	98	97	97	97
AJ831417	94	99	98	99	99	x	97	99	98	98	98	98	98	98	98	98	98	98	97	97	97
AJ781318	86	86	85	99	86	85	x	80	85	84	85	85	85	85	84	85	84	85	85	78	84
AJ748320	89	94	93	94	94	94	97	x	98	98	98	98	98	98	98	98	98	98	97	97	97
X15343	94	99	98	99	99	99	98	94	x	97	99	99	99	99	98	98	98	99	98	98	98
AJ780923	92	97	96	97	97	97	96	92	97	x	97	97	97	97	97	97	97	97	96	96	96
AJ564638	94	99	98	99	99	98	97	93	99	96	x	99	99	99	98	98	98	98	98	98	97
AJ564641	94	99	98	99	99	98	97	93	99	96	99	x	98	98	98	98	98	98	98	97	98
AF103784	93	98	98	98	98	98	97	93	99	96	98	98	x	98	98	98	98	98	98	97	97
U43905	93	98	98	98	98	98	98	93	99	96	98	98	98	x	98	98	98	98	98	98	98
AJ748277	93	98	98	98	98	98	97	93	98	96	98	98	97	97	x	98	98	97	97	97	97
AJ516059	93	98	98	98	98	98	97	93	99	96	98	98	98	98	97	x	98	98	98	97	97
AJ564640	93	98	97	98	98	98	97	93	98	96	98	98	98	98	97	98	x	98	98	97	97
AF015286	93	98	97	98	98	98	97	93	98	96	98	98	98	98	97	98	97	x	97	97	97
AY326460	94	98	98	98	98	98	97	93	99	96	98	98	98	98	97	98	98	98	x	97	97
AJ585052	87	92	91	92	92	91	97	94	92	89	91	91	91	91	91	91	91	91	91	x	97
AJ564639	93	98	97	98	98	98	96	93	98	96	98	99	98	98	97	98	97	97	98	91	x

