MOLECULAR CHARACTERIZATION OF BEGOMOVIRUSES ISOLATED FROM DIFFERENT AREAS AROUND FAISALABAD

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Molecular Characterization of Begomoviruses Isolated from Different Areas Around Faisalabad

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CERTIFICATE

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“I dedicate this effort to my parents for their constant love and support, my friends who motivated and encouraged me and my supervisor, without his inspiration, coaching and enthusiasm none of this would have been possible.”

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Abbreviations

ATP	Adenosine triphosphate
AC1/Rep	Replication initiator
AC2/TrAP	Transcription activator protein
AC3/REn	Replication enhancer protein
ACMV	*African cassava mosaic virus*
ALCCMA	*Ageratum leaf curl Cameroon alphasatellite*
ALCCMB	*Ageratum leaf curl Cameroon betasatellite*
ALCCMV	*Ageratum leaf curl Cameroon virus*
ACMV	*African cassava mosaic virus*
A-Rich	Adenine rich
α	alpha
AYVV	*Ageratum yellow vein virus*
AYLCuB	*Ageratum yellow leaf curl betasatellite*
β	Beta
BLAST	Basic local alignment tool
BGYVV	*Bitter gourd yellow vein virus*
Bp	base pair
BYVMV	*Bhindi yellow vein mosaic virus*
$CaCl_2$	Calcium chloride
CLCuMB	*Cotton leaf curl Multan betasatellite*
CLCuMA	*Cotton leaf curl Multan alphasatellite*
CoGMV	*Corchorus golden mosaic virus*
CLCrV	*Cotton leaf curl virus*

CLCuGB	*Cotton leaf curl Gezira betasatellie*
ChLCB	*Chili leaf curl betasatellite*
ChiLCV	*Chili leaf curl virus*
CLCuMV	*Cotton leaf curl Multan virus*
CLCuMuB	*Cotton leaf curl Multan betasatellite*
CLCuRV	*Cotton leaf curl Rajasthan virus*
CLCuAV	*Cotton leaf curl Allahabad virus*
CLCuGV	*Cotton leaf curl Gezira virus*
CLCuBV	*Cotton leaf curl Bangalore virus*
CLCuBuV	*Cotton leaf curl Burewala virus*
CLCrV	*Cotton leaf Crumple virus*
CLCuKV	Cotton leaf curl Kokhran
CLCuD	*Cotton leaf curl disease*
CLCuV	*Cotton leaf curl virus*
CR	Common region
CP	Coat protein
CroYVMB	*Croton yellow vein mosaic betasatellite*
CSD	*Chickpea stunt disease*
CTAB	Cetyl trimethyl ammonium bromide
CYVMV	*Croton yellow vein mosaic virus*
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphates
dsDNA	Double stranded Deoxyriboncleic Acid
EACMV	*East African cassava mosaic virus*
EDTA	Ethylene diamine tetraacetic acid

EpYVB	*Eupatorium yellow vein betasatellite*
G	Gram
GMusSLA	*Gossypium mustelinium symptomless alphasatellite*
GZ	Gezira
HR	Hypersensitive response
H_2O	Water
HCL	Hydrochloric acid
ICMV	*Indian cassava mosaic virus*
IPTG	Isopropyl-beta-D-thiogalactopyranoside.
IToLCV	*Indian tomato leaf curl virus*
Kbp	kilobase pair
LB Broth	Lauria Bertani Broth
LYMV	*Legume yellow mosaic virus*
MYMV	*Mungbean yellow mosaic virus*
MYMIV	*Mungbean yellow mosaic India virus*
MSV	Maize streak virus
mM	Mili molar
ml	Mili liter
Min	Minute
$MgCl_2$	Magnesium Chloride
Mg	Milligram
NIBGE	National Institute of Biotechnology & Genetic Engineering
NCBI	National Centre of Bioinformatics
nptII	Neomycin phosphotransferase
nos	Nopaline synthase

ng	Nanogram
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
Nm	Nano meter
NS	Nonanucleotide sequence
NW	New World
OW	Old World
ORFs	Open reading frames
OD	Optical Density
PYMV	*Potato yellow mosaic virus*
PYMLkV	*Pumpkin yellow mosaic Lucknow virus*
PepLCLV	*Pepper leaf curl Lahore virus*
PeHV	*Pepper huasteco virus*
PedLCV	*Pedilanthus leaf curl virus*
PaLCuV	*Papaya leaf curl virus*
PCR	Polymerase chain reaction.
PaLCuV	*Papaya leaf curl virus*
PVX	*Potato virus X*
PKC	Protein kinase C
PSV	*Panicum streak virus*
RaLCD	*Radish leaf curl disease*
RaLCV	*Radish leaf curl virus*
RhYMIV	*Rhynchosia yellow mosaic India virus*
rpm	Revolutions per minute
RFLP	Restriction fragment length polymorphism

RF	Replicative forms
RDR	Recombination-driven replication
RCA	Rolling circle amplification.
SCR	Satellite conserved region.
SDS	Sodium dodecyl sulfate.
SiGMV	*Sida golden mosaic virus*
SiYMoV	*Sida yellow mottle virus*
SLCCNV	*Squash leaf curl China virus*
SPLCV	*Sweet potato leaf curl virus*
ssDNA	Single-stranded DNA
SYLCV	*Solanum yellow leaf curl virus*
Tris-HCl	Tris-Hydro choleric Acid
ToYVSV	*Tomato yellow vein streak virus*
ToYMoV	*Tomato yellow mottle Virus*
ToMoV	*Tomato mottle virus*
ToLCPKV	*Tomato leaf curl Pakistan virus*
ToLCV	*Tomato leaf curl virus*
ToLCNDV	*Tomato leaf curl New Dehli virus*
ToLCNDV-IN	*Tomato leaf curl New Dehli virus India*
ToLCGV	*Tomato leaf curl Gujarat virus*
ToYLThB	*Tomato yellow leaf curl Thailand betasatellite*
ToLCNDV	*Tomato leaf curl New Delhi virus*
ToLCCMA	*Tomato leaf curl Cameroon alphasatellite*
ToLCPMV	*Tomato leaf curl Palampur virus*
ToLCuBV	*Tomato leaf curl virus Bangalore*

ToLCBDV	*Tomato leaf curl Bangladesh virus*
ToRMV	*Tomato yellow vein streak virus*
ToLCOMV	*Tomato leaf curl Oman virus*
ToYMV	*Tomato yellow mosaic virus*
ToYLCV	*Tomato yellow leaf curl virus*
ToYLCV	*Tomato yellow leaf curl virus*
TbLCB	*Tobacco leaf curl betasatellite*
ToLCV-Ind	*Tomato leaf curl virus India*
ToLCrV	*Tomato leaf crumple virus*
ToGMV	*Tomato golden mosaic virus*
TE	Tris- Ethylenediaminetetraacetic acid
T-DNA	Total DNA
Taq	Thermococcus aquaticus
TAE	Tris-Acetate EDTA
UV	Ultraviolet
US $	United States Dollar
U	Units
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-(galactopyranosidase)
°C	Degree centigrade
µl	Micro liter
µg	Micro gram
$(NH_4)_2SO_4$	Ammonium Sulphate
%	Percent
µM	Micromolar

ABSTRACT

Emergence of begomoviruses is one of the biggest limitation to the cultivation and production of crops in different countries including Pakistan. Pakistan being an agriculture country is very important for the production of wheat and cotton, and cotton is the widely grown crop in the areas of Punjab and Sindh Province. Pakistan is the fourth largest grower of cotton. The main threat to cotton production is CLCuD, caused by a complex of begomoviruses with a single known betasatellite (CLCuMB). Identification of begomoviruses on weeds and fruit plants is important to detect because these are alternative hosts and may provide shelter and inoculum source to other field crops. *Begomovirus* belongs to family *Geminiviridae* and transmits through white fly (*Bemisia tabaci*). Leaf samples of four different plants Duranta (*Duranta erecta*), Black nightshade (*Solanum nigrum),* Papaya *(Carica papaya*) and Granda (*Carissa opaca*) showing leaf curl and mosaic symptoms were collected from different areas of Faisalabad in 2015. Total DNA was extracted from symptomatic leaf samples by CTAB method. The DNA was subjected to PCR and expected band size (approx. 2.8 kb) was obtained by using a set of abutting primers. Attempts to identify the presence of betasatellite in the infected samples by using universal betasatellite primers (Beta01/Beta02) produced an approximately 1.3 kb product. DNA B was also identified in Papaya by using KTBF/KTBR and produced an approximately 2.8 kb product. Partial nucleotide sequence of DNA B from papaya showed 95% sequence identity with *Tomato leaf curl New Delhi virus* (ToLCNDV) and betasatellite from Black nightshade showed 93%identity with *Cotton leaf curl Multan betasatellite* (CLCuMB). Granda belongs to family Apyocynaceae, which shows that begomovirus not only effects solanaceae family but also non solanaceous hosts. Begomovirus was isolated first time from Granda plant. Isolation of begomovirus from alternative hosts is important because they provide shelter for recombination during the off-season.

INTRODUCTION

1.1. Geminiviruses

Geminiviruses belong to the family *Geminiviridae*, which is the family of plant viruses and first described by the Goodman in 1977. They are obligate intracellular viruses, which is feature characteristic of all viruses (Goodman 1977). All Geminiviruses have circular ssDNA genome of approximately 2.5-3.0 kb length which is encapsidated within a geminate particle (Figure: 1.1). Family *Geminiviridae* is one of the only two plant virus families that have DNA genome and replicate through DNA intermediates.

Geminiviruses are important for drastic losses in wide variety of agricultural crops like cotton, maize, tomato, wheat and cassava (Hanley-Bowdoin *et al*., 1999; Mansoor *et al*., 2003). Geminiviruses have small size of genome and overlapping genes. These are necessary for efficient coding of proteins, replication, movement and controlled expression of genes and encapsulation of virus. *Geminiviridae* was established in 1995 (Murphy et al., 1995).

Due to infection of Geminiviruses economic losses were observed worldwide during previous two decades. In Pakistan, loss of 5 billion US $ for cotton (Briddon and Markham, 2001), In Africa 1.3-2.3 billion US $ for cassava (Thresh and Cotter, 2005), In India, loss of 300 million US $ for grain legumes (Varma and Malathi, 2003) and in Florida loss of 140 million US $ for tomato (Moffat, 1999) was observed.

1.1.1 Classification of family *Geminiviridae*

The family *Geminiviridae* is divided into eight genera based on genome organization, type of host range and insect vector (Table: 1.1). Members of these genera cause an infection on a wide range of host plants, which lead to worldwide crop losses

(Morales and Anderson 2001; Mansoor 2003). Mastreviruses contain monopartite genome and are transmitted by leaf-hopper. They infect either monocotyledonous or dicotyledonous plants. Curtoviruses have monopartite genome, transmitted by leaf hopper and can infect dicotyledonous plants only. The genus *Topocuvirus* consisting of monopartite genome can infect dicotyledonous plants and are only transmitted by tree hoppers (Briddon, Bedford et al. 1996). The genus *Begomovirus* has mono- or bipartite genome, infect dicotyledonous plants only and transmitted exclusively by a single species of whitefly (*Bemisia tabaci*). *Turncortovirus* has monopartite genome and infect dicots only. *Capulavirus*, *Becurtovirus* and *Eragrovirus* contain monopartite genome and infect monocotyledonous plants only.

1.2. *Begomovirus*

Largest genus of the family *Geminiviridae* is begomovirus and it comprised of 288 species. Term begomovirus emerged from *Bean golden mosaic virus* which is now called *Bean golden yellow mosaic virus* (BGYMV). This virus is exclusively transmitted by whitefly (*Bemisia tabaci*) in persistent and circulative manner (Figure:1.2).

Begomovirus causes huge losses to dicotyledonous plants with estimated losses of several billion dollars per year (Malathi and Varma, 2003). The Begomoviruses cause many types of symptoms like leaf curling, vein yellowing, mosaic, leaf distortion, leaf enation, vein thickening and stunting of plants (Briddon and Markham, 2001). Many Recombinant viruses are also reported. For example, *Cotton leaf curl Burewala virus* is a recombinant of *Cotton leaf curl Multan* and *Cotton leaf curl Khokhran virus*. It is a significant example of breaking resistance in begomoviruses, which is due to development of new strain of Cotton leaf curl disease (CLCuD) (Amrao *et al*., 2010).

Table 1.1. Eight Genera of *Geminiviridae* (Varsani *et al.,* 2014; Roumagnac *et al.,* 2015).

