Molecular characterization of Begomoviruses isolated from areas around Azad Kashmir

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Under the Supervision of

Dr. Muhammad Tahir

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CERTIFICATE

It is certified that the contents and the format of the thesis entitled "**Molecular characterization of Begomoviruses isolated from areas around Azad Kashmir**" submitted by **Mr. Aamir Lal** has been found satisfactory for the requirement of the **Master of Science** degree in **Plant Biotechnology.**

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All of my work is dedicated to

My Loving Parents

&

My Supervisor

"To what heights can we not rise with their attentions and prayers"

(Aamir Lal)

BISMILLAH

In the Name of Allah

Most Beneficent & Most Merciful

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"Mentioning (speaking of) the favors of **Allah** is (a show of) gratefulness leaving it (the favor) is ingratitude. Whoever does not thank (for) the little will not thank the much. And he who does not thank the people does not thank Allah" (on the authority of Nu' man Ibn Basheer, Hadith No. 5325 in Al-Jammi Assaghir).

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Aamir Lal

CONTENTS

LIST OF ABBREVIATIONS

AEV	Ageratum enation virus
A-Rich	Adenine-Rich
AYLCB	Ageratum yellow leaf curl Betasatellite
AYVV	Ageratum yellow vein virus
AYVV-P	Ageratum yellow vein virus- Pakistan
ALCCMB	Ageratum leaf curl Cameroon betasatellite
AYLCuB	Ageratum yellow leaf curl betasatellite
ChLCB	Chili leaf curl Betasatellite
CLCuBV	Cotton leaf curl Burewala virus
CLCuD	Cotton leaf curl Disease
CLCuMB	Cotton leaf curl Multan Betasatellite
CroYVMB	Croton yellow vein mosaic betasatellite
CLCV-SD	Cotton leaf curl virus-Sudan
C^o	Centigrade
DiAYVB	Digeraarvensis yellow vein Betasatellite
EACMKV	East African Cassava mosaic Kenya virus
ICMV-SG	Indian Cassava mosaic virus- Singapore
JcMD	Jatropha Curcas mosaic Disease
JLCV	Jatropha leaf curl virus
JLYMKV	Jatropha leaf yellow mosaic Katerniaghat virus
JMIV	Jatropha mosaic India virus
JMLV	Jatropha mosaic Lucknow virus
JMNV	Jatropha mosaic Nigeria virus

mM	millimolar
ml	milliliter
Min	minute
MgCl2	Magnesium Chloride
mg	milligram
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
Nm	nano meter
NS	Nona-nucleotide sequence
NW	New World
OW	Old World
OMoV	Okra mottle virus
PaLCV	Papaya leaf curl virus
PepLCLV	Pepper leaf curl Lahore virus
PGMV	Pouzolzia golden mosaic virus
PrLCV	Premna leaf curl virus
RaLCV	Radish leaf curl virus
RCA	Rolling Circle Amplification
RhYMV	Rhynchosia yellow mosaic virus
RoLCuV	Rose leaf curl virus
SAP	Shrimp Alkaline Phosphatasase
SYVSV	Spinach yellow vein Sikar virus
TLCYnV	Tomato leaf curl Yunnan virus
ToLCHJB	Tomato leaf curl Hajipur Betasatellite
ToLCNDV	Tomato leaf curl New Delhi virus

ToYLThB	Tomato yellow leaf curl Thailand betasatellite
TbLCB	Tobacco leaf curl betasatellite
TYLCCNV	Tomato yellow leaf curl China virus
TYLCV	Tomato yellow leaf curl virus
TE	Tris-Ethylene diamine tetra acetic acid
T-DNA	Total DNA
Taq	Thermococcus aquaticus
TAE	Tris-Acetate EDTA
UV	Ultraviolet
U	Units
VeYVV	Vernonia yellow vein virus
VeYVVB	Vernonia yellow vein virus betasatellite
w/v	Weight/Volume
X-gal	5-bromo-4-chloro-3-indolyl-(galactopyranosidase)
ZLCV	Zinnia leaf curl virus
°C	Degree centigrade
µl	micro liter
µg	micro gram
$(NH4)_2SO4$	Ammonium Sulphate
%	Percent

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ABSTRACT

Begomovirus of family *Geminiviridae* has been a considerable hindrance in producing high agricultural yields and causes severe economic losses in the majority of agro-based countries, including Pakistan. Among them only the cotton leaf curl disease (CLCuD) caused over US$ 5 billion loss to the Pakistan economy during the mid to late 1990s. Begomovirus also affects about 80 to 85 percent ornamental plants. This group of plant viruses is transmitted by whitefly (*Bemisia tabaci*) and infects dicotyledonous plants only. This group is composed of single stranded, circular DNA genome. A lot of data with reference to begomovirus diversity is available from Pakistan but scientific reports related to begomovirus from Azad Kashmir area are scanty. The project was designed: (I) to collect begomovirus suspected symptomatic and asymptomatic leaves samples from areas around Azad Kashmir; (II) to perform diagnostic PCR (Polymerase Chain Reaction) for the presence or absence of begomovirus; (III) molecular characterization of begomoviruses. About twenty five begomovirus suspected plant samples were collected. Total DNA was extracted, from both symptomatic and asymptomatic samples, using CTAB (Cetyl Tri methyl Ammonium Bromide) method. PCR was performed for diagnosis and amplification of begomoviruses. Only two samples i.e., Turmeric and Bottle gourd showed the amplification with universal core protein (CP) primers. The amplified partial products, DNA A and DNA betasatellite, of Turmeric and Bottle gourd respectively, were sequenced. This confirms the begomoviruses presence in these samples. Since Pakistan is an agricultural country, the diverse studies of begomoviruses, especially from far flung regions of the country, will contribute significantly for the development of scientific strategy for the problem.

Chapter 1

INTRODUCTION

1.1 GENERAL

The term "virus" is a Latin word meaning "poison". Viruses are obligate intracellular parasite which use host molecular machinery for their replication. They can be transferred horizontally between the cells. The most reasonable definition of virus is "virus is a set of one or more nucleic acid template molecule, usually encased in a protein or lipoprotein protective coating, which is only able to organize their own replication in a suitable host cell. Martinus Beijielinke discovered the first virus in 1898 that was the tobacco mosaic virus (TMV).

Most plant viruses (about 90%) having the RNA (single stranded or double stranded), as genetic material, while other viruses have viral DNA genome, which can be single-stranded (ssDNA) or double-stranded (dsDNA). They need some kinds of vector for transmission from one host to another. These vectors can be, fungi, nematodes and other insects (aphids, whiteflies, mites, leafhoppers, and treehoppers) etc. Their means of transmission include pollen, seeds and soil. However, there are mechanical means of transmission as well. Plant viruses have been characterized on the basis of their host range, source of transmission, comparison of the nucleic acid sequence and serological relationship between them.

International Committee on Taxonomy of viruses (ICTV) in its 8th report of classification and nomenclature of viruses has approved 5 orders, 82 families, 11 subfamilies, 307 genera and 2083 species of viruses. There are many plants which yield loss due to virus and the vectors which are responsible for virus spread. Therefore, these vectors and the weeds which are alternate host for viruses also are targeted to control the effects. Viral replication is

dependent on host cell replication machinery. Changes in viral nucleic acid molecule are caused by the variant. But plants have developed defense mechanisms to combat these viruses. All of these viruses cause huge losses in economically important crops, thus affecting the economies of many countries throughout the world.

1.1. GEMINIVIRUSES

Geminiviruses are one of the largest groups of monocots and dicots viruses which cause significant loss of economically important crops. The genome of Geminiviruses consists of one or two circular ssDNA, which are icosahederal packaged into the capsid (diameter 18-20 nm) and seen as twins particles under an electron microscope can be seen below in fig.1.1. The length of its genomes varies from 2.8 kb to 5.6 kb. These viruses replicate by double-stranded DNA intermediates in host plant cell nucleus. These viruses are dependent on host proteins for replication purpose because they can encode a few proteins only, not sufficient to carry out this process. Transmission of these viruses takes place by the insect vectors in a circulative, persistent and non propagated manner. Geminiviruses are mostly phloem limited viruses and remain restricted to the vascular tissues, whereas, some can escape to the mesophyll cells.

There are eight genera of family Geminiviruses: *Becurtoviruses, Begomoviruses, Curtoviruses, Eragroviruses, Masteviruses, Topocoviruses, Turncurtoviruses* and Capulavirus. *Capulavirus*is the most recent discovered genus of Geminiviruses with only three viruses categorized in it. This classification is based upon their host range, source of transmission, comparison of the nucleic acid sequence and serological relationship between them as shown:

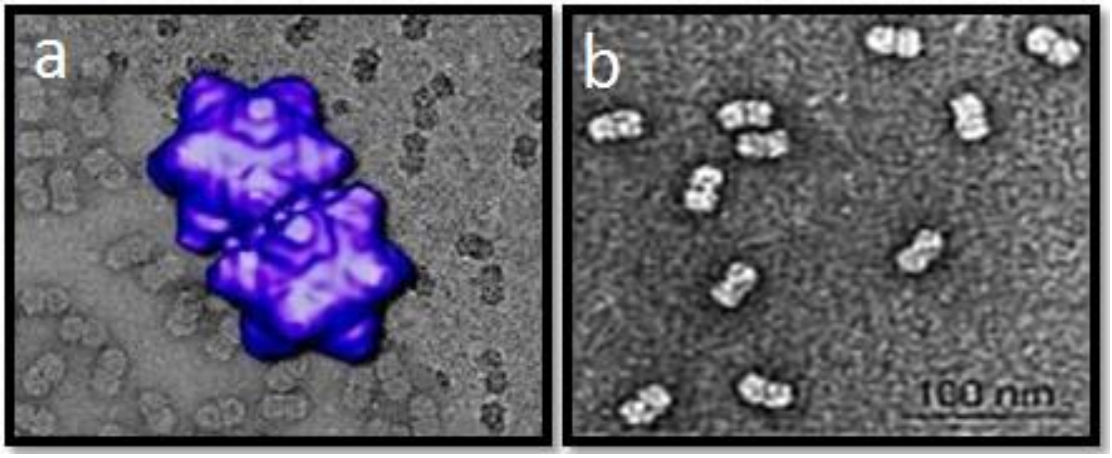

Figure 1.1: Structure of geminivirus (a) Computer simulated model. (b) Paired icosahedra, germinate particles (20 × 30 nm) under electron microscope.

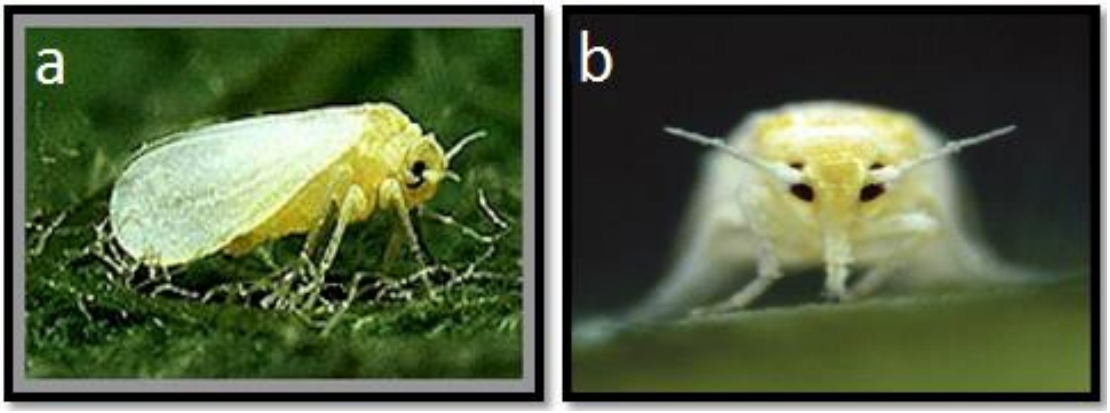

Figure 1.2 Sole vector of Begomovirus (a) Whiteflies on plant leaf, (b) whitefly transmitting the begomovirus into the host plant.

1.2. BEGOMOVIRUSES

Begomoviruses are the largest genus of family *Geminiviridae* and transmitted by white fly *Bemesia tabaci*, sole vector of begomovirus which can be seen in fig.1.2. The name "Begomovirus" was derived from Bean Golden Mosaic Virus (BGMV).

The earliest record of plant disease in 752 AD have been caused by virus i.e., begomovirus. Begomovirus only effects dicots, but little evidences of infection in monocots are also there. It has about 300 species members. Symptoms of infection of begomovirus are leaf curling, mosaic pattern, vein yellowing, enation, and stunting of plants growth, vein thickening, crinkling and many others as shown in fig.1.3.

In the last few decades, it has become the most significant plant viruses group in both regions, tropical and subtropical. Begomoviruses cause unprecedented devastating yield losses to economically important crops, including cotton, bean, cassava, tomato and cucurbits

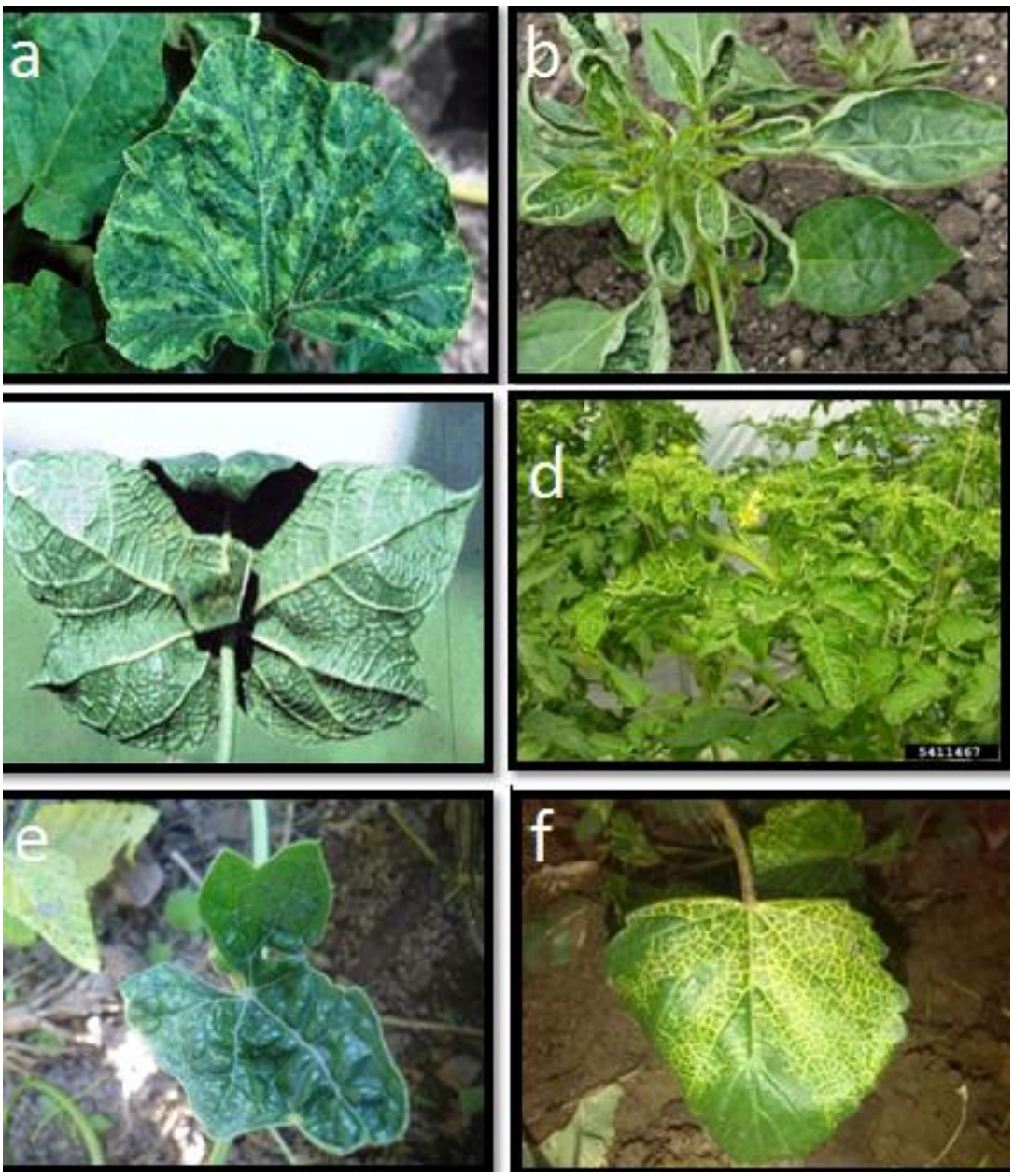

Figure 1.3.: Symptoms of Begomovirus, (a) Mosaic, (b) Leaf Curling, (c) Enation and Vein thickening, (d) Stunt growth, (e) Crinkling and (f) Vein yellowing and Mosaic.