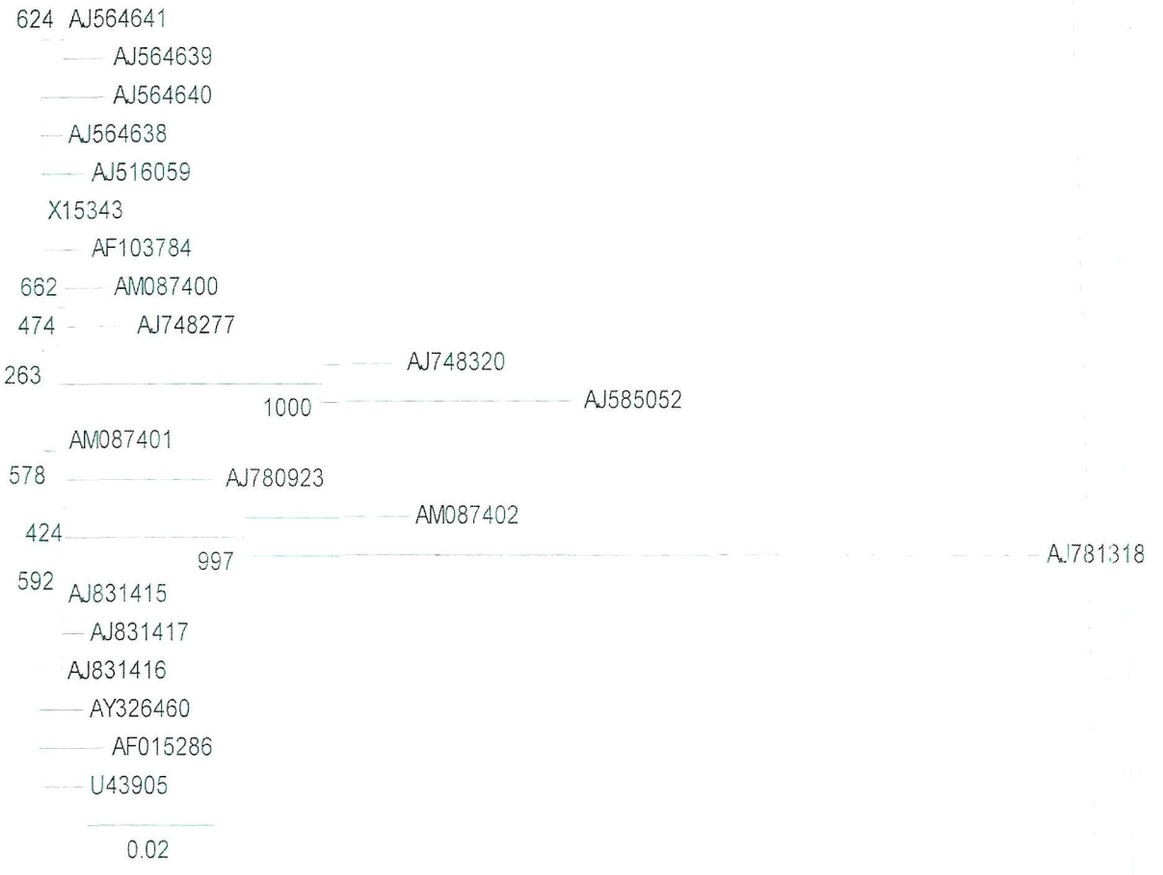
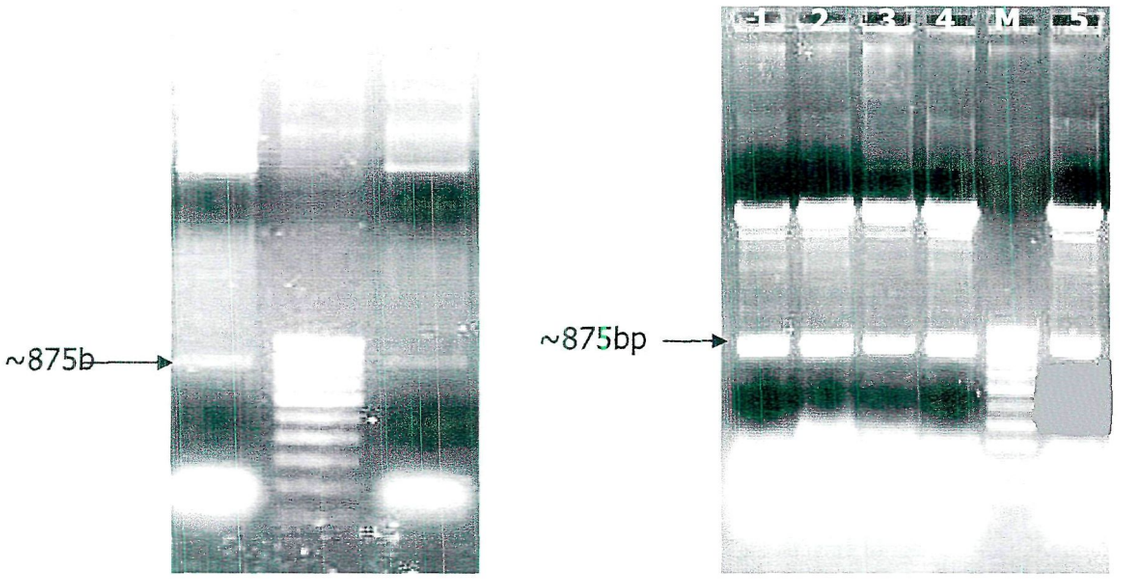


Fig: 4.12 Phylogenetic tree based on nucleotide sequences of Indian isolates of LSV infecting lily and tulip with other reported LSV isolates



(a) LSV-CP cloned in pGEM-T Eassy vector

(b) LSV-CP cloned in pET32 (+) expression vector

Fig. 4.13 (a) Gel photograph showing PCR of LSV CP gene cloned in pGEMT®-T Easy Vector using the designed expression primers to get the amplified product with new restriction site that are compatible for the expression vector (pET32 (+)). Gel showing the amplification of ~875 bp in lane 1-2 and lane M: DNA marker (λ DNA.