Genera	Virus type Specie	Genomic Organization	Host Range
Begomovirus	Bean golden mosaic virus (BGMV)	Mono or Bipartite	Dicots
Becurtovirus	Beet curly top Iran virus *(BCTIV)*	Monopartite	Monocots
Curtovirus	Beet curly top virus *(BCTV)*	Monopartite	Dicots
Capulavirus	Alfalfa leaf curl virus *(ALCV)*	Monopartite	Monocots
Eragrovirus	Eragrostis Curvula streak virus (ECSV)	Monopartite	Monocots
Mastevirus	Maize streak virus *(MSV)*	Monopartite	Monocots
Turncurtovirus	Turnip curly top virus *(TCTV)*	Monopartite	Dicots
Topocuvirus	Tomato pseudocurly top virus *(TPCTV)*	Monopartite	Dicots

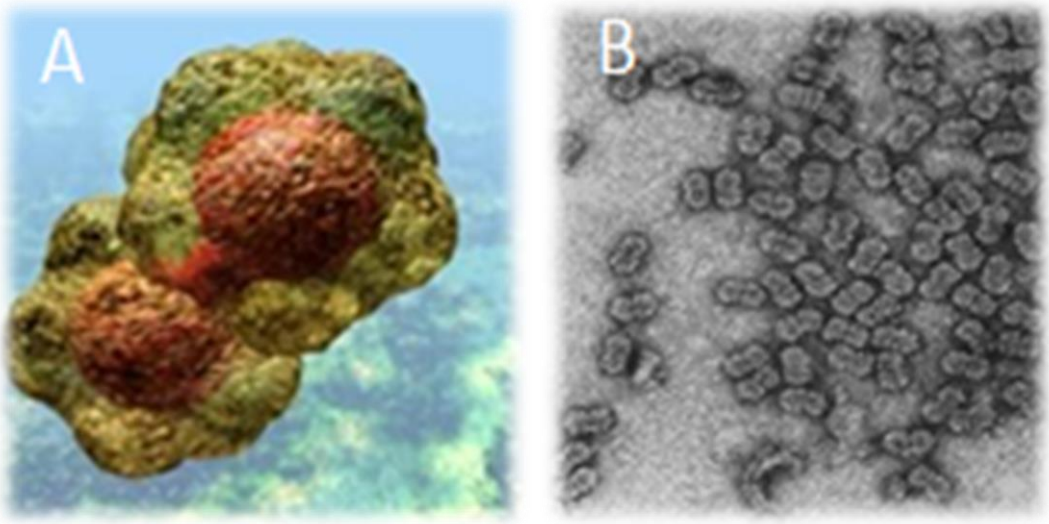

Figure1.1: Structure of a typical geminiviruses **A,** computer simulated model. **B,** scanning electron micrograph (Picture taken from internet "rybicki.wordpress.com").

Figure1.2: vector of begomovirus, Whitefly transmitting begomovirus into host plant.

1.2.1 Genome Organization of Begomovirus

Begomoviruses have single stranded and circular DNA genome, which have been categorized into two types, monopartite and bipartite on the bases of their genome organization. In bipartite begomoviruses two genomic components are present. Both of these are circular, single stranded and closed molecules of approximately 2.5kb to 2.9kb. In monopartite begomoviruses only one genomic component is present. alphasatellites and betasatellites may also present with DNA A (Briddon *et al*., 2004).

1.2.1.1 Bipartite Begomovirus

Two genomic components DNA A and DNA B are present in bipartite begomoviruses. Both genomic components have circular and single stranded DNA of approximately 2.8 kb (Figure 1.3). In bipartite begomoviruses six coding ORFs and two genomic molecules are basic identifiers. Genes on DNA A have important role in replication and encapsidation. DNA B has genes which show important role in movement. 200 nucleotides are common within the intergenic region of DNA A and DNA B (Lazarowitz, 1992).

1.2.1.2. Monopartite Begomovirus

In monopartite begomoviruses single genomic component DNA A is present, which is similar to DNA A in bipartite begomoviruses (Figure 1.4). Satellite molecules (Alphasatellite, Betasatellite) may also be present in most of monopartite and some bipartite begomoviruses. *Cotton leaf curl Multan virus* (CLCuMV) is an important example of monopartite begomovirus with an associated betasatellite. Monopartite genomes are present in curtoviruses, topocuviruses and mastreviruses. Begomoviruses may contain bipartite or monopartite genome.

1.2.2. Satellite DNA

In most monopartite begomoviruses, alphasatellites and betasatellites are present. Size of each satellite is approximately 1.4kb. Alphasatellites can replicate themselves in their host, as they have rep gene. Betasatellite cannot replicate itself and depend on DNA A for their replication. Betasatellite contribute to the symptom induction. Alphasatellites depend on helper begomovirus for transmission and systemic infection in host. Alphasatellite have no contribution in symptom induction or causing infection (Cui *et al*., 2004; Saunders *et al*., 2008).

1.2.2.1 Alphasatellites

Alphasatellites encode their own Rep protein and hence they are self-replicating components. In all Alphasatellites an A-rich region is common which is of 153-169 nucleotides (Figure 1.5 a). Alphasatellites contributes in modifying symptoms along with monopartite begomovirus and causes less DNA to accumulate, process is still unknown (Briddon *et al*., 2004; Idris *et al*., 2011).

1.2.2.2 Betasatellites

Betasatellites have a single ORF and encode βC1 protein which plays a significant role in pathogenicity determination, suppresses plant defense system and accelerates helper DNA replication (Saunders *et al*., 2004; Cui *et al*., 2005). Betasatellites depends on the Rep gene of helper virus for replication. On both satellites an A-rich region is found (Figure 1.6 b). Betasatellite have a conserved region called satellite conserved region (SCR).

SCR functions are still unknown but due to its location near the hairpin structure, it is supposed to play a role for transcription and replication in begomoviruses. The βC1 protein determines movement and host range (Saundres et al., 2000; Zhou et al., 2003).

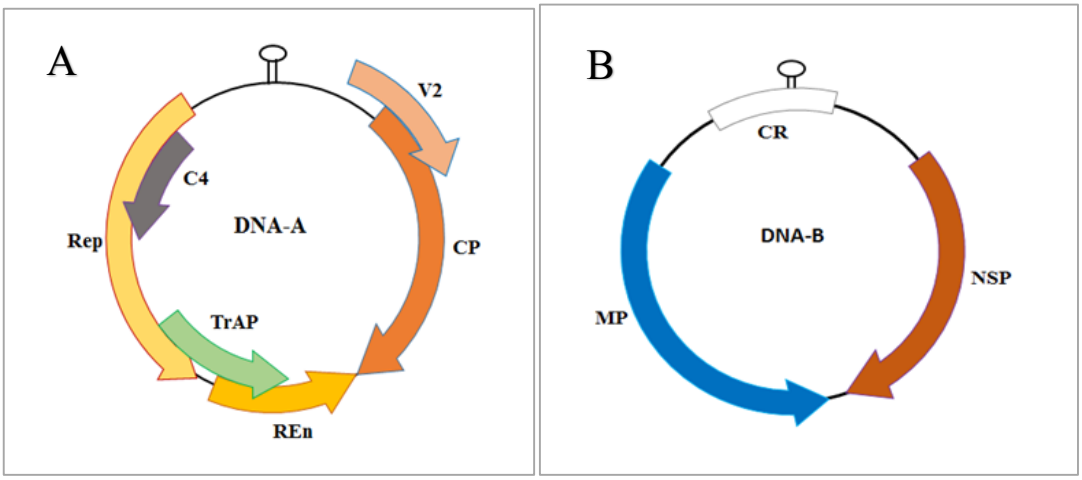

Figure 1.3: Genomics components of bipartite begomoviruses, **A,** DNA A of begomoviruses along with the genes they encode. DNA A has 6 ORFs. **B,** Genomics components DNA B of begomoviruses along with genes they encode.

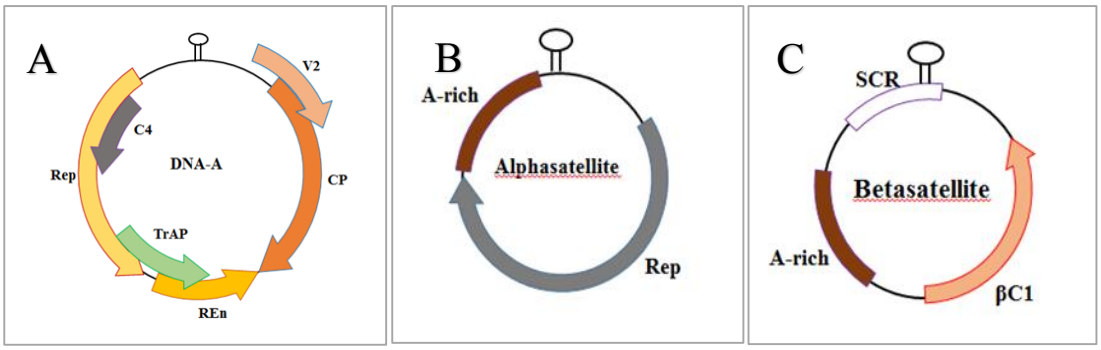

Figure 1.4: **A,** Genomic component of monopartite begomovirus DNA A. Satellite molecules, Alphasatellite and Betasatellite of begomoviruses along with the genes they encode. **B,** Alphasatellite comprises of single ORF encoding replication protein and are autonomously replicated. **C,** encodes only 1 gene βC1.

1.2.3. Proteins encoded by begomoviruses

Six open reading frames are present in DNA A of begomovirus, two in complementary strand and four in virion strand. Begomoviruses encode different proteins. Stem loop structure represents nonanucleotide which have specific sequence (TAATATTAC). This intergenic region encompasses a stem loop of conserved 30 nucleotides which serve as an origin of replication (Sunter and Bisaro, 1991). Other genes are Coat protein (CP), Pre-coat protein (*V2*), transcription activator (TrAP/*C2*), C4 protein (*C4*), a rolling-circle replication initiator (Rep) and replication enhancer (REn/*C3*). DNA B contains two proteins, movement protein (MP) and nuclear shuttle protein (NSP). Coat proteins have various functions like stability of genome, transmission via vectors, strength of infectivity and spread of virus in entire plant (Lazarowitz, 1992). In Old World begomoviruses the pre-coat protein is encoded by V2, have a role in the movement of bipartite begomoviruses (Padidam *et al*., 1996).

Rep is a multifunctional protein. Main functions are repression of its promoter (Eagle *et al*., 1994), helicase activity (Pant *et al*., 2001) and binding to iterons (Fontes *et al*., 1994). Another important protein present on DNA B component is transcriptional activator protein (TrAP), modulates movement and expression (Sunter and Bisaro, 1991). TrAP activates virion sense gene expression and is located in nucleus. It is also pathogenicity determinant and suppresses host defense pathways (Sunter *et al*., 2001).

Replication enhancer protein (REn) involved in virus replication by activating coat protein gene (Azzam *et al*., 1994). Nuclear shuttle protein (NSP) is encoded by DNA B. It restricted to the host cell nucleus, binds it to single standard DNA and moves it in cytoplasm (Pascal *et al*., 1994). Movement protein (MP) changes the anatomy of plasmodesmata and hence accelerates the movement of host cells (Pascal *et al*., 1993). MP also involved in symptom severity (Duan *et al*., 1997)

Table 1.2: Different functions performed by the proteins encoded by DNA A, DNA B and betasatellite.