1.3. GENOME ORGANIZATION OF BEGOMOVIRUSES

Genome of begomovirus is small, circular single–stranded DNA. Its length varies from 2.5kb to 2.8kb. Begomoviruses can be monopartite and bipartite. Those have single genomic component are known as monopartite and those begomviruses which have two genomic components are called bipartite. Bipartites have two genomic components (DNA-A and DNA-B).

1.4.1. Bipartite Begomoviruses

Bipartite begomoviruses consists of two genomic components i.e. DNA-A and DNA-B each of size 2.6kb approximately as shown below in Figure-1.4. Many of them belong to the new world begomovirus. Both these components are essential part for plant infectivity. Viral proteins for replication, encapsidation, insect transmission and gene expression regulation, are encoded by DNA-A while proteins for viral movement either intracellular or intercellular in plant and symptom induction are encoded by DNA-B. For replication DNA-B is dependent on DNA-A. DNA-B also helps the DNA-A component in infection spreading.

There are two sets of genes of DNA-A and DNA-B which diverge away into opposite directions i.e., one in clockwise direction (virion sense) and the other in anticlockwise direction (complementary sense). Both of these sets overlap each other which are divided by an intergenic region (IR). The non-coding region which contains an identical region is known as common region (CR). This region includes the origin of replication (ori). This origin of replication has a conserved nonanucleotide sequence (TAATATTAC). This conserved sequence makes a hairpin like structure. There is an initiation site in this structure for DNA replication, where binding of REP protein (replication associated protein) with virus specific

iterated sequences takes place. These sequences present on upstream of origin of replication and referred to, as iterons.

Total six open reading frames (ORFs) are present in DNA-A, four of them are in complementary sense while two in virion sense, as shown in fig. 1.4(a). Replication initiation protein (Rep), Replication enhancer protein (REn), Transcriptional activator protein (TrAP) and C4 are ORFs of complementary sense. Coat protein (CP) and V2 are ORFs of virion sense. Proteins for genome encapsidation and transmission are encoded by CP while V2 is important in symptom expression and systemic spread.C4 is involved in suppressing host defence of post transcriptional gene silencing (PTGS). Initiation of DNA replication is done by Rep. TrAP nullifies gene silencing induced by host, up-regulates transcription of late virus genes usually coat protein gene and suppresses cell death due to hypersensitivity

DNA- B contains two ORFs, one in complementary sense and other in virion sense, as shown in Fig. 1.4(b). Movement protein (MP) presents in the complementary sense which helps in cell to cell movement while nuclear shuttle protein (NSP) presents in virion sense which is responsible for encoding protein for genomic transport across nuclear pore.

1.4.2 Monopartite Begomoviruses

Monopartite begomoviruses contain a single genomic component i.e. DNA-A. Many of them belong to the old world begomoviruses and shown in fig. 1.5. All the viral activities i.e. replication, infection, transmission, gene expression in host etc. are performed by only DNA-A. Satellite DNA of two types is associated with monopartite begomoviruses, betasatellites and alphasatellites.

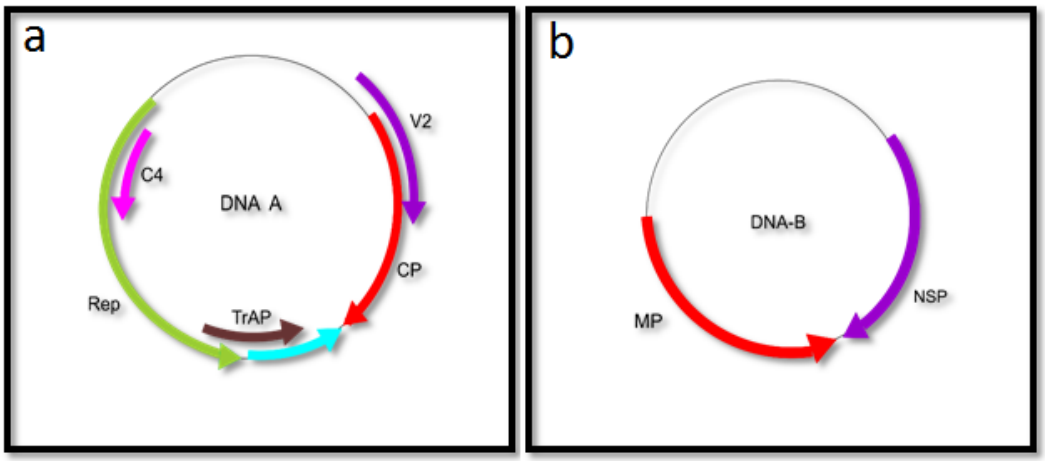

Figure-1.4: Genome organization of Bipartite Begomoviruses: (a) DNA A, (b) DNA-B

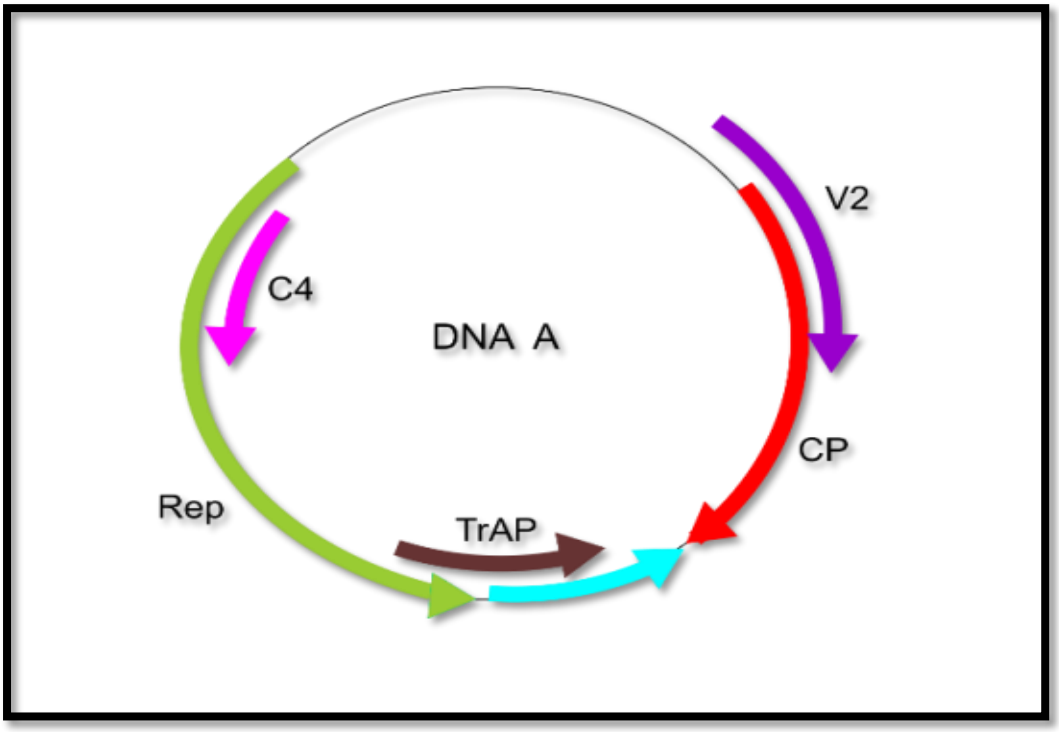

Figure 1.5: Genome organization of Monopartite Begomoviruses. (Replicated from Hanley-Bowdoin., et al 1999)

1.4.3. Betasatellites

Betasatellites are small, circular, single stranded satellite molecules OF approximately 1.4 kb and consists of only single ORF (βC-1) and can be seen in fig.1.6.For transmission, replication, encapsidation and movement, these betasatellites depend upon their helper begomoviruses. ORF of DNA betasatellites is adenine rich region (240 nucleotides). Betasatellites also consist of satellite conserved region (SCR) and about 220 nucleotides of SCR remains conserved among all DNA betasatellites. These 220 nucleotides of SCR are located near hairpin loop which is the binding site for Rep and probability is there of its involvement in trans-replication of begomoviruses. Betasatellites plays vital role as they are diverse in nature. βC-1 is the pathogenecity determinant and suppresses the PTGS. It also assists virus movement and upregulates the viral DNA level in plants.

1.4.4. Alphasatellites

A circular, single stranded DNA component associated with monopartite begomoviruses is called alphasatellite. The length of alphasatellites is approximately 1350 bp and is shown in the fig. 1.7. Contrary to betasatellites, alphasatellites are ‘self-replicating DNA molecules. But they also depend upon associated monopartite begomoviruses for encapsidation, movement and transmission. Previously, alphasatellites were associated with old world begomoviruses only but now, they also have found associated with the new world begomoviruses. The DNA alphasatellites consists of adenine rich, conserved sequence Rep gene and the nonanucleotide (TAGTATTAC), hairpin structure.

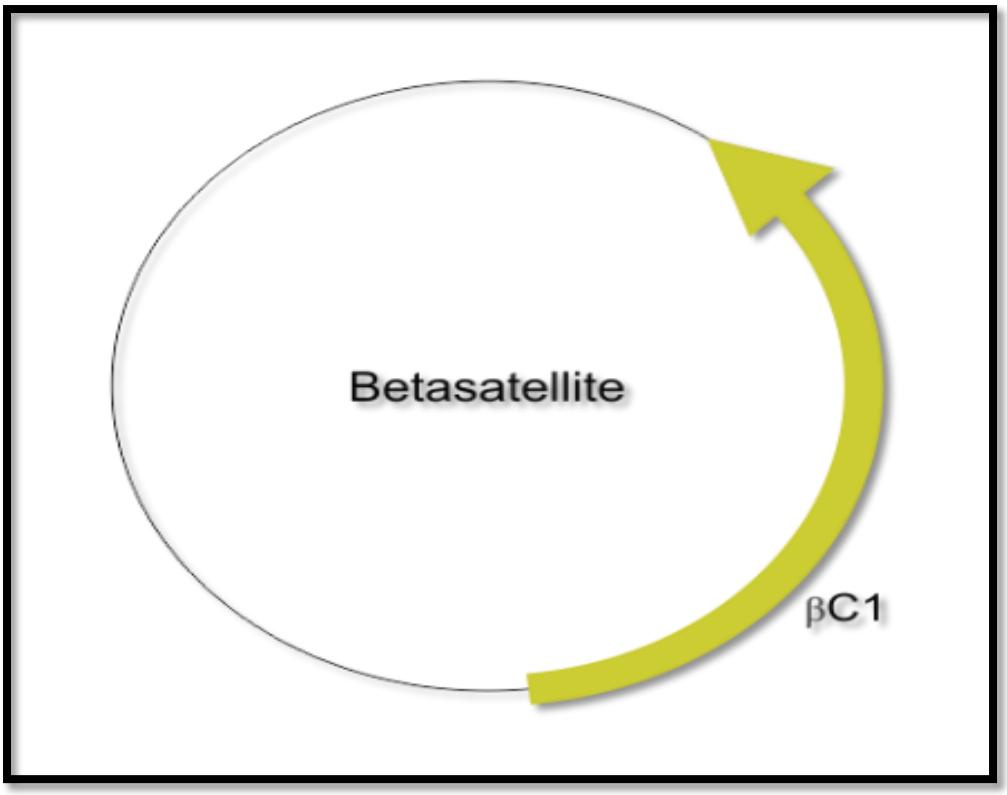

Figure 1.6: Molecule of a DNA betasatellite

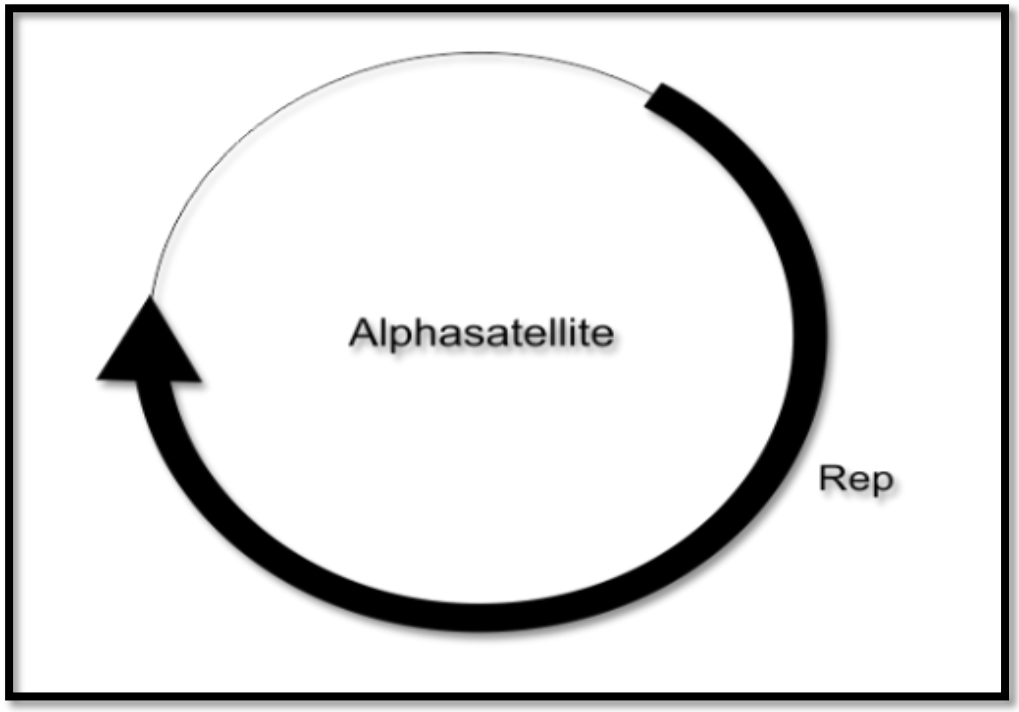

Figure 1.7: Molecule of a DNA alphasatellite.