Fig. 4.13 (b) Agarose gel electrophoresis (1.0%) showing restriction digestion of recombinant pET32 (+) vector with *Eco* RI and *Hind* III to check the identity of clones. Lane 1-5: Clones contain the LSV insert. Lane M: DNA marker (λ DNA). The approximately 875 bp band in lane 1-5 after restriction digestion reaction confirms presence of LSV insert in clones.

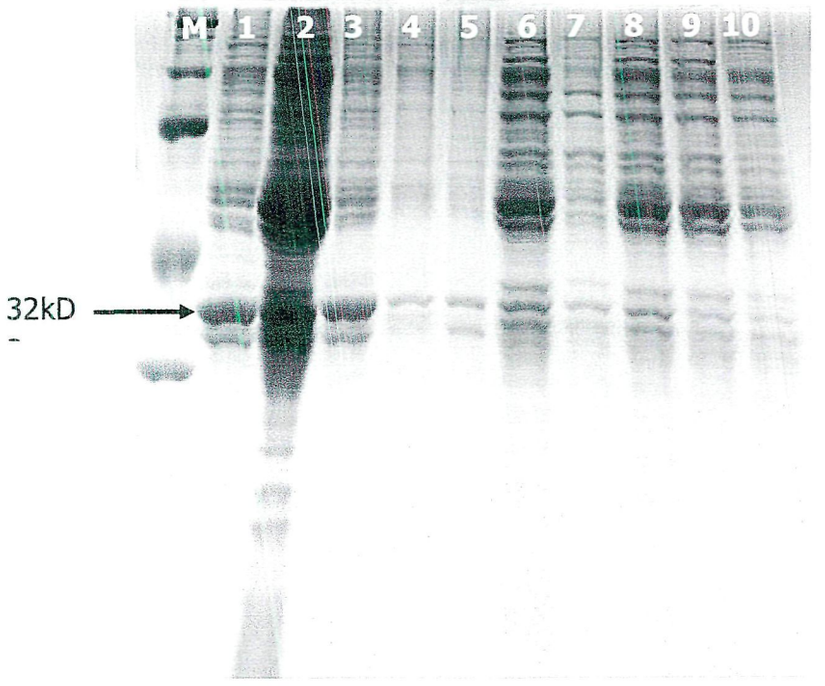


Figure 4.14 **SDS-PAGE showing expressed coat protein gene of LSV in pET32 (+) expression vector Lane 1-5: Showing approximately 32 kDa coat protein expressed after different IPTG concentration treatment, respectively; Lane 1: low range protein marker (BioRad). Lane 6-10: Showing uninduced coat protein that is expressing at basic level .**

for LSV CP (Fig. 4.13a). The PCR product was then eluted, digested with appropriate restriction enzyme, cloned in predigested pET32 (+) expression vector and transformed to *E. coli* (BL-21) competent cell cells. Several recombinant clones of pET32 (+) vector containing LSV CP insert were checked with restriction detestation for the presence of desired fragment. Gel photograph of restriction detestation revealed the restriction digestion product of same size (~875 bp) (Fig. 4.13b) in 5 out of 5 clones checked. Several positive clones were checked by sequencing for the inframe cloning and single colony containing the insert in frame to the coding sequence of the vector was used for the standardization of conditions of optimal expression.

4.7.2 Standardization of optimal expression conditions for LSV CP

Several temperature and IPTG concentrations were tried in different combinations in order to standardize the conditions for optimum expression of LSV CP. The best expression level of LSV CP in solution was observed when the protein was expressed at particular temperature with a particular IPTG concentration and sampled after particular hours of IPTG addition (Fig. 4.14).

