INTERACTION AND REPLICATION OF ssDNA VIRUSES AND ASSOCIATED SATELLITES

By

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First revelation of Allah Almighty received in the cave of Hira by the Holy Prophet Hazrat Muhammad (PBUH) was “Read in the name of thy Lord, who created man from a clot of blood. Read! The Lord is most bounteous Who taught by pen.” (Al-Alaq: 1-4)

So I dedicate my elfin endeavor to the last prophet of Almighty “Muhammad (peace be upon him)” who is the real source of knowledge for the whole mankind,

And

Then to my parents, my wife and my boys.
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# LIST OF CONTENTS

Acknowledgements

List of Contents

List of Tables

List of Figures

Abstract

## Chapter 1 (INTRODUCTION)

1.1 Plant Viruses

1.2 Geminiviruses

1.3 Genome organization of Geminiviruses
   1.3.1 Mosaicviruses
   1.3.2 Topocuviruses
   1.3.3 Curvoviruses
   1.3.4 Begomoviruses
   1.3.5 Bemovirus
   1.3.6 Tumouroviruses
   1.3.7 Ermoviruses
   1.4 Functions of Geminivirus proteins
   1.4.1 Replication associated Protein
   1.4.2 Transcriptional activator protein
   1.4.3 replication enhancer protein
   1.4.4 ACP Protein
   1.4.5 Cost Protein
   1.4.6 Precer Protein
   1.4.7 Nuclear Shuttles Protein
   1.4.8 Movement Protein

## Chapter 2 (REVIEW OF LITERATURE)

1.5 Satellite Molecules: In Geminivirus-Plant Disease Complex

1.5.1 Alphasatellites

1.5.2 Betasatellite

1.6 Replication of Geminiviruses and Associated Satellites

1.7 Interaction of Satellites with DNA-A

1.8 Role of betasatellite in the disease complex

1.9 Eclipta prostrata and its associated viruses

2 Chapter 2 (REVIEW OF LITERATURE)

vi
1.3 Satellite Molecules: In Geminivirus-Plant Disease Complex 20
1.3.1 Alphasatellites 21
1.4 Replication of Geminiviruses and Associated Satellites 24
1.5 Interaction of Satellites with DNA-A 26
1.6 Role of betasatellite in the disease complex 28
1.7 Eclipta prostrata and its associated viruses 28

2 Chapter 2 (REVIEW OF LITERATURE) 30

3 Chapter 3 (MATERIAL AND METHODS) 46
3.1 DNA Manipulation 46
3.1.1 Sample Collection 46
3.1.2 DNA extraction from plant sample 47
3.2 Amplification of DNA 47
3.2.1 PCR amplification of DNA 47
3.2.2 Rolling-circle amplification 47
3.3 Cloning of amplified DNA 47
3.3.1 Cloning of PCR product 48
3.3.2 Cloning of RCA product 48
3.4 Transformation of heat-shock competent E. coli cells 48
3.5 Transformation of competent Agrobacterium tumefaciens cells 49
3.6 Plasmid Isolation 49
3.7 Digestion of plasmid DNA 50
3.8 DNA Analysis 50
3.8.1 Agarose-gel electrophoresis 50
3.8.2 Southern Blot analysis 50
3.9 Purification of DNA 53
3.9.1 Gel extraction and PCR product purification 53
3.9.2 Phenol-chloroform treatment of DNA 54
3.10 Microbiological Techniques 54
3.10.1 Preparation of heat shock competent *Escherichia coli* cells  
3.10.2 Preparation of electro competent *Agrobacterium tumefaciens* cells  
3.10.3 Agrobacterium-mediated inoculation  
3.11 Plant growth conditions  

4  Chapter 4 (RESULTS)  

4.1 Symptomatology  
4.2 Detection of Geminiviruses  
4.2.1 Detection of components of Begomovirus disease complex through PCR  
4.3 Detection of satellites in the Begomovirus disease complex through PCR  
4.3.1 Detection of Alphasatellites  
4.3.2 Detection of Betasatellite  
4.4 Detection of components of begomovirus disease complex using RCA  
4.5 Cloning and sequencing of full length viral molecules  
4.6 Sequence and Phylogenetic analysis  
4.6.1 Sequence and Phylogenetic analysis of Alternanthera yellow vein virus  
4.6.2 Sequence and Phylogenetic analysis of Alphasatellites i.e., ChLCuA and AlYVA  
4.7 Synthesis of viral infectious molecules  
4.8 Infectivity assay  

5  Chapter 5 (DISCUSSION)  

6  Chapter 6 (SUMMARY)  

7  LITERATURE CITED
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Serial #</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Classification of Geminiviruses with respect to their host, genome type, genome size and vector involved in transmission.</td>
<td>12</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Features of Begomovirus isolated from Eclipta prostrata.</td>
<td>59</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Representing the inoculation of plants with combinations with alphasatellites and betasatellites</td>
<td>69</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Serial #</th>
<th>Title</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1.1</td>
<td>Typical genome organization of <em>Mastrevirus.</em></td>
<td>4</td>
</tr>
<tr>
<td>Fig 1.2</td>
<td>genome organization of <em>Tomato pseudo curly top virus.</em></td>
<td>6</td>
</tr>
<tr>
<td>Fig 1.3</td>
<td>Genome organization of <em>Beet Curly Top Virus. Circalifer tenellus</em>, vector for <em>Beet Curly Top Virus.</em></td>
<td>7</td>
</tr>
<tr>
<td>Fig 1.4</td>
<td>Typical genome organization of bipartire begomovirus, DNA-A and DNA-B molecule.</td>
<td>9</td>
</tr>
<tr>
<td>Fig 1.5</td>
<td>Genome organization of BCTIV</td>
<td>15</td>
</tr>
<tr>
<td>Fig 1.6</td>
<td>Genome organization of TCTV.</td>
<td>13</td>
</tr>
<tr>
<td>Fig 1.7</td>
<td>Genome organization of ECSV</td>
<td>14</td>
</tr>
<tr>
<td>Fig 1.8</td>
<td>Genome organization of a typical Alphasatellite.</td>
<td>24</td>
</tr>
<tr>
<td>Fig 1.9</td>
<td>Genome organization of a typical betasatellite</td>
<td>25</td>
</tr>
<tr>
<td>Fig 4.1</td>
<td>Eclipta prostrata plants , normal and infectious</td>
<td>58</td>
</tr>
<tr>
<td>Fig 4.2</td>
<td>Map of Punjab.</td>
<td>59</td>
</tr>
<tr>
<td>Fig 4.3</td>
<td>Amplification and cloning of viral molecules associated with vein yellowing disease of <em>Eclipta prostrata</em></td>
<td>63</td>
</tr>
<tr>
<td>Fig 4.4</td>
<td>Phylogenetic dendrogram based upon selected complete sequences of AlYVV.</td>
<td>64</td>
</tr>
<tr>
<td>Fig 4.5</td>
<td>Phylogenetic dendrogram based upon selected complete sequences of alphasatellites molecules.</td>
<td>66</td>
</tr>
<tr>
<td>Fig 4.6</td>
<td>Recombinant Alphasatellite AlYVA.</td>
<td>68</td>
</tr>
<tr>
<td>Fig 4.7</td>
<td>Flow chart representing how to synthesize an infectious molecule.</td>
<td>70</td>
</tr>
<tr>
<td>Fig 4.8</td>
<td>Confirmation of dimeric molecule</td>
<td>71</td>
</tr>
<tr>
<td>Fig 4.9</td>
<td>A) Agroinfiltration of ALYVV and associated satellites on N.benthamiana plants, (B) Southern blot analysis of inoculated <em>Nicotiana benthamiana</em> plants</td>
<td>74</td>
</tr>
<tr>
<td>Fig 4.10</td>
<td>PCR amplification from inoculated, systemic plants</td>
<td>75</td>
</tr>
</tbody>
</table>
ABSTRACT

Whitefly-transmitted geminiviruses (Family Geminiviridae; genus Begomovirus) were known to occur in the Indian subcontinent for a long time but have recently emerged as major pathogens on food and fiber crops. Highly diverse begomoviruses have been reported from the Indian subcontinent could be resulting from multiple infections, recombination, component capture/exchange and emergence of whiteflies as major pest with extended host range. The sources of resistance are limited and are often prone to breakdown due to emergence of resistance breaking strains (Amrao et al., 2010). This study was carried out to find out the diversity among the existing genome components of begomoviruses found associated with the weed Eclipta prostrata is aperennial weed found along the water channels and crop fields in china, India and Pakistan. During the last ten years extensive knowledge about the diversity of begomoviruses found on crops in the Indian subcontinent has been generated. However, knowledge about begomoviruses on weeds that often serve as alternate hosts for the crop infecting begomoviruses and also the chances of recombinations due to multiple infections. Diversity of begomovirus components on Eclipta prostrata, found commonly around field crops and water channels was studied. Ecliptaprostrata samples with typical symptoms of infection i.e; vein yellowing were collected from different districts of Punjab province, Pakistan. Rolling Circle Amplification (RCA) and Plymerase Chain Reaction (PCR) were used to amplify the full length molecules and their associated satellite molecules. Sequencing of full-length viral molecules after cloning, Phylogenetic studies and recombination analysis were carried out using different softwares like DNA Star and MEGA 5. These analyses showed that this weed plant is source of multiple begomovirus components found on crop plants. This weed host carries Alternanthera yellow vein virus (AlYVV), causing typical symptoms of begomovirus infection i.e; vein yellowing and stunting along with associated satellite molecules in different plant hosts, with twodistinct Alphasatellite molecules i.e; Chili leaf curl alphasatellite and Alternanthera yellow vein alphasatellite. Mesta yellow vein virus showed 83% sequence homology with AlYVA, so it was proposed as a new Alphasatellite species. No betasatellite molecule was found to be associated with vein yellowing disease of Eclipta prostrata. Analysis of all isolated full length viruses of showed that AlYVV is recombination free in all plant samples. Most interestingly there was a recombination among Malvaceous and non-Malvaceous alphasatellites co-existing along with ALYVV infecting Eclipta prostrata. This suggests that this weed is important source of recombination. Begomovirus genome sequences characterized from Eclipta prostrata available in the databanks were aligned and analyzed. The study of interaction of alphasatellite molecules with the helper viral molecules for the onset of disease was also studied. In order to fulfill Koch’s postulate, infectious molecules of the full-length viruses and satellite molecules were constructed for infectivity analysis. Southern blot analysis confirmed the viral movement in Nicotiana benthamiana plants, which were used in the infectivity analysis for AlYVV. While alphasatellites were coinoculated with AlYVV, their systemic movement in the leaves was confirmed via PCR. The data presented in this thesis would help in understanding that how weeds can play a vital role in speedy evolution of begomoviruses and how understanding the diversity of begomoviruses in non-host plants could help in devising control strategies against begomoviruses.
Chapter 1: INTRODUCTION

1.1 Plant Viruses
Viruses are obligate parasites, which need a living host for multiplication. Plant viruses are also obligate parasites like all other viruses, which require plant cellular machinery to replicate. Plant viruses infect a range of cultivated, non-cultivated and higher plants as well. Viral classification explains that at the present time there are 3 orders, There are currently three orders, 73 families, 9 subfamilies, 287 genera and 1941 viral species (Emerson, Anderson et al. 2004). The majority of plant viruses are RNA viruses; while the viruses having DNA as their genetic material accounts for a minor portion of all the plant infecting viruses. Plant viruses with DNA genomes fall into two categories, those with single-stranded DNA (ssDNA) as their genome replicated by a dsDNA intermediate via rolling circle replication (RCR) method (Geminiviridea and Nanoviridae) (Gutierrez 1999; Jeske, Lütgemeier et al. 2001), those with a double-stranded DNA (dsDNA) genome replicated through an RNA intermediate via reverse transcription (Culimoviruses and Badnaviruses) (Gutierrez 1999; Jeske, Lütgemeier et al. 2001).

1.2 Gemini viruses
The family Gemini viridea includes DNA viruses, which counts for the major portion of plant-infecting viruses, which infect cultivated crops and ornamental plants including monocots and dicots causing considerable yield losses. The family Gemini viruses is named so because of the geminate (twinned) structure of its member viruses. Members of family Gemini viruses have either one or two single stranded circular components of approximate size of 2.6 – 3.1 Kb (Fauquet and Fargette 2005). Gemini viruses replicate their ssDNA genome in the nucleus of the infected cell by a double-stranded replicative form with the help of the Gemini virus encoded replication associated protein (Rep) and plant DNA replicative machinery (Hanley-Bowdoin, Bejarano et al. 2013).