1.4 IMPORTANCE OF BEGOMOVIRUSES

Begomoviruses (family Geminiviridae) are main obstacles in high agricultural yields and are responsible for causing severe losses in many of the agro-based countries, also including Pakistan. Over US$ 5 billion loss is reported due to Cotton leaf curl disease (CLCuD) in Pakistan during 1994-1999 and it is still a very serious threat to Pakistan economy. About 80 to 85 percent ornamental plants are affected by begomovirus. Pakistan is an agro-based country and highly populated country as ranked sixth in the world. Agriculture constitutes the largest sector of Pakistan's economy. This sector contributes 21.4% to its GDP, provides 40% employment and 60% exports. The horticulture sub-sector (Fruits and vegetables etc.) contributes 11% to the total value addition in agriculture sector. The current national horticulture exports are about 400 million US$ (2013-14). Pakistan is a poor, highly populated Provision of healthy and plenty of food to 190 million people is a big challenge. Vegetables are relatively cheap and healthy source of food. Losses of vegetables due to viral infections especially begomovirus are very high. Begomoviruses have become a serious threat to vegetables production in tropical and subtropical region of the world.

Turmeric (scientific name: *Curcuma longa*), is a rhizomatous herbaceous perennial plant belongs to the ginger family "*Zingiberaceae*" and is common in India and Southeast Asia. In addition to its culinary use, turmeric has remained a mainstay herb in botanical medicine. On a worldwide basis, about 800,000 tons of turmeric produced each year, with over 75% of this total amount coming from India. After India, the greatest turmeric production currently occurs in Bangladesh, Pakistan, Sri Lanka, Taiwan, China, Myanmar, and Indonesia. Turmeric is also grown commercially in many Central and South American countries**.** Overall Decreased Cancer Risk, cardiovascular Benefits, the

anti-inflammatory and antioxidant effects of turmeric are really beneficial for human beings.

Bottle gourd (*Lagenaria siceraria*), belongs to family *Cucurbitaceae*, native to tropical Africa but cultivated in warm climates around the world for its ornamental and useful hard-shelled fruits. The young fruits are edible and are usually cooked as a vegetable.

Isolation of begomoviruses from these vegetables are really threatening to meet the food requirements in near future.

1.4. **Objectives**

The main purpose of the study was:

- Molecular diagnostics of begomoviruses circulating in Azad Kashmir.
- DNA Sequencing and Sequence analysis.
- Phylogenetic relationship of the characterized viruses.

Chapter 2

REVIEW OF LITERATURE

Begomoviruses are the major limitation to the cultivation of economically important crops in the whole world during the last 3 decades. Evolution of begomoviruses due to recombination and mutation makes huge impact as up- gradation and the validation of the previous published literature and conducted research becomes the utmost need of hour. Scientists did great efforts to meet the challenge. Summary of the work done around the whole world including Pakistan is given below;

Srivastava *et al.*, (2015a) observed *Jatropha curcas* having leaf yellow mosaic symptoms from Katerniaghat wildlife sanctuary India. About 15-20 infected leaves were collected. Extraction of total DNA from infected leaves was done and then by using specific primers presence of begomoviruses was confirmed. Rolling Circle Amplification (RCA) was used for identification of its complete genome. After cloning of the amplified product, 86% sequence identity was found with *Jatropha mosaic India virus* (JMIV). So, it was named as *Jatropha leaf yellow mosaic Katerniaghat virus* (JLYMKV).

Tahir *et al.*, (2015) collected symptomatic leaf samples of *Sonchusoleraceu*s, *Ageratum conyzoides* and turnip (*Brassica rapa*var. rapa) from Nepal and Pakistan. Full-length genome was cloned along with DNA betasatellite from the infected leaf samples. The full-length DNA clone on sequencing showed 89.1% similarity to *Ageratum enation virus* (AEV) which confirmed these, the isolates of *Ageratum enation virus* (AEV). Whereas, cloned DNA betasatellites were found 90% identical so it was confirmed that these were isolates of *Ageratum yellow leaf curl betasatellite* (AYLCB). Inoculation of infectious clones of *Ageratum enation virus* (AEV) along with *Ageratum yellow leaf curl betasatellite*

(AYLCB) was done through agrobacterium *N. tabacum*, *N.benthamiana*, *A. conyzoides*, *S. lycopersicon*. Only *N. Benthaminana* showed the leaf curl symptoms. Hence, it was proved that *Ageratum enation virus* (AEV) infects crop but is a weed virus, because no symptoms were displayed by the inoculum of either infectious clones of *Ageratum enation virus* (AEV) or *Ageratum yellow leaf curl betasatellite* (AYLCB).

Gupta *et al.*, (2015) collected infected samples of chili *(Capsicum* genus) from Tirupati (India). Leaf curling was observed in these samples. Total DNA was extracted from samples. Then, a DNA fragment of 1300 nucleotide was amplified by using specific primers in the PCR. They found that chili leaf curl disease was caused by the begomoviruses.

Wyant *et al*., (2015) observed plants with symptoms: vein yellowing, leaf curling and mosaic pattern, on *Asystasiagangetica* (*Acanthaceae)* plants. First, amplification of viral DNAs was done by Rolling Circle Amplification (RCA) and then cloned through restriction based cloning. On Sequencing of RCA products, two Geminiviruses were obtained: Asystasia begomovirus 1 (bipartite genome) and *Asystasia* begomovirus 2 (monopartite genome) with defective DNA together. In a plant or a single leaf these investigated relationship of symptoms with virus distribution under different conditions of light and found uncommon separation of symptoms and viruses.

Sahu *et al*., (2015) studied the spinach leaves with symptoms: vein yellowing and reduced of size in India. Then, they cloned the DNA A and the DNA betasatellites from samples of spinach. On Sequencing *Spinach yellow vein Sikar virus* (SYVSV) showed 88% nucleotides identity with *Papaya leaf curl virus (PaLCV).* So, a new species of begomovirus was discovered.

Khatri *et al*., (2014) studied an infected ornamental plant: rose (*Rosa chinensis).* Infected leaves were collected with symptoms: leaf curling and stunted growth. For viral DNA amplification, PCR and RCA were used. Then they cloned and on sequencing of this amplified product it was found that *Rose leaf curl virus* (RoLCuV) was 83% identical to Tomato leaf curl Pakistan virus. Whereas, DNA betasatellite was found to be 96% identical to the *Digera arvensis yellow vein betasatellite* (DiAYVB).

Shuja *et al,*. (2014) reported a distinct strain of the *Cotton leaf curl Burewala virus* (CLCuBuV), which has no transcriptional activator genome (TrAP) gene in its genome. This virus is a recombinant of *Cotton leaf curl Kokhran virus* and *Cotton leaf curl Multan virus.* It is the only virus which prevalent after resistance breaking disease the outbreak of cotton in 2001. On sequencing, this virus showed less than 93% identity with CLCuBuV, it was a new strain while the betasatellite associated to CLCuBuV showed 97.6% identity with *cotton leaf curl Multan betasatellite*(CLCuMB).

George *et al*., (2014) did the molecular characterization of a complete monopartite begomovirus from an infected plant of *Amaranthus*. Symptoms of the infected leaves were: leaf distortion, leaf curling, yellow leaf margins and leaf crinkling. On sequencing, it was found that DNA alphasatellite was identical to *Chilli leaf curl virus*, *Chilli leaf curl alphasatellite*and DNA betasatellite showed identity with *Tomato yellow leaf curl Thailand betasatellite*.

Wang *et al.*, (2014) reported the complete nucleotides sequence of a new isolate of the *Indian cassava mosaic virus* collected from Singapore (ICMV-SG). This displayed severe Jatropha curcas mosaic disease (JcMD) in *N. Benthamiana*and *Jatropha curcas*as compared to previous reported ICMV. No systemic symptoms were observed on the induction of agroinfectious DNA-A of ICMV-SG, in *Jatropha curcas* while symptoms were observed in

the case of *N. benthamiana*. So, DNA-B along with DNA-A is required to infect *Jatropha curcas.*

She *et al.,* (2013) studied monopartite begomovirus' DNA-A of 2753 nucleotides long and then cloned and sequenced. This DNA-A was the major cause of infection in Creek Premna (*Premnaserratifolia*). Observed symptoms were: leaf curling, enation and vein swelling. It showed 83% homology with an isolate IN:Pusa:Tb:10 of *Tobacco leaf curl Pusa virus*. According to updated taxonomic criteria and the phylogenetic relationship named this was characterized as a new specie and named as *Premna leaf curl virus* (PrLCV).

Harimalala *et al.,* (2013) characterized cassava plant with mosaic symptoms. Isolated DNA alphasatellites had 80% sequence homology with *Cotton leaf curl Gezira alphasatellite.* This *Cotton leaf curl Gezira alphasatellite* was furthermore, associated with the *East African Cassava Mosaic Kenya Virus* (EACMKV). Due to 80% sequence identity *cassava mosaic alphasatellite* was classified as new specie.

Kamaal *et al.,* (2013) observed French bean (*Phaseolus vulgaris*) with leaf curl symptoms. DNA-A showed 80% sequence homology with *Cotton leaf curl Bangalore virus* and consists of 2741 nucleotides and six ORFS. So, it was new begomovirus specie according to new demarcation criteria and named as *French bean leaf curl virus.* Its associated betasatellite showed 80% similarity with *Papaya leaf curl betasatellite* and consists of one ORF i.e., bC1 and 1379 nucleotides. No recombination was seen in DNA-A and betasatellite of FbLCV.

Tang *et al.,* (2013) studied *Pouzolzia zeylanica* plant with golden mosaic symptom, *Pouzolzia golden mosaic virus* (PGMV) was found in it which was a monopartite begomovirus with 2723 nucleotides genome. In its genome two ORFs were encoded (CP and

AV2) in virion sense while five (AV1-AV5) ORFs were in t complementary sense. PGMV showed 78.5% nucleotide homology with *Ageratum yellow vein virus* (AYVV). So, PGMV was placed in a new specie category of begomovirus.

Phaneendra *et al.*, (2013) collected samples i.e., pumpkin leaves (*cucurbita moschata*) with leaf curl symptoms from Indian Agriculture Research Institute. Extraction of DNA was done and then by using CP primers which were specific to the *Tomato leaf curl New Delhi virus* (ToLCNDV), PCR was used for amplification purpose. The amplified DNA genome then showed the maximum sequence similarity with *Tomato leaf curl New Delhi virus* (ToLCNDV). Hence it was proved that leaf curl disease was caused *Tomato leaf curl New Delhi virus* (ToLCNDV) in Northern India..

Sidra *et al.*, (2012) studied *Codiaeum variegatum*(Croton) which belongs to *Euphorbiaceae,* family of evergreen shrub common in Pakistan. The virus isolated showed 99.1% sequence homology with *Clerodendron yellow mosaic virus* (ClYMV). Due to abundant presence of croton in Pakistan, it can host many begomoviruses which can cause multiple infections and in case of recombination probability of complications are very threatening.

Mubin *et al.*, (2012) isolated the DNA betasatellite and DNA alphasatellite as well as two begomoviruses from *Xanthium strumarium,* a common weed. One isolate of them had genetic homology with *Cotton leaf curl Burewala virus* (CLCuBV) while other with *Tomato leaf curl Gujrat virus* (ToLCGV). So, betasatellites were first time reported in Pakistan. These isolates were actually of *Tomato yellow curl Thailand betasatellite*. DNA alphasatellite associated with the *X. strumarium* infection was now identified also in many weeds and potato as *Potato leaf curl alphasatellite.*

Hina *et al.*, (2012) cloned viral genomes which had maximum homogeneity of 98.9-99.55% with *Cotton leaf curl Burewala virus* (CLCuBV) but surprisingly one of the cloned genomes did not have TrAP region in it. Hina et al. used symptomatic cotton plants for screening disease and sequence analysis, which revealed that CLCuBV is a single infectious agent of leaf curl disease in cotton including other cash crops.

Singh *et al.*, (2012) found that, in India, two monopartite begomoviruses and their associated DNA betasatellite is the major cause of *Radish leaf curl disease* (RaLCD). *Radish leaf curl-Varanasi* was recombinant specie, newly identified, which showed 87.7% homology to *Tomato leaf curl Bangladesh virus* and the second identified radish leaf curl disease pataudi was 95.8% identical to *Crotton yellow vein mosaic virus* (CYVMV)-India. By RDP analysis they also came to know that RaLCV was a recombinant of *Euphorbia leaf curl virus* and *Papaya leaf curl virus*. In order to investigate, the interaction between radish begomovirus and their associated satellite with *Tomato leaf curl Gujrat* and *Tomato leaf curl New Delhi virus* then both of the tomato leaf curl viruses exhibited contrasting relationship with DNA satellites. So they assumed that, when DNA betasatellite is exchanged with other begomovirus then it results in a new threatening disease to crops.

Malik *et al.*, (2011) studied the complete sequence of DNA-A and DNA-B genome of begomovirus which was isolated from muskmelon and it had maximum homology to ToLPMV. *Nicotiana benthamiana* showed leaf curl symptoms when both genomic component (DNA-A component of ToLCPMV and DNA-B component of ToLCNDV) were inoculated to it. DNA-B component of ToLCPMV clone was defective and it could not lead to infectivity. And when the DNA-A of either viruses were inoculated with DNA-B of ToLCPMV then it systemically moved. Along the veins Hypersensitivity response was seen when DNA-A inoculated with DNA-B of ToLCNDV. In addition, when NSP gene of

ToLCNDV with its construct was infiltered for the expression through agrobacterium in muskmelon under the control of 35s promotor induced hypersensitivity response. So, they concluded defective NSP gene causes HR in ZYMV, remains avirulent in muskmelon.

Tahir and Mansoor (2011) used PVX vector for the induction of common begomovirus by *Chili leaf curl betasatellite* (ChLCB) in *N. benthamiana*. BC1 was expressed by the PVX vector which then developed same symptoms as caused by *Cotton leaf curl Multan betasatellite* (CLCuMB) which were: leaf curl and leaf like enations. So, final conclusion revealed that BC1 of ChLCB and PVX vector displayed same symptoms which are produced by *Cotton leaf curl virus*.