Gene	Protein	Function	Refrences
AV2	Pre-coat protein(V2)	Involved in movement, act as a suppressor of RNA silencing, distribution of begomovirus by association with cytoplasmic strands and endoplasmic reticulum.	(Yadava *et al.*, 2010; Zrachya *et al.*, 2007;Rojas *et al.*, 2005; Sharma *et al.*, 2011; Sharma and Ikegami, 2010)
AV1	Coat protein (CP/V1)	Involved in insect transmission and movement in plants, encapsidation and stabilizes genome	(Hanley-Bowdoin, Settlage et al. 1999; Rojas, Hagen et al. 2005; Sharma and Ikegami 2009; Yadava, Suyal et al. 2010))
AC1/Rep	Replication associated protein (Rep)	Binding to iterons, DNA replication, interaction with plant proteins ad control cell cycle	(Hanley-Bowdoin, Settlage et al. 2004; Choudhury, Malik et al. 2006; Nash, Dallas et al. 2011).
AC2/TrAP	Transcriptional activator protein (TrAP)	Regulates expression of CP and MP, Silencing suppressor, viral transcription and suppress host defense mechanism	(Gopal *et al.*, 2007; Hussain *et al.*, 2007; Trinks *et al.*, 2005; Mubin *et al.*, 2010;
AC3/Ren	Replication enhancer protein (Ren)	Enhance viral replication, increase binding affinity of Rep, interacts with plant proteins.	(Pedersen and Hanley-Bowdoin1994;Pasumarthy, Mukherjee et al. 2011).
AC4	C4	Suppression of gene silencing, symptom determination, movement and also act as virulence factor	(Vanitharani, Chellappan et al. 2004; Pandey, Choudhury et al. 2009).
βC1	βC1	Suppresses host defense and up regulates viral replication, movement and symptom induction	Cui *et al*., 2005; Saunders *et al*., 2004; Zhou *et al*., 2003; Saeed *et al*., 2007
V1/C1	Nuclear shuttle Protein/Movement protein (NSP/MP)	NSP transport viral DNA from nucleus to cytoplasm and MP involved cell to cell movement.	(Sanderfoot, Ingham et al. 1996; Malik, Kumar et al. 2005).Jeffrey, Pooma et al. 1996) Hussain, Mansoor et al. 2007).

1.3. Importance of the Present Work

Isolation of begomovirus from weeds, shrubs and other alternative hosts is very important because these alternative hosts provide shelter and chances of recombination to different strains of begomoviruses. In Pakistan, the most agricultural province is Punjab where wheat and cotton are the most grown. The main threat to Cotton is begomoviruses. Faisalabad was selected for sample collection because it is major cotton growing region of Pakistan and called cotton belt. Begomovirus not only infect cotton crop but also many other important plants which have nutritional and medicinal values i.e Papaya *(Carica papaya*) is a fruit plant which is found in Punjab and Sindh province. Papaya is one of healthiest food in the world which is rich in vitamin A &C, folate, magnesium, potassium and calcium. Granda (*Carissa opaca*) is also an important medicinal plant found in hilly areas of Pakistan. It belongs to the family Apyocynaceae and it can be used for treatment of eye disorders and fever. Isolation of begomovirus from these samples is important to find diversity of begomoviruses due to recombination.

1.4 Aims of Study

Main focus of research is detection of begomovirus from different samples.

- Molecular characterization of a begomovirus from different samples by extraction of Total DNA from symptomatic and non-symptomatic samples, using CTAB. Performing PCR for diagnosis and amplification of begomovirus(es).
- T/A cloning of PCR product and sequencing of DNA.
- Phylogenetic relationships of characterized virus with the rest of the world

REVIEW OF LITERATURE

Begomoviruses are serious threat to crop plants reducing both quality and quantity of plant product. A lot of work has been completed on isolation of begomovirus, characterization and development of resistance against begomoviruses. Some previous work on begomoviruses is reported below.

Eui-Joon *et al.* (2016) reported seed transmission of *tomato yellow leaf curl virus* (TYLCV) from tomato first time. TYLCV is an important begomovirus of tomato plant which is transmitted by whitefly. Seed transmission is reported for RNA viruses but begomovirus transmission through seeds is reported first time. TYLCV was isolated from young plants which were germinated from fallen fruits of previous season infected plants while there was no whitefly-mediated transmission. TYLCV-Israel was also found in seeds. TYLCV-IL was transmitted by whitefly and agro-inoculation. Transmission rate through whitefly was 84.62% and through agro-inoculation 80.77%. Seed transmitted tomato plants, healthy tomato plants and non viruliferous whiteflies were kept in cages and then observed that healthy plants were also infected. Hence seed transmitted begomovirus can also act as inoculum for healthy plant. It was first report of TYLCV through seeds.

Karyana *et al.* (2016) isolated begomoviruses and their associated satellites from whiteflies. This metagenomics survey was done from different areas of California (USA), Israel, Guatemala, Puerto Rico and Spain. Alphasatellites and betasatellites, were identified in Puerto Rico, Israel, and Guatemala. Novel alphasatellites were detected in samples from Puerto Rico and Guatemala, which led to the depiction of a phylogenetic clade (DNA-3-type alphasatellites). In addition, a diversity of small satellite DNAs (~640–750 nucleotides) which were similar to satellites linked with begomoviruses which infects Ipomoea spp. were detected in Spain and Puerto Rico.

Another class of satellite molecules called gamma satellites of size 1kb was also discovered. Hence it shows satellite DNA's have great genetic diversity.

Sohrab *et al.* (2016) developed resistant transgenic plants of Cotton by using βC1 gene. *Cotton leaf curl virus* is a major threat of Cotton crop in major cotton growing areas. The βC1gene was used to develop transgenic cotton (*Gossypium hirsutum* cv.Coker 310) plants. For this purpose, antisense orientation gene which was drived by nos (nopaline synthase) terminator, Cauliflower mosaic virus-35S promoter and mediated by somatic embryogenesis system and Agrobacterium tumefaciens transformation. Southern blot hybridization and polymerase chain reaction (PCR) was used for molecular confirmation. Resulted plants remain symptomless through their growth period. Hence they were proved to be resistant for CLCuV.

Srivastava *et al.* (2016) reported first time *Cotton leaf curl Multan virus* (CLCuMV) and *Cotton leaf curl Multan betasatellite* (CLCuMB) from india. After observing symptoms (curling, leaf enation, vein thickening) on a perennial ornamental plant Hibiscus (*Hibiscus rosa-sinensis*), four healthy and 13 diseased samples were collected. Presence of begomovirus was confirmed by using C-P Forward (5′-GCATCTGCAGGCCCACATYGTCTTYCCNGT-3′) and CP- reverse (5′-AATACTGCAGGGCTTYCTRTACATRGG-3′). Then full length virus (2.7kb) was amplified by using Rolling circle mechanism. Nucleotide sequence showed that virus had close phylogenetic relationship and 90-93% identity with *Cotton leaf curl Multan virus* (CLCuMV). Betasatellite was checked by using primers Beta-01(5′-AGCCTTAGCTACGCCGGAGC-3′) and Beta-02 (5′-GCTGCGTAGCGTAGAGGTTT-3′). This virus showed close phylogenetic relationship and nucleotide identity (98%) with *Cotton leaf curl Multan betasatellite* (CLCuMB). Natural occurrence of CLCuMB and CLCuMV has reported from China

and *Cotton leaf curl Burewala virus* (CLCuBV) with CLCuMB from Pakistan. Whoever it was first report from India.

Dhriti *et al*. (2015) reported the damages due to betasatellite on functional and structural integrity of chloroplast which causes symptom formation and photosynthesis inhibition. To understand the interaction between host and betasatellite to cause infection was studied thoroughly. For this purpose, systematic study on *Nicotiana benthamiana* plant on which Radish leaf curl disease (RaLCB) were induced through betasatellite. βC1 protein are present on chloroplast of host. RaLCB on which βC1is not present have no effect on chloroplast structure and showed no symptoms. Betasatellites also suppress the genes expressions which are involved in chlorophyll biosynthesis, plastid translocation and chloroplast development. Genes that were involved in chlorophyll damage remain unaffected. The plastoquinol pool size and numbers of active reaction centers in leaves were severely reduced due to infection of betasatellite hence showing vein clearing symptoms. This was the first report of chloroplast damage due to DNA virus which damaged the structure and function and induced symptoms.

Brown *et al*. (2015) revised the taxonomy of begomoviruses based on sequence comparison. Begomoviruses are emergent pathogens from different tropical and subtropical region of all over the world. Begomoviruses have small DNA genome, easily cloned and low cost sequencing, that's why large number of begomoviruses has been sequenced. Most of these viruses are isolated from cultivated crops and plants which are small and phylogenetically unrepresentative. Another problem of assigning species to new viruses has highlighted. On the bases of 3123 full length genome analysis of begomovirus (DNA A components) available on public database, a new set of classification and guidelines was proposed. This Guideline considers biological

characteristics on genus level and results were obtained by using Sequence Demarcation Tool and standardized classification tool. These guidelines are dependable with the newly published recommendations for the genera Curtovirus and Mastrevirus of the family Geminiviridae. Genome-wide identities of 91 % for species and 94 % for strain were proposed for Begomoviruses classification.

Tahir *et al*. (2015) isolated *Ageratum enation virus* which is an important begomovirus of weeds and infect crops. Two infected plant samples *Sonchus oleraceus* and *Brassica rapa var. rapa* from Nepal and Pakistan were collected. Four Full length clones of begomovirus and three betasatellite clones were isolated. Sequences of these begomoviruses shown greater than 89.1 % identity with *Ageratum enation virus* (AEV) and hence they were isolates of 26AEV. Sequences of three betasatellites shown 90 % identity with Ageratum yellow leaf curl betasatellite (AYLCB) and hence they were isolates of 18AYLCB. For these virus strains two names were suggested. Isolates from India, Pakistan and Nepal were suggested Nepal strain and isolates from only India were suggested India strain. Infectivity of these clones with their associated betasatellite was checked by inoculation of Agrobacterium to *Nicotiana benthamiana, Solanum lycopersicon, N tabacum* and *A. conyzoides. N. benthamiana* plants that were infected with AEV or only betasatellite remained symptomless. *N. benthamiana* plants that were infected with AEV and associated betasatellite showed clear symptoms. Hence it was proved that AEV is a common virus of weeds and that can infect crops with associated betasatellite. Betasatellite show important role in symptom induction and make etiology more complex than only virus.

Srivastava *et al.* (2015) reported new species of begomovirus named Jatropha mosaic Lucknow virus. Symptoms like yellow mosaic on Jatropha multifidi, Jatropha podagrica and Jatropha integerrima ornamental plants in Lucknow, India were

observed. Monopartite begomovirus were detected through PCR by using universal primers. Seven ORFs of DNA-A containing 2.844 kb were amplified through an important mechanism, rolling circle amplification (RCA). After cloning when sequencing of RCA products was done, it showed 94-95% identity with one another and 61% identity was shown with Jatropha leaf curl virus (JLCV). Because that was first report of new species of begomoviruses so the name Jatropha mosaic Lucknow virus (JMLV) was suggested.

Briddon *et al.* (2014) reported the genetic changes in begomovirus which break the resistance in Cotton crop. In 1990's new Cotton varieties were introduced that were resistance to begomoviruses. But that resistance was also break by *Cotton leaf curl Burewala virus* (CLCuBuV). It was reported that the transcriptional activator gene (TrAP) was not intact in CLCuBuV. TrAP gene is very important in host virus interaction and it was present in all begomoviruses and gave gene product of ~134 amino acids. In recent studies the difference between CLCuBuV and earlier viruses has been explained which causes the resistance break in Cotton crop.

Shuja *et al.* (2014) isolated new species of *Cotton leaf cul burewala virus* (CLCuBuV). Virus and its associated betasatellite were amplified through PCR by using BurNF/BurNR for full length virus and Beta01/Beta02 for betasatellite. When analysis was done it was seen that virus contained sequence fragments from two different viruses, CLCuKoV and CLCuMuV. Virus didn't have intact TrAP and instead of encoding 35 amino acids it was encoding 39 amino acids. Betasatellite was an isolate of CLCuMB but when analysis was done deeply it was revealed that it was isolate of CLCuMBBur which was involved to break resistance of Cotton leaf curl disease.

Marval *et al.* (2014) reported in-silico analysis of Ageratum enation viruse (AEV) which was isolated from Merigold, ornamental plant. On the bases of similarity

in sequence alignment, six recombination events were shown by AEV using Recombination Determination program (RDP). According to first recombination the sequences were from CrYVMV and PedLCV, coordination between starting and ending nucleotide was at 2665-287. Second recombination was shown between CrYVMV and TLCuKV, coordination between nucleotides was at 388-935. Third recombination was shown between TLCuRV and AEV, coordination between nucleotides was at 938-1042. Fourth recombination was between TLCuRV and AEV, coordination between nucleotides was at 1042-1200. Fifth recombination was between minor CrYVMV and major parent TbCuSV, coordination between nucleotides was at 1237-1366. Final recombination was between *bhendi yellow vein Bhubhaneswar virus* and *euphorbia leaf curl virus* at 22074-2210 nucleotide position.

Lake *et al*. (2013) isolated recombinant virus which was infecting Okra from West Africa. That Okra plant virus was recombinant because of having sequence similarity with OYCrY and CLCuGeV. The nucleotide sequence identity with CLOCuGeV was 87.8%. Hence that was proposed as new species of begomovirus. From those infected plants different strains of Alphasatellites and betasatellites were also isolated. Sequance identity of CLCuGeV betasatellite was 93.3% with CLCuGeV and hence named CLCuGeB. Identity of alphasatellite was 97.3% and named CLCuGeA. Another alphasatellite also showed 95.2% identity with okra leaf curl Burkina Faso alphasatellite and was named OLCuBFA. Hence it proved that okra plants have satellites complexes and variety of begomoviruses in Africa.