Currently there are 7 genera of Gemini viruses which include Topocoviruses, Mastreviruses, Curtoviruses, Begomo viruses, Turncurto viruses, Becurtovirus sand Eragro viruses (Varsani, Navas-Castillo et al. 2014). Begomovirus is named after Bean Golden Yellow Mosaic Virus (BGYMV; formerly called Bean Golden Mosaic Virus). This genus includes dicot infecting white fly (Bemisia tabaci) transmitted viruses. Mastreviruses includes leaf hopper (Cicadulina mbila) transmitted ssDNA viruses infecting monocots,
which include Wheat Dwarf Virus (WDV) and Maize Streak Virus (MSV). Beat Curly Top Virus (BCTV) is the most prominent example of the leafhopper transmitted monopartite viruses that infect dicots. The genus Topocovirus includes Tomato Pseudo Curly Top Virus (TPCTV) as the only example. This dicot infecting virus is transmitted by tree hopper (BRIDDON, BEDFORD et al. 1996). Family Nanoviridae is classified into two genera depending upon their host range. Genus babuvirus includes monocot-infecting viruses; Banana Bunchy Top Virus (BBTV) is the most studied example of this genus which infects the cultivated banana in the Sindh region of Pakistan and banana plantations in the temperate zone. The members of the genus nanovirus include the dicotyledonous infecting viruses with Faba bean necrotic yellow vein virus as a typical example of this genus (Gronenborn 2004).

1.3 Genome Organization of Gemini viruses:

1.3.1 Mastrevirus:
Mastreviruses are monopartite ssDNA viruses, infect dicotyledonous as well as monocotyledonous plants. The vector for the transmission of mastreviruses is leaf hoppers (Cicadulina mbila). Mastreviruses are largely phloem limited viruses. Mastreviruses are found mostly in the Old World (OW). Maize Streak Virus (MSV) and Wheat dwarf Virus (WDV) are the most well characterized members of the genus Mastrevirus (Willment, Martin et al. 2007).

![Typical genome organization of Mastrevirus. The orientation and the position of genes are indicated. Cicadulina mbila vector for Maize streak virus.](image)
There are four ORF in the Mastrevirus genome. The complimentary strand ORF C1 and C2 encode for the Rep A and Rep proteins, while Virion strand ORF V1 and V2 encode for Coat Protein (CP) and movement protein (MP) (Nahid, Amin et al. 2008). Splicing feature of Mastrevirus Rep during expression is a unique feature among geminiviruses (Gao, Gordon-Kamm et al. 2004). Along with replication function Virion sense strand regulation is also done by Rep. Rep A has its role in the cell cycle control. The presence of the intron is also the unique character of the Mastreviruses. CP and MP perform the function of encapsidation and systemic movement (cell to cell movement) of the viral molecule.

Opposite to each other there are located two intergenic regions, large intergenic region (LIR) and the small intergenic region (SIR) having regulatory elements. The LIR contains the promoter (consensus) sequence for both the C1 and V1 genes (Palmer and Rybicki 1998). A begomovirus like origin of replication (ori) for regulation of virion strand, is also present in the LIR (Willment, Martin et al. 2007). SIR has the ori for complementary strand synthesis.

1.3.2 Topocuvirus:

Topocuviruses are monopartite dicot infecting viruses that are transmitted by the treehopper (Micratalis malleifera). As far as the vector specificity is concerned, Topocuviruses are the only one having unique vector i.e; treehopper, unlike other viruses having whitefly or leafhopper as vectors.

Tomato Pseudo Curly Top Virus (ToPCTV) is the only example (BRIDDON, BEDFORD et al. 1996). The ToPCTV contain the feature typical of both the Begomoviruses and the Mastreviruses which is a solid clue for it being a natural recombinant. Keeping in view this hypothesis, ToPCTV can trans-compliment the movement of the DNA-A component of two begomoviruses when their corresponding DNA-B component is absent (Briddon and Markham 2001).
Fig. 1.2: Typical genome organization of Tomato Pseudo Curly Top Virus. Position and orientation of genes are indicated. *Micrutalis malleifera*, Treehopper vector for Tomato pseudo curly top virus.

1.3.3 Curtovirus:

The most-studied example of genus Curtovirus is *Beet Curly Top Virus* (BCTV). Curtoviruses are monopartite viruses that infect dicots and are transmitted by the leafhopper vector (Soto and Gilbertson 2003). Curtoviruses are mainly phloem-limited. Inside the nuclear region the virion formation as well as the gene expression takes place. (Esau 1977; Latham, Saunders et al. 1997). Curtoviruses have circular genome size of 2.9-3.0 kb (Baliji, Black et al. 2004).

Fig. 1.3: Typical genome organization of (BCTV). The position and orientation of genes are indicated. *Circulifer tenellus*, vector for (BCTV).
The intergenic region (IR) of curtoviruses is not sufficient for full expression of C1, unlike other geminiviruses; however, the 3’ portion of the C1 coding region contains the transcriptional activator elements for the expression of the C1 (Hur, Buckley et al. 2007). In order to identify the promoter motif involved in the Curtovirus sense-gene expression in the transgenic Arabidopsis it was evident that Curtoviruses late gene expression by the virion-sense promoter depends on the development stage of the host plant and the number of the Conserved Late Element (CLE) motifs present in the promoter (Hur, Choi et al. 2008; Singh, Malik et al. 2008). A wide range of plant species is infected by limited number of curtoviruses.

An unusual geminivirus has recently been identified from the Iran, named Beet Curly Top Iran Virus (Yazdi, Heydarnajad et al. 2008) and it was tentatively placed in the genus Curtovirus. It has a typical coat protein of a geminivirus but its vector and the genetic arrangement in the complementary-sense strand is unusual. It encodes a typical viral Rep but has only one downstream ORF that may possibly be expressed by splicing with the Rep (in many aspects similar to the Mastreviruses). This very unusual arrangement depicts that it may have the recombinant origin (a possible progenitor of the Curtoviruses that has recombined with the Begomoviruses to yield the existing Curtoviruses). There have been suggestions from the geminivirus community that a new genus may need to be established, which can accommodate this virus but debates are still continued.

1.3.4 Begomovirus:

The genus Begomovirus has the 318 species (215 reported and 103 candidate) (King, Adams et al. 2012). Begomoviruses as group have a very broad range of the hosts infecting mostly dicotyledonous hosts.
Fig.1.4: Typical genome organization of bipartite begomovirus, DNA-A and DNA-B molecule. The position and orientation of genes are indicated. *Bemisia tabaci*, whitefly vector for begomoviruses.

Begomoviruses are transmitted to their host by a whitefly vector (*Bemisia tabaci*). Begomoviruses infect a wide range of important agronomic crops like cotton, chilies, tobacco, cassava, tomatoes, beans and squashes. While infected by the begomoviruses the infected plants show a range of the symptoms like leaf curling, stunted growth, vein yellowing and poor yield (Briddon 2003).

Majority of begomoviruses infecting plants have bipartite genome, with two genomic components one is called DNA-A and the second one as DNA-B. (Stanley and Gay 1983; Howarth, Caton et al. 1985). Except a highly conserved region called Common Region (CR) there is no sequence identity between these two components. This common region has the origin of replication and also maintain the integrity of the bipartite genome (Stanley, Boulton et al. 2001). The CR has the specific motifs, which are needed for control and regulation of gene expression, specifically the Iteron motif and the stem loop structure containing the nonanucleotide (TAATATTAC) that is the origin of replication for the virion strand.

Both viral components encode for the proteins that are necessary for the replication of the genome, encapsidation, and movement. Viral replication and encapsidation is performed by the proteins encoded by DNA-A, while the intercellular and intracellular movement is controlled by the proteins encoded by DNA-B. There are total six ORF of DNA-A
component, among these six, complementary sense strand has four ORF, while Virion strand possess two ORF. (Dry, Rigden et al. 1993). AC4 protein, Replication enhancer protein (REn), Transcriptional activator protein (TrAp.) and Replication associate protein (Rep.) are encoded by the complementary sense strand. Coat protein (CP) which is responsible for the formation of the coat protein complex which is used for spread of virus and insect transmission and pre-coat protein (AV2) are encoded by the virion sense strand. AV2 is absent in most of the new world begomoviruses. Rep. is essential for the replication of viral genome, when mutated there was no viral replication. REn helps in the viral replication. When REn is mutated there is massive reduction in the viral replication and it results in attenuated and much delayed symptoms (Elmer, Brand et al. 1988; Etessami, Callis et al. 1988; Gardner 1991; Faria, Albino et al. 2006).

Two ORF are encoded by DNA-B. For systemic movement (cell to cell), Movement Protein (MP) is encoded by the complementary sense strand. (Noueiry, Lucas et al. 1994; Ward, Medville et al. 1997) and the nuclear shuttle protein (NSP) which binds and transports the ssDNA across the nuclear envelope(Pascal, Sanderfoot et al. 1994; Sanderfoot, Ingham et al. 1996). For the onset of a systemic infection both the components of begomovirus are necessary.

There are some Old World (OW) begomoviruses with their genome as a single component (Kheyr-Pour, Bendahmane et al. 1991; Navot, Pichersky et al. 1991; Dry, Rigden et al. 1993; Noris, Accotto et al. 1994; Mansoor, Khan et al. 1999). DNA-A component of bipartite begomoviruses is homologous to genome of these monopartite viruses, which is evident to its role in evolution.

Many of the monopartite begomoviruses are found associated with a DNA satellite or the satellite like molecules. These viruses produce infection but the produced symptoms are not severe. Cotton Leaf Curl Multan Virus (CLCuMV) and Ageratum Yellow Vein virus (AYVV) from Singapore represent such examples(Briddon and Markham 2000; Saunders, Bedford et al. 2002).

Satellites are single-stranded molecules found associated mostly with monopartite begomoviruses and are half the size (~1350 bp) of helper viral molecules. The satellite
molecule which was formerly also known as DNA-1 is called alphasatellite and encodes for
the Rolling circle initiator protein (Rep) as the only product for the replication of its circular
genome. This Rep of alphasatellite has high identity with the Rep of the nanoviruses
(Mansoor, Khan et al. 1999; Aronson, Complainville et al. 2002). Alphasatellite depend
toally on helper virus for its encapsidation and transmission while it apparently have no
role in the disease onset and severity.

The second DNA satellite-like molecule is the betasatellite that is a symptom-modulating
molecule and plays a role in the severity of the disease by interacting with the helper
molecule. The betasatellite perform almost the same function as DNA-B of bipartite
begomoviruses. For replication and encapsidation and transmission betasatellite depend
totally on the helper virus as it does not has a Rep protein. Betasatellites have one ORF that
encodes for the βC1 protein, which perform vital functions as a pathogenicity determinant
protein and it also serves as a suppressor of RNA silencing (Qazi, Amin et al. 2007; Saeed,
Zafar et al. 2007).

1.3.5: Becurtovirus:

Becurtovirus is the newly added genus in the family Geminiviridea including the Beat Curly
Top Iran Virus (BCTIV) (Yazdi, Heydarnejad et al. 2008) as the first studied example and
Beat Curly Top Arizona Virus (BCTAV) (Brown and Hernandez-Zepeda 2011) as the recently
discovered species. The genome of Becurtovirus is unique and has features of both Curto-
like and Mastre-like (Heydarnejad, Keyvani et al. 2013).

The unique features of the Becurtovirus include the presence of the CP which is closely
similar to the Curtoviruses while the Rep of it is more closely related to the Mastreviruses.
Vector for transmission of Becurtoviruses is still unknown (Varsani, Navas-Castillo et al.
2014). Becurtovirus has the circular genome ~2.9 kb. The genome is split into virion sense
region and the complementary region. Virion region, which is Curto-like transcribe for the
proteins responsible for movement and encapsidation. While the Mastre-like complementary
region transcribes for proteins responsible for replication and cell cycle progression.
Fig.1.5: Genome organization of BCTIV. The genome organization and the orientation of the genes is indicated. Vector transmitting BCTIV is unknown.

1.3.6: Turncurtovirus:

*Turnip Curly Top Virus* (TCTV) is the only example of Turncurtovirus. Turncurtovirus is the dicot infecting monopartite virus (Varsani, Navas-Castillo et al. 2014). Virion region of the TCTV encodes for the CP and the MP. While the complementary region of the TCTV encodes for the Trap, REn, Rep and (sd) symptom determinant proteins. Vector transmitting this virus is still unknown.
**Fig.1.6:** Genome organization of TCTV. The genome organization and the orientation of the genes is indicated. Vector transmitting TCTV is unknown.