Discussion

Chapter V

DISCUSSION

Lily (*Lilium spp.*) and tulip (*Tulipa spp*) comes within top five commercially important potential flower crops in the world. Various pathological agents namely fungi, bacteria and virus infect lily and tulip in nature (Lawson and Hus, 1996). Amongst them viruses are one of the major pathogens of lily and tulip, because as a routine practice lily and tulip being multiplied by vegetative propagation *viz.* bulbs and bulb-lets allowing the perpetuation of virus too. The bulbs if once infected by viruses, subject to carryover virus infection, produce several distinctive, damaging symptoms including irregular mottling, flecking of leaves, reduced plant size, distorted and twisted growth. colour breaking in flowers and concentric brown ring patterns on bulbs scales. There are number of viruses that infect lily and tulip which are LSV, TBV, CMV, ArMV, TMV, TRV, TRSV and LVX. They often limit the quality and quantity of planting material and flower. Lily and tulip infected with viruses are weakened and become susceptible to other diseases.

5.1 Viral symptomatology

During the survey of *L. longiflorum* (Ester lily), *L. tigrinum* (Tiger lily), *Hymenocallis spp.* (Spider lily), *Zentedeschia spp.*(Cala lily) and 21 cultivars of Oriental and Asiatic hybrid lilies for virus infection, it was found that virus infected plants are widely spread in Kangra, Chamba, Solan and Chail region of Himachal Pradesh. The virus symptoms on plant were observed in very young stage of plant. In the field lilies apparently seen healthy, however sometimes leaves might show vein clearing or light green stripes on the leaves. At the flowering stage, small light brown spots appear on the

adaxial surface of the leaves which turned into yellow, brown spots and pass on abaxial surface, which finally lead to premature death of plant at the end of growing season. The observations which were observed during the survey also correlated with findings made by Derks and Hendricks (1990), that the symptoms in lilies infected by LSV alone differ in severity from cultivar to cultivar and growing condition. However, few workers have reported that when lilies were infected only by LSV, sometime virus like symptoms did not appear i.e. they remain symptomless (McWhorter and Brierley, 1963; Ballantne, *et al.,* 1979, Blake and Wilson, 1996; Schouten *et al.,* 1997). Boontjes (1983) also explained that many cultivars remained symptomless and plants show reduced growth with smaller flowers.

During this survey, the severely infected plants of Asiatic and Oriental Hybrids lily cultivars *viz.* London, Romano, Alaska, Nova cento, Pollyanna showed symptoms like mild mosaic of leaves, vein clearing, leaf curling, stunting growth and brown rings on some bulb scales. The symptoms may be due to mixed infection of LSV with LMoV and CMV. Similar results were also observed by Lee *et al.* (1996) and Asjes (1997) Asjes *et al.* (1973). According to them leaf symptoms of LSV varied from vein clearing, leaf mottle, leaf mosaic, narrowing, reddish-brown necrotic spots and differ according to susceptibility and sensitivity of the cultivar. Mokra (1976) also reported that infection by LSV and TBV caused streak mottle on leaves in Enchantment and Harmony cultivars and severe mosaic and stunting in *L. speciosum.*

Mixed infection of LSV, LMoV and CMV was found in Asiatic and Oriental hybrid lilies. Plants showed severe mosaic, curling of leaves and drastic reduction in flower production. Similarly it has also been reported (Duineveld *et al.,* 1980; Brierley

and Smith, 1944a, b, Asjes *et al.,* 1973), that LSV occurs in lily along with *Tulip breaking virus* and *Cucumber mosaic virus* and produced similar symptoms like mosaic, malformation, vein chlorosis and chlorotic spots in infected lily.

Tulip was also taken for diagnostic studies as they are also found to be infected by LSV. During the investigation, two LSV isolates of tulip from different geographical locations (Palampur and Srinagar) showed symptoms like chlorotic streaks and silver line pattern on leaves and stunted growth of plant.