Shahid *et al*., (2013) collected papaya (Carica papaya) leaf samples which were infected and showing symptoms like vein thickening, leaf curling, reduction in leaf size and vein darkening from Rampur, Nepal. Full length monopartite, DNA alphasatellite and DNA betasatellite were amplified by using PCR and cloned. They showed more

than 99% sequence identity with an isolate of Ageratum Yellow Vein Virus (AYVV). DNA betasatellite complete sequence was identified which showed more than 89% identity with Indonesian isolate of Tomato leaf curl java betasatellite. DNA alphasatellite showed 92% genome identity with Sida yellow vein china alphasatellite.

Gaba *et al.* (2013) inoculated virus through particle bombardment which was an effective method. PCR products and DNA of full length infective clones are inoculated in virus for transcription (in vitro) in this technique. Under vacuum bombardment, soft leaves are not inoculated usually. So rolling circle amplification (RCA) for begomoviruses inoculation is used which is followed by vacuum less bombardment.

Mubin *et al.* (2012) isolated begomovirus from *Xanthium strumarium* with it associated betasatellite and aplhasatellite. One isolate showed identity with *Cotton leaf curl Burewala virus* (CLCuBuV) and second virus showed identity with *Tomato leaf curl Gujarat virus* (ToLCGV). That begomovirus was reported as bipartite previously. But there was no other genomic component (DNA B) in *X. strumarium*. Another betasatellite, *Tomato yellow leaf curl Thailand betasatellite* was isolated, first time that satellite was reported from Pakistan, but alphasatellite that was identified recently in many weeds and potato as a potato leaf curl alphasatellite was linked with infection of *X. strumarium*.

Fiallo-Olivé *et al.* (2012) conducted many surveys for search of begomoviruses reservoirs. They identified a bipartite begomovirus in Cuba from infected plants of *sida rhombifolia* during last years. Those plants were infected by *Sida yellow mottle virus* (SiYMoV), after sequencing they observed that DNA A was of 2622 nucleotides (nt) and showed 87.6% identity with *sida golden mosaic virus* (SiGMV). In Florida, snap beans (*Phaseolus vulgaris*) were infected by this virus. DNA B was of 2600 nucleotides

(nt) in length and it showed 75.1% n identity with a virus named *corchorus yellow spot virus* (CoYSV).

Hina *et al.* (2012) screened cotton plants which were diseased and checked the presence of begomoviruse which was infecting Cotton crop. The product which was amplified was cloned and then sequenced. The sequence analysis showed that *cotton leaf curl Burewala virus* (CLCuBuV) was the major cause of cotton leaf curl disease. A single virus was infecting the most economically important crops in all over the World which was CLCuBuV. Isolates were checked through sequence comparison and they showed 98.9% to 99.55% maximum similarities with published sequences of CLCuBuV but it was surprising that intact TrAP which was present in all begomoviruses consistently was not present in one of isolate from cotton.

Tahir and Briddon (2011) collected leaf samples of *Ageratum conyzoides* (ACL) and *Sonchusoleraceous* (SOL) which were showing symptoms of begomoviruses. Genomic sequences of full length begomoviruses were isolated from ACL and SOL and they showed 92.5% to 98.7% sequence identity of nucleotides to each other. It was also observed that DNA A of isolates ACL and SOL shared 92.2% to 97.8% nucleotide identity with Nepalese isolate (CAN) AEN [Nepal: 01], On the other hand betasatellite was also isolated from ACL and SOL, which shared 93.5% to 96.0% nucleotide sequence identity and shared 80.9% to 96.0% identity with another betasatellite named *Ageratum yellow leaf curl betasatellite* (AYLCuB). That was first report of AEV in Pakistan. Partial repeat constructs were also produced for agro inoculation. Those constructs were produced from isolates ACL and SOL with associated betasatellite. Then those constructs were inoculated to *Solanumlycopersiconand Ageratum conyzoide, Nicotianatabacum,*

Nicotianabenthamiana, and Koch's postulates were observed for AEV which was causing the disease in *Ageratum conyzoidesare*.

Tahir and Mansoor (2011) studied induction of symptoms of typical begomovirus by PVX vector. Symptoms were induced in *N. benthamiana* through Chili leaf curl betasatellite (ChLCB). It was also observed that expression of *β*C1 developed severe curling of leaves and leaf enations by PVX vector. Those symptoms were same as induced by Cotton leaf curl Multan betasatellite (CLCuMB). Finally it was concluded that *β*C1 of ChLCB showed same symptoms of CLCV phenotypically in the existence of PVX vector.

Hussain *et al.* (2011) identified begomovirus complete sequences from Croton (*bonp-landianus*) and associated betasatellite. The sequence analysis showed 88.9% nucleotide identity with Papaya leaf curl virus. That was then characterized a new species of begomovirus and the new name *Croton yellow vein virus* (CYVV) was proposed for it. While betasatellite nucleotide identity was showed 82-98.4% with six sequences, that also showed 48.7-52.5% sequence identity with *tomato leaf curl Joydebpur betasatellite.* Then it was proposed that betasatellite and other six sequences which were present in the database were isolate of that newly isolated virus. The name which was proposed was *Croton yellow vein mosaic betasatellite* (CroYVMB).

Tahir *et al.*(2011) collected cotton samples from Southern Pakistan and isolated *cotton leaf curl Gezira virus* (CLCuGV) from symptomatic leaves. Another begomovirus which was associated with CLCuD in Africa was identified. Associated *Cotton leaf curl Gezira betasatellite* was not found but two Asian betasatellites were identified, *Chilli leaf curl betasatellite* (ChLCB) and *Cotton leaf curl Multan betasatellite* (CLCuMB). It was also observed that in *N. benthamiana*, agro-inoculation

from CLCuGV was retained with both ChLCB and CLCuMB. But it was observed that typical enation was induced by CLCuGV in the occurrence of CLCuMB.

Jyothsna *et al.* (2011) cloned yellow mosaic virus from a common weed *Rhynchosia minima.* Length of DNA A component was 2727 nucleotides and length of DNA B was 2679 nucleotides. That was an Old World begomovirus having bipartite genome. When sequence analysis was done, virus from *R. minima* showed 84% nucleotide identity with (*Velvet bean severe mosaic virus*. While with other viruses infecting legumes showed < 73% identity. Name proposed for that virus was *Rhynchosia yellow mosaic India virus*.

Tahir *et al.* (2010a) cloned begomoviruses from infected samples of Capsicum from central Pakistan. These begomoviruses were distinct from earlier characterized viruses. They recommended this as a new species name suggested was *Pepper leaf curl Lahore virus* (PepLCLV). The resulted sequence of PepLCLV was recombinant of tow viruses, *Chili leaf curl virus* (ChiLCV) and *Papaya leaf curl virus*.Associated betasatellite was *Chili leaf curl betasatellite* (ChLCB) in Capsicum. That was reported first time in Chilli but previously it has been reported to infect Potato in Pakistan.

Tahir *et al.* (2010b) collected leaf samples of *Momordica charantia* vegetable around Lahore in 2004. Both healthy and infected (yellow vein) leaves were collected. Full length begomoviruses from samples were cloned and sequenced. Complete nucleotide sequences showed the sequence similarity with Old world begomovirus genes. Nucleotides sequence identity of DNA A was 86.9 % with *Tomato leaf curl New Delhi virus* (ToLCNDV), the name proposed for that was *Bitter gourd yellow vein virus* (BGYVV). There was Inter-specific recombination in *tomato leaf curl Bangladesh virus* (ToLCBDV) and ToLCNDV resulted virus was BGYVV. The DNA-B component showed 97.2% nucleotide similarity with *Squash leaf curl China virus*, Indian strain.

Ilyas *et al*. (2010) characterized 44 components (23 DNA A, 2 betasatellite and 19 DNA B) from legume samples. It was resulted that in legumes only *Mungbean yellow mosaic India virus* (MYMIV) is present in Pakistan. MYMIV was identified only in weed and then first time observed that it combined with *Legume yellow mosaic viruses* (LYMVs). It was also observed that interspecific recombination was shown between LYMVs and begomovirus, which were not infecting legumes. It was concluded that recombination of non-legume viruses or their interaction with associated particles caused more effective strains of viruses, increase host range and pathogenicity.

Tahir *et al.* (2009) isolated begomovirus from *Pedilanthus tithymaloides* (Redbird flower) which is an ornamental plant showing enation and leaf curl symptoms. Sequence analysis showed 90.3% nucleotide identity with begomovirus that was previously isolated from tomato and showed 86.3% nucleotide identity with *Radish leaf curl virus.* Associated betasatellite showed 97% sequence identity with *Tobacco leaf curls betasatellite*. That was reported first time and name suggested for that was *Pedilanthus leaf curl virus* (PedLCV).

Ilyas *et al.* (2009) isolated *legume yellow mosaic viruses* (LYMVs) from weed (*Rhynchosia minima*) from Pakistan. When sequence analysis was done it shared 69.5% nucleotides sequence identity with Mungbean yellow mosaic virus. Name suggested for that virus was *Rhynchosia yellow mosaic virus* (RhYMV). Infectious cloned were made and it was checked that clones were not infectious when injected in *N. benthamiana*. In soybean infection depended on varieties. In var. Ig6 mild infection occurred, while in var. FS-85 severe symptoms occurred that caused necrosis, showed hypersensitive response. Hence it was proved that resistance was present against RhYMV in germplasm of soybean.

Briddon *et al*. (2008) compared all betasatellites which were available and showed that the minimum no. of pairs shared sequence identity 78% which were suggested species distinction threshold for all betasatellite. That threshold rate was present in fifty one different betasatellite species. Naming conversion system was also proposed for satellites like all geminiviruses.

Haider *et al*. (2008) isolated begomovirus from ornamental plant (*Vinca minor L*) from School of Biological Sciences (SBS), Lahore, Pakistan. Both healthy and diseased samples were collected and presence of begomovirus was confirmed with polymerase chain reaction (PCR) by using universal primers (Highly conserved for CP). When sequence analysis was done, Virus showed 93% nucleotide sequence identity with *Pedilanthus leaf curl virus* (PedLCV). That was reported first time in Pakistan.

Haider *et al.* (2007) isolated begomovirus from shrub plant *Vinca minor L* (common name periwinkle). Infected samples were amplified through PCR and then cloned and sequenced. Sequence analysis showed highest 90% nucleotide sequence identity with DNA-A of *Tomoato leaf curl Joydebpur virus*.

Tahir *et al.* (2006) isolated begomovirus from ornamental plant (*Duranta repens*) from Multan, Pakistan. Amplification was done through PCR by using specific primers which were designed by using conserved regions of Rep protein and Coat protein (CP). When sequence analysis was done, DNA A showed 91% nucleotide sequence identity with *croton yellow vein mosaic virus*. DNA B showed 94% sequence identity with movement protein of *Tomato leaf curl New Dehli virus*. It was concluded that *D. repens* leaf curl disease is associated with bipartite begomovirus.

Bull *et al*. (2006) showed that begomovirus in Kenya causing Cassava mosaic disease (CMD) was diverse genetically. Through Restriction Fragment length

Polymerase genetic diversity of begomoviruses was checked. When full length clones were sequenced, they showed the presence of *East African cassava mosaic virus* and *East African cassava mosaic Zanzibar virus.* It was suggested new species of begomovirus and named *East African cassava mosaic Kenya virus*. DNA B was less diverse. This showed that viral movement supported diversification.

Tahir *et al*. (2005a) isolated begomovirus first time from Bitter gourd. Samples were collected from Pakistan and *tomato leaf curl New Delhi virus* was isolated from infected samples. CP gene was amplified through PCR by using universal primers designed for CP. When sequence analysis was done it shown 95% identity with *Tomato leaf curl New Delhi Virus*.

Tahir *et al*. (2005b) isolated a monopartite virus, *Bell pepper leaf curl virus* from infected samples of bell pepper. Infected samples showing leaf curl symptoms were amplified through PCR by using CP primers. When sequence analysis was done maximum sequence identity was shown with tomato leaf curl New Delhi Virus.