Tahir and Briddon (2011) collected *Sonchusoleraceous* (SOL) and *Ageratum conyzoides*'(ACL) infected leaf samples and then full length genome sequences of begomovirus was isolated with 92.5% to 98.7% sequence identity with each other. DNA –A and DNA betasatellite of SOL and ACL isolates had 92.2% to 97.8% nucleotide identity with Nepalese isolate (CAN) AEN [Nepal: 01] and 93.5% to 96.0% nucleotide identity with *Ageratum yellow leaf curl betasatellite* (AYLCuB). In Pakistan AEV was first time reported. Isolates of SOL and ACL along with their DNA betasatellite were used for the production of partial repeat constructs. Then the produced constructs were inoculated to *Nicotiana benthamiana, Solanum lycopersicon, Nicotiana tabacum*, and *Ageratum conyzoides* and they finally observed the Koch's postulates for AEV causing disease in *Ageratum conyzoides.*

Tahir *et al.*, (2010a) screened *Capsicum* for begomoviruses which was then cloned and sequenced. They found *Pepper leaf curl Lahore virus* (PepLCLV) which was a new specie. PepLCLV was a recombinant of *Chili leaf curl virus* and *Papaya leaf curl virus* and moreover its DNA betasatellite was also similar to *Chili leaf curl betasatellite* (ChLCB) which was shown by the results. So, *Capsicum* is infected by ChLCB and ChLCB in

association with ChiLCVwere infecting potatoes in Pakistan. Geographically, PepLCLV and ChLCB are separate and Capsicum adapted similar of DNA betasatellite due to them.

Tahir *et al.*, (2010b) collected leaves of *Momordica charantia* with yellow vein symptoms from Lahore. Using PCR, they cloned full length sequence of bipartite begomovirus. Identity of nucleotide sequence was 86.9% with *Tomato leaf curl New Delhi virus* (ToLCNDV). *Bitter ground yellow vein virus* (BGYVV) was a proposed name for this distant specie. Recombination between ToLCNDV and *Tomato leaf curl Bangladesh virus* (ToLCBDV) is shown in BGYVV. DNA-B component was 97.2% homogenetic with *Squash leaf curl China virus,* Indian strain.

Tahir *et al.*, (2010c) cloned DNA-A and DNA-B of bipartite begomovirus isolated from *Cucurbita pepo* which was sampled from Lahore. DNA-A was 98.4%, while DNA-B was 89.6% identical to *Squash leaf curl China virus* (SLCCNV) Indian strain.

Azhar*et al.*, (2010) grew cotton, under field conditions, in the herbarium at the Central Cotton Research Institute (CCRI), Multan and compared it to cotton which was grown in the field. Level of virus was found much more symptomatic in cotton grown in the field relative to the cotton grown in research center. Among herbarium cotton species: *Gossypium robinsoni, Gossypium anomalum,* and *Gossypium somalense* were detected with high level of begomovirus by the help of Real-time PCR.*G. somalense* and, *G. anomalum* were two species which were characterized with DNA betasatellite. So, their results announced that *Cotton leaf Multan betasatellite* (CLCuMB) was related with two wild species of cotton while Malvaceous DNA betasatellite were associated with CLCuD, as well as *Chili leaf curl virus.*

Tahir *et al.*, (2009) isolated full length begomovirus and associated DNA betasatellite from a *Pedilanthus tithymaloides*. 86.3% of nucleotide sequence identity of an isolated begomovirus was with *Radish leaf curl virus*, but the maximum homology i.e. 90% was with begomovirus isolated from tomato. Isolated DNA betasatellite was 97% genetically identical to *Tobacco leaf curl betasatellite*. Since they reported it first time, so *Pedilanthus leaf curl virus* name was proposed.

Nawaz-ul-Rehman *et al.*, (2009) first studied New World begomovirus to which *Cotton leaf curl Multan betasatellite* (CLCuMuB) was associated. Both of them combined were causing severe disease of cotton in all parts of Indian subcontinent. When DNA betasatellite was inoculated in *N. benthamiana* in the presence of CLCuMuB and NW cabbage leaf curl virus then severe observable symptoms were seen. *Chili leaf curl betasatellite*, nevertheless, did not interact with CbLCuV. They observed many mutations when they isolated first offspring after inoculation of CbLCuV together with CLCuMuB. After isolation they again inoculated it to *N. benthamiana* in order to get infections with more satellite DNA level. These results disclosed that DNA betasatellite can adapt too fast for trans-replication by a new helper begomovirus.

Ilyas *et al.*, (2009) identified new virus which were not infectious for *N. benthaminana* and its 69.5% homology with *Mung bean yellow mosaic virus* confirmed that it was new specie and finally *Rhynchosia yellow mosaic virus* (RhYMV) name was proposed. When RhYMV was inoculated to soybean var. Ig6 then it showed mild symptoms unlike var. FS-85 which showed some degree of resistance to it in soybean germplasm.

Haider *et al.*, (2008) collected leaf samples which included apparently healthy and symptomatic leaves both. *Vinca minor* was an ornamental plant in which presence of begomovirus was confirmed first time in Pakistan. They amplified coat protein (CP) of

begomovirus. Finally, they came to know that sequence homology was 93% with *Pedilanthus leaf curl virus* (PedLCV).

McLaughlin *et al.*, (2008) collected 171 plant samples including squash, string bean, pepper, red kidney bean, tomato and weeds. Symptoms observed were: vein yellowing, leaf curling, mosaic, mottling and stunted growth. *Pepper golden mosaic virus* found in pepper and tomato while *Tomato mottle virus* showed association with sweet pepper and tomato only. *Merremia mosaic virus* infected the weed i.e., *Euphorbia heterophylla. Euphorbia* and hot pepper were found associated with *Euphorbia mosaic virus.*

Saunder *et al.*, (2008) studied *Ageratum yellow vein virus* (AYVV) and *Eupatorium yellow vein virus* (EpYVV) and the functional interaction among DNA betasatellite and *Ageratum yellow vein virus (*AYVV), *Honeysuckle yellow vein virus* (HYVV), *Cotton leaf curl Multan virus* (CLCuMV) and *Eupatorium yellow vein virus* (EpYVV). It was found that HYVV interacts only with its own satellite and its trans-replication with other satellites is not possible. CLCuMV could interact with other distinct satellites. Trans-replication in DNA betasatellite in association with AYVV was described by using deletion mutagenesis. In case of additional deletion, a new region was reported in DNA betasatellite which capable it to interact with other begomoviruses species.

Haider *et al.*, (2007a) took leaf samples of *Vinca minor L.* After DNA extraction, DNA-A was cloned by using PCR. The sequencing of DNA-A displayed maximum sequence identity 90% with the *Tomato leaf curl Joydebpur virus* segment DNA-A.

Haider *et al.*, (2007b) took infected samples of *Solanum nigrum, Ageratum conyzoides,* and *Zinnia elegans* with mild symptoms. To analyse conserved region of coat protein (CP), specific primers were designed. By using of two degenerate primers, CP region

of all three viruses i.e., *Solanum yellow leaf curl virus* (SYLCV), *Zinnia leaf curl virus* (ZLCV) and *Ageratum yellow vein virus*- Pakistan (AYVV-P) were amplified. On comparison of sequences in a phylogenetic tree, *Ageratum yellow vein virus*- Pakistan (AYVV-P) showed close relation with *Indian cassava mosaic virus* (ICMV), *Tomato leaf curl virus*-India (TLCV-Ind) and *Cotton leaf curl virus* (CLCuV). *Solanum yellow leaf curl virus* (SYLCV) showed close relation with ZLCV and TLCV-Ind, hence confirmed as a new strain of AYVV-P.

Saeed *et al.*, (2007) observed the association of a monopartite DNA betasatellite, associated with cotton leaf curl disease (CLCuD), with a bipartite *Tomato leaf curl New Delhi Virus* (ToLCNDV). The function of DNA betasatellite and DNA-B was similar. Like DNA-B, bC1 encoded by betasatellite provides movement function. More systemic infection was induced by the co-infection of DNA-A and DNA betasatellites. Inoculation of only DNA-A produced no symptoms. Similarly, mutant bC1 inoculation produced no symptoms. So, association of betasatellites with mono or bipartite begomoviruses leads to emergence of the new infections.

Tahir *et al.*, (2006) took *Duranta repens* (Pigeon berry) an ornamental plant common in Pakistan. Both healthy and infected leaf samples were collected and mild symptoms were observed. 1.5kb amplification product was obtained when a primers pair was used designed by Mansoor*et al*. Sequence identity of DNA-A was 91% with *Croton yellow vein mosaic virus* while 94% identity was observed between DNA-B and *Tomato leaf curl New Delhi virus* B segment. So, leaf curl disease in *Duranta repens* caused by bipartite begomovirus present in it.

Bull *et al.*,(2006) studied distribution and genetic variability of *Cassava mosaic viruses* in Kenya. It causes Cassava mosaic disease, a major threat to economy in Africa. Due

to genetic diversity of *Cassava mosaic virus*, they cause great epidemics. On molecular analysis, these were categorized into different groups. On further analysis, *East African Cassava mosaic virus*, *East African Cassava mosaic Zanzibar virus* and *East African Cassava mosaic Kenya virus,* new specie, were identified. These viruses had specific geographical distribution and showed restricted movement from native locations.

Ogbe *et al.*, (2006) visited about 418 farms and found moderate and severe symptoms on about 48% cassava. 52% mild symptoms were observed on cassava. The samples with 74.1% symptoms had African cassava mosaic virus (ACMV), EACMV on samples with 0.3% symptoms, 24.4% symptoms on samples were due to two viruses and samples with 1.2% symptoms did not react with primers. South African cassava mosaic virus and Indian cassava mosaic virus were not detected in any sample. ACMV was detected in case of single infection and also in mixed case with EACMV to produce severe symptoms on samples. EACMV and ACMV were also detected in new hosts i.e., *Combretum confertum* (weed plant) and *Senna occidentalis*.

Tahir and Haider (2005) collected samples of *Momordica charantia* (bitter gourd) from Lahore. Yellow blotch symptoms were observed on it. To amplify the viral DNA, CP primers were used in PCR. Then sequenced the cloned product and it was found that it had 95% nucleotide with *Tomato leaf curl New Delhi virus* (ToLCNDV). This isolated virus from *Momordica charantia* was confirmed as a new strain of ToLCNDV.

Khan *et al.*, (2005) studied ornamental plants with yellow vein net disease of *Calendula,* in India. DNA was extracted from both healthy and infected leaves and then coat protein region of *Tomato leaf curl New Delhi virus* was amplified by using PCR, followed by the cloning and sequence analysis. To cross check the PCR authenticity, took ToLCNDV DNA-A probe and cross- hybridized. The virus showed 95% identity with *Tobacco curly*

shoot virus, 94% with *Ageratum enation virus* and 93% with *Tomato leaf curl Bangladesh virus.* So, *Calendula officinalis* infected by begomovirus was reported for the first time.

Tahir *et al.*, (2005) collected samples of bell pepper having leaf curl symptoms and isolated a monopartite virus, *Bell pepper leaf curl virus* from it. The 750 bp CP gene was amplified. On sequencing the clone, it was found that it showed maximum sequence identity with *tomato leaf curl New Delhi Virus.*

Briddon *et al.*, (2004) observed that both DNA 1 components and the satellite molecule, DNA beta, were required for the induction of in some hosts. DNA 1 with DNA beta causes Okra leaf curl disease (OLCD) and Cotton leaf curl disease (CLCuD) in Pakistan while causes *Ageratum yellow vein disease* (AYVD) in Singapore. DNA 1 molecule of 17 different plants species were cloned from different areas and it was found that maximum of the DNA 1 present in complex with DNA betasatellites. Origin and functions DNA 1 component were discussed in these findings.

Wang *et al.*, (2004) collected six samples of papaya having leaf curl symptoms. According to collection point of sample, these were divided into two groups: first group comprises of virus isolates, G2, G4, G5, G28 and G29 collected from Guangxi province, second group comprises of GD2 collected from Guandong province. *Papaya leaf curl China virus* was observed in first group while *Papaya leaf curl Guandong virus* was observed in second group. 97.7% and 94.2% identity was observed with *Papaya leaf curl virus* by G2 and GD2 respectively. Comparison revealed the common ancestor among all viruses for coat protein.

Mansoor *et al.*, (2003) reported that in a bipartite begomovirus for trans-replication of DNA-B, DNA-A encodes replication-associated protein (Rep). A common region (CR) of

about 200 nucleotides, highly conserved presents in bipartite genome. Iterons, binding site for Rep present in common region (CR) and forms a part of the origin for replication. In the Indian subcontinent, Cotton leaf curl disease (CLCuD) caused by *Cotton leaf curl virus* is the major limitation in cotton production. *Cotton leaf curl Multan virus* (CLCuMV) with no iteron trans-replicate DNA satellite molecules called as CLCuD DNA-beta. Then, CLCuD DNA beta was interacted to three clones of the monopartite begomoviruses isolated from crop i.e., cotton. In all cloned viruses trans-replication of the DNA satellite molecule was observed with induction of symptoms in the cotton. They deduced that DNA beta was detected only in cotton rich areas in Pakistan, which had the ability to be taken on not by related begomoviruses.

Jose & Usha., (2003) stated that okra plant are really threatened in India due to the interaction of *Bhendi yellow vein mosaic virus* (BYVMV) with DNA betasatellites. They manufactured partial repeat construct of DNA betasatellite and BYVMV and inserted them into binary vector for the purpose agro-inoculation. Mild leaf curl symptoms were produced by alone BYVMV while yellow vein, mosaic symptoms were observed in the case of both BYVMV and DNA betasatellite.

Briddon *et al*., (2002) first time found a conserved region located at upstream of hairpin loop. A set of abutting primers was designed for amplification purpose of DNA beta of about 1350 nucleotides from the infected plants. DNA beta needs a helper begomovirus for replication purpose in host plants and needs trans-encapsidation for the insect transmission.

Idris and Brown, (2002) took collected okra, cotton and Sidalba samples from Shambat, Gezira and Sudan. These samples were cloned and then sequenced the monopartite begomovirus had 2761 nucleotides DNA genome. Its sequence identity with *Cotton leaf curl virus-Sudan* was 99.3-99.5%. CLCuV-SD isolated from okra was not transmitted to cotton by

Bemisia tabaci, but in case of transmission from okra to okra are done by *M. parviflora* and hollyhock whitefly. While CLCuV-SD an isolate of cotton infected hollyhock and cotton but did not infect okra. CLCuV-SD showed 99.5% identity with *Okra enation virus* (OkEV).