5.2 Virus detection

5.2.1 Detection by immunoassay

Since the development of ELISA, it is one of the most popularly used techniques till today for detection of viruses and routine testing of bulbs (Zaidi & Mukharjee, 1991, Zaidi *et al.,* 1993). *Lily symptomless virus* (LSV) is highly immunogenic so serological method of detection can be a reliable method for its detection. ELISA has been found to be a best way for large scale routine testing of viruses due to its rapid, cost effective, simple and fast assay properties. When ELISA was performed by using leaves of lily, out of *L. longiflorum* (Ester lily), *L. tigrinum* (Tiger lily), *Hymenocallis spp.* (Spider lily), *Zentedeschia spp.* (Cala lily) and 21 cultivars of Oriental and Asiatic hybrid lilies, 10 were found positive of which three were *Lilium longiflorum*, *L. tigrinum* and *Hymenocallis spp.* (Spider lily) and 7 were Asiatic and Oriental hybrid lily cultivars. The positive Asiatic and Oriental hybrid cultivars were London, Romano, Alaska, Nova cento, Pollyanna, Galeili, White Mero Star indicating 33.3% virus incidence. These results were found to be in correlation with that described by Beijersbergen and Van der Hulst (1980), Van Schadewijk (1986), Asjes (1990, 1997)

and Kim *et al.* (1998) who showed that ELISA proved satisfactory to detect LSV in bulb scales of cultivars of Asiatic and Oriental hybrid lilies. Differences in the efficiency of detection were apparent with ELISA, DBIA and TBIA (Derks, 1992; Hsu *et al.*, 1995a, b; Kim *et al.*, 1995a, b; Nimi *et al.*, 1999). High virus concentration can be easily detected by ELISA, DBIA and TBIA, whereas for plants with mild mosaic symptoms having low virus concentrations, tested negatively by ELISA, there is a need to develop other detection techniques like PCR, nucleic acid hybridization etc. Virus concentration depends on the growth stage and was uneven in all organs and tissues of lily (Kim et al., 1995b). But DAS-ELISA was still used by the flower bulb inspection service to test several thousands of bulbs and leaves per season (Asjes, 1990; 1997; 2000)

5.2.2 Detection by nucleic acid

5.2.2.1 Northern Hybridization

Northern hybridization is very sensitive technique. It has found to detect virus concentration in pg compared to µg in ELISA. Northern hybridization with specific DNA probes is an alternative technique for identification and detection of viruses. It can help in further characterization of pathogens (Boonekamp *et al.* 1990). To screen different lily cultivars for LSV, cloned LSV CP gene (~875bp) was used radioactive probe. Out of *L. longiflorm* (Ester lily) and 14 cultivars of Asiatic and Oriental hybrid lilies, 6 were positive in which one was *L. longiflorm* and five were the cultivars of Asiatic and Oriental hybrids namely Corida, Brunello, Nova Cento, London, Cavi and positive control Tiger-lily RNA (Fig. 4.1) indicating 40% incidence. The percentage of infection was observed using Northern Hybridization is greater than ELISA which confirmed that Northern Hybridization is more sensitive method than ELISA.

5.2.2.2 Detection by RT-PCR

Limitation of ELISA to detect the virus may be overcome by the use of RT-PCR technique, which is more sensitive than ELISA and can detect as low as 50-200 ng concentration of virions (Wang *et al.*, 2004). In the present study, out of four cultivars of lily, three were positive for LSV. The numbers of cultivars found to be positive for LSV were more through RT-PCR in comparison to ELISA. The result of RT-PCR showed that some plants which were not positive for DAS-ELISA were found to be positive through RT-PCR, showing that RT-PCR is more reliable and sensitive than ELISA based detection. Similar results were also reported by Joung *et al.*, (1996) and Lee *et al.* (1998) using RT-PCR for detection of LSV. RT-PCR analysis was done for samples of leaves, bulbs and stem with different symptoms in lily plant. RT-PCR was also used for detection of LSV from the plants showing stripe, yellow mottle and mosaic symptoms.