**1.3.7:Eragroivirus:**

*Eragrostiscurvula streak virus* (ECSV) is the only one species representing the genus *Eragroivirus* (Varsani, Navas-Castillo et al. 2014). *Eragroivirus* is the monopartite monocot infecting virus, which has been found associated to only one host *Eragrostiscurvula* (weeping love grass). Vector transmitting this virus species is still unknown.

The genome of ECSV is ~ 2.8kb in size and composed of a virion region and a complementary region. The Virion sense region encodes for the V1 (CP) and V2 (MP) while the complementary region of ECSV genome encodes for C1 (Rep) and C2 (Trap) proteins.
Fig. 1.7: Genome organization of ECSV. The genome organization and the orientation of the genes is indicated. Vector transmitting ECSV is unknown.
<table>
<thead>
<tr>
<th>Genus</th>
<th>Vector</th>
<th>Type of Plant Infecting</th>
<th>Size</th>
</tr>
</thead>
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<tr>
<td>Mastrevirus</td>
<td>Leafhopper</td>
<td>Monocots</td>
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<tr>
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<td></td>
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<td>Leafhopper</td>
<td>Dicots</td>
<td>2.9-3.0kb</td>
</tr>
<tr>
<td>i.e; BCTV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Begomoviruses</td>
<td>Whitefly</td>
<td>Dicots</td>
<td>2.8kb</td>
</tr>
<tr>
<td>i.e; BGMV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topocuvirus</td>
<td>Treehopper</td>
<td>Dicots</td>
<td>2.8kb</td>
</tr>
<tr>
<td>i.e; TPCTV</td>
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<tr>
<td>Becurtovirus</td>
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<td>Dicots</td>
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<td>Eragrovirus</td>
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<td>Dicots</td>
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<tr>
<td>i.e; TCTV</td>
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Table 1.1: Classification of Geminiviruses with respect to their host, genome type, genome size and vector involved in transmission.
1.4 Functions of Geminivirus proteins:

1.4.1 Replication associated protein:

Replication associated protein (Rep) also called AL1, AC1 or C1 (Kong, Orozco et al. 2000). Complementary strand of viral genome encodes for the Rep. This protein performs many important biological functions: It initiates and then terminates rolling circle replication (RCR) of the viral circular genome and after nicking it religates the origin of replication of viral DNA (Laufs, Traut et al. 1995), and it also represses its own gene transcription (Eagle, Orozco et al. 1994).

C1/AC1 protein of all Geminiviruses have higher homology and is also have highly conserved sequence. Among four of its functional domains. N-terminal domain (amino acids 1-120) is responsible for the startup of RCR for all the geminiviruses (Orozco, Kong et al. 2000), AC1 initiates RCR by creating a nick at a specific site within the nona nucleotide region TAATATTAC (Heyraud-Nitschke, Schumacher et al. 1995). The recognition site for the startup of transcription is present between the TATA box and the initial sequence of AC1 site, which also serve as a negative regulator for Rep gene transcription. (Eagle, Orozco et al. 1994), the oligomerization domain (amino acid 121-180) leading to interaction with its own (Orozco, Kong et al. 2000) and the host factors (Hanley-Bowdoin, Settlage et al. 2004). Replication and Rep mediated transcription repression are affected if there is any mutation induced in oligomerization domain. (Orozco, Kong et al. 2000), The ATPase domain (amino acid 181-330), which is identified by the presence of Carboxyl terminal domain and P loop (amino acid 331-359) with no known function but in case of Tomato Golden Mosaic Virus (TGMV) it is necessary for the viral replication (Orozco, Kong et al. 2000). There has been identified a motif, which is highly conserved and prescribed as Geminivirus Rep Sequence (GRS) and is necessary for the startup of viral replication (Nash et al. 2011).

There is a common element among all the RNA viruses and small DNA viruses, known as ATPase domain in case of geminiviruses (Gorbalenya, Koonin et al. 1990) and it is identified by the presence of C motif, walker B and the walker A in the P loop. The binding of Rep during RCR between the 7th and 8th nucleotide of invariant nonanucleotide sequence 5'
TAATATTAC3’ (Laufs, Traut et al. 1995; Stanley 1995). Rep binding to the Iteron sequence is observed to be highly ordered and precise (Singh, Malik et al. 2008).

1.4.2 Transcriptional Activator protein (TrAP):

In case of monopartite begomoviruses Transcriptional activator protein (TrAP) is encoded by C2 while in case of bipartite begomoviruses it is encoded by AL2 but in case of Mastreviruses this protein is absent and in case of Curtoviruses a related protein AC2 plays a different role rather C1 protein in Mastreviruses performs the function of the AC2 (Palmer and Rybicki 1998).

TrAP is observed to be a multitasking protein in begomoviruses performing the functions of suppression of gen silencing, gene activation and virus pathogenicity (Chowda-Reddy, Dong et al. 2009; Coll, Epple et al. 2011). The transcriptional activation of MP and CP is also done by the TrAP in the non-virus-specific way (Yang, Baliji et al. 2007).

Inside the phloem tissue the overriding of putative host repressor is done by the TrAP to regulate tissue-specific expression. Being a factor of pathogenicity hypersensitive response (HR) is also countered by TrAP, which is also a form of programmed cell death (PCD) inferred as a way of resistance to the infecting pathogen around the area of infection (Postel and Kemmerling 2009; Coll, Epple et al. 2011). But in case of Tomato Leaf Curl New Delhi Virus (ToLCNDV) the nuclear shuttle protein (NSP)-induced HR is overcome by the TrAP (Hussain, Mansoor et al. 2007).

A phenomenon referred as suppression of RNA silencing is present in many plant viruses, which protect plants from viruses, transposable elements and also regulates the gene expression (Pantaleo 2011; Wang, Masuta et al. 2012). RNA silencing is proved to be suppressed by both C2 from monopartite and TrAP of bipartite geminiviruses (Dong, van Wezel et al. 2003; Trinks, Rajeswaran et al. 2005; Chowda-Reddy, Dong et al. 2009). The process of production of small interfering RNAs (siRNA) has been affected in Beet Severe Curly Top Virus (BSCTV) by lowering down DNA methylation, which in turn triggers the process of RNA silencing (Yang, Fang et al. 2013). Following these results transient expression of BCTV C2 and TGMV TrAP increase susceptibility of tobacco to these viruses (Sunter, Sunter et al. 2001; Hao, Wang et al. 2003).
1.4.3 Replication enhancer protein (REnh)

In case of monopartite begomoviruses the ORF L3/C3 and in case of bipartite begomoviruses the ORF AL3/AC3 encodes the replication enhancer protein (REnh), while REnh is absent in Mastreviruses (Stanley, Latham et al. 1992). A monodirectional promoter present inside the AC1 sequence controls the expression of AC3 (Shivaprasad, Akbergenov et al. 2005).

Being not necessary for replication of viruses, the accumulation of viral DNA and modulation of infection symptoms are enhanced by the REnh, which was demonstrated in an experiment with mutated REnh resulting less accumulation of circular viral molecules (Sunter, Hartitz et al. 1990; Sung and Coutts 1995).

In Tomato Yellow Leaf Curl Sardinia Virus and TYLCV REnh interacts with Rep to increase the accumulation of the viral DNA (Castillo, Collinet et al. 2003; Settlage, See et al. 2005). In case of heterologous begomoviruses the complementation studies of AC3 has been successful. (Sunter, Stenger et al. 1994). In order to enhance the efficiency of the viral replication REnh assists Rep (Settlage, See et al. 2005).

1.4.4 AC4 Protein

In bipartite Geminiviruses the ORF AC4/AL4 and C4/L4 ORF in case of monopartite geminiviruses (except mastreviruses where C4 is absent) are present completely but with a different frame within AC1 ORF (Fondong 2013). With its least homology among all the geminiviruses, this protein is said to have a variety of functions.

The induced mutation in ORF C4 in Tomato leaf curl virus, (Rigden, Krake et al. 1994), the TYLCV (Jupin, De Kouchkovsky et al. 1994) and the BSCTV (Teng, Chen et al. 2010) produced the decrease in symptom development and least viral DNA, which is evident of its role in symptom development and accumulation of viral DNA. The development of typical symptoms of vein swallowing by BCTV while infecting plants is mainly due to C4 (Stanley and Latham 1992; Mills-Lujan and Deom 2010).

AC4 protein of TGMV is not found to be necessary for viral infection (Pooma and Petty 1996) but do have a role in the movement of virus. (Elmer, Brand et al. 1988). Induced
mutation in AC4 of Cassava infecting geminiviruses has clearly no effect on symptoms in *N. benthamiana* (Bull, Briddon et al. 2007).

The role of C4/AC4 is proven as a suppressor of RNA silencing for a number of both bipartite and monopartite geminiviruses. The same has been observed in Cassava infecting viruses by suppressing RNA silencing (Fondong, Reddy et al. 2007) by binding microRNAs (miRNA) and siRNAs (Chellappan, Vanitharani et al. 2004).

**1.4.5 Coat Protein (AV1)**

The ORF AR1, AV1, V2 or V1 encode the single CP constituting capsid in geiminviruses depending upon the genome type (Zhang, Olson et al. 2001). Being the only structural gene, CP is a late gene (Stanley and Gay 1983). Along with its function of packaging, insect transmission and many other functions are also performed by the CP (Briddon, Pinner et al. 1990; Boulton, Pallaghy et al. 1993), in case of monopartite geminiviruses the function of shuttling of viral DNA across the nuclear membrane (Liu, Saunders et al. 1999) as well as involved in the spread if virus from one cell to another and systemic movement of the virus (Boulton, Steinkellner et al. 1989; Liu, Boulton et al. 1997; Pitsakhtheepong 1999). For bipartite geminiviruses that ask of systemic spread does not depend on CP at all (Gardiner, Sunter et al. 1988; Unseld, Frischmuth et al. 2004). CP work as a functional substitute when replaced with NSP ORF of DNA-B. (Ingham, Pascal et al. 1995). Binding of ssDNA and dsDNA by the CP is independent of sequence specificity (Lazarowitz and Beachy 1999). CP is required for the virus transmission through the insect vector. The ability of CP being necessary for the transmission of viruses by insects was performed by using leafhopper transmitted BCTV and Whitefly-transmitted ACMV. In a chimeric molecule of ACMV with replaced CP of BCTV the transmission via leafhopper was observed (Briddon, Pinner et al. 1990). The central part of the CP possessed the sequence required for the insect transmission of viruses and (Kheyr-Pour, Bananej et al. 2000; Höhnle, Höfer et al. 2001; Liu, Lucy et al. 2001; Unseld, Höhnle et al. 2001) it also have a role in multimerization and hence in insect transmission (Hallan and Gafni 2001; Zhang, Olson et al. 2001).

The role of CP in movement of viral DNA across the nuclear membrane in case of some monopartite geminiviruses like TYLCV and mastreviruses (Liu, Saunders et al. 1999; Liu,
Lucy et al. 2001), N-terminal of CP binds necessarily to the ssDNA to transport it across the nuclear membrane (Pitaksutheepong, Vimoltat et al. 2007). The Coat Protein Nuclear Localization Signal (NLS) is said to be present at the middle region of this protein (Unseld, Höhnle et al. 2001). The movement of viral DNA-protein complex between the nucleoplasm and the cytoplasm is mediated via a structure known as Nuclear Pore Complex (NPC), this movement is supported by host factors of nuclear protein trafficking system (Guerra-Peraza, Kirk et al. 2005). The GroEL protein of insect gets attached with NLS region of the CP, supposedly for the protection of the virion inside the insect vector, while transmitting (Yaakov, Levy et al. 2011; Rana, Singh et al. 2012).

When CP of bipartite begomoviruses gets mutated there is infection but there is delay in the symptom induction and the severity of the disease (POOMA, GILLETTE et al. 1996; Sudarshana, Wang et al. 1998), Which is evident that some other proteins have replaced the function of the CP.

In case of monopartite begomoviruses like mastreviruses along with movement of viral DNA across the nuclear membrane, (Liu, Lucy et al. 2001) the CP is also responsible for the systemic spread of the virus and also movement from one cell to another cell (Rigden, Dry et al. 1993; Padidam, Beachy et al. 1996). Interaction of MP with CP affects the movement of viral particle (Priyadarshini, Ambika et al. 2011), as in case of TYLCV and cotton leaf curl khokhran virus. CP of bipartite begomoviruses might not be necessary for the onset of infection, although it enhances systemic spread of the viral particles across the plant as in case of SqLCV (Ingham, Pascal et al. 1995). Bean dwarf mosaic virus (BDMV) (Seo, Gepts et al. 2004) and CaLCV (Carvalho, Machado et al. 2008). CP is not essential for the viral replication but mutation or the absence of the CP result in decreased level of ssDNA of viruses without changing the level of the dsDNA of viruses (Padidam, Beachy et al. 1996).