Yin *et al.*, (2001) studied the transmission of monopartite *Tomato yellow leaf curl China virus* (TYLCCNV) having 2734 nucleotides in genomic DNA, by white fly (*Bemisia tabaci*). Six open reading frames (ORFs) encode for V1, V2, C1, C2, C3 and C4 were present in its circular genome. Molecular weight of each protein, translated was greater than 10KD. As TYLCCNV was an old world begomovirus so could be compared only with monopartite begomoviruses genome or bipartite DNA-A. On agroinoculation, TYLCCNV could replicate itself and could infect plants i.e., nicotina and tomato as well.

Adkins (2001) collected samples of hibiscus (*Hibiscus rosa-sinensis*) from Florida and observed some chlorotic and ring spots on it. He, then, transferred the viral agents into five different hosts (*Chenopodium amaranticolor, Chenopodium quinoa, Nicotiana glutinosa, Nicotiana benthamiana*and *Nicotiana tabacum cv. Xanthi*). Chlorotic local lesions were observed in *C. quinoa, N. glutinosa* and *C. amaranticolor* without any kind of systemic infections. At the end, rod-shaped particles were seen on the three infected leaves of the host plants.

Briddon *et al.*, (2000) collected the samples of the infected cotton and *Nicotiana benthamiana* having symptoms: vein yellowing, mild leaf curling and stunting. They got DNA-A when *Cotton leaf curl virus* (CLCuV) was cloned. They proved that, alone CLCuV could not cause *Cotton leaf curl disease.*

Lotrakul *et al.*, (2000) characterized a bipartite begomovirus from *Dicliptera sexangularis,* an ornamental plant found in Florida. The viral infection was detected by the

whiteflies, inoculation either by grafting or mechanical followed by PCR usage for amplification and then the southern blotting. DNA-A and DNA-B were extracted and transformed into the DH5α cells after cloning. The virus observed was *Dicliptera yellow mottle virus* (DiYMV) which showed resemblance with *Dicliptera yellow mottle virus* (DiYMV) of new world on the basis of phylogenetic analysis, based on replication initiation protein, coat protein and nuclear shuttle protein. On sequence comparison different ORFs showed different percentage of identity with DiYMV. DiYMV displayed close relationship with begomovirustype II isolates on basis of NSP and Rep ORFs. Whereas, on common region and AC4 amino acids basis it showed resemblance with PYMV-VE, which could be due to possibility of recombination occurrence among new world begomoviruses.

Chapter 3

MATERIALS AND METHODS

3.1. SAMPLE COLLECTION

About twenty five different begomovirus infected samples were collected from areas around Azad Kashmir i.e., Sehnsa, Kotli, Samror. Symptoms like mosaic pattern, leaf curling, leaf distortion, vein yellowing and vein thickening were clearly observable in these samples. All samples were stored at -30^{o}C until DNA extraction.

3.2. TOTAL DNA EXTRACTION

First of all, total DNA extraction from the plant samples was done by using cetyl tri methyl ammonium bromide (CTAB) method. This method was first introduced by Doyle In 1990. Leaf samples of approximately 1 g were taken and then by using liquid nitrogen were grounded thoroughly in mortar and pestle. Then small amount of 60^{o}C hot CTAB isolation buffer (2% Cetyltrimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mMTris-HCl, and 0.2% mercaptoethanol) was added in the powdered form leaf tissue mass. The resulting slurry was transferred to labeled 50ml falcon tube. Then added more CTAB isolation buffer in the mixture upto 25ml in the falcon tube and mixed well. This mixture was incubated at 60°C on a shaker for 30 minutes. After incubation, this mixture was kept in fume hood to cool for 3 minutes.

Now, an equal volume of chloroform: isoamylalcohol (24:1) was added and mixed. Then poured the mixture into two falcon tubes of 50 ml, each containing 25 ml of the mixture. Then centrifugation of both tubes was done at 6500 rpm for 15 minutes at room temperature. The upper aqueous phase from both the falcon tubes was removed and

transferred into a new 50 ml falcon tube. About 20 ml of this upper aqueous phase was transferred. Ice cold isopropanol of equal volume was then added to the falcon tube and mixed gently by inversion. After this, this tube was incubated overnight at room temperature.

Next day, mixture was centrifuged at 6500 rpm for 15 minutes and removed the supernatant. 1 ml cold wash buffer (76% ethanol, 10 mM ammonium acetate) was added to the pellet, agitated and incubated at room temperature for 20 minutes. Again centrifuged at 6500 rpm for 15 minutes and obtained pellet was transferred to 1.5 ml eppendorf tube and then dried at 37^{o}C in incubator for 30-60 minutes. At the end this dried pellet was re suspended in1ml of TE buffer (10mM Tris-HCl, 1mM EDTA) and stored at -20°C.

3.3. QUANTIFICATION ANALYSIS OF DNA

5µl of DNA was loaded on 1% agarose gel along with DNA ladder (1 kb DNA ladder, Fermentas). DNA quantity was analysed by using spectrophotometer. Quantification analysis of DNA was done using spectrophotometer. For DNA, 260 nm wavelength of ultraviolet light is used and distilled water is used as blank sample. By using following formula DNA was quantitatively analyzed:

DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (50 µg DNA/ml) / (1 OD_{260} unit).

3.4 DILUTION OF TOTAL DNA FOR PCR

10 µl total DNA was added in 90 µl PCR water in 1.5 ml micro-centrifuge tube so the total DNA was diluted in 1:10 ratio.

3.5. POLYMERASE CHAIN REACTION

Presence of begomovirus in sample is checked by performing Polymerase chain reaction. Total reaction mixture for PCR is 50 µl and contained: 10X Taq buffer $(NH_4)SO_4$ (5µl), 1.5 mM $MgCl_2$ (3µl), 2mM dNTPs (2µl), 2 µl of each 50µM primers (both Forward and Reverse), 100 ng of total DNA, 0.5 units *Taq Polymerase*(1µl). ProFlex™ PCR System (Life Technologies) was used to place PCR reaction and particular temperature was set for the amplification of specified region.

3.6. POLYMERASE CHAIN REACTION CONDITIONS

ProFlex™ PCR System (Life Technologies) was used to place PCR reaction was placed by ProFlex™ PCR System (Life Technologies) and particular conditions for amplification of specified region used for Polymerase chain reaction are given in Figure 3.1 and 3.2.

3.7. AGAROSE GEL ELECTROPHORESIS

To prepare 1% (w/v) agarose gel, 0.7g agarose was added in 70 ml of 1x TAE buffer (25 mMTris, 1mM EDTA, 5Mm NaOH) in 250 ml flask. Then covered the flask with aluminium foil and kept in microwave oven until solution became clear. Cooled it for about 10 minutes. Then 7µl of 0.1% (w/v) ethidium bromide was added in melted agarose. Gel was poured in gel tray with an appropriate comb. DNA loading buffer was mixed in PCR product and loaded in well along with DNA ladder. The gel was run at 80 volts and switched off on well separation of bands. To visualize DNA bands, UV-trans-illuminator with short wave ultraviolet light was used.

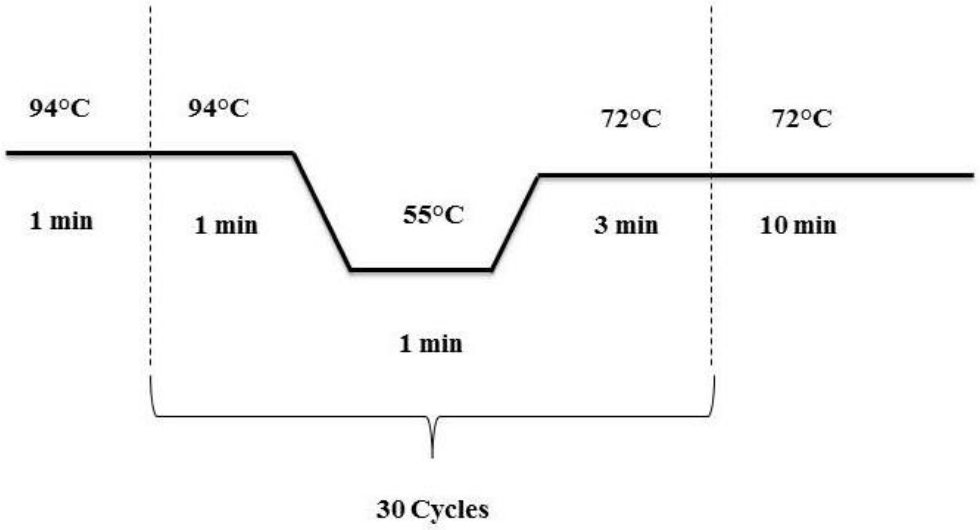

Figure 3.1: PCR condition for amplification of full length virus i.e., DNA A and DNA B of begomovirus.

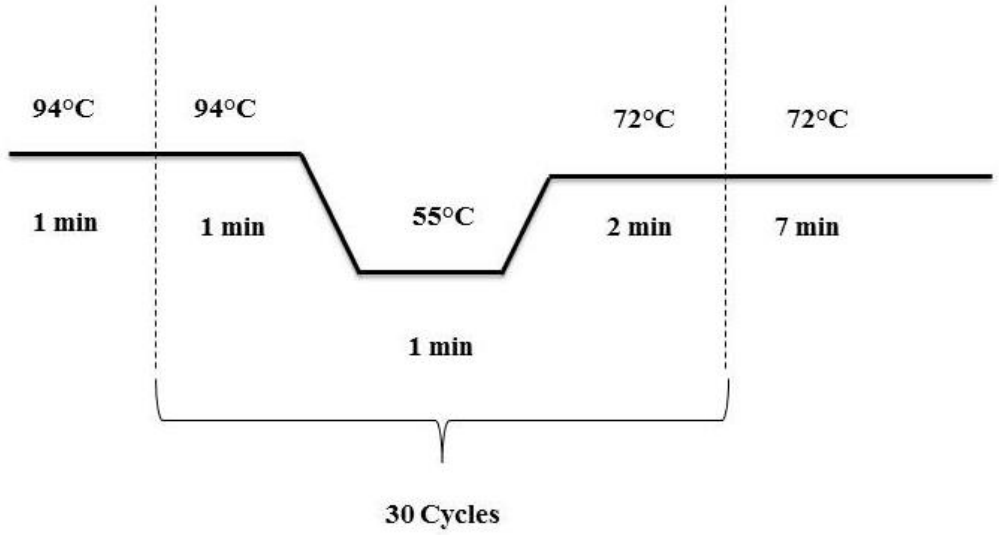

Figure 3.2: PCR condition for amplification of betasatellite of begomovirus.

3.8. GEL ELUTION OF PCR PRODUCT

Gel elution of PCR product is done by using Silica Bead DNA Gel Extraction kit. By using a sterile blade, required band from gel was excised and added in a weighed empty eppendorf tube (1.5ml) and weighed again. Incubation of mixture was done at 55°C for dissolution of gel after addition of 1ml DNA binding buffer to the eppendorf tube. Now addition of re-suspended silica powder suspension in the mixture is done before incubation at 55°C for 5 minutes. This mixture was centrifuged at 12000 rpm for 1 minute. Then washing of the resultant pellet was done by adding 500 µl of cold wash buffer and centrifuged again at same speed and time. Washing was done twice in the same pattern. Dissolved pellet in cold wash buffer, repeating this step twice, was again centrifuged at 10625 rpm for 60 seconds. Washed pellet is then incubated at 55°C for 5 minutes after the addition of 20 µl 1X TE buffer. Centrifuged it and transferred the supernatant to a new eppendorf tube for the ligation process.

3.9. T/A CLONING INTO pTZ57R/T VECTOR

To ligate the eluted PCR product pTZ57R/T cloning vector was used. For ligation purpose, 30µl mixture is prepared by addition of reagents in different amounts as shown

A total of 30 µl ligation mixture was incubated at 4^{o}C overnight for the completion of the ligation process.

3.10. PREPARATION OF DH-5α COMPETENT CELLS

A colony of *E. coli* DH5α strain was inoculated with 10 ml of *LauriaBertani* Broth (LB Broth) (tryptone; 1% w/v, sodium chloride 0.5%w/v and yeast extract 0.5% w/v, autoclaved at 121°C for 15 minutes) and incubated at 37^{o}Cin a shaker for overnight. After

incubation, 1 ml of culture was inoculated in 50 ml LB broth containing 250ml flask. This mixture was shaked continuously at 37^{o}C for 2 hours. Then transferred this culture into a 50ml falcon tube and cooled on ice for 10 minutes. This tube was then centrifuged at 4000 rpm for 15 minutes at 4^{o}C.10 ml of 50mM $CaCl_2$ was added in obtained pellet and kept on ice again for 15 minutes. Again centrifuged this suspension at the same conditions applied before in previous step. Now, supernatant was removed and 2ml of ice cold 50mM $CaCl_2$was added in the pellet. Aliquots of competent cells 50µl each were prepared and for further use, stored at -80^{o}C.

3.11. TRANSFORMATION

Heat shock method was used for the transformation purpose of DH5α cells. Addition of 30 µl ligation mixture in DH5α is done in safety cabinet and then kept on ice for 30 minutes. To give heat shock, mixture was placed for 2 minutes exactly at 42^{o}C and again kept for 2 minutes on ice. Transformation mixture with addition of 800 µl LB media was then added in mixture and incubated for 2-3 hours at 37^{o}C. LB media plates containing IPTG (isopropyl-beta-D-thiogalactopyranoside), X-Gal (*5-bromo-4-chloro-3-indolyl-β-galactopyranosidase*) (20 ml each), ampicillin and nutrient agar were taken and then transformed cells were spread on it and these plates were incubated overnight at 37^{o}C.

3.12. PREPARATION OF GLYCEROL STOCKS

Bacterial cultures were preserved by the preparation of glycerol stocks. In 1.5ml eppendorf tubes, added 200µl sterilized glycerol and 800µl cell culture was mixed in it and stored these tubes at -80^{o}C. By using sterile loop, streaking of culture in small amount is done on culture plates and incubated at suitable temperature. In this way the bacterial culture can be recovered.

3.13. ISOLATION OF PLASMID DNA

Three 50ml falcon tubes were taken and 10ml LB broth was added along with 100µg/ml ampicillin. A single white colony was added in each falcon tube and incubated overnight in shaker for at 37^{o}C. Plasmid isolation is done by Alkaline Lysis Method. 5ml overnight incubated culture was centrifuged at maximum speed for 1 minute and supernatant was discarded leaving behind small amount of supernatant. 250µl resuspension solution was then added. This mixture was transferred into new 1.5ml eppendorf tube.250 µl Lysis solution was then added and mixed by inversion for 4-5 times till solution became viscous. After it, 350 µl neutralized solution was added and by immediately inversion for 4-6 times, the solution was mixed. Centrifuged it for4-5 minutes. Then transferred the supernatant to gene jet spin column very carefully, not allowing white precipitates to enter. Centrifuged it for only 1 minute and the supernatant in the collection tube was discarded. 500 µl wash solution was then added and centrifuged for 1 minute (this washing step was repeated twice). Then transferred this spin column in a new 1.5ml eppendorf tube. 50µl elution buffer was then added. After this, it was allowed to incubate at room temperature only for 2 minutes. At last, centrifuged it for 2 minutes. Discarded the column and the purified plasmid obtained was then stored at -20^{o}C. This purified plasmid was confirmed on 1% agarose gel.