5.3 Cleaved amplified polymorphic DNA (CAP) analysis for differential study of LSV isolates

To check the variability of CP gene of different isolates of cleaved amplified polymorphic (CAP) DNA analysis was done. Although sequencing is more reliable and accurate but also a costly affair for differential studies of various isolates of LSV, so to check the homology between different clones restriction digestion analysis was performed with two 4 bp cutters *viz.* *Hae*III, *Taq*I. One restriction enzyme analysis may not be productive enough so the two enzymes were used. Restriction pattern with these enzymes was helpful in differentiating various isolates of LSV on the basis of their sequences. In present study CAP DNA analysis of four different lily isolates one *Lilium longiflorum* isolate two tulip isolates and one Asiatic Hybrid lily isolate was done. It was observed that two isolates from tulip and one Asiatic lily isolate has pattern which is very

similar while *L. longiflorum* isolate from Palampur was quite different from others. The results found to be correlated with Singh *et al.*, (2005b), who found that *L. longiflorum* isolate has unique stretches in the middle portion of the protein not found in other isolates, even the Indian one.

5.4 Cloning and sequencing

The seven LSV CP clones of different isolates were sequenced in an automated sequencer. The sequence of CP gene of LSV was found to be of 875 bp in size encoding 294 amino acids. Similarly Takamatsu *et al.* (1994) have reported that on basis of CP sequence LSV isolates that it comprised of 875 bp (294 amino acids). The sequencing data were obtained and submitted to EMBL database with accession number AJ831416 for Asiatic hybrid lily isolate from Solan, AJ831415 for Asiatic hybrid lily isolate from Chamba, AJ831417 for Asiatic hybrid lily isolate from Kangra, AM087402 for tulip isolate from Srinagar, AM087401 for tulip isolate from Palampur, AM087400 for Oriental hybrid lily isolate from Palampur and AJ 781318 for tiger lily isolate from Palampur.

5.5 Sequence analysis

The nucleotide and deduced amino acid sequence of seven Indian (lily) isolates of LSV were aligned with 14 different isolates of LSV reported from various parts of the world, infecting different crops and causing various symptoms on their respective hosts. The sequence analysis revealed that one tulip isolate from Srinagar having 10 amino acid long variable region in its C-terminal region. This may be due to that Srinager tulip is indigenous of Northern Himalayan region and all other Indian isolates are imported from the Netherlands. Another isolate which was showing

variability was LSV CP isolate of tiger lily from Palampur. It showed unique residues toward the C-terminal of the protein; residues at position 210-206, 220-238 and 240-235. These portions were variable and unique to this isolate. As these unique residues were in stretches, they might have been acquired from other genomes, probably through recombination (Bonnet *et al.*, 2005; Tanne and Sela 2005). Sequence analysis also revealed that C-terminal region of LSV-CP is highly variable while all other regions mostly conserved. The Indian tiger lily isolate of LSV showed least homology 85-99% at amino acid and 74-85% at nucleotide level with all other isolates of LSV reported from other countries and other parts of India due to unique residue in its sequence. Singh *et al.* (2005b) also reported that *Lily symptomless virus* (LSV) isolates infecting *Lilium longiflorum*, *Liium tigrinum*, *Hymenocalis littoralis* (spider lily) and Asiatic and Oriental hybrid lilies isolates showed 78-98% homology with each other. When compared with LSV isolates reported from elsewhere in the world, the Indian isolates showed 83-98% homology.

5.6 Expression of LSV CP in *E. coli*

5.6.1 Amplification, cloning and transformation of cloned fragment in *E. coli*

Primers were so designed to amplify the complete CP gene of LSV that could be ligated inframe with in the expression vector and expressed inframe. For inframe expression, the restriction enzyme sites were added in upstream and downstream primers, respectively. The CP gene thus amplified (~875 bp) was successfully cloned in the expression vector. Similarly, Hammond and Crosslin (1995), Thomas and Baneyx (1996), Kadkhodayan *et al.* (2000), Bicka *et al.* (2001), Petrzik *et al.* (2001), Jacob and Usha (2002) and Saini and Vrati (2003a and b) have used various restriction enzyme sites for inframe cloning of the gene of interest into the *E. coli* expression vectors.