These results and findings are evident that the CP of the bipartite begomoviruses conserved their functions over the course of evolution from monopartite progenitors (Fondong 2013). The results are evident from the evolutionary pathway that the small genome of the viruses was compensated with the ability of the proteins to be multitasking and the course of evolution brought it to current status (Walsh and Mohr 2006).
1.4.6 *Precoat Protein* AV2

For NW bipartite begomoviruses ORF AV2 encodes for the pre coat protein with no clear clue for its function while V2 ORF in case of monopartite (OW) geminiviruses is said to be involved in the viral movement (Rojas, Jiang et al. 2001; Selth, Randles et al. 2004; Bull, Briddon et al. 2007). In bipartite geminiviruses the induced mutation in the AV2 protein, when analyzed, it was found to be involved in the viral movement (Padidam, Beachy et al. 1996). Phloem is not the only area of accumulation of protein AV2 when it is moving from one cell to another cell (Rothenstein, Krenz et al. 2007).

Absence of AV2 in the New World monopartite geminiviruses is an evidence for the OW geminiviruses are the true progenitors of the bipartite geminiviruses.

Plants inoculated with the ToLCNDV and ToLCV with induced mutations respectively in AV2 and in V2 showed that there is infection but lower level of viral DNA in the tissues of infected plants (Padidam, Beachy et al. 1996; Selth, Randles et al. 2004). With *Cotton leaf curl Khokhran virus* and *Papaya leaf curl virus* almost same inferences were derived (Mubin, Amin et al. 2010). Suppression of RNA silencing in EACMCV by AV2 (Chowda-Reddy, Achenjang et al. 2008) and V2 protein for monopartite geminivirus AYVV support the recent findings (Sharma and Ikegami 2010; Sharma, Ikegami et al. 2010). Almost the same results were obtained for the V2 protein of TYLCV supposedly (Glick, Zrachya et al. 2008) its interaction with the protein known as suppressor of gene silencing 3 protein (SGS3) (Glick et al., 2008).

1.4.7 *Nuclear Shuttle Protein* (NSP)

DNA-B of bipartite geminiviruses encodes two ORF, between these two ORF one is BR1/BV1, which encodes for the Nuclear Shuttle Protein (NSP). NSP performs the vital function of transporting viral ssDNA across the nuclear membrane (Noueiry, Lucas et al. 1994; Ward and Lazarowitz 1999) where a complex is formed as viral DNA-NSP (Ward and Lazarowitz 1999). There is no sequence of binding of NSP with viral DNA while making viral DNA-NSP complex (Hehnle, Wege et al. 2004). For movement of viral DNA from one cell to another cell the viral DNA-NSP complex make an interaction with MP when this complex is moving from cytoplasm of a cell to the neighboring cell through the cell wall for
the beginning of the new replication phase in the new cell (Noueiry, Lucas et al. 1994; Lazarowitz and Beachy 1999).

NSP is not required at all for the viral infection because CP performs the function of nuclear import and export, which results the inference that there is same origin of evolutionary course and functional redundancy for both the proteins (Zhou, Garrido-Ramirez et al. 2007). The point of localization for both the proteins is nucleolus (Zhou, Rojas et al. 2011). Along with its function of viral DNA movement across the nuclear membrane, NSP also serves as a determinant of avirulence in few hosts. ToLCNDV-NSP in case of Tomato and Nicotiana tabacum (Garrido-Ramirez, Sudarshana et al. 2000) (Zhou, Garrido-Ramirez et al. 2007), also BDMV-NSP in case of Phaseolus vulgaris demonstrated the induction of HR (Hussain, Mansoor et al. 2005; Hussain, Mansoor et al. 2007).

1.4.8 Movement Protein

BL1/BC1 is one of the two ORF of bi partite geminiviruses DNA-B component, while in case of monopartite geminivirus the ORF V2 encode for the movement protein with no sequence identity with BV1 protein. With a combined interaction of NSP with the MP, this complex is known to be involved in the movement of viral DNA from one cell to another cell and also across the nuclear membrane (Noueiry, Lucas et al. 1994; Ward, Medville et al. 1997). MP–NSP cooperation, serves as a source of transportation of viral DNA in the nascent form (Zhang, Wege et al. 2001; Hehnle, Wege et al. 2004; Frischmuth, Wege et al. 2007), where viral DNA-NSP binds around the plasma membrane for movement of this complex across the neighboring cell (Kleinow, Tanwir et al. 2009). Middle region of MP is the point where viral DNA-NSP complex develop a binding connection (Frischmuth, Wege et al. 2007). As evident by the example of SqLCV, (Pascal, Sanderfoot et al. 1994) there are some viruses where MP make no binding or connection with the DNA (Hehnle, Wege et al. 2004). Unlikely, there has been observed a great affinity of binding (Rojas, Noueiry et al. 1998) for both dsDNA and ssDNA with the movement proteins of BDMV and MYMIV (Radhakrishnan, Splitter et al. 2008).
1.5 Satellite Molecules: In Geminivirus-Plant Disease Complex:

There are found small ssDNA, circular molecules along with begomoviruses, these smaller molecules are almost half the size of begomoviruses with which these molecules are found together. These include DNA satellites, one is Nanovirus like molecules called Alphasatellites, second one is the true satellite molecule called Betasatellite (Briddon and Stanley 2006). Size of this true satellite called betasatellite is almost half (~1350) of the begomovirus with which it is found together in the disease complex, while there is at all no sequence homology with the helper begomovirus; except nonanucleotide sequence TAATATTAC present in the stem loop structure (Briddon, Bull et al. 2003). A segment of 100nt, which is highly conserved, called Satellite Conserved Region (SCR), possessing the stem loop of betasatellite. Other than this betasatellite encode for the only one ORF, coding a protein called βC1, and this ORF is found in the complementary way in the betasatellite molecule, and there is an adenine rich region (A-rich). All the functions of the betasatellite are controlled and executed via βC1 gene. This βC1 gene performs multy tasks, which include suppressor of post transcriptional gene silencing (PTGS), pathogenicity determinant, viral movement inside the plant, interaction with a variety of host factors (Briddon, Mansoor et al. 2001) and up regulation of virus DNA level in the plant cell (Eini, Dogra et al. 2009).
1.5.1 Alphasatellite:

Alphasatellites are not true satellites, but are satellite like molecules found along with the begomoviruses while infecting plants in the disease complex. Alphasatellites, formerly known as DNA 1; have the capability to replicate themselves by its own Rep. Alphasatellites have very conserved genome organization with only one ORF encoding Rep present in the virion sense orientation, a nonanucleotide sequence TAGTATT/AC present inside the hairpin structure and adenine rich region. (Mansoor, Khan et al. 1999).

![Fig.1.8: Genome structure of a typical Alphasatellite.](image)

The alphasatellite molecules encode only one protein called Rep protein, which has the highest resemblance with the Rep of nanoviruses (Xie, Wu et al. 2010). Alphasatellites are autonomous in replication but for insect transmission and encapsidation depend totally upon helper virus (Idris, Shahid et al. 2011).

Alphasatellite molecules when present along with the helper virus and their associated betasatellite molecules; can potentially attenuate the symptoms and can also affect the level of betasatellite DNA (Idris, Shahid et al. 2011).
1.5.2 Betasatellite:

Betasatellites are ssDNA circular molecules with a size approximately half the size (1.4kb) of helper begomovirus. Betasatellite encodes only one protein called βC1, which is a pathogenicity determinant. Betasatellite was first of all discovered associated with *Tomato leaf curl virus* (ToLCV) in Australia. For encapsidation and replication betasatellite are found to be totally dependent on helper begomovirus. It has no observable effects on the viral replication and in development of the symptoms by ToLCV (Dry, Krake et al. 1997).

**Fig.1.9: Genome structure of a typical betasatellite**

*Cotton Leaf Curl Multan Virus* (CLCuMV) and *Ageratum Yellow Vein Virus* (AYVV) are found to be infectious even without their satellites but the development of specific symptoms of leaf curling and vein yellowing can only be seen when these viruses are present along with their respective betasatellites.

Detailed analysis of betasatellite sequence showed that it has almost half size (~ 1350 nt) of the begomovirus it is associated with, having no sequence homology with the helper begomovirus except the highly conserved nonanucleotide TAATATTAC sequence present in the hair pin loop (Nawaz-ul-Rehman, Mansoor et al. 2009). Betasatellite require the DNA- A Begomovirus molecule for their replication, encapsidation, vector transmission and
movement inside the host plant after the onset of the infection (Singh, Chattopadhyay et al. 2012). Structural analysis of the betasatellite reveals its three features: a satellite conserved region (SCR), a $\beta$C1 gene and A-Rich region. The DNA-A component of the Begomoviruses has the potential to transreplicate betasatellite of different origin (Patil and Fauquet 2010; Sivalingam, Malathi et al. 2010). *Indian Cassava Mosaic Virus* (ICMV) and *African Cassava Mosaic Virus* (ACMV) can potentially transreplicate the *Eupatorium Yellow Vein Betasatellite* (EpYMVB) and *Ageratum yellow vein betasatellite* (AYVB). This demonstration shows that the DNA-A mediated replication has less specificity and all CGMs have almost the same behavior towards transreplication of betasatellite (Patil and Fauquet 2010). Many begomoviruses has the capability to transreplicate several types of the betasatellites. Knowing most importantly how DNA-A Rep protein can identify these satellite molecules and transreplication of satellites by DNA-A (Saunders, Bedford et al. 2002; Saunders 2008).

These satellite molecules are found to be widespread in the OW monopartite begomoviruses. In a study 26 betasatellite molecules were related with different plant species obtained from the diverse geographical regions. Sequence analysis revealed a highly conserved organization of the genome of betasatellite molecules consisting of a highly conserved ORF called $\beta$C1, A-Rich region and a region which is highly conserved called the Satellite Conserved Region (SCR). Phylogenetic analysis of the betasatellites divided them into two groups. The first group originated from the hosts in the *Malvaceae* family while the second group was found to be originated from the host in the *Solanaceae* and *Compositae*. Within both groups the betasatellites present the similarity depending upon both the origin and the geographical area. These results highly favor the co-adaptation of the betasatellites with their helper begomoviruses (Briddon 2003).

Betasatellites are found to be pathogenicity determinant along with the begomoviruses when present but surprisingly betasatellites are found to be playing the role of pathogenicity determinant along with the Mastreviruses. Some defective satellites and some full-length beta satellites like *Ageratum yellow leaf curl betasatellite* (AYLCB) were cloned along with the *Wheat Dwarf India Virus* (WDIV) from the same infected sample (Kumar, Kumar et al. 2014).
A-Rich region (Sivalingam, Malathi et al. 2010) of betasatellite is supposed to have some role, but apparently no role in the trans-replication and encapsidation. A-rich region mutants produced symptoms during the infection which neglects the role of the A-rich region in the trans-replication and encapsidation. In the presence of the betasatellite in Ageratum, begomoviruses accumulate to its normal level suggest that the satellite may have role in both movement and replication of begomovirus in the host or it may suppress the host defense mechanism. βC1 encodes the only protein of the betasatellite which has a key role in the begomovirus disease complex (Sivalingam and Varma 2012) but exact information of the fundamental functions of it is not known yet. βC1 may also interact with the miRNA pathways. The activity of DICER-like proteins, which are nuclear proteins required for the biogenesis of miRNA and siRNA and act in the silencing pathway is affected by the βC1 (Amin, Hussain et al. 2011). βC1 can also down regulate the transcription of the proteins having role in PTGS pathways in cytoplasm or the βC1 can also activate the transcription of the host PTGS inhibitors (Cui, Li et al. 2005).

1.6 Replication of Geminiviruses and Associated Satellites:

In the first step of the RCR, while forming “minus-strand” as well as the “plus-strand” is used as a template to produce a replicative form (RF) which is double stranded. During the next step RF form serves as a template for the formation of the plus strand to produce free ssDNA molecules. At this step Rep is the extremely important component (Laufs, Traut et al. 1995). Rep binds to the repeated Rep binding motif in the intergenic region in the start of RCR (Behjatnia, Dry et al. 1998). The virion sense strand is cleaved by Rep, downstream of this motif, within the conserved mononucleotide region (TAATATTAC) inside the loop of the hairpin structure (Laufs, Traut et al. 1995; Stanley 1995) and hence the viral genome multiplication is started in this way (Hanley-Bowdoin, Settlage et al. 1999).