Tahir and Haider (2005) isolated new strain of *Tomato leaf curl New Delhi virus* (ToLCNDV) from bitter gourd (*Momordica charantia*) samples collected from Lahore, Pakistan. Yellow blotch symptomatic plants were collected and amplified through PCR by using specially designed primers of Coat protein (CP). When sequence analysis was done it showed 95% nucleotide sequence identity with *Tomato leaf curl New Delhi virus* (ToLCNDV). Hence it was concluded that virus is strain of (ToLCNDV) in *M. charantia.*

Gutierrez *et al*. (2004) showed that proteins of geminivirus interact with DNA replication factors and proteins of host cell cycle. Yeast two hybrid assays were used to study these interactions. It was seen that host plant protein retinoblastoma related (RBR) interact with Rep A protein of wheat dwarf virus. It was also seen that Rep

protein of virus did not affect RBR interaction. But the C terminal of Rep protein is deleted which perform same function like Rep protein suggests by interaction of that domain with RBR binding motif (LXCXE), that can be confirmed by analysis of secondary structure.

Briddon *et al*. (2004) studied diversity of betasatellite found in South East and East Asia. *Okra leaf curl virus* and *Cotton leaf curl virus* were isolate from Pakistan while *Ageratum yellow vein virus* (AYVV) was isolated from Singapore. For study of diversity 17 β satellites were collected, isolated and then cloned. DNA β was present in all samples, only β satellite was absent in two samples which were collected from East Asia. Then it was resulted that in both architecture and sequence β satellites are conserved.

Mansoor *et al*. (2003) concluded that betasatellite is compulsory for accumulation of helper virus and symptoms expression in a review paper. They are regulator elements and highly conserved region. Around Asia and Africa, satellites have been isolated causing diseases in vegetables weeds, fiber crops and ornamental plants. Their transmission totally depends on vector (whitefly). It was seen that disease complexes were causing serious threat in all over the world.

Briddon (2003) observed that begomovirus are affecting cotton and many *Malvaceous* species. Many symptoms were observed on cotton like leaf enation, curling of leaf and stunt growth. In cotton, for induction of symptoms betasatellite is compulsory. But for symptom development, additional DNA component like satellite is not compulsory. Insecticide treatment against whitefly, avoiding ratoon crop, using resistant varieties and infected plants removal can control the disease.

Briddon *et al.* (2002) identified hairpin structure of betasatellite which showed the highly conserved region of betasatellite. Butting primers were also designed to amplify full length betasatellite component through PCR from infected samples.

Idris and Brown (2001) extracted DNA from symptomatic leaves of hollyhock, cotton, sida spp. and okra from Gezira (GZ) and Shambat (SH) Sudan. Then samples were amplified through PCR and cloned. When analysis was done 92.1-97% nucleotide sequence identity was shown by DNA betasatellite, except DNA satellite of hollyhock-SH, which showed 43.1- 44.9% nucleotide sequence identity to others. All those DNAs were showing 36.4 -38.5% nt identity to *Ageratum Yellow Vein Virus*-associated satellite. It was concluded that DNAs satellite were causing pathogenicity and play important role in symptoms development.

MATERIALS AND METHODS

3.1 Samples Collection

Different samples showing typical begomoviral symptoms like vein thickening, mosaic and leaf curl have been collected from different areas of Faisalabad, Pakistan. These samples were cleaned properly, well labeled and stored at -20 °C until further processing.

3.2 DNA Isolation

Infected plant sample DNA was isolated by using of protocol given by (Doyle 1990). Approximately, 1 gram of infected plant sample tissue was weighed in a digital weighing balance. The infected plant tissue was ground in mortar and pestle thoroughly by using liquid nitrogen until it made a fine powder. A small amount of CTAB isolation buffer (2% Cetyl trimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, and 0.2 % β-mercaptoethanol) which was preheated upto 60 °C was added into the mortar to make the slurry. The resulting slurry was transferred into a labeled blue capped tube (50 milliliter) and made a total volume of 25ml with CTAB buffer. The mixture was well mixed and then incubated at 60 °C on a shaker for 30 minutes.

This mixture was allowed to cool in a fume hood for 3 minutes and extracted one time with same volume (25ml) of cloroform-isoamyl alcohol (24:1). The slurry was mixed well and poured into two 50 ml (Sterile) falcon tubes. The resulted slurry was well mixed and filled into two 50 ml (sterile) tubes. Tubes were centrifuged (Eppendorf centrifuge 5804 R) at 12000g at ambient temperature for 15 minutes to separate the phases. The upper portion of aqueous phase approximately 13 ml was removed and transferred to fresh 50 ml tube. Equal volume of ice cold isopropanol was added to the supernatant.

The tubes were incubated at room temperature for overnight and then centrifuged at 12000g for 15 minutes. The supernatant was poured off carefully and pellet was washed with 1 ml of cold wash buffer (76% ethanol, 10mM ammonium acetate) . Then pellet was transferred to 1.5 ml Eppendorf tube, agitated gently and incubated at room temperature for 20 minutes. Eppendorf tube was centrifuged at 5976 g for 5 minutes. The supernatant was removed and the pellet was dried in a 37°C incubator for 30-60 minutes. Finally the pellet was resuspended in 1ml TE buffer (10mM Tris-HCl, 1mM EDTA) (Doyle JJ & Doyle JL, 1990) and stored at -20° C.

3.3 Analysis of DNA

DNA analysis was done by following two methods.

3.3.1. Agarose Gel Electrophoresis

The isolated DNA quality was analyzed on 1% Agarose gel. 0.4 grams (g) agarose was heated in 40 ml of 1x TAE (5mM Glacial Acetic acid, 1mM EDTA, 25 mM Tris, pH 8.0) in a microwave oven in a reagent bottle for 2 minutes. Finally, a transparent solution was obtained. After cooling the solution 0.01% ethidium bromide was added. The solution was poured into a gel caster. Require comb was selected. When the gel set completely at ambient temperature, it was placed in an electrophoresis tanker contained sufficient amount of 1X TAE buffer and comb was removed carefully. DNA samples were dissolved with 10X loading buffer (0.4% xylene cyanol FF, 25 % Ficol and 0.4% bromophenol blue). The DNA samples and standard markers [Fermentas 1 kilo base pair (kb)] were loaded into 1X TAE solidified agarose gel wells. Gel was allowed to run at 80 V for 45 minutes. The DNA bands were visualized using Dolphin-Doc Plus Image System.

3.3.2. Quantification of DNA

DNA was quantified spectrophotometrically by taking wavelength of 260 nm and using water as blank sample. The quantities of DNA were calculated according to this formula.

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

3.4. Polymerase Chain Reaction (PCR) Amplification of DNA

A total volume of 50 µl PCR reaction was made for all samples. The reaction consists of 10X Taq buffer [$(NH_4)_2SO_4$ and 25 mM $MgCl_2$] 2mM dNTP's, 100µM primers (both forward and reverse), 1µl Taq Polymerase and DNA depending upon its concentration. Amplification, primers β01 and β02 were used (*Briddon et al*., 2002). PCR reaction was placed in Dual Block PCR machines (ESCO, Swift.Max.Pro) and following PCR reaction was set for the amplification of specified region (Figure: 3.2, 3.3)

Then whole PCR product was then loaded on 1% agarose gel and allowed to run for 45 minutes at 80 Volts.

3.5. Gene Elution from PCR product

DNA was isolated from the PCR product using Silica Bead Gel Extraction Kit for DNA (Fermentas) according to manufacturer's instruction. The amplified PCR product of required size was cut from gel using clean razor blade and shifted to pre-weighed 1.5 ml Eppendorf tube. In Eppendorf tube, 3:1 volume of binding buffer was added and thoroughly mixed. It was incubated for 15 minutes at 55^{o}C in heat block, then in this mixture 8 µl of silica bead powder was added and vortexed to mix it properly. Then it was incubated for 5 minutes at 55^{o}C. The reaction mixture was centrifuged at 12000rpm for 2 min to get a pellet.

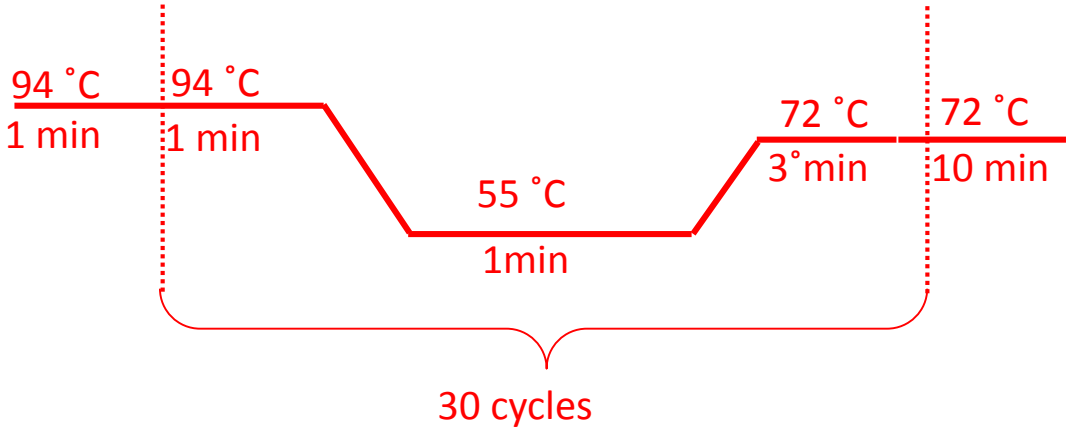

Figure 3.2: PCR conditions used for amplification of required fragments with SONAF/SONAR, BurNF/BurNR and KTBF/KTBR primers.

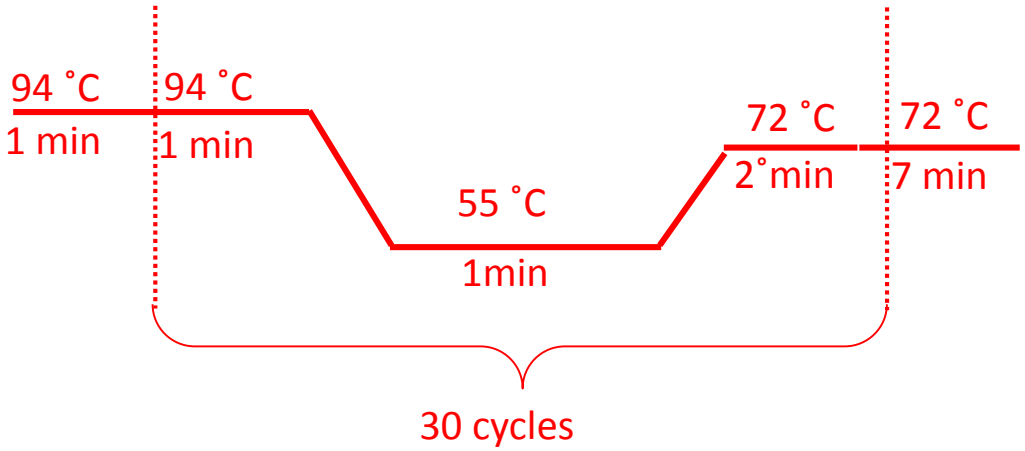

Figure 3.3: PCR conditions used for amplification of betasatellite with β01/β02 primers. The difference is in primary extension time.

The supernatant was discarded and pellet was resuspended in 600 µl diluted cold washing buffer which was provided with kit. It was centrifuged again at 12000rpm for 1 min and supernatant was discarded. Same washing step was repeated. Then pellet was dried and resuspended in 25 µl of 1X TE buffer and again incubated for 5 minutes at 55ºC. It was again centrifuged at 120000 rpm for 2 min. Then supernatant was transferred in a fresh labeled Eppendorf tube and this gene clean product was used for ligation.

3.6. T/A Cloning into pTZ57R/T vector

The purified DNA product was ligated with pTZ57R/T vector. InsTAclone™ PCR Cloning Kit, Fermentas was used, by using protocol of manufacturer. Unique restriction sites are present on vector. In an Eppendorf 30 µl reaction was prepared containing Vector pTZ57R/T (3 µl), 5X Ligation Buffer (6 µl), PCR product (20µl), T4 DNA Ligase (1 µl). The reaction was incubated overnight in a water bath at 4 ^{0}C and processed next day for transformation in already prepared DH5α competent cells.