A number of various expression vectors viz. pGEX vector (Kadkhodayan *et al.*, 2000 and Liu *et al.*, 2001), pET vector (Kadkhodayan *et al.*, 2000; Liu *et al.*, 2001; Bragard *et al.*, 2000; Petrzik *et al.*, 2001), pTrcHis vector (Hammond and Crosslin, 1995), pTBG(H) vector (Thomas and Baneyx, 1996), pT7This vector (Bicka *et al.*, 2001), pProEX HTb vector (Jacob and Usha, 2002) and pVEX based vector (Saini and Vrati, 2003a and 2003b) have been used for the respective gene study.

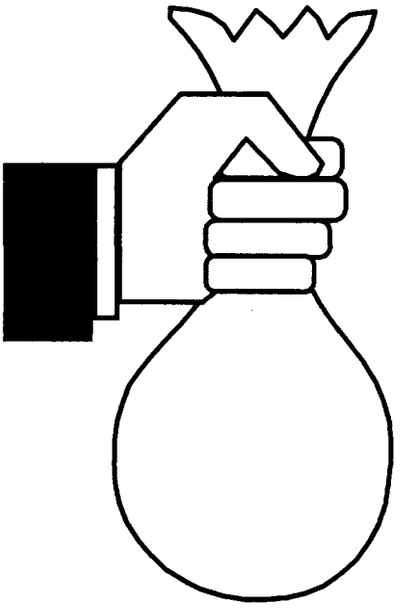
Digested vector and LSV CP gene were then ligated and transformed in *E. coli* BL-21 strain (which possess more efficient RNA polymerase) for expression. *E. coli* BL 21 has been widely used for expression of gene of interest (Bragard *et al.*, 2000; Liu *et al.*, 2001; Petrzik *et al.*, 2001; Jacob and Usha, 2002; Saini and Vrati, 2003a and b).

5.6.2 Standardization of optimal expression conditions for LSV CP

The expression conditions were standardized against time of induction, temperatures and IPTG concentrations for optimal expression of LSV CP gene in *E. coli*. The conditions which are standardized favored the most of the expressed protein to be present in soluble fraction in the cell lysate, while at higher temperature most of the expressed protein was present as insoluble fraction. Koschorreck *et al.* (2005) have also reported that when large sized proteins were expressed in *E. coli* or when small sized proteins were over expressed they formed insoluble protein complexes which are difficult to hydrolyze, so lower temperature was recommended for their expression. SDS-PAGE of the expressed protein showed maximum level of expression of the expected size of coat protein (~32 kDa) which confirms the expression of LSV CP.

Various IPTG concentrations have been used with different genes of interest viz. 1 mM (Kadkhodayan *et al.*, 2000; Bragard *et al.*, 2000; Thomas and Baneyx, 1996; Saini and Vrati, 2003a and b) 0.4 mM (Jacob and Usha, 2002) for expression of

Cardamom mosaic virus CP and 0.1 mM (Petrzik *et al.*, 2001) for expression of PNRSV CP in *E. coli*. Similarly, the duration for expression of gene of interest at a particular IPTG concentration may vary according to the gene of interest or by other conditions, as 37°C for 3-4 h has been used for the expression by Bragard *et al.* (2000), Liu *et al.* (2001), Jacob and Usha (2002) and Saini and Vrati (2003a and b), and 16°C for overnight by Kadkhodayan *et al.* (2000).



Summary

Chapter VI

SUMMARY

Lily and tulip are commercially important flower crops, their flowers are quite diverse in form and colour. They ranked third and fourth among the top ten flowers of the international floriculture trade. Lily and tulip infected by number of viruses that cause significant losses to lily and tulip cut flower production. Among these, one of the most impotent virus is *Lily symptomless virus*. LSV is a Carlavirus having elongated, slightly flexuous rod shaped practical, approximately 640 nm long and 17-18 nm in diameter. Its nucleic acid constitutes 8.3% of total particle weight. The genome of LSV is monopartite linear, positive sense (+), single standard RNA with genomic sizes of approximately 8.3 kb excluding poly A sequences. The 3' terminus has a poly (A) tail and the 5'- terminus sometimes has a methylated nucleotide cap, or a monophosphate. Carlavirus genomic RNA is encapsulated by a single type of coat protein (CP) with a Mr of 32 kDa.