Another method of replication of geminiviruses and their associated satellites has been observed recently, which involves recombination dependent replication (RDR) (Alberter, Ali Rezaian et al. 2005) and this is based on the observation of replication intermediates of ToLCV, ACMV, TGMV, BCTV and a betasatellite (CLCuMV) with the help of electron microscopy and two dimensional gel electrophoresis.
Other than the RCR intermediate, a number of other intermediates suggest another additional pathway which is alternate of RDR pathway of bacteriophage T4 (Kreuzer 2000) that is called as “join-copy pathway” (Jeske, Lütgemeier et al. 2001), “bubble migration synthesis” and “break induced replication.” This RDR model is accomplished in four steps (Kreuzer 2000; Jeske, Lütgemeier et al. 2001):

1. Interaction of the incomplete ssDNA with the homologous site of the cccDNA
2. A recombination between the homologous sites
3. Loop displaces ahead and elongation of the ssDNA
4. This elongation along with the formation of complementary strand results into the synthesis of dsDNA

In this model of RDR elimination of the supercoiling of the parent strands is not required at all while synthesis of the new strand (Kreuzer 2000). Geminiviruses replications via RDR do not need the participation of the Rep for the recognition of the cognate virus molecule and for nicking of the ssDNA in the nonanucleotide region. (Lin, Behjatnia et al. 2003) supported this hypothesis in a study where ToLCV and its associated satellite lost its ability to bind with the rep but still were able to produce infection. This RDR model explains many biological phenomena such as how frequent and rapid recombination are observed. The RDR model also has many advantages for the geminiviruses such as to aid in replication when normal replication is hindered by is shortage of nucleotides, digestion of the viral DNA by the host enzymes, problem between replication and transcription, the productive infection can be achieved by recovery of the DNA molecules through the homologous recombination in the form of full length viral molecules (Kowalczykowski and Eggleston 1994; Jeske, Lütgemeier et al. 2001).

In the geminivirus plant disease complex where geminiviruses are associated with satellite molecules, which include the betasatellite and satellite like molecules called Alphasatellites. Betasatellites are found in the OW viruses only (Briddon and Stanley 2006; Briddon, Brown et al. 2008). Alphasatellites is not true satellite because of its ability to self-replicate. But for encapsidation and vector transmission, it depends on the helper begomovirus. Alphasatellites
have been found to have no role in the disease modulation. Betasatellite is a true satellite as it depends totally on the helper virus for replication because the betasatellites have no Rep protein and it has a single ORF encoding for a protein βC1. Replication of the betasatellite is also accomplished by the helper molecule. Betasatellite has a considerable role in symptom induction and disease modulation in the begomovirus plant disease complex. These disease and symptom modulating betasatellites are mostly found linked with OW geminiviruses for efficient infection in the host (Briddon and Stanley 2006; Nawaz-ul-Rehman, Mansoor et al. 2009). Begomoviruses have another advantageous feature of transreplication, *Cotton leaf Curl Multan Betasatellite* (CLCuMuB) can interact with a NW begomovirus. Disease symptoms of the NW cabbage leaf curl virus (CaLCuV) were enhanced when there is also present CLCuMuB. This finding suggest that for transreplication betasatellites are quickly and efficiently adapted(Nawaz-ul-Rehman, Mansoor et al. 2009). In another study (Saunders, Briddon et al. 2008) showed that AYVV when co-inoculated on *N. benthamiana* can transreplicate the betasatellites associated with *Honeysuckle Yellow Vein Virus* (HYVV), *Eupatorium Yellow Vein Virus* (EpYVV) and cotton leaf curl multan virus (CLCuMuV). This shows the elasticity in the ability of begomoviruses to interact with distinct betasatellites to produce successful infection.

**1.7 Interaction of Satellites with DNA-A:**

Satellite molecules are typically found associated with the RNA viruses. These satellite molecules can autonomously replicate but are unable to encapsidate so they use the coat protein of the helper RNA virus for encapsidation and possess least sequence homology with their helper viruses (Murant and Mayo 1982). ToLCV, the first geminivirus found associated with a ssDNA satellite molecule originated from Australia(Dry, Krake et al. 1997). Satellite molecules rely totally upon helper begomovirus for insect transmission, replication and encapsidation.

Betasatellites associated with Begomoviruses are unable to self-replicate but rather depend on helper begomovirus for its replication because the only ORF betasatellites have, transcribes a protein βC1, which is determinant of pathogenicity that contributes to symptom modulation and movement of the helper virus and also serves as suppressor of PTGS(Cui, Li et al. 2005).
Majority of geminiviruses in eastern hemisphere (EH) are monopartite begomoviruses, whereas the bipartite begomoviruses are found in both EH and western hemisphere (WH) (Whitham and Wang) (Rojas, Hagen et al. 2005). The monopartite component is found to be homologous to the DNA-A component of bipartite begomoviruses. These monopartite begomoviruses found associated with two classes of satellites referred to as alphasatellites (Mansoor, Khan et al. 1999; Briddon, Bull et al. 2004) and betasatellites (Briddon, Brown et al. 2008) are found more frequently in the EH. Bipartite begomoviruses found in the WH are also found associated with the alphasatellites in conjugation (Paprotka, Boiteux et al. 2010; Romay, Chirinos et al. 2010). Alphasatellites, as studied until now, having no visible role in the development of disease or disease severity (Saunders, Bedford et al. 2000; Briddon, Bull et al. 2004; Briddon and Stanley 2006). DNA-2 is a subclass of alphasatellites, which is very rare having least sequence homology with DNA-1 molecule been found associated only with AYVV isolated from Singapore (Saunders, Bedford et al. 2002), overall these two kind of molecules have no apparent contribution in the disease development when found together with begomoviruses. In a study (Idris, Shahid et al. 2011) Tomato Leaf Curl Oman Virus (ToLCOMV) and its associated alphasatellite which is closely related to Ageratum Yellow Vein Singapore Alphasatellite (AYVSGA) and Tomato yellow leaf curl Oman strain (TYLCV-OM) with its associated betasatellite tomato leaf curl betasatellite (ToLCB) were inoculated to N.banthamiana and to the original host. When betasatellite is agro-infiltrated with any of the helper virus it enhances the disease severity and spread. When the alphasatellite with any of the two helper viruses and betasatellite were co-inoculated there was a considerable reduction in the DNA of betasatellite and that of helper virus.

In bipartite begomoviruses, the component tDNA-A is able to encode proteins required for virus transmission by insect vector, replication of DNA, and for control of gene expression (Nawaz-ul-Rehman, Briddon et al. 2012). While DNA-B possesses only two ORF, which are responsible for movement of viral DNA across the nuclear membrane and for long distance transmission of the virus. Betasatellites when combined with DNA-A can complement DNA-B and the disease development, symptom severity and the virus spread is enhanced. In contrast alphasatellites when present with the DNA-A molecule do not contribute to the induction of disease symptoms but rather the accumulation of the DNA-A considerably decreases.
1.8 Role of betasatellite in the disease complex:

Betasatellites contribute considerably along with the helper virus and are important agents in the plant-virus disease complex. In bipartite Begomoviruses like *Tomato leaf curl New Delhi Virus* (ToLCNDV), *Cabbage leaf curl leaf curlvirus* (CbLCuV) it has been proven that bipartite begomoviruses can transreplicate betasatellites and betasatellites can modulate the disease symptoms. In a study CbLCuV was co-inoculated with the CLCuMuB on *N. benthamiana* and the disease symptoms were enhanced by the betasatellite but CbLCuV was unable to transreplicate Chili Leaf Curl betasatellite (CLCuB). Simultaneously it has been pointed out that the ability of different helper viruses to transreplicate different betasatellites is different.

Whereas in case of OW monopartite begomoviruses like *Tomato Leaf Curl Virus*, *Tomato Yellow Leaf Curl Virus*, *Cotton Leaf Curl Multan Virus* the one DNA component is homologous to the DNA-A component of the bipartite begomoviruses. *Cotton Leaf Curl Multan Virus* (CLCuMV), which is a monopartite virus, when inoculated alone cannot produce detectable symptoms but when co-inoculated with cotton leaf curl betasatellite it produces typical symptoms of vein swelling, leaf curling, enations and vein darkening and. The betasatellite encodes for only one protein, βC1, which is a pathogenicity determinant and it modulates the symptoms in host and also acts as suppressor of PTGS.

1.9 Eclipta prostrata and its associated viruses:

The *Eclipta prostrata* belongs to family *Asteraceae*, and is an annual herb (DiTomaso and Healy 2003). It is found both in subtropical and tropical regions of the world. *E. prostrata* is able to grow and thrive in the varied geographical conditions, mainly found around the wet crop fields and moist places (Holm, Plucknett et al. 1977). It widely grows almost in all the continents except Antarctica (Holm et al. 1977, Flora of North America).

Ecliptaprostrata while infected shows typical symptoms of vein yellowing caused by the begomoviruses. Earlier the vein yellowing disease was linked with Tomato Leaf Curl New Delhi Virus (TLCNDV) (Haider, Tahir et al. 2006), which was transmitted by the vector *Bemisia tabaci*. This vein yellowing disease was seen in Eclipta prostrata in Guangdong China in 2005. Full-length virus was isolated and cloned from the infected plant showing the
typical symptoms of disease. When the whole sequence of cloned virus was analyses, it was found to have a typical genome arrangement of a begomovirus. Blast analysis of the sequence revealed that it has more than 95% sequence homology with AlYVV (He, Mao et al. 2008).

In Pakistan *Eclipta prostrata* is found mostly around water channels, having vein yellowing disease symptoms. Objective of this study is to uncover the component viruses and possibility of variation in the disease complex.
Chapter 2: REVIEW OF LITERATURE

Inspite of the huge advances and progress in the modern technology our agriculture and food security is facing adverse and the constantly emerging problems, which include both biotic and abiotic ones. These include changing weather, decreasing water availability and the fiercest one is the changing mode of the pathogens i.e.; change in host selection, diversity in host range and resistance against human developed strategies against the pathogens to overcome the disease (Strange and Scott 2005). Pakistan is an agricultural country that faces every year huge losses due to biotic and abiotic constraints. The largest proportion of our population is dependent on agriculture directly or indirectly, so loses to agriculture affects the dependent population directly (Jaleel, Manivannan et al. 2009). Wheat, cotton, tobacco, maize, rice, chilies, potato and tomato are important agricultural commodities of our country, which contribute in feeding as well as in earning foreign exchange through exportation (Murgai, Ali et al. 2001). The main constraint to our agricultural yield is diseases to our crops. Pathogens like bacteria, fungi, nematodes and viruses, cause diseases and affect the production of the crop partially or completely (Oerke 2006). Agricultural experts have been introducing resistance against these pathogens to our existing varieties but the course of evolution and adaptation of pathogens is also parallel (Hammond-Kosack and Jones 1997). Existence of alternate hosts, abundance of the vector to transmit pathogens and the availability of the favorable environment accelerates the evolution of the pathogens (Dinoor and Eshed 1984). This suggests that the development of the resistance against the pathogen is not a singular job, but along the passing time the course of resistance changes as well (Grover and Gowthaman 2003). Looking at the ability of the pathogens to evolve new strains and isolates we see that some pathogens evolve faster enough in this regard while some are bit slower and year after year there is no change in these pathogens (Fritz and Simms 1992). ‘Fungal pathogens, as we see in our cropping system, show less variation and develop resistance and the mode of treatment against these pathogen remain effective over the years until the arrival of an exotic strain (Staskawicz, Ausubel et al. 1995). Bacterial disease and the nematodes similarly, show a slower rate of variation.

The case is different with viral pathogens which infect a variety of cultivated, ornamental and weed plants, and are considered to be the most damaging pathogens to agriculture around the
globe (Huang, Xie et al. 2013). For instance we see severe food security concerns in Africa where a comprehensive proportion of the world’s population is; these frequently are due to the viral epidemics to the agricultural commodities like cassava (Thresh, Otim-Nape et al. 1997). There have been efforts to develop strategies like development of resistance via breeding or gene manipulations to the existing germplasm of the cassava (Zhang, Vanderschuren et al. 2005), but along with these efforts there are number of new strains of the viruses which are developing day by day and it is quite difficult to bring resistance against the viral pathogen (Pita, Fondong et al. 2001).

The same is true about the Asian region where cotton infecting viruses have been appearing in epidemic forms with consecutive intervals since 1992 (Farooq, Farooq et al. 2011). This is because there is availability of suitable environment, abundance of the vector, numerous alternative hosts where these viruses have the chance to recombine and to come into being in the new more virulent forms and hence there is the breakage of the resistance against the already known viruses and the viral strains (Shih, Tsai et al. 2013).