3.14. CONFIRMATION THROUGH RESTRICTION ENZYMESDIGESTION

To confirm the digestion through restriction enzyme30µl volume of reaction mixture was prepared and incubated it at 37^{o}C for 3 hours.

Approximately band of 1.4 kb (insert) and 2.8 kb (vector) was seen on 1% agarose gel which confirmed the digestion products in case of betasatellite while in cases of Turmeric (CH1) and Bottle gourd (CH2) both bands were found of size2.8kb.

3.15. DNA SEQUENCING

For sequencing purpose, isolated plasmid was sent to Macrogen, Korea. Universal primers M13F and M13R were used for sequencing purpose.

Chapter 4

RESULTS

4.1. SAMPLE COLLECTION

Leaf samples, both infected and normal, of about 25 different plants were collected from different areas of Kotli, Azad Jammu and Kashmir. These samples were collected on the basis of symptoms shown by begomovirus i.e., mosaic, Vein thickening, vein yellowing, crinkling, leaf curling, leaf distortion etc. as shown in table 4.1. All these samples were labeled properly and stored at -30^{o}C until DNA extraction.

About twenty five begomovirus suspected plant samples were collected from areas around Azad Kashmir. Total DNA was extracted, from both symptomatic and asymptomatic samples, using CTAB (CetylTrimethyl Ammonium Bromide) method. PCR was performed for diagnosis and amplification of begomoviruses. Only two samples i.e., Turmeric and Bottle gourd showed amplification with CLCV primers. Turmeric and Bottle gourd were only processed then among these samples and labeled as:

- **Turmeric :** **CH1**
- **Bottle gourd:** **CH2**

4.2. EXTRACTION OF DNA

By using CTAB method, total DNA from the samples was extracted and then stored this extracted DNA at -20^{o}C. The total DNA quality was then analyzed by using agarose gel electrophoresis. This extracted DNA was compared with 1kb marker on 1% agarose gel under UV trans-illuminator.

Table 4.1. Collected samples with codes, symptoms, location and date of collection

Sr. #	Plant Samples	Codes	Symptoms	Area	Date of collection
1	**Bitter Gourd**	BGa	Leaf distortion	Kalyal House Samror,Kotli	07-11-15
2	**Chilli**	CHIa	Leaf Distortion	Kalyal House Samror,Kotli	07-11-15
3	**Tomato**	TOM	Curling	DeraNawab Khan, Sehnsa, Kotli	22-07-15
4	**Indian Round Gourd**	IRGa	Mosaic Vein Thickening	Zubair Farms, Sehrmandi,Kotli	22-07-15
5	**Cucumber**	CUCa	Vein Yellowing Curling Crinkling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
6	**Cestrum**	CESa	Curling Leaf Distortion	MorhaSamror,Kotli	16-10-15
7	**Turmeric**	CH1	Mosaic	Zubair Farms, Sehrmandi,Kotli	22-07-15
8	**Bottle gourd**	CH2	Mosaic	DeraNawab Khan, Sehnsa, Kotli	22-07-15
9	**Brinjal**	BRIa	Mosaic Curling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
10	**Raddish**	RADa	Crinkling Leaf Distortion Curling	Zubair Farms, Sehrmandi,Kotli	22-07-15
		RADb	Crinkling Leaf Distortion	Kalyal House Samror,Kotli	07-11-15
11	**Mint**	MIN	Crinkling Curling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
13	**Ageratum**	AGE	Mosaic Curling	Kalyal House Samror,Kotli	07-11-15
14	**Mustard**	MUSa	Mosaic Vein Yellowing	Kalyal House Samror,Kotli	07-11-15
15	**Spinach**	SPI	Mosaic	Zubair Farms, Sehrmandi,Kotli	22-07-15
16	**Salvia**	SAL	Mosaic Curling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
17	**Ridged Gourd**	RGD	Curling Crinkling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
18	**Jasmine**	JAS	Mosaic Curling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
19	**Malvestrum**	MAL	Curling Mosaic Vein Yellowing	SaleemHouse,Samror, Kotli	30-09-15
20	**Vinca**	VIN	Mosaic Curling	Khuiratta, BnahValley,Kotli	07-11-15
21	**Alianthus**	ALI	Curling Crinkling	Suswah,nearSamrorKotli	30-09-15
22	**Shirin**	SHI	Curling Crinkling	ASAB parking,NUST	31-10-15
23	**Syngonium**	SYN	Crinkling Curling	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
24	**Salvia**	SAL	Mosaic	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15
25	**Irosine**	IRO	Curling Leaf Distortion	B.H.ShahMiniparadiseSarhota,Kotli	29-09-15

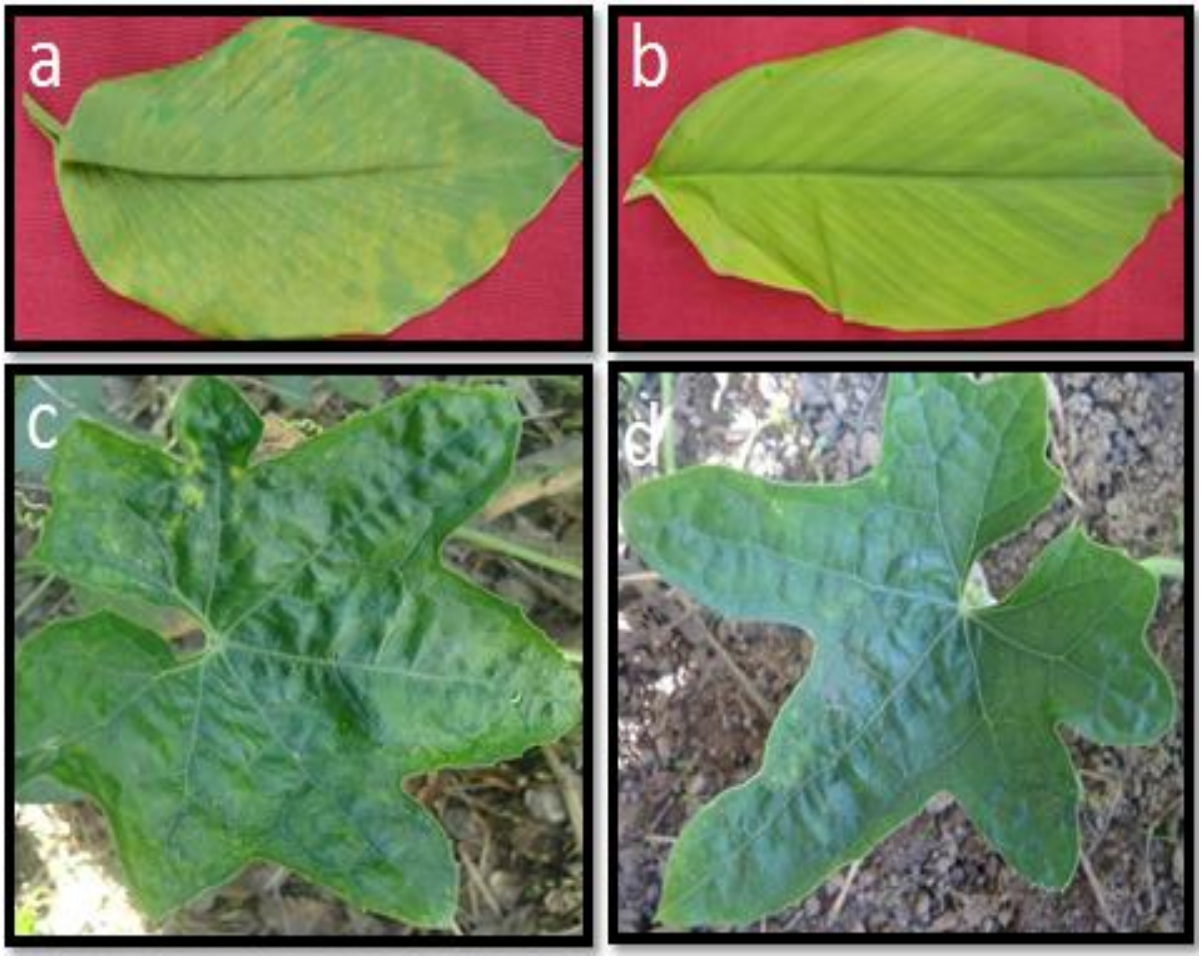

Figure 4.1: (a) Symptomatic (b) Asymptomatic leaf samples of Turmeric (CH1), (c) Symptomatic (d) Asymptomatic leaf samples of Bottle gourd (CH2).

4.3. PCR AMPLIFICATION

To confirm the presence of begomovirus in the samples Coat Protein (CP) and Cotton Leaf Curl Virus Primers (CLCV) i.e., both forward and reverse were used. Amplification in the samples confirmed presence of Begomovirus in both samples. Amplified products obtained are shown in Figure 4.2.

To detect DNA A in both samples i.e., Turmeric (CH1) and Bottle gourd (CH2), different abutting primer pairs were used but the abutting primer pairs BURXF/R and BURNF/R were the only primers which showed bands of about 2.8 kb in Bottle gourd and turmeric respectively. These amplified products of approx. 2.8 kb, correspond to DNA A of begomovirus were obtained and visualized under UV trans-illuminator as shown in Figure 4.3. To identify the second component of DNA, many attempts were made by using specific abutting primers KTBF/KTBR and DNA betasatellite specific primers Beta01/Beta02. In case of KTBF/KTBR primers no amplified product was obtained in both samples i.e., bottle gourd and turmeric while Beta01/Beta 02 yield amplified product i.e., approx. 1.4kb in both samples as shown in Figure 4.4. The present study showed the association of bipartite begomoviruses with Turmeric and Bottle gourd.

4.4. DNA GEL ELUTION OF PCR PRODUCT

All the expected size bands of DNA A and DNA Betasatellites were excised from the agarose gel and purified through Silica Bead DNA Gel Extraction Kit. The purified PCR products i.e., gene clean were then compared with standard 1kb marker. Bands were exactly on the same places as were observed on 1% agarose gel in PCR amplification.

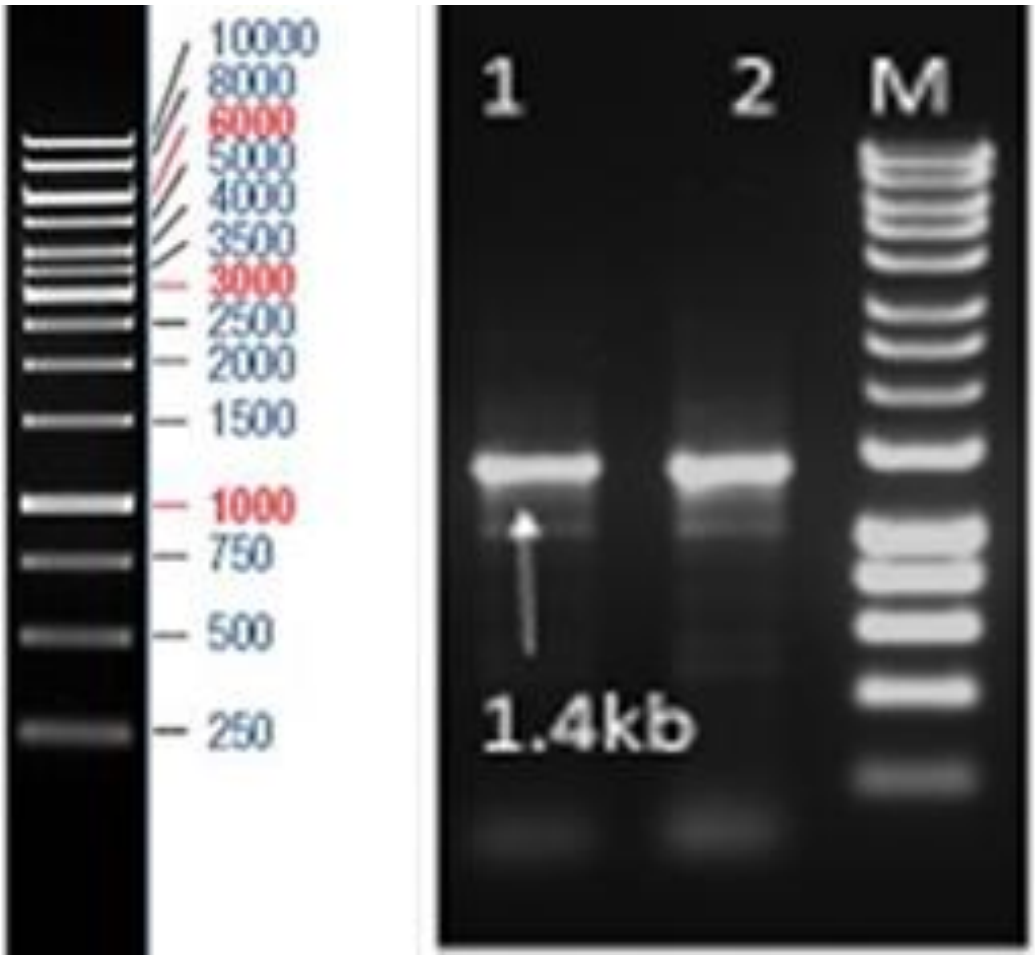

Figure 4.2: Agarose gel Electrophoresis of amplified gene of Turmeric (CH1) by CLCV. Bands on lane-1 and lane-2 (control) are of approximately 1.4kb with reference to M (standard 1 kb gene ruler).

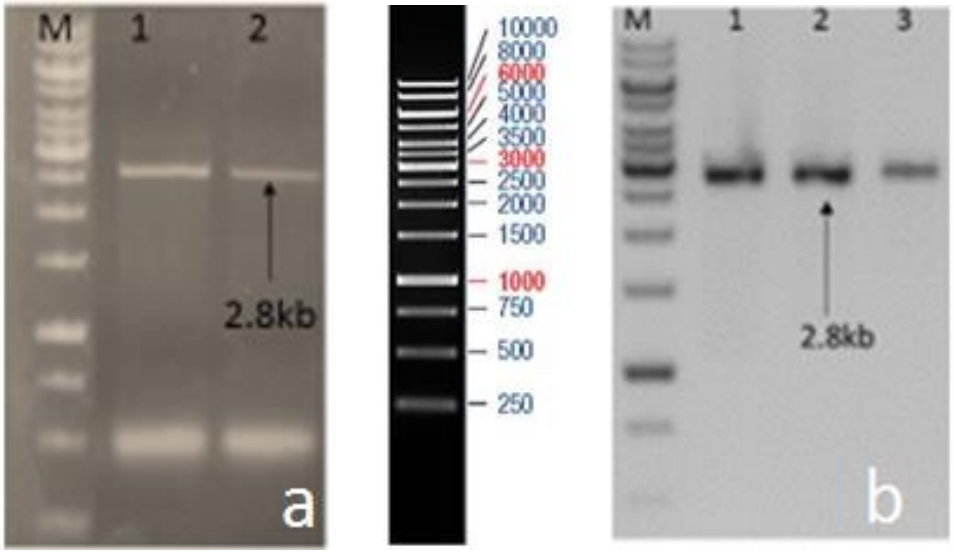

Figure 4.3 (a): Agarose gel Electrophoresis of amplified DNA A. (a) Bottle gourd (CH2), Bands on lane-1 by BURXF/R and lane-2 (control) are of approximately 2.8kb, (b) Turmeric (CH1), Bands on lane-1, lane-2 by BURNF/R and lane-3 (control) are of 2.8kb with reference to M (standard 1 kb gene ruler).