3.7. Preparation of DH5α competent cells

Competent cells were prepared by *Escherichia coli* DH5α strain. Single colony of freshly grown *Escherichia coli* DH5α strain was taken and inoculated in 10 ml of Lauria Bertani 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) in sterile condition and incubated at 37°C overnight at continuous shaking. Culture was refreshed by transferring 2ml in 50 ml LB broth next day. Refreshed culture was again incubated at 37ºC for 2 hours in a shaking incubator. Then culture was transferred into a 50ml falcon tube in sterile condition and cold on ice for 10 minutes. Centrifugation was done at 4000rpm for 15 minutes at 4ºC. Supernatant was removed and cells were suspended in 10 ml ice cold 50 mM $CaCl_2$ solution (sterile) and left on ice for 15 minutes. The suspension was again

centrifuged at 4000rpm for 15 minutes at 4°C. The supernatant was again discarded, Ice cold 2 ml sterile 50mM $CaCl_2$ solution was taken again and cells were resuspended. Aliquots of 50 µl of cells were made in Eppendorf tubes and stored at -80°C for further use.

3.8. Transformation of DH5α Cells

E. coli cells were transformed by method which was described by Sambrook and Russell (2001). Heat shock method was used for transformation. 30µl of ligated product was transferred in Eppendorf tubes containing DH5**α** competent cells and mix gently. The reaction was incubated for 30 minutes on ice. Then reaction mixture was heat shocked at 42°C on heat block for 2 min and allowed to chill on ice for 2 minutes. After that 800 ul of LB medium was added to the mixture and incubated for 1-3 hours at 37°C. Transformation mixture was spread on LB media plates having nutrient agar as solidifying agent, ampicillin (added to a final concentration of 100ug/ml, 20 µl of 5-bromo-4-chloro-3-indolyl-β- galactopyranosidase (X-gal) (stock; 20 mg/ ml), 20 µl of isopropyl-beta-D-thiogalactopyranoside (IPTG) (stock ;100mM/ ml) for blue/white screening of colonies. Plates were incubated for overnight at 37°C.

3.9. Blue and White Colonies Selection

3.9.1 Isolation of Plasmid DNA

White colonies were inoculated in different flasks (one at a time) having 10 ml LB medium and ampi (100 µg / ml). Incubate in incubator at 37 °C for 16 hours with continuous shaking in water bath. DNA plasmids were isolated by using of Alkaline Lysis Method, after 16 hours of growth. In centrifuge tubes 5 ml of overnight culture were taken for plasmid preparation. Centrifugation was done at 14800 rpm for 1 min and the supernatant was removed. Pellet was resuspended in 100 µl solution A containing (25 mM Tris-HCl, 10 mM EDTA and pH maintained at 8.0). Then 200 µl

solution B (1M NaOH, 10 % SDS) was added and mixed by inverting the tubes gently. After that 150 µl of solution C (28.5 ml H_2O, pH 4.8, 6 ml of 3 M potassium acetate, 11.5 ml acetic acid) were added, Vortexed gently to mix. Then again centrifuged at full speed for 10 minutes, supernatant was transferred in a new tube and 1 ml of 100% chilled ethanol was added to the supernatant. After that incubated for 1 hour and centrifuged for 10 min at full speed. Supernatant was discarded and the pellet was dried at 37^0C. Then pellet was resuspended in 40 µl of RNase A water (containing 20µg/ml RNase A). Then extracted plasmid was checked by running 3µl of plasmid DNA mixed with loading dye in 1 % agarose gel (Sambrook & Russell, 2001).

3.9.2 Confirmation using restriction analysis

Clones were confirmed by digestion with *Apa*1, *Nco*1*, Kpn*1*, Pst*1 and *Eco*R1 (Fermentas), total volume of 30 µl using 5U of each enzyme along with recommended Buffer and 5µL of plasmid. Finally, these mixtures were incubated for 3 to 5 hours at 37 °C and the digestion products were confirmed by 1 % agarose gel.

3.10. Sequence and phylogenetic analysis

Plasmid isolation for sequencing was done via kit (**FavorPrep™ Nucleic Acid Extraction**) according to manufacturer's protocol. Clones containing the desired transgene were streaked on agar plate. Then white colonies were picked and inoculate in LB medium before extraction of plasmid.20 µl of extracted plasmid was sent to Korea for sequencing.

Resulting sequences were analyzed in BLASTN for local alignment based on similarity search. Complete sequences of viruses and betasatellites were arranged and Aligned sequences were selected and phylogenetic tree was built using CLUSTALX2. Trees were edited and viewed by using Coral Draw and tree view packages.

RESULTS

4.1 Sample Collection

Three plant samples Papaya *(Carica papaya,* S1), Black nightshade (*Solanum nigrum,* S2) *and* Duranta (*Duranta erecta,* S3) were collected from different areas around Faisalabad, Pakistan and Granda (*Carissa opaca,* S4) was collected from Trail 3, Islamabad, Pakistan (Figure 4.1). All samples were stored at -80°C until further processing.

4.2. DNA isolation

DNA was extracted through CTAB method from symptomless and symptomatic plant samples. Total DNA was analyzed on 1% agarose gels. The total DNA was kept at -80°C until further process. Quality of DNA was checked by comparing with 1kb DNA ladder and proved that of good quality DNA was obtained.

4.3. PCR Amplification of DNA

Samples were amplified by using full length abutting primers. DNA-A of Duranta was amplified by using SonAF/SonAR with band size approx. 2.7kb. DNA-A of Black nightshade, papaya and Granda were amplified by BurNF/BurNR with bands size of approx. 2.8kb. DNA-B was also amplified from papaya by using KTBF/KTBR with band size of 2.8kb. Betasatellite was amplified from black nightshade and granda with band size of 1350 base pairs.

Figure 4.1: A, infected sample of Papaya *(Carica papaya*) with leaf curling symptoms, **B**, infected sample of Black nightshade (*Solanum nigrum)*, **C,** infected sample of Duranta (*Duranta erecta)* and **D,** infected sample of Granda (*Carissa opaca*).

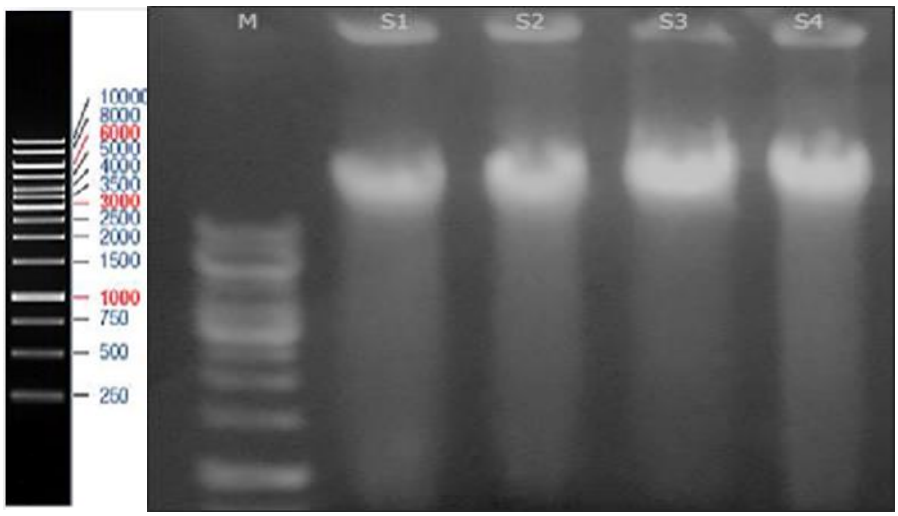

Figure4.2: Agarose gel electrophoresis showing total DNA extracted from symptomatic leaf samples. **S1** is sample from Papaya, **S2** is sample from Black nightshade, **S3** is sample from Duranta and **S4** is showing Granda sample. **M** is showing 1kb standard DNA marker.

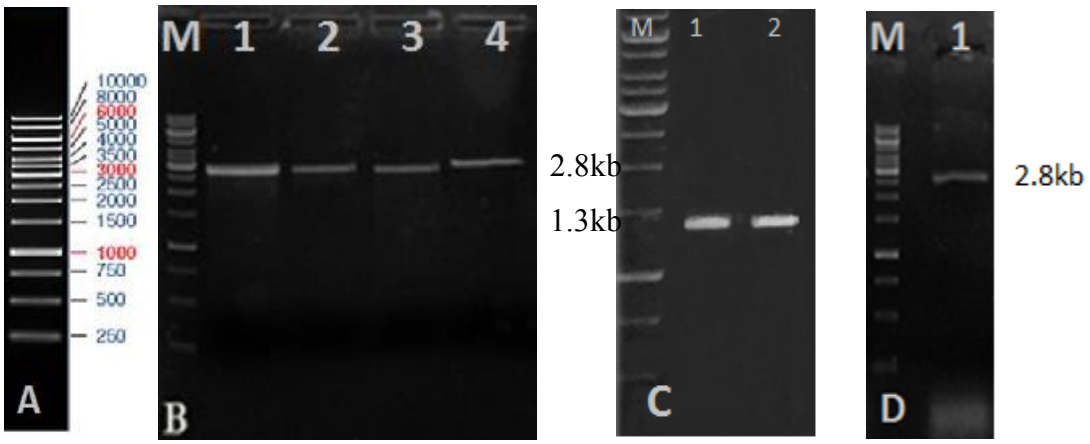

Figure 4.3: A, M is1kb DNA marker. **B,** Gel picture of full length amplified product of DNA-A at 2.8kb. Lane 1,2,3,4 is amplified product of Papaya, Black nightshade, Duranta and Granda respectively. All samples were amplified by using abuting primer BURNF/R except Duranta. Duranta was amplified with SONF/R. **C,** Gel picture of amplified product at 1.3kb by using abuting primers beta01/beta02. Lane 1 is amplified product of Black nightshaade while lane 2 is amplified product of Granda. **D**, Gel picture of full length amplified product at 2.8kb from Papaya by using abuting primers KTBF/R.

4.4. T/A Cloning of begomovirus

Required PCR bands were cut and purified through Gel Extraction Kit by using manufacturer's protocol. Then purified product was checked on UV trans-illuminnator and thick bands of good quality were observed. Resulted product was then ligated in pTZ57R/T vector. Strain of E. coli, DH5α was used for transformation of ligated product. Then spreading of transformed cells was done on agar plates containing X-gal, IPTG and ampicillin.

4.5. Colonies selection

Next day many blue and white colonies were appeared on plates. White colonies were selected and inoculated for plasmid isolation. Next day plasmid was isolated and subjected to digestion analysis.

4.6. Clones confirmation through Restriction Digestion analysis

Clones of Papaya *(Carica papaya*), Black nightshade (*Solanum nigrum)* and Granda (*Carissa opaca*) were digested through *EcoR1* and *Nco1*. Clones of Duranta (*Duranta erecta)* were digested through *EcoR1* and *Apa1* (Figure 4.4). Enzymes used for digestion were different because primers used for PCR were different. Each primer has specific digestion enzyme. SONF/R gives ideal digestion results with *Apa1*while BURNF/R gives ideal results with *Nco1*.

Clones of DNA-B component from papaya were digested with *Eco1* and *Pst1* (two bands on 2.8kb), Beta clones from *S. nigrum* and Granda were digested with *Eco1* (single band on 4.1kb) and *Kpn1* (two bands on 2.8kb and 1.3kb).

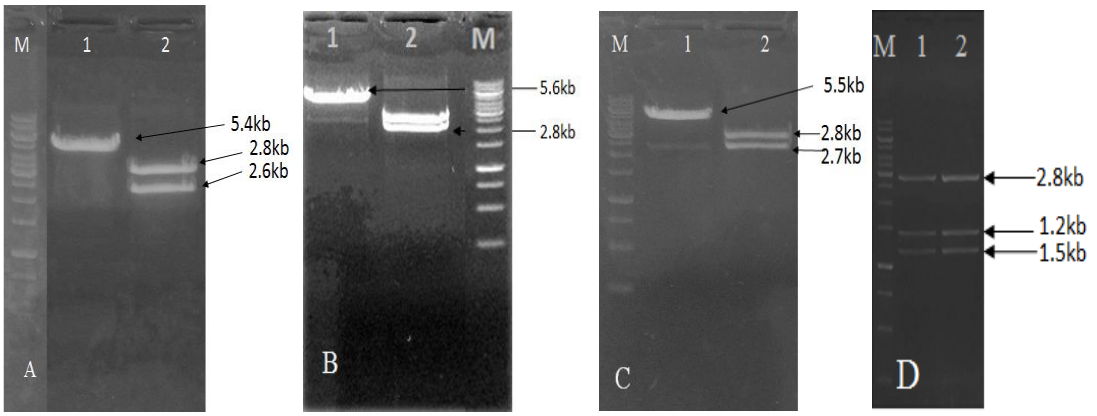

Figure 4.4: Agarose gel showing digestion of DNA A of four samples. **A,** Rows 1,2 are showing the digestion of Papaya with *EcoR1* and *Nco1* respectively. **B,** Rows 1,2 are showing the digestion of Black nightshade with *EcoR1* and *Nco1* respectively. **C,** Rows 1,2 are showing the digestion of Duranta with *EcoR1*and *Apa1*enzyme respectively. **D,** Rows 1,2 are showing the digestion of Granda with *EcoR1* and *Nco1* respectively.