With their limited genomes viruses can cause serious infections and can cease and alter metabolic pathways of the living cell (Gutierrez 1999). There is a great range and diversity in the plant infecting viruses including RNA viruses, ssDNA viruses, ds DNA viruses. Plant viruses are not as investigated as the animal viruses are. Tobacco mosaic Virus (TMV) was the first plant virus to be isolated. Plant infecting viruses can cause more than US 60 billion losses to the yield of the cultivated crops around the world every year. Plant viruses are arranged into 73 genera, 49 families and with an increasing number of species day by day. As compared to the viruses infecting cultivated plants, the viruses, that infect wild plants and the weed hosts are poorly studied (Roossinck 2011).

Plant viruses are particularly important in this regard that they can interrupt our food security causing great deal of problems for the human beings. They are capable to spread readily and to infect variety of non- cultivated and cultivated plants (Cooper and Jones 2006). Rice, wheat, maize, cassava, cotton, tobacco, chilies and vegetable crops are severely affected by the plant viruses. Cassava being the fourth largest food crop grown over the planet has been affected by the Begomoviruses and the cassava infecting viruses has been in epidemic form in the African region (Rybicki and Pietersen 1999).
Plant infecting viruses include both DNA and RNA viruses where the greater portion is occupied by the plant infecting RNA viruses. While most of the living beings contain double-stranded DNA but in case of the viruses the dsDNA viruses constitute only a minority, while most of the DNA viruses are ssDNA viruses. RNA viruses infecting plant are in great majority, which is largely comprised of ssRNA viruses having the same polarity (positive sense) as that of the mRNA of the cell (Gergerich and Dolja 2006).

As we all know that the plant viruses are biotrophic and obligate parasites and depend totally on the host cell for their replication while for the transmission the viruses need some vector to be transmitted to the host plant or via some mechanical injury to enter the host plant body. Vectors play a vital role in the transmission, maintenance and survival. We can say in the biodiversity of the viruses in a particular region depend on the diversity of the vectors found over there. This is because that for different viruses the specificity of the vector is strictly followed. White fly can transmit most of the Begomoviruses while the Mastreviruses are being transmitted by the leaf hopper (Andret-Link and Fuchs 2005).

Transmission is the fundamental property of the viruses. Viruses can be transmitted vegetatively via rootstocks, seeds, tubers or bud woods like in case of Citrus tristeza Virus (CTV) (Bar-Joseph and Lee 1989). Viruses can also be transmitted through nematodes, fungi and the insects. Occurrence and the diversity of the viruses in a particular plant population or in the particular region depend on the presence and abundance of the vector for that virus over there. *Tomato spotted wilt virus* (TSWV) transmitted via thrips vector infect more than one-thousand plant species (Parrella, Gognalons et al. 2003) showing the diversity and the range of the virus.

Among the DNA viruses there are three classes of the DNA viruses which infect plants. Theses DNA infecting viruses include *Geminiviridae, Nanoviridae*, *Caulimoviridae*. These DNA viruses have the special adaptations by which these viruses make their survival best on the planet. We see in case of *Geminiviridea* there are bipartite geminiviruses while all the other known DNA viruses in case of the vertebrates, invertebrates, prokaryotes are monopartite. So this bipartite nature may be an advantageous way for making their survival (Rojas, Hagen et al. 2005).
Fact about the viruses that how successful they are, can be inferred by the importance how economically important they are for human beings. DNA viruses in this context are the most important because these viruses have great impact on human life and the economy. Other things which make the DNA viruses more successful is their genetic make-up: have smaller genome; this smaller genome is capable of performing multiple jobs, replication via dsDNA. These characteristics make the DNA viruses most suitable for investigation of the replication studies in the plant (Matthews 2012). DNA viruses were less characterized as compared to the other viruses but increasing world population, increases in trade and the global movement of the plant and the plant materials responsible for the spread of these viruses from one place to another and hence it demands now to be investigated thoroughly. Reason for this increasing trend of investigation towards the DNA viruses is their economic importance and their great impact on the economy and the food security (Salati, Nahkla et al. 2002; Legg and Fauquet 2004).

Geminiviridae include the plant infecting ssDNA viruses with genome size of ~2.5- 3.0 kb, and accumulatively the genome size may range from ~ 2.5-5.0 kb. The twinned icosahedral structure for which the family name was derived from the Latin word “geminus” that means “twin”. Reviewing all the plant infecting viruses, it is evident that Geminiviruses are with the smallest possible genome, which can perform its independent replication (Harrison 1985). Being so successful plant infecting viruses, Geminiviruses have many structural and functional adaptations, which make these viruses to cope with the much larger cells and genome to make its survival successful over the past thousands of years. Geminiviruses have compact overlapped genome but with separate start codon for each ORF, which may be a structural adaptation to perform more functions with the limited genome. Coat protein of the geminiviruses is highly conserved for the virions but have higher level of specificity as well in sense of its interaction with the vectors transmitting these viruses (Briddon, Pinner et al. 1990). A single viral protein named Replication associated protein Rep, which has the conserved sequence (one, Rep; in case of bipartite begomoviruses while two Rep and Rep A in case of monopartite begomoviruses) is necessary for the viral replication (Gutierrez 1999; Hanley-Bowdoin, Settlage et al. 1999; Gutierrez, Ramirez-Parra et al. 2004; Hanley- Bowdoin, Settlage et al. 2004).
One or two Intergenic Regions, IR (SIR or LIR) are present in all the Geminiviruses, in one of which Origin of Replication (ori) is located with the stem loop structure having the invariant nonanucleotide region which serves as the beginning signal in case of Rolling Circle Replication (Hanley-Bowdoin, Settlage et al. 1999).

Geminiviruses have a promoter, which is bidirectional helps in the transcription of both the Virion and the Sense strand. This is an adaptation of the viruses to transcribe the overlapping genes on both the Virion and complementary sense strand. Along with this geminiviruses also show a great variety in context to their host vector interaction, genome structure and host range. On the basis of these properties Geminiviruses are classified into seven genera including Mastreviruses, Curtoviruses, Topocuviruses, Begomoviruses, Becurtoviruses, Turncuviruses and Eragroviruses. This variety of genome sequences, in the host vector interaction and host range is a reflection of a long evolutionary pathway.

Among all mentioned genera of Geminiviruses, Begomoviruses are the most important as these have economic importance because of their invasion to economically important crops. Among all the ssDNA viruses Begomoviruses are the extensively studied viruses which show their importance. Begomoviruses are the single stranded DNA viruses which are either with monopartite or bipartite genome and are transmitted by insect vector whitefly Bemisia tabaci (Brown 1994; Brown, Frohlich et al. 1995). Begomoviruses are categorized in two groups according to their evolutionary pathway i.e Old World (OW) begomoviruses and the New World (Kleinow, Tanwir et al.) begomoviruses. Begomoviruses infecting most of economically important crops include vide majority of the plant infecting Geminiviruses.

ssDNA viruses has narrow host range as compared to the RNA viruses with an exception of curtoviruses, its few members infect a wide variety of hosts (Rojas, Hagen et al. 2005).

As compared to plan RNA viruses the plant infecting ssDNA viruses are most widely studied because of their ability to infect more than one host and ability to recombine when more than one virus has been together in a single host. That is why ssDNA viruses can break down resistance and can possibly come into new forms and new strains are evolved. Cotton leaf curl Burewala strain was evolved in this way by the recombination of the cotton lead curl Multan virus and cotton leaf curl khokhran virus (Mansoor, Amin et al. 2003; Mubin, Briddon et al. 2009).
Begomoviruses which are with bipartite genome are composed of two circular molecules of ssDNA which has equal size of ~ 2.8kb each. These molecules are referred as DNA-A and DNA-B having no sequence identity with each other with an exception of a ~200bp Conserved region (CR) which has >90% sequence identity (Harrison 1985). This conserved region contains sequences which help to control the activities of transcription and translation (Garrido-Ramirez, Sudarshana et al. 2000) and hence believed to maintain the fidelity of both the DNA components of the bipartite begomoviruses (Hanley-Bowdoin, Settlage et al. 1999). DNA-A Component possesses four ORF which help in transcription, encapsidation and translation while the DNA-B component has only two ORF’s involved in the induction of symptom and the movement of viral particles. DNA-A and DNA-B both are necessary for the efficient development of the systemic infection (Whitham and Wang 2004).

Monopartite begomoviruses possess a single circular DNA molecule which is homologus to DNA-A molecule of bipartite begomoviruses, which depicts their possible role as early progenitor. Monopartite begomoviruses encode up to 6 proteins controlling all the functions of transcription, translation, encapsidation, movement and the pathogenicity (Wartig, Kheyr-Pour et al. 1997; Liu, Lucy et al. 2001).

For replication and transcription, geminiviruses have only a few proteins and depends totally on the host RNA or DNA polymerases for its transcription and replication (Hanley-Bowdoin, Settlage et al. 1999). Geminiviruses, being circular and ssDNA, replicate via rolling circle replication (RCR) inside the nucleus of the infected host cell. RCR is accomplished in two steps with leading strand and lagging strand synthesis and these two steps are entirely distinguished (Kornberg and Baker).

OW begomoviruses are found associated with small circular ssDNA molecules which are approximately half the size of the helper molecule (~ 1.4kb). These ssDNA molecules are called satellites which are of two types Alphasatellite and Betasatellite. These satellites depend totally on begomovirus for transmission through the vector and the replication (Mansoor, Briddon et al. 2003).

These satellite molecules have no sequence identity with the helper begomovirus except for the invariant nonanucleotide sequence TAATATTAC, apparently having no effect on the
viral replication and on symptom induction (Dry, Krake et al. 1997). Alphasatellite seems to have a parasitic nature because there is no evidence of its involvement in the replication of the viral molecule.

Alphasatellite is not a true satellite rather it is a satellite like molecule because alphasatellite has its own Rep and much similarity with the nanoviruses which are also ssDNA and circular molecules (Saunders and Stanley 1999; Briddon, Bull et al. 2004). Alphasatellites can perform self-replication but for encapsidation and transmission through vector it totally depends on the helper molecule (Mansoor, Khan et al. 1999; Mansoor, Briddon et al. 2003). Alphasatellites are distributed in the 5 classes depending upon their hair pin structure (Xie, Wu et al. 2010). Tomato Leaf Curl Oman Virus (ToLCOMV) and betasatellites related to it like Tomato Leaf Curl Betasatellite were accompanied by Ageratum yellow vein Singapore Alphasatellite which is far found in Singapore. When Nicotiana benthamiana was inoculated with all three molecules the Alphasatellite molecule attenuated the symptoms and also the accumulation of the betasatellite molecule.

Alternethera Yellow Vein Virus (AIYVV) which is first of all reported from China is found to infect Eclipta prostrate (He, Mao et al. 2008). AIYVV is accompanied with Alphasatellite molecules which apparently seem to have no effect on symptom induction and the disease severity.

Alphasatellites are reportedly having their role in the post transcriptional gene silencing. Two alphasatellite molecules isolated from the exotic species of cotton including Gossypium darwinii symptomless alphasatellite (GDarSLA) and Gossypium mustelinium symptomless alphasatellite (GMusSLA) which are unusual molecules having no identity with the already existing molecules. In a finding it was evaluated that Rep (replication associated protein) encoded by the alphasatellite interact with the C4 protein and the Rep protein encoded by the helper virus molecules Cotton leaf curl Rajasthan virus (CLCuRaV) while studied their interaction in the yeast two hybrid analysis. Rep protein of both the satellite molecules have strong gene silencing suppressor activity as compared to βC1 encoded by the betasatellite and the C4,V2 and C2 proteins encoded by the CLCuRaV (Nawaz-Ul-Rehman, Nahid et al. 2010).
Alphasatellite also called DNA-1 molecule, its biological role while it is associated with the begomovirus- satellite complex yet cannot be confirmed. Alphasatellite associated with *Tobacco curly shoot virus* isolate Y35 (TbCSV-Y35) attenuated the typical symptom of leaf curling. In case of Y35 symptoms of upward leaf curling were appeared in 9dpi while in case of plants inoculated with the Y35 + alphasatellite the symptoms were appeared in the 12dpi. The symptoms appeared in case of Virus Y35 accompanied by its associated alphasatellite were milder and the viral titer was also lower as compared to the plants inoculated only with the Y35 (Wu and Zhou 2005).

Tomato plantation in Oman was usually infected by *Tomato yellow leaf curl virus –Oman* strain (TYLC-OM) and this virus is accompanied by its associated betasatellite, which is an isolate of *Tomato leaf curl betasatellite* (TLCB). *Tomato leaf Curl Oman Virus* (ToLCOMV) and its associated alphasatellite *Ageratum Yellow Vein Singapore Alphasatellite* (AYVSGA) is a new combination recently isolated from the infected plants. *Tomato leaf Curl Oman Virus* (ToLCOMV). Nicotiana benthamiana plants were when inoculated with TYLC-OM, TLCB, ToLCOMV and AYVSGA it was found that along either of the helper virus TLCB produced more severe symptoms while when co-inoculated with alphasatellite the symptoms were milder as were observed with betasatellite (Idris, Shahid et al. 2011).