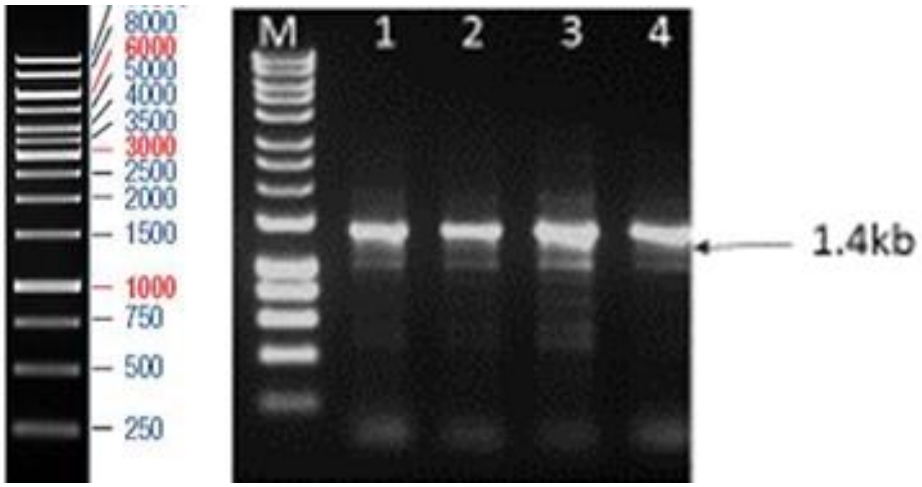

Figure 4.4. Agarose (1%) gel Electrophoresis of amplified DNA betasatellite. lane-1: 1.4 kb band of Bottle gourd (CH2), lane-2, 3: 1.4 kb band of Turmeric (CH1) and lane-4 is the control with reference to M (standard 1 kb gene ruler).

4.5. T/A CLONING

The PCR products after gene elusion were ligated into expression vector i.e., PTZ57R/T for ligation process. These ligated DNA with recombinant vector were transformed with into DH5α competent cells of *E. coli* strain by heat-shock method. For selection and the screening of clones these transformants were spread on LB agar medium and incubated overnight at 37^{o}C. Blue and white colonies were appeared on the plates. Only white colonies were picked and inoculated for about 16 hours. Then plasmid DNA were isolated and subjected to the restriction enzyme digestion.

4.6. SELECTION AND SCREENING OF CLONES/ PLASMID ISOLATION

Only white colonies were picked and inoculated them in LB media and incubated overnight to allow growth. Then Transformed plasmids were isolated and subjected to the restriction enzyme digestion.

4.7. CONFIRMATION OF CLONES THROUGH RESTRICTION ENZYME DIGESTION

Transformed plasmid containing pTZ57R/T vector (approximately 2.8 kb size) containing cloned DNA fragment (approximately 0.78 kb size) were digested with *Eco R1* and *Hind-III endonuclease* restriction enzymes. Clear couple of bands, of the double digestion, of the mentioned size on the agarose gel confirmed the presence of desired cloned DNA fragment as shown in Figure 4.6.

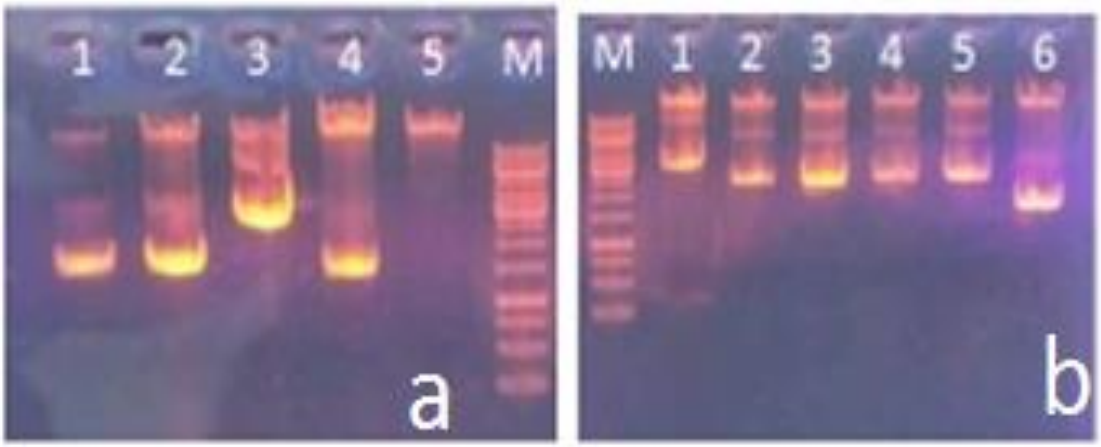

Figure 4.5. Agarose gel Electrophoresis of Isolated Plasmids (a) lane-1, 2 and 3 are DNA A plasmids by BURNF/R, 4 and 5 are DNA betasatellite plasmids of Turmeric (CH1), (b) lane-1, 2 and 3 are DNA betasatellite plasmids and lane-4, 5 and 6 show DNA A plasmids by BURXF/R of Bottle gourd (CH2), with reference to M (standard 1 kb gene ruler).

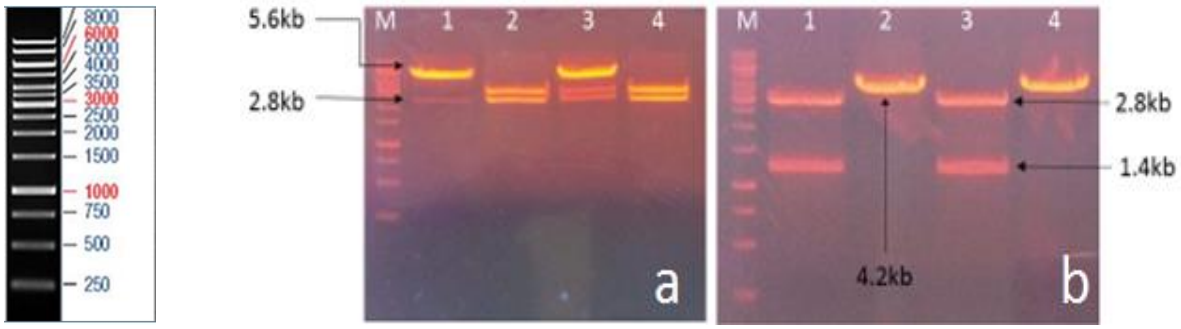

Figure 4.6. Agarose Gel Electrophoresis of Restriction Enzymatic product: (a) lane-1 and 2 show the bands when digested with EcoR1 and nco1 in case of Turmeric (CH1) respectively, lane-3 and 4show the bands when digested with EcoR1 and xho1in case of Bottle gourd (CH2) respectively. (b) lane-1 and 3 show the bands when digested with kpn1 and lane-2 and 4 show the bands when digested with EcoR1 in cases of Turmeric (CH1) DNA betasatellite and Bottle gourd (CH2) DNA betasatellite respectively.

4.8. DNA SEQUENCING AND ITS ANALYSIS

The sequences in the contigs form were assembled to determine the nucleotide sequences. To detect the complete sequence identity BLAST was used. It was found that DNA A of Turmeric (CH1) and DNA betasatellite of Bottle gourd (CH2) showed significant sequence identity with Cotton leaf curl Burewala virus and chilli leaf curl virus DNA betasatellite respectively. DNA A of Turmeric (CH1) was partially sequenced. The other two clones i.e., Bottle gourd DNA A and Turmeric DNA Betasatellite could not be identified properly on sequencing. CH1 and CH2 components obtained are shown below:

4.9. PHYLOGENETIC ANALYSIS

Neighbour joining phylogenetic dendogram was constructed based upon alignment of complete nucleotide sequences of beta clones in databases. Phylogenetic analysis was performed to check the relationship of obtained partial DNA A with 25other sequences from database. Analysis showed that it has close relationship with cotton leaf curl virus.

Phylogenetic analysis was performed to check the relationship of obtained beta-satellite with 33 other sequences from database. Analysis showed that it has close relationship with chilli leaf curl virus satellite DNA beta and chilli leaf curl betasatellite India-Palampur.

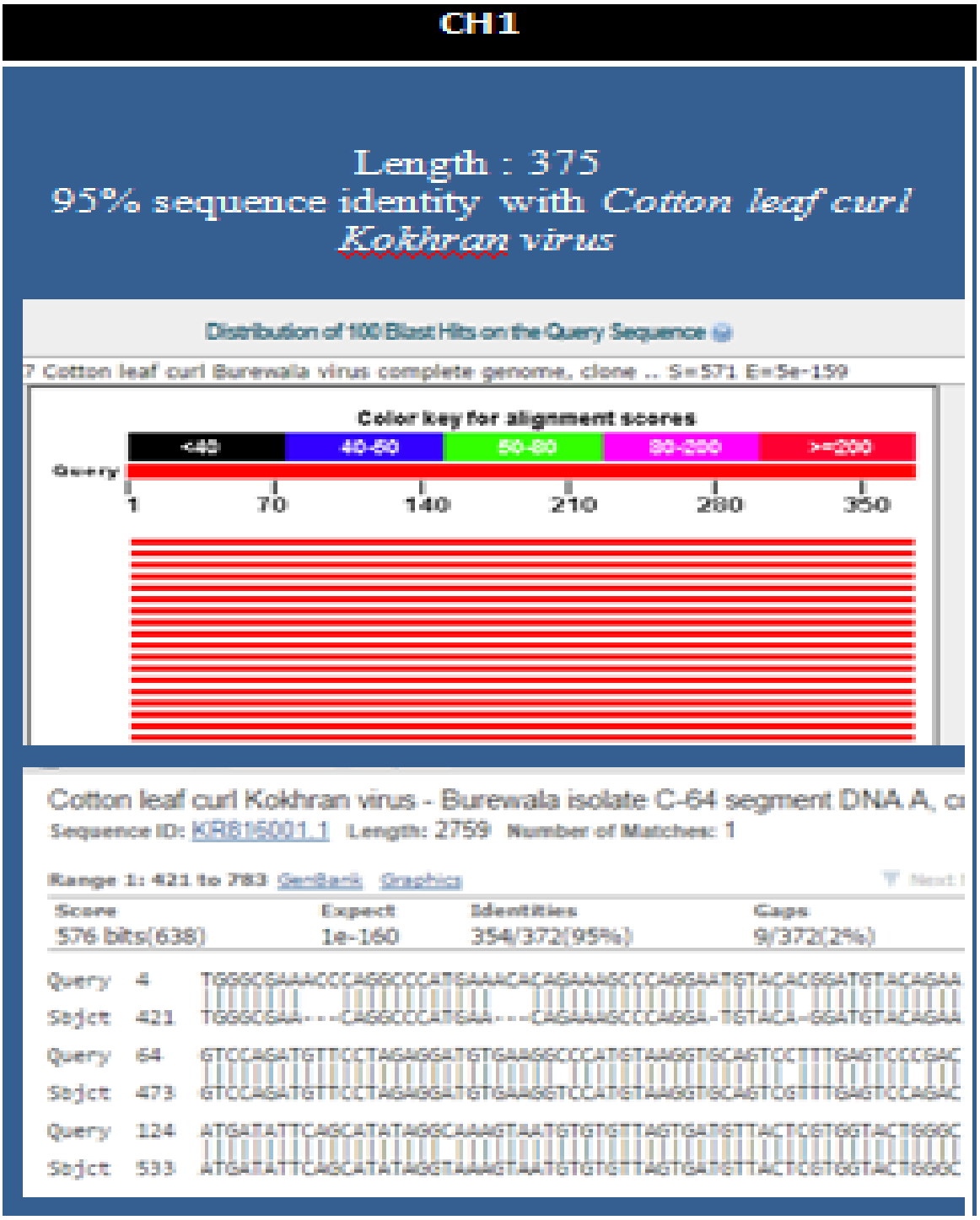

Figure 4.7. Blast sequence of Turmeric (CH1). 95% sequence identity was found with *Cotton Leaf Curl Kokhran Virus-Burewala* [CLCuKoV-Bu].