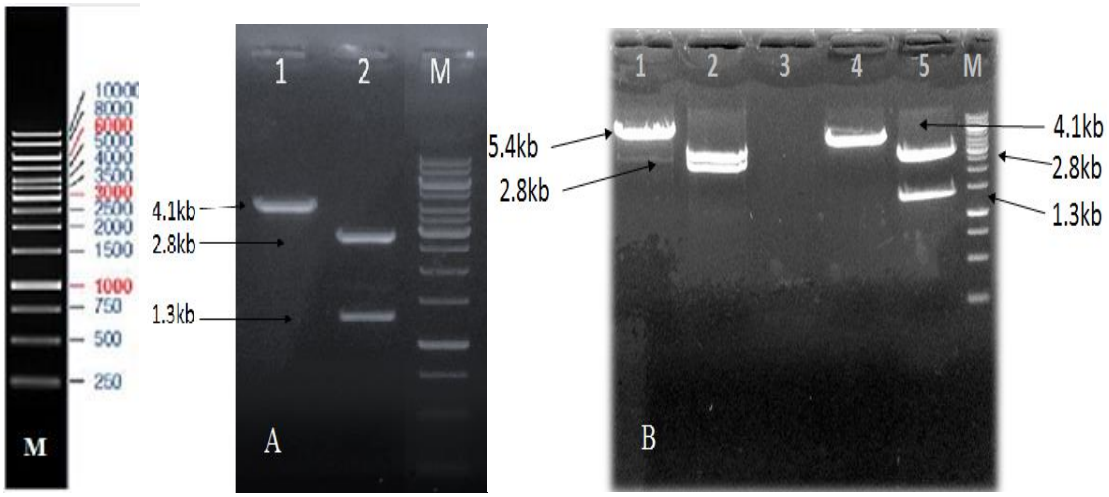

Figure.4.5: Gel picture of digestion of DNA B component and betasatellite. **A,** Row 1 is showing digestion of betasatellite from Granda with *EcoR*1 and row 2 with *Kpn1*. **B,** Row 1 is showing digestion of DNA B component which was isolated from papaya with *EcoR*1 enzyme. **2** is showing the digestion of same component with *pst1* while **4** is showing the DNA digestion of betasatellite from Black nightshade with *EcoR*1 and **5** with *Kpn1*. **M** is showing 1kb DNA marker.

4.7. DNA sequencing

All samples were sent to Macrogen for sequencing. Two samples S1 and S2 were positive. Other two samples S3 and S4 were negative, may be due to some sequencing error. Two positive samples were DNA B of S1 and Betasatellite of S2.

4.7. Phylogenetic Analysis

Phylogenetic analysis was performed to check the relationship of DNA B and betasatellite with other sequences from database. To check the most similar sequences in database, phylogenetic trees were constructed. Analysis of DNA B showed that it has close relationship with *Tomato leaf curl New Delhi virus* (ToLCNDV) isolate from Lahore and (ToLCNDV) isolate from Faisalabad and located in same cluster. Analysis of betasatellite showed that it is closely related to *Cotton leaf curl Multan betasatellite* (CLCuMB) isolates from Pakistan and India.

Table 4.3: Samples, their PCR results, cloning and enzyme digestion.

PCR results		**S1**	**S2**	**S3**	**S4**
DNA A		+	+	+	+
DNAB		+	-	-	-
betasatellite		-	+	-	+
Cloning		✓	✓	✓	✓
Confirmation	Restriction Enzyme	+	+	+	+
	DNA sequencing	+	+	-	-

S1

Length: 1289

95% sequence identity with *Tomato leaf curl New Delhi virus* (ToLCNDV)

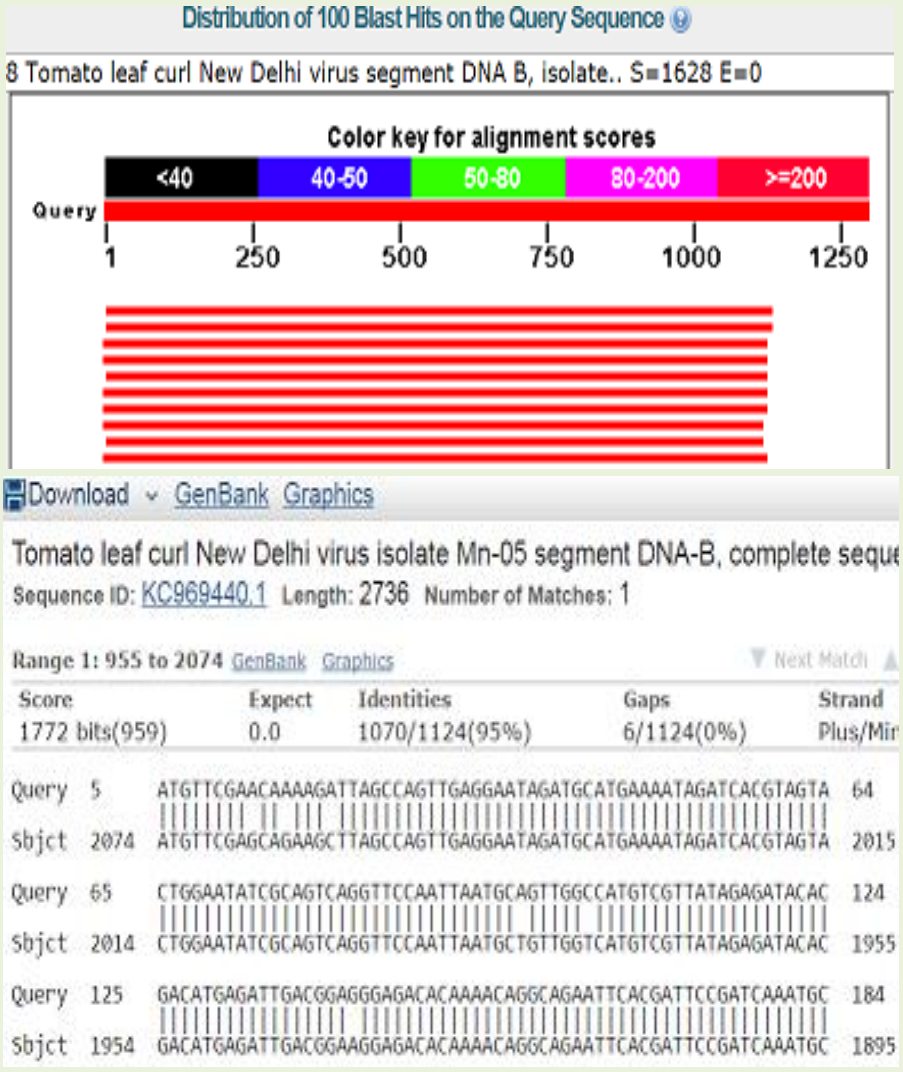

Figure: 4.6 Sequence identity and Blast sequance of DNA B from Papaya (S1). It shows 95% sequence identity with *Tomato leaf curl New Delhi virus* (ToLCNDV).

S2

Length: 1281

93%identity with *Cotton leaf curl Multan betasatellite* (CLCuMB)

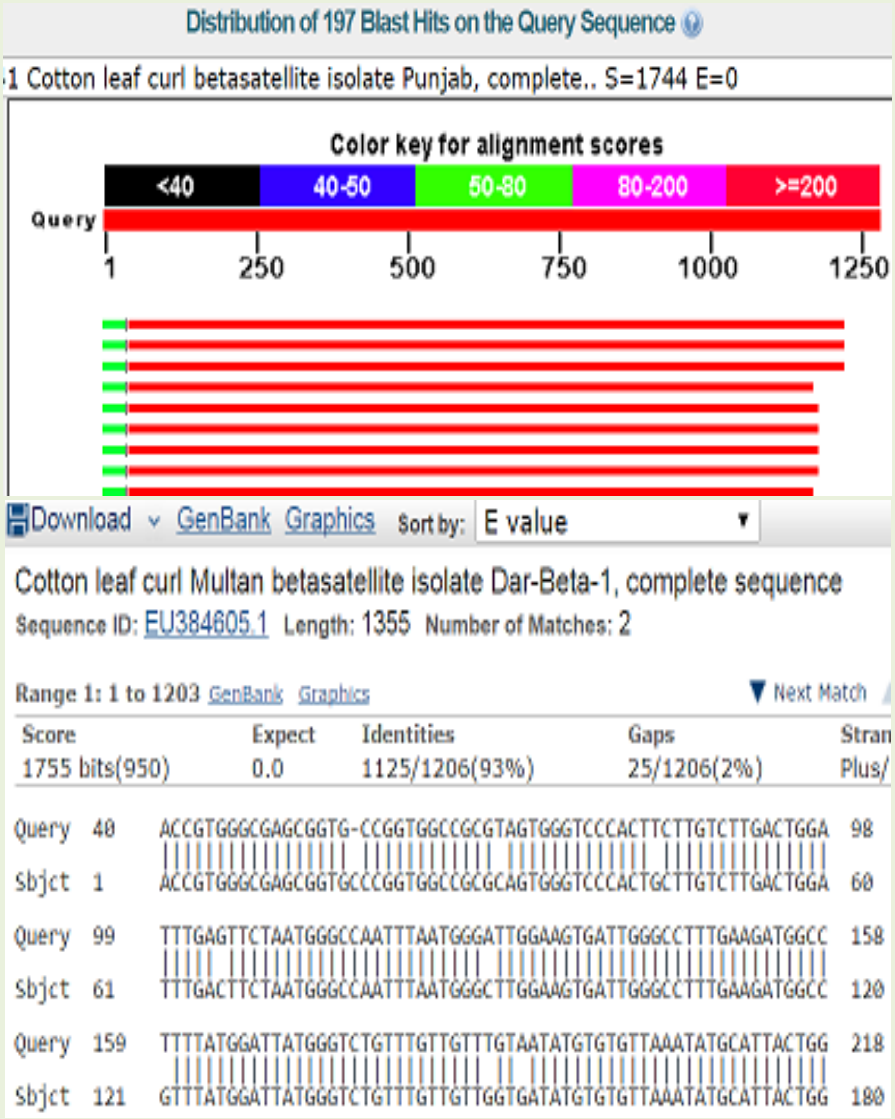

Figure: 4.7 Sequence identity and Blast sequance of betasatellite from Black nightshade (S2). It shows 93%identity with *Cotton leaf curl Multan betasatellite* (CLCuMB)