Association with the satellites is a feature of most of the OW monopartite begomoviruses but some NW begomoviruses are also found associated with the satellite viral molecules. In brazil two bipartite begomoviruses *Cleome leaf crumple virus* (CILCrV) and *Euphorbia mosaic virus* (EuMV) were isolated and these bipartite begomoviruses were found associated with the DNA1 (alphasatellite) like molecules. When *Arabidopsis thaliana* and the original plants were coinoculated with EuMV and CILCrV separately along with the associated satellite molecules. Both the EuMV and CILCrV showed the mild symptoms but CILCrV isolate trans-replicated the alphasatellite associated with it (Paprotka, Metzler et al. 2010).

From Cuba similar reports from two malvaceous plant species, which are infected by the bipartite begomoviruses accompanying satellite molecules including both the alphasatellitess and betasatellites. While plants were coinfected with helper virus and the alphasatellite there was milder symptoms and the viral titer was also low depicting that perhaps alphasatellite interfere of with helper virus.
Diversity and recombinations also exist in case of Alphasatellites. In a research conducted by (Saunders, Bedford et al. 2002) there has been observed two types of alphasatellite molecules DNA 1 and DNA 2 molecules. This DNA 2 molecule has a genome size which is almost same to that of DNA 1 molecule (DNA 2 1360 bp & 1367 bp DNA 1) and a typical nonanucleotide region (TAGTATTAC), which is identity of alphasatellite and DNA 2 molecule has the same kind of Rep that helps in replication of this satellite molecule. DNA 2 molecule also have an A-Rich region which is extensive and is immediately downstream the Rep of the satellite molecule. This DNA 2 molecule has the least sequence identity with the other alphasatellite molecules i.e.; it shares only 47% sequence identity with the AYVV DNA 1 molecule while 49 % sequence identity with the CLCuD DNA1 molecules respectively. This diversity is even shown at the protein level where proteins encoded by both the satellite molecules have less homology with each other as the proteins of two DNA 1 molecules have.

OW (monopartite) begomoviruses are accompanied by another kind of subviral molecule called betasatellite (DNA- β). This is a true satellite as it depends totally on the helper molecule for its complete lifecycle (Briddon and Stanley 2006).

Okra leaf curl disease in the West Africa is a major problem which is caused by a begomovirus complex including Okra Yellow Crinkle Virus (OYCrV), a recombinant isolate of Cotton Leaf Curl Gezira Virus (CLCuGV), a betasatellite and a DNA1 satellite associated with the OLCD in the Mali. Infectious molecules of OYCrV and CLCuGV produced symptoms in Nicotiana benthamiana but could not produce OLCD in Okra. But when both of these DNA-A molecules were coinoculated with the CLCuGB DNA successful symptoms were induced in both Nicotiana benthamiana and in Okra. Both of the begomoviruses-trans-replicated the CLCuGB and DNA1. DNA-1 decreases the DNA of helper begomovirus but there is no change in the symptoms produced. Presence of CLCuGB increased the DNA level of both the helper begomoviruses and DNA1 (Kon, 2009 #340).

*Tomato Yellow Leaf Curl China Virus* (TYLCCNV) isolates collected from *Siegesbeckia orientalis*, tomato plants and tobacco plants from china were found associated with the DNAβ molecules. Full-length infectious molecules of DNA-A and DNAβ were agroinoculated to petunia, *Nicotiana benthamiana* and *Nicotiana glatinosa* plants. It was
observed that TYLCCNV can systemically infect the plants but symptoms were not induced. For induction of the symptoms and typical leaf curl disease along with TYLCCNV DNA betasatellite is required. When C1 gene of TYLCCNV was mutated there was no replication of betasatellite and no symptoms were produced. *N. benthamiana* transgenic were produced using a construct having a C1 gene controlled by 35Spromoter produced the leaf curl symptoms (Cui, 2004).

An important vegetable corps subcontinent called Okra or bhindi (*Abelmoschusesculentus*) is being infected by yellow vein mosaic disease as a serious threat to its production. Yellow vein mosaic disease was produced by a complex of monopartite begomovirus and a betasatellite. *Bhendi Yellow Vein Mosaic Virus* (BYVMV) on agroinoculation can systemically infect the okra plants but with only mild symptoms of leaf curling. When okra plants were coinoculated with BYVMV and betasatellite, typical symptoms of leaf curling were produced (Jose and Usha 2003).

In Pakistan, production of Cotton is facing serious threat from cotton infecting begomoviruses. Infectious clones of monopartite begomoviruses infecting cotton, producing disease are alone unable to show the leaf curling symptoms and vein yellowing. A betasatellite is associated with the CLCuD complex. When CLCuV was coinoculated with the betasatellite, typical symptoms of leaf curling vein swelling were produced (Briddon, Mansoor et al. 2001).

A monopartite begomovirus and its associated satellite produce a disease in Radish. In this first study of RaLCD it was observed that the disease severity of the disease is due to associated betasatellite. *Radish Leaf Curl Virus* (RaLCV), *Croton Yellow Vein Mosaic Virus* CYVMV were monopartite viruses associated with TbLCB and CroYVMB satellites. *Croton Yellow Vein Mosaic Virus* (CYVMV) was originally related to its weed host “Croton”. It’s shifting to a cultivated host show the potential of viruses for changing hosts (Singh, Chattopadhyay et al. 2012).

ToLCNDV is a potential pathogen affecting the production of tomato. A survey of tomato, potato and cucurbit fields was conducted where plants were showing the symptoms of yellow mosaic, leaf curling, vein yellowing, distortion and leaf puckering. Among all the
begomoviruses isolated from the infected plants 44% showed the association with the betasatellites with LuLDB and CLCuMuB more common. DNA-B and DNA components of ToLCNDV were coinoculated to tomato (Solanum lycopersicum) and Nicotiana benthamiana plants with or without LuLDB and CLCuMuB. The level of virus genome was detected by using Real Time PCR at different stages of the infection. Plants coinfectected with the betasatellites were showing the more symptom severity and the enhanced levels of DNA A and DNA B. Betasatellites and the DNA B interacted antagonistically with each other. DNA-B level was observed to be enhanced in the presence of betasatellite by 16 times and level of betasatellite was reduced by 60% (Jyothsna, 2013).

Gene silencing or RNAi is an innate defense against viruses invading plants. Viruses encode suppressors as a counter defense that allows the viruses to effectively invade the plants. CLCuMV, a monopartite begomovirus and its associated betasatellite, CLCuMB cause CLCuD and alongwith this complex CLCuMA have the ability to suppress the gene silencing in the Nicotiana benthamiana. The CLCuMV is unable to suppress the gene silencing efficiently but in the presence of betasatellite the gene silencing was completely blocked [Amin, 2011 #345]

A tomato infecting virus was isolated from Japan that has highest sequence identity with the Tomato Yellow Leaf Curl Israel Virus (TYLCV-IL). Interestingly it was not found associated with any other pathogenicity determinant like DNA B or Betasatellite. For investigation of its interaction with satellite molecules, tobacco leaf curl Japan betasatellite and honeysuckle yellow vein mosaic betasatellite. TYLCV-IL transreplicated both the betasatellites and the symptom in tomato and Nicotiana benthamiana were more severe than alone TYLCV-IL(Ito, Kimbara et al. 2009).

A monopartite begomovirus isolated form West Bengal was found to have closest homology (93.6%) with tomato leaf curl Joydebpur virus (ToLCJoV). ToLCJoV was accompanied by a betasatellite identified as tomato leaf curr Joydebpur betasatellite. When ToLCJoV was alone agroinfiltrated to Solanum lycopersicum and N. benthamiana it produced severe disease symptoms in both Solanum lycopersicum and N. benthamiana. However, on coinoculation of Solanum lycopersicum and N. benthamiana plants with ToLCJoV and its cognate
betasatellite more severe symptoms were produced. When ToLCJoV was coinoculated with non-cognate betasatellite molecules, ToLCJoV successfully transreplicated all betasatelites with more severe symptoms. As ToLCJoV can alone produce systemic infection so it might be a satellite free virus (Tiwari, Singh et al. 2013).

Tomato Leaf Curl Gujarat Virus (ToLCGuV) isolated from Maharashtra, India has not DNA B as compared to Varanasi isolate of ToLCGuV. It was like typical OW monopartite begomovirus and was associated with only one kind of Betasatellite identified as tomato yellow leaf curl Thailand betasatellite (TYLCTHB). ToLCGuV is alone infectious and can produce systemic infection and clear disease symptoms in Nicotiana benthamiana and tomato. However, on agroinfiltration of both the ToLCGuV and TYLCTHB there was an enhanced disease severity and decreased time for it (Jyothsna, Rawat et al. 2013).

Chilli is an important vegetable crop in Pakistan being infected by the begomovirus disease complex. A chilli infecting virus was isolated from the Lahore region of Pakistan and on sequencing it was found to be a monopartite begomovirus which is recombinant of Chili leaf curl virus (ChLCV) and Papaya leaf curl virus (PaLCV) two species which are already reported from Pakistan. This newly characterized virus was proposed a name Pepper Leaf Curl Lahore Virus (PepLCLV). It was also found associated with already known satellite, Chili leaf curl betasatellite (ChLCB). Being first example of association of betasatellite with its cognate virus, which is a recombinant (Tahir, Haider et al. 2010).

Pedilanthus tithymaloides (Redbird flower) is an ornamental plant that showed the symptoms of enation and leaf curling in Pakistan. The symptoms were typical of begomovirus. On isolation and sequencing a monopartite begomovirus was isolated that has highest sequence identity with TYLCV (90.3% homology) and Radish leaf curl virus (86.3%). The betasatellite found associated with the begomovirus was Tobacco leaf curl betasatellite. This Pedilanthus infecting virus was found to be a distinct virus so a name Pedilanthus leaf curl virus (PedLCLV) was proposed (Tahir, Haider et al. 2009).

ToLCNDV a bipartite begomovirus infecting tomato plants. An experiment was conducted to unravel the role of betasatellite in disease induction, systemic movement and infection in leaf curl disease caused by begomoviruses. It was observed in the experiment that alone DNA A
of ToLCNDV produced mild symptoms in Nicotiana benthamiana and tomato. But when DNA A was coinoculated with DNA B or CLCuMB typical leaf curl symptoms were produced. The produced symptoms were even more severe when the plants were infected with all the three components which showed the role of betasatellite in the pathogenesis of ToLCNDV. There was reduced level of viral DNA in the plants infected by DNA A alone but it was increased by many folds when coinfected with DNA-B/ betasatellite. Plants infected with all the three components have 20 times less betasatellite accumulation than the plants infected with betasatellite and DNA A (Sivalingam and Varma 2012).

Watermelon in Venezuela is infected by a NW bipartite begomovirus, melon chlorotic mosaic virus (MeCMV). A putative alphasatellite Mellon Chlorotic mosaic alphasatellite (MeCMVa1) was found associated with the MeCMV. This alphasatellite is the first instance of association of an alphasatellite with some NW bipartite begomovirus. MeCMVa1 is atypical one having a putative ORF2 with in the Rep binding ORF1 (Romay, Chirinos et al. 2010).

A monopartite begomovirus and its associated Tomato leaf curl Java betasatellite (ToLCJB) causes the Ageratum yellow vein disease. AYVV can alone symptomatically infect the plants and induces upward leaf curling. ToLCJB is required for the induction of severe downward curling and the vein yellowing in Nicotiana benthamiana. ToLCJB having a frame shift mutation in the βC1 protein when coinoculated with the AYVV could not produce the severe symptoms in Nicotiana benthamiana. Infectivity analysis of AYVV and its associated ToLCJB encoded genes was carried out using potato virus X (PVX) vector in Nicotiana benthamiana which revealed that V2 of AYVV and βC1 of betasatellite are symptom determinant. It was also identified that V2 of AYVV and βC1 of betasatellite are efficient suppressors (PTGS) (Sharma, Ikegami et al. 2010).

Pouzolzia zeylanica (L.) Benn is a weed and was infected by begomovirus showing typical yellow mosaic symptoms in Guangdong, China (CD) and Vietnam (VN). Sequences of viruses isolated from both the regions were compared with each other. GD1 has 86.2% sequence identity with the VN. Both VN and GD1 shared the highest nucleotide sequence identity 91.4% and 86.7% respectively with the isolate of pouzolzia golden mosaic virus (PGMV-TY01), which has already been isolated from Pouzolzia zeylanica. Phylogenetic
analysis of PGMV, VN and GD1 showed that all these three viruses belong to different clade. According to the ICTV species demarcation guidelines GD1 belongs to a new species. While VN is strain of PGMV and it was a recombinant of Ageratum Yellow Vein China Virus (AYVCNV) and PGMV (Tang, Du et al. 2014).