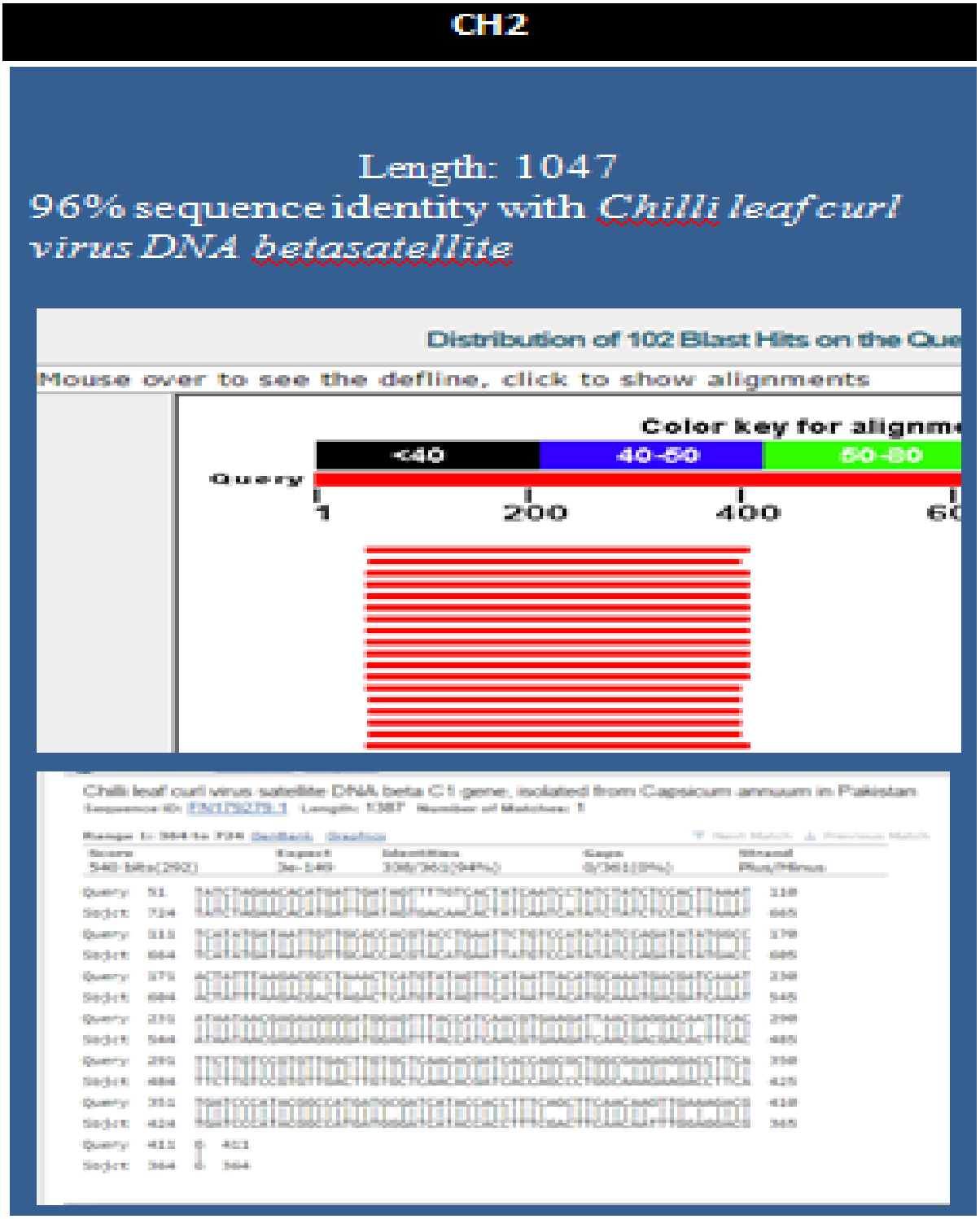

Figure 4.7(b). Blast sequence of Bottle gourd (CH2). 96% sequence identity was found with *Chilli leaf curl virus DNA betasatellite*.

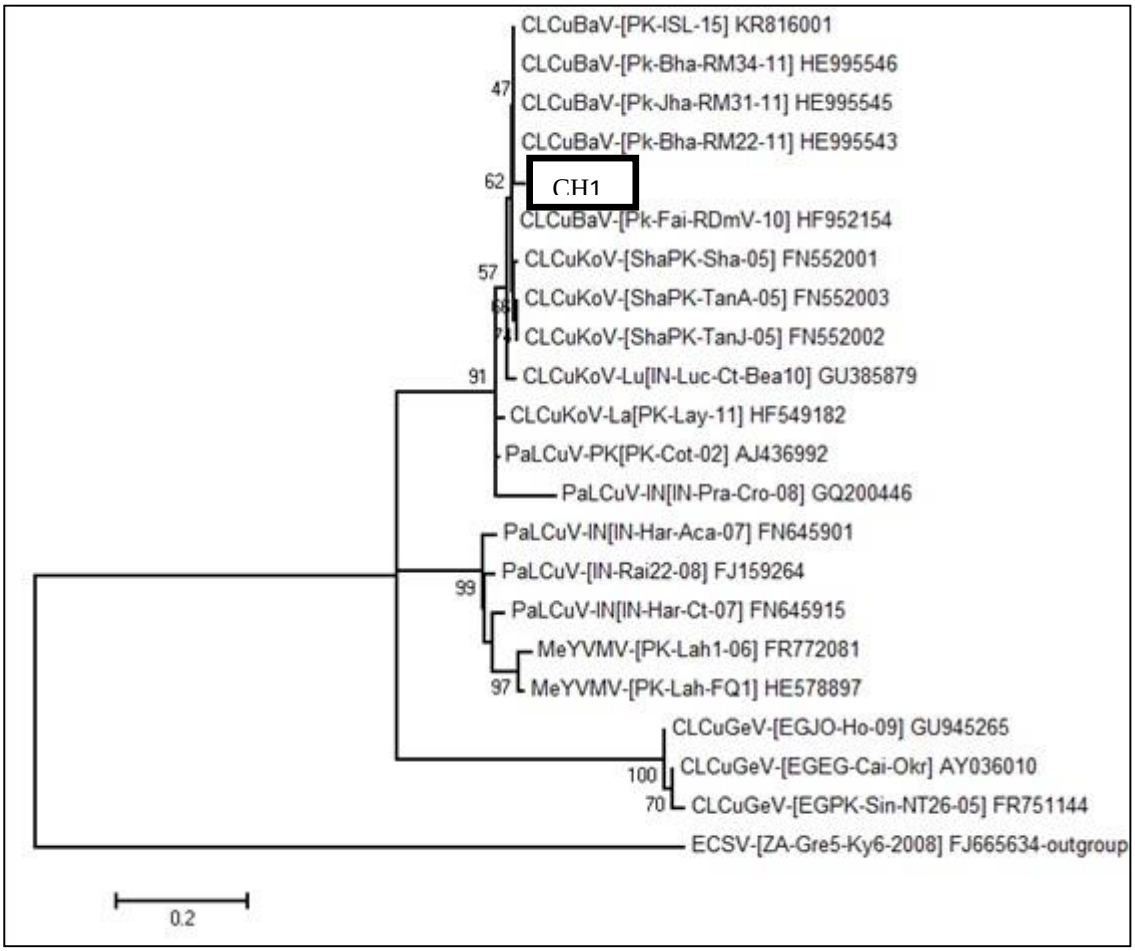

Figure 4.8. Phylogenetic analysis of DNA A of CH1 (Turmeric). Neighbour joining phylogenetic dendogram was constructed based upon alignment of partial nucleotide sequences in databases. Phylogenetic tree is showing DNA A related components in cluster. In this study *Eragrostiscurvula streak virus* (ECSV) was used as outgroup. Nodes values show bootstrap values. Other viruses which were used are: *Cotton leaf curl Bangalore virus* (CLCuBaV), *Cotton leaf curl Kokhran virus* (CLCuKoV), *Papaya leaf curl virus* (PaLCuV), Mesta yellow vein mosaic virus (MEYVMV) and *Cotton leaf curl Gezira virus* (CLCuGeV).

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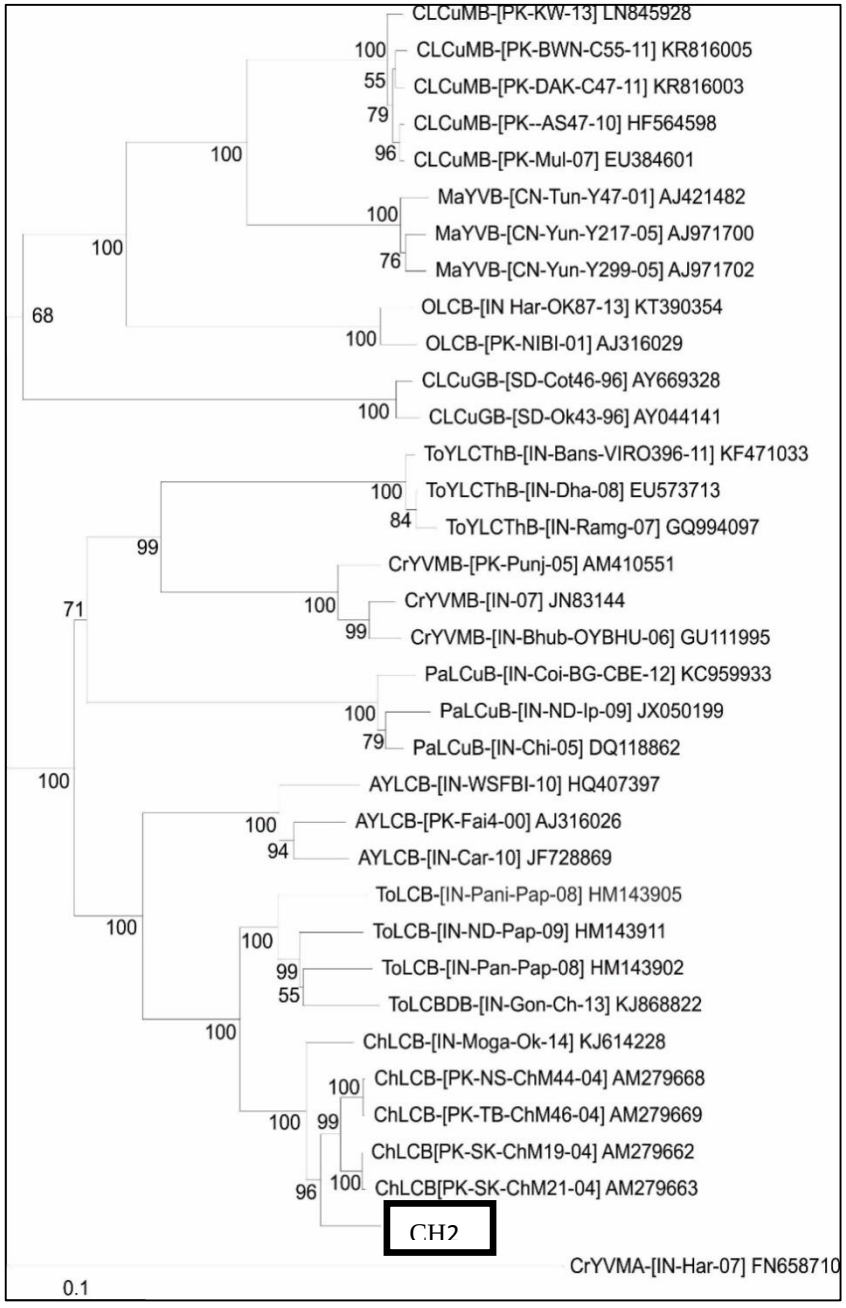

Figure 4.9. Phylogenetic analysis of DNA betasatellite of CH2 (Bottle gourd). Neighbour joining phylogenetic dendogram was constructed based upon alignment of complete nucleotide sequences of beta clones in databases. Phylogenetic tree is showing DNA betasatellite with closely related components in cluster. In this study *Croton yellow vein mosaic alphasatellite* was used as outgroup. Nodes values are showing bootstrap values. Other viruses which were used are *Cotton leaf curl Multan betasatellite* (CLCuMB), *Croton yellow vein mosaic betaasatellite* (CrYVMB), *tomato leaf curl Bangladesh betasatellite* (ToLCBDB), *Ageratum yellow leaf curl betasatellite* (AYLCB).

Chapter 5

DISCUSSION

Geminiviruses are amongst the largest plants viruses which infect both monocots and dicots. They can be monopartite and bipartite genome. Geminiviruses are encapsidated by a double geminated icosahedral capsid.Leafhoppers, Whiteflies, Treehoppers and Aphids are the vectors of *Geminiviruses.* Currently, geminiviruses are divided into eight genera: *Becurtoviruses, Begomoviruses, Curtoviruses, Eragroviruses, Masteviruses, Topocoviruses, Turncurtoviruses* and *Capulavirus*on the basis of genome type, insect vector and host range. *Capulavirus*is is only genera, amongst these eight, which are transmitted by aphids and is the recently added new genera.

*Begomovirus*are the largest genus of family *Geminiviridae* and transmitted by white fly *Bamesia tabaci*. *Begomovirus* only affects dicots, but little evidences of infection in monocots are also there. It has about 300 species members. Symptoms of infection of begomovirus are leaf curling, mosaic pattern, vein yellowing, enation, and stunting of plants growth, vein thickening, crinkling and many others. In the last few decades, it has become the most significant plant viruses group in both regions, tropical and subtropical. *Begomoviruses* cause unprecedented yield losses of many economically important crops, including cotton, bean, cassava, tomato and cucurbits.

Its threatening diversification emergence through recombination and mutations, explains the Emerging Infectious Diseases (EIDs) which is an alarming problem in many crops like: vegetables, cotton, cassava etc.

Vegetables like Bitter gourd, Pumpkin, Bottle gourd, Tomato, Okra, Squash, Cucumber, and Turmeric etc. are some of the popular vegetables cultivated in Asia.

Begomoviruses use these vegetables as a host and different species of begomoviruses like: *Pumpkin yellow mosaic virus* (PYMV), *Bitter gourd yellow vein virus* (BGYVV), *Pepper leaf curl Bangladesh virus* (PepLCBDV), *Squash leaf curl China virus* (SLCCNV), *Tomato leaf curl New Delhi virus* (ToLCNDV) etc. are the biggest limitation agents to the production of these important crops. Previous studies reveal that, begomoviruses were affecting these important vegetables in China, India, Pakistan, Vietnam, Philippines, Thailand and Taiwan from the beginning of current century.

The purpose of the present study was to characterize the bottle gourd and turmeric infecting begomoviruses. Bottle gourd belongs to *Cucurbitaceae* family, so begomovirus characterization from this vegetable is of great importance. Symptomatic leaves of bottle gourd plants having crinkling, leaf distortion symptoms were found and collected from areas around Azad Kashmir. .Full length DNA-A component and DNA betasatellite were amplified through PCR by using BURX (F/R) and Beta (01/02) respectively. Cloned products were then sequenced. Nucleotides sequence identity showed 96% sequence identity with Chilli leaf curl virus DNA betasatellite. Many crops in Bangladesh, India, Pakistan and Southeast Asian countries are affected by Chilli leaf curl virus. Emergence of new variety of begomoviruses involves the mechanism of recombination. DNA A of pumpkin couldn't be found identical to other viruses because of its irregular sequencing.

Turmeric (*Curcuma longa*) is a rhizomatous herbaceous perennial plant of the ginger family, *Zingiberaceae*. It is native to southern Asia, requiring temperatures between 20 and 30 °C. Turmeric is grown by people and it grows wild in the forests Asia and is one of the key ingredients in Asian dishes. Turmeric is medically very important. It is used as coloring agent in South Asian cuisine.

Numerous attempts were made for the amplification of full length of DNA-A from both bottle gourd and turmeric. Only DNA A of Turmeric and DNA betasatellite of Bottle gourd were obtained, none of the other part was amplified which concludes that DNA-A of Bottle gourd, might have been recombinant: this could be cause of non-attachment of primers, or mishandling that as a result was not able to amplify the required components.

Result of this study confirms that vegetables can be good homing site for the recombination of different species of viruses to generate new species of viruses. Different vegetables can harbor more than one begomoviruses, which may be the cause of generation of new specie.

CONCLUSION:

Present research reveals that both bottle gourd and turmeric harbors bipartite begomoviruses. Infectivity studies, in future, will reveal the disease caused by begomoviruses in these plants along with mode of transmission, recombinant behavior etc.

FUTURE PROSPECTS:

- Present research can be used for disease management and control.
- It can also be used for targeting virus, white fly and alternate hosts.
- Collection of large number of samples to evaluate the diversity of begomoviuses.
- Development of infectious clones are used for:

    1. Infectivity
    2. Host range
    3. Koch postulate

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