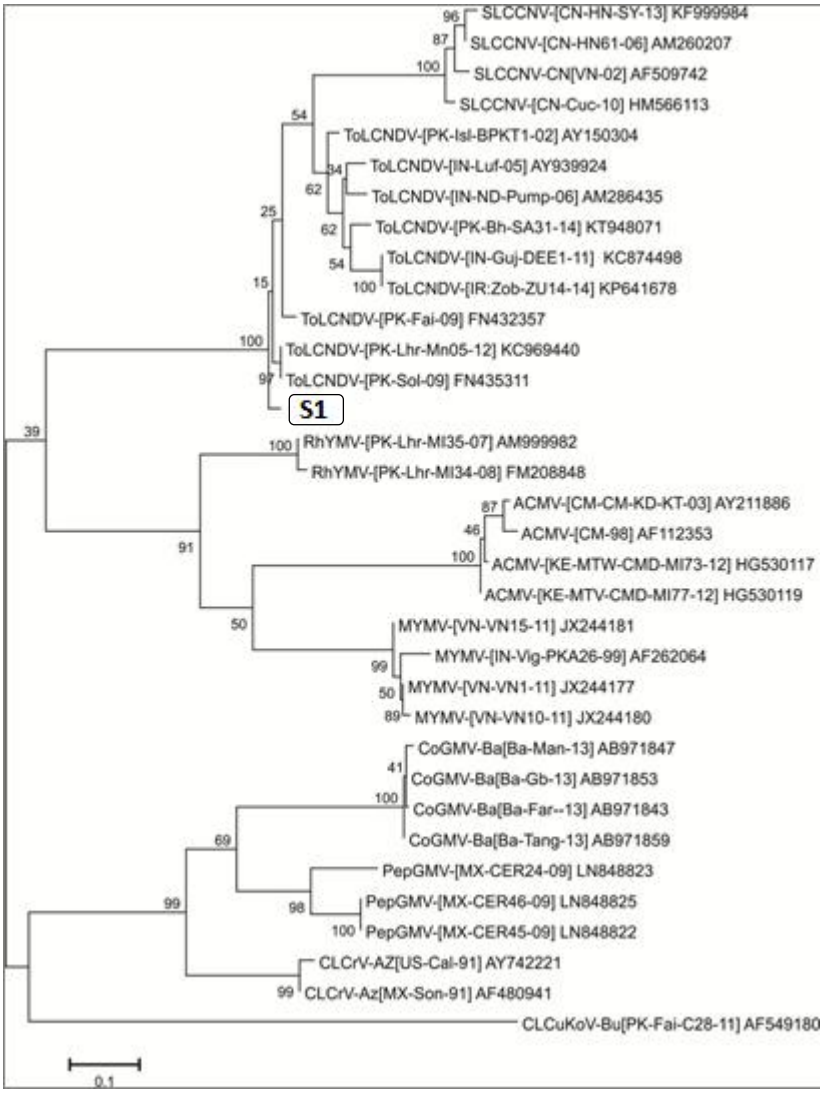

Figure 4.6: Phylogenetic tree (phylogram) is showing DNA B with closely related components in cluster. DNA B was isolated from sample **S1.** This S1 was in same cluster with *Tomato leaf curl New Delhi virus* (ToLCNDV) isolates from Lahore and Faisalabad. In this study *Cotton leaf curl Kokran virus* (CLCuKoV) was used as outgroup. Nodes values are showing bootstrap values. Other viruses which were used are *Squash leaf curl China virus* (SLCCNV), *Tomato leaf curl New Delhi virus* (ToLCNDV), *Rhynchosia yellow mosaic virus* (RhYMV), *African cassava mosaic virus* (ACMV), *Mungbean yellow mosaic virus* (MYMV), *Corchorus golden mosaic virus* (CoGMV), *Pepper golden mosaic virus* (PepGMV) and *Cotton leaf curl virus* (CLCrV).

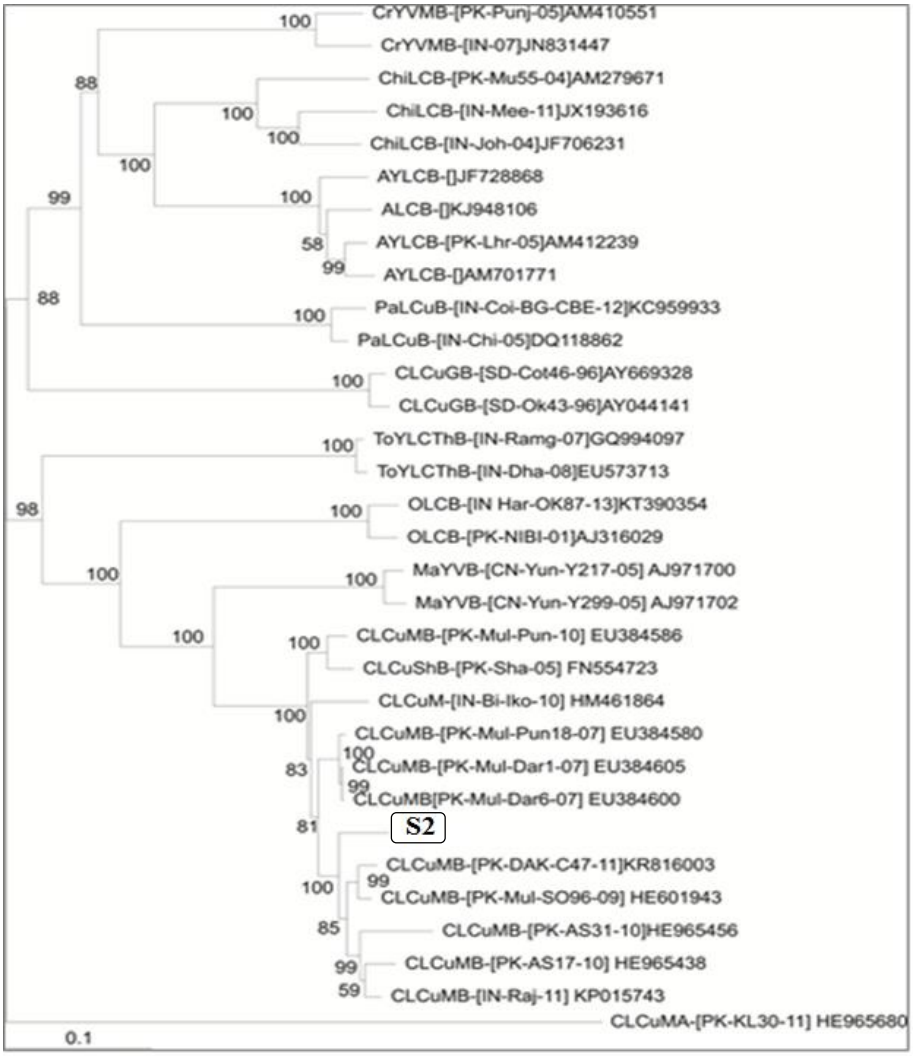

Figure 4.7: Phylogram showing cluster of closely related species with betasatellite. Betasatellite was isolated from sample **S2**. S2 was in same cluster with *Cotton leaf curl Multan betasatellite* (CLCuMB) isolates from Pakistan and India. *Cotton leaf curl Multan alphasatellite* (CLCuMA) was used as outgroup. Values at nodes are bootstrap values. Other viruses which were used are *Chilli leaf curl betasatellite* (ChiLCB), *Ageratum yellow leaf curl betasatellite* (AYLCV), *Papaya leaf curl betasatellite* (PaLCuB), *Cotton leaf curl Gezira betasatellie* (CLCuGB), *Tomato yellow leaf curl Thailand betasatellite* (ToYLCThB), *Okra leaf curl betasatellite* (OLCB), *Malvastrum yellow vein betasatellite* (MaYVB) and *Cotton leaf curl Multan betasatellite* (CLCuMB).

DISCUSSION

Crops, ornamental plants, weeds and vegetables are infected mainly with largest genus of family *Geminiviridae* called begomoviruses. These viruses cause serious destruction and devastating crop losses (Tahir *et al*., 2010; Hussain *et al*., 2011). These viruses infect only dicotyledonous plants and are transmitted through whitefly (*Bemisia tabaci*). Begomovirus is the largest threat to Cotton crop in Pakistan. Up to 20% losses in Cotton crop were estimated due to begomovirus causing Cotton leaf curl disease (CLCuD) in 2010. Weeds, vegetables and ornamental plants are alternative hosts for begomoviruses when Cotton crop is not present. Hence these alternate hosts provide shelter and chances of recombination.

Different group of viruses are present in Pakistan which are infecting chili pepper and tomato (Shih *et al.*, 2003; Mansoor *et al.*, 1997; Hussain *et al.*, 2004), watermelon, radish and okra (Mansoor *et al.*, 2000a; Mansoor *et al.*, 2000b; Mansoor *et al.*, 2001), Potato (Mubin *et al.*, 2009), *Rhynchosia minima* (Ilyas *et al.*, 2009), Squash, *Momordica charantia, Capsicum* (Tahir *et al.*, 2010a; Tahir *et al.*, 2010b; Tahir *et al.*, 2010c) Mungbean (Hameed and Robinson, 2004 ; Bashir *et al.*, 2006). *Solanum nigrum* and *Ageratum conyzoides* (*Haider et al., 2007*) .

Most important crop in Pakistan is Cotton which is infected by Cotton leaf curl disease (CLCuD) (Mansoor *et al.*, 2006; Khan and Khan, 2000; Saeed *et al.*, 2005). Cotton leaf curl disease is not seed-borne. So both insect vector and pathogen survive on alternative hosts (Briddon and Markham, 2000). Hence alternative hosts are as important as main crop. It was showed that only *Cotton leaf curl Burewala virus* (CLCuBuV)was present in Pakistan (Amrao *et al.*, 2007).

In present study begomovirus was isolated from different samples of weeds and ornamental plants which were collected from Faisalabad. Faisalabad was selected for

collection of samples because this area is very important for production of Cotton and called cotton belt. One sample was collected from Margla hills Islamabad which was Granda. Granda was showing typical begomoviral symptoms (Mosaic, Leaf curling) and begomovirus was not reported from this plant. Granda (*Carissa opaca*) is a medicinal plant and belongs to family Apyocynaceae. It cures fever and it is good in eye disorders.

In gemninviruses a lot of mutation has been occurred which causes recombination. This is the main cause of evolution and highly virulent viruses are produced. CLCuBuV is a recombinant virus and intact Transcriptional activation protein (TrAP) is not present in it (Amrao *et al.*, 2010). Ornamental plants and weeds provide reservoirs for recombination and hence cause evolution of new viral strains. That's why alternative hosts always remain in focus of researchers. In many reports begomoviuses were isolated from alternative hosts like *Malvastrum coromandelianum* (Stanley, 2004; Chowda Reddy *et al.*, 2005), *Dicliptera sexagularis* (Echemendia *et al.*, 2003), *Solanum nigrum* (García-Andrés *et al.*, 2006), *Duranta stramonium* (Kashina *et al.*, 2002).

CLCuBuV was different from other begomoviruses and hence considered as new speice and named *cotton leaf curl Burewala virus*. CLCuBuV has ability to break resistance of cotton and cause disease. However, many horticultural crops are also infected by this virus. Weeds are important reservoir for different viral species than host crops (Webster *et al.*, 2007). Recently, many recombinant betasatellites isolated from *Digera arvensis* which is a common weed with an isolate *cotton leaf curl Rajasthan virus* (Mubin *et al.*, 2009). It can also be seen in many reports that Malvaceous hosts caused recombination of many begomoviruse, It can be checked by examining the recombination and evolution between *Cotton leaf curl Kokhran virus* (CLCuKV) and

Cotton leaf curl Multan virus (CLCuMV) resulted *Cotton leaf curl Burewala virus* (CLCuBuV) (Amrao *et al.*, 2010). Many malvaceous species and cotton are also infected with non malvaceous begomoviruses and many *cotton leaf curl viruses of Pakistan* (CLCuV-PK) also infect non malvaceous plants causing multiple-infection. Betasatellite which is associated with CLCuBuV has same gene C1 as encoded in CLCuMuV strain, Aithough betasatellite is recombinant which consists of SCR fragment and *tomato leaf curl betasatellite* (ToLCuB). Effects of this recombination are not clear but possibility is that replication is better due to this recombination (Saunders *et al.*, 2008).

Papaya *(Carica papaya*) is a fruit plant which is found in Punjab and Sindh province. Duranta (*Duranta erecta*) is an ornamental shrub. Mostly it is cultivated as a hedge. Other names are Sky flower, Golden dewdrop, Aussie gold, Sheena's gold, and Geisha girl. Solanum is one of largest and hyper diverse genera which belong to family Solanaceae. Solanum is represented by fifteen species in Pakistan, from which 11 species have medicinal importance. *Solanum nigrum* is also garden huckleberry, garden nightshade, petty morel, hound's berry and black nightshade. Granda (*Carissa opaca*) is found in hilly areas of Pakistan. It is a medicinal plant. It is used to cure fever and it is also good in eye disorder. Begomovirus was isolated from all these samples which show the diversity of host. Granda belongs to family Apyocynaceae, which shows that begomovirus not only effects solanaceae family but also non solanaceous hosts. It has been observed in present study that Granda plant also works as a reservoir, which harbors new viruses and it has become good host for survival of begomoviruses during off season. This is first report of occurrence of begomovirus in Granda.

Betasatellites were also isolated from *Solanum nigrum* and *Carissa opaca* which has 99% resemblance with *Cotton leaf curl Multan betasatellite* (CLCuMB)

isolate from Multan, Pakistan. DNA B component was also isolated from Papaya which has 97% resembles with *Tomato leaf curl New Delhi virus* (ToLCNDV) isolate from Lahore, Pakistan. Hence it can be seen that begomoviruses are biggest threat to cotton crop and Pakistan economy because Pakistan is biggest exporter of cotton. Now it is need of time to make transgenic plants of cotton which should be resistant to begomovirus otherwise cotton crop will be destroyed and economy of Pakistan is in danger.

FUTURE PROSPECTS:

- Collection of more samples for evaluating diversity of begomoviuses.
- This research can also be used for disease control and management.
- Development of infectious clones for:'

    1. Host range
    2. Infectivity
    3. Koch's postulate

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