To fulfill the aim of work "Assessment of variability in coat protein gene of *Lily symptomless virus* infecting lily and tulip" the studies on incidence of virus infecting lily and tulip (Detection of virus by using various techniques like ELISA, Northern Hybridization and RT-PCR), sequencing and sequence analysis (to assess the variability) and expression of LSV coat protein gene in *E. coli* were performed.

During the survey of the field of Floriculture Division, IHBT Palampur and leaf samples collected from different district of H.P. and J&K, lily were found to be infected with LSV. The infected lily cultivars exhibiting various kinds of symptoms like leaf mosaic and dwarfing, vein clearing or light brown spots on the lower side of the leaves, reduced growth with smaller flowers size.

A total of 21 cultivars of Asiatic and Oriental lily and four other lilies were tested by ELISA for the presence of LSV. The per cent infection was observed to be 33.3% for LSV using ELISA. A total of 16 cultivars of Asiatic and Oriental lily were also screened for the presence of LSV by Northern Hybridization using ^{32}P probe. The percent infection was observed to be 40% using Northern Hybridization. Thus the percent infection by Northern Hybridization was found to be greater then ELISA, it shows that Northern Hybridization is more sensitive detection technique than ELISA and is able to detect the lower concentration of virus in the infected tissue.

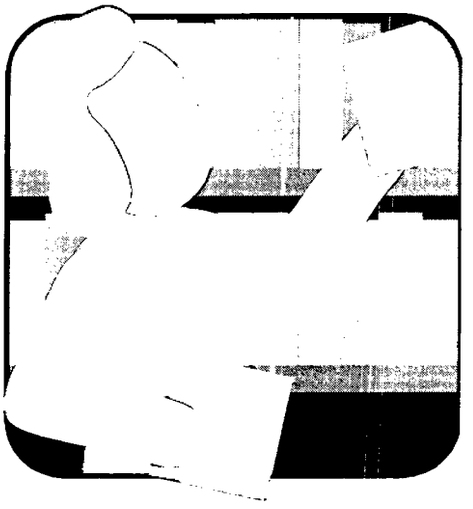
RT-PCR was also standardized for the detection of LSV from the infected lily and tulip leaf samples. The present study showed that RT-PCR is more sensitive method for the detection for the virus in various tissues.

Primer which were used for RT-PCR studies were further used for the amplification of complete coat protein (CP) of LSV. RT-PCR successfully amplified the product of expected size *viz.* approximately 875bp. PCR amplified products of CP genes of different isolates were cloned in pGEM-T Easy vector and sequenced. It was revealed that LSV CP is 876bp long by sequencing. The nucleotide sequence of LSV CP of different isolate have been submitted to EMBL database with the accession number AJ831416 for Solan Isolate, AJ831415 for Chamba isolate, AJ831417 for Kangra isolate, AM087402 for tulip isolate from Srinagar, AM087401 for tulip isolate from Palampur, AM087400 for lily isolate from Palampur and AJ781318 for tiger lily isolate from Palampur.

On sequence analysis of LSV CP, it was found that two isolates one from tulip (Acc. No AM087402) and one from Tiger lily (Acc. No. AJ781318) showed variability. They contain a stretch of variable amino sequence in their C-terminal end of coat protein.

The tulip isolate having 11 amino acid long stretch and tiger lily isolate contain 21 amino acid stretch. This finding also confirmed by Phylogenetic tree analysis, in Phylogenetic tree analysis both above isolates deviate from other known isolates.

Complete CP gene of LSV was expressed in *E. coli* BL-21 strain using pET32 (+) expression vector and the conditions for optimal expression were standardized.



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