*Sauropus androgynous* (L.) Merr. is a weed which showed leaf curling and vein yellowing symptoms typical of begomoviruses in Thailand. 17 samples were taken from different infected plants and on amplification through PCR DNA A was amplified from all the samples. No DNA B was present in any of the sample which showed the monopartite nature of the virus. Study of genome organization showed that all the DNA A molecules has genome structure of monopartite begomoviruses with six open reading frames. Sequence comparison of the isolated viruses showed that one belongs to Tomato leaf curl New Delhi virus, 12 belong to Ageratum yellow vein virus while 4 isolates belong to distinct species, with tentative name of Sauropus leaf curl virus. One of these four isolated was believed to arise through recombination event. The experiment showed that the *Sauropus androgynous* serves as a reservoir for accumulation and recombination between different begomoviruses (Shih, Tsai et al. 2013).

*Amaranthus* Amaranth, a perennial plant that is used as vegetable and also for ornamental purpose in many regions of the world including India. *Amaranthus* plant having vein yellowing, vein thickening, leaf distortion and leaf crinkling were collected from Banswara district of Rajasthan, India. Amplification yielded full length monopartite viral molecules, alphasatellite and betasatellite. Nucleotide sequence of the begomovirus revealed it as typical OW monopartite begomovirus with highest nucleotide sequence identity with Chilli leaf curl virus and is an isolate of Chilli leaf curl virus. The nucleotide sequence comparisons of alphasatellite and betasatellite showed the highest nucleotide sequence identity with ChLCuA and TYLCTHB respectively. First case was found where a Chilli viruses and satellites on a totally diverse and new host (George, Kumar et al. 2014).

A monopartite begomovirus was isolated from the infected samples of *Hedyotis uncinella*. This virus was previously named as *H. Uncinella Yellow Mosaic Virus* (HUYMV). Sequence comparison of the newly isolated virus presenting higher homology with Premna leaf curl virus (PLCuV ) and Soybean crinkle leaf virus (SbLCV) at 81.9% and
82.1%. respectively. According to ICTV criterion for species demarcation in begomoviruses this is a new species. HUYMV sequence shows the recombination with *Tomato leaf curl Philippines virus* (ToLCPV) as minor parent and an unknown virus as a major parent. HUYMV was also found associated with a betasatellite which presented higher homology with *Tomato leaf curl Philippine betasatellite* (70%) and was proposed a name *H. uncinella* yellow mosaic betasatellite (Du, Chen et al. 2014).

*Justicia adhatoda* and *Tecoma stans* plants showing leaf curling symptoms were collected for viral amplification from Sikar, Rajasthan. CP of DNA A was amplified from the infected samples using universal primers through PCR. Sequence analysis showed that the virus infecting *Justicia adhatoda* has the highest sequence identity with *Vinca Yellow Vein Virus* (VYVV) (79%) and the one infecting *Tecoma stans* has highest nucleotide sequence similarity with *Lantana yellow vein virus* (100%) (Sahu et al. 2013).

*Ageratum conyzoides*, *Datura stramonium*, *Croton bonplandianum* and *Jatropha curcas* are the common weeds grown in and around the cultivated fields of sub Himalayan region. Vein yellowing, leaf enations, vein thickening, leaf curling, stunted growth and the distortion are the symptoms observed in the above mentioned weed plants. CP is the conserved sequence and often in begomovirus identification and classification CP sequences are useful. Universal primers for CP were used for the amplification from the infected weed hosts. Three begomoviruses were amplified from *A. conyzoides*, *C. bonplandianum* and *A. indica* using universal primers. Sequence analysis of the amplified viruses revealed that there is a remarkable variation among the present sequences although the symptoms were similar. But the interesting aspect was that the weed infecting viruses showed less variation in sequences as compared to the crop infecting begomoviruses (Saha et al. 2013).

Begomovirus disease complex involving satellites is becoming a serious issue. Recombinations among the viruses and satellites inside the host has a vital role in evolution of viruses. In a study a novel defective satellite (RecSat) was found associated with the *Tobacco leaf curl Yunnan virus* (TbLCYNV). Sequence analysis showed that the RecSat have a size of 754 nt and it is a chimera molecule having segments both form alphasatellite and betasatellite with CR from betasatellite and stem loop from alphasatellite. Recombination analysis revealed that RecSat evolved via recombination from three recombination events
involving yellow vein China betasatellite, Tobacco curly shoot alphasatellite and Tomato yellow leaf curl China betasatellite and Ageratum. When *Nicotiana benthamiana* plants were coinoculated with RecSat and TbLCYNV the plants showed the severe disease symptoms than alone with TbLCYNV (Huang, Xie et al. 2013).

For diversity studies of begomoviruses infecting weed especially *Sida* spp. Samples were collected from different regions of Brazil. RCA was used for the amplification of begomoviruses. Sequence analysis of the cloned viruses showed existence of 10 different begomoviruses. Virus molecules amplified from *Blainvilllea homboidea* belong to the same species, *Blainvillea* yellow spot virus (BIYSV), suggesting that BIYSV might be the only virus infecting this weed host. Among all the isolates four represent the new species with proposed names as: *Sida yellow mosaic Alagoas virus* (SiYMAV), *Sida yellow blotch virus* (SiYBV), *Sida yellow vein virus* (SiYNV) and *Sida mottle Alagoas virus* (SiMoAV). SiYNV and SiYBV isolates showed recombination. This study represent the high diversity of begomoviruses in weeds (Tavares, Ramos-Sobrinho et al. 2012).

A mono partite begomovirus and an alphasatellite were isolated from *Xanthium strumarium* L. Sequence analysis revealed that isolated alphasatellite has the highest nucleotide similarity (96.7%) to an *Gossypium darwinii* symptomless alphasatellite and 85.8% with *Papaya leaf curl alphasatellite*. A tentative name was proposed as *Gossypium darwinii* symptomless alphasatellite isolate *Xanthium* (Akram, Azeem et al. 2013).
Chapter 3: MATERIAL AND METHODS

3.1.2 Extraction of DNS from plant samples

Extraction of DNA from infected plant leave samples was done by the following protocol,

DNA was extracted from leaf samples by the CTAB method described by Doyle and Doyle (1990). 100 to 200mg of leaf tissue was ground in liquid nitrogen in a pestle and mortar. In a microcentrifuge tube the powdered tissue was mixed with 700μL of pre-warmed CTAB buffer (100mM Tris-HCl [pH 8.0], 20mM EDTA, 1.4M NaCl, 2% [w/v] Cetyl Triethyl Ammonium Bromide (CTAB) and 0.02% (v/v) β-mercaptoethanol) and incubated at 65°C for 30 min. After lowering the temperature of the samples to room temperature, an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed well and centrifuged at 9000 rpm for 10 min at room temperature in a microfuge (Eppendorf model 5414D). The upper aqueous, DNA-containing phase was taken into a new microcentrifuge tube and mixed with 0.6 volume isopropanol to precipitate the DNA. DNA was pelleted by centrifugation at 13,200 rpm for 10 minutes and, after discarding the supernatant, the pellet was washed with 70% (v/v) ethanol and air dried. Finally the pellet was dissolved in sterile distilled water (SDW).

3.2 Amplification of DNA

3.2.1 PCR amplification of DNA

For amplification of DNA by PCR a reaction mixture of 25μL containing 10pg-1μg template DNA, 2.5μL 10X Taq polymerase buffer (Fermentas), 0.5μL 10mM dNTP mix., 1.5mM MgCl₂, 0.5μM each of primers and 1u Taq DNA polymerase (Fermentas) was prepared in 0.25mL or 0.5mL PCR tubes. The reaction mixture was incubated in thermal cycler (Eppendorf Model, Hamburg, Germany or Creacon technologies Model).

The thermal cycler was programmed for a preheat treatment of 94°C for 5 minutes followed by 35 cycles of 94°C for 1min, 48°C to 52°C for 1min and 72°C for varying times (dependent upon the length of fragment to be amplified; typically 1 min per 1000 nucleotides to be amplified), followed by a final incubation of 10 min at 72°C and routinely the machine was set to hold at 4°C until the samples were removed. Universal as well as specific primers were used for DNA amplification from all samples. For diagnostic PCR the volume of the reaction mixture was 25μL per tube by using the ingredients accordingly as mentioned above.
3.2.2 Rolling-circle amplification (RCA)

For amplification of circular DNA molecules by RCA a reaction mixture of 20μl containing 100 to 200ng genomic DNA of infected plant samples, 50 μM random hexamer primers, 2μl 10X Φ29 DNA polymerase reaction buffer (330mM Tris-acetate [pH 7.9] ), 100mM magnesium acetate, 660mM potassium acetate, 1% (v/v) Tween 20, 10mM DTT) was prepared and incubated at 94°C for 3 minutes to denature double stranded DNA.

The mixture was cooled to room temperature and mixed with 1mM dNTPs, 5-7 units of Φ 29 DNA polymerase and 0.02 unit of pyrophosphatase (to eliminate inhibitory accumulation of pyrophosphate) and incubated at 28°C for 18 to 20 h. The following day Φ29 DNA polymerase was inactivated at 65°C for 5 minutes.

3.3 Cloning of amplified DNA

3.3.1 Cloning of PCR product

PCR amplified fragment was cloned using the PCR Cloning Kit (Fermentas) according to the instructions given by the manufacturer. In brief, a reaction mixture of 30μL containing 18 to 540ng PCR product (depends upon the length of DNA fragment), 3μL vector (pTZ57R/T), 6μL 5X ligation buffer and 5u T4 DNA Ligase, was prepared in a 1.5mL micro centrifuge tube and incubated at 16°C overnight. The following day the ligation mixture was transformed to competent cells of E.coli (DH5α/Top10) by the heat-shock method.

LB agar media plate with 100μg/mL ampicillin, spread with 20μL (50mg/mL) X-Gal and 40μL (20mg/mL) IPTG were used for plating-out the transformed cells and incubated at 37°C for 16 hours. On appearance of colonies white colonies were picked by sterile tooth picks, inoculated in 5mL LB (1% [w/v] tryptone, 0.5% yeast extract and 1% [w/v] NaCl) liquid media in autoclaved test tube and grown at 37°C in a shaker overnight. The following day plasmids were isolated from E. coli cultures by the miniprep method and screened by restriction analysis.
3.3.2 Cloning of RCA product

RCA product was digested with unique cutter enzymes into monomers. Cloning vector (usually pTZ57/RT) was also restricted with same enzyme. Restricted RCA product and vector were treated with phenol-chloroform or pass through the column, using the kit by Wizard SV Gel and PCR Clean-Up System (Fermentas) by the method described by the manufacturer to remove salts. Vector and insert were ligated in a reaction mixture of 20 μL containing vector and insert in 1:3 ratios, 4μL 5X ligation buffer and 1u T4 DNA ligase. Ligation mixture was kept at 16°C overnight and next day transformed into competent E. coli cells.

3.4 Transformation of heat-shock competent E. coli cells

Transformation of competent E. coli cells was carried out by the methods described by Sambrook, Frisch and Maniatis (1989). The ligation mixture was added to thawed competent E. coli (200μl), mixed gently and incubated on ice for 15min. The cells were shocked at 42°C a dry bath-water bath. After 45 seconds cells were transferred to ice and incubated for two min. 1mL LB liquid medium was mixed in each tube and put on shaking at 37°C for 45min. Transformed cells were spread on solid LB media plates with appropriate antibiotics and kept at 37°C in an incubator overnight.

3.5 Transformation of competent Agrobacterium tumefaciens cells

2μL of the plasmid was mixed with electro-competent A. tumefaciens cells (GV3101) on ice and wrapped with aluminium foil and dipped in the liquid nitrogen for 5min. After 5min thawed on ice for 5min and then added 200μl of liquid LB media and then placed at RT for 2-3 hrs. Transformed cells were spread on LB medium with appropriate antibiotics kanamycin (30μg/ml); rifampicin (50μg/ml) and Gentamycin 12μg/ml (for GV3101, for pGreen0029, pCambia0013) wrapped with aluminium foil and incubated at 28°C for 48h.
3.6 Plasmid isolation

Using a sterile toothpick, a single bacterial colony from a plate was picked and inoculated into 5mL LB broth with appropriate antibiotic selection in a sterile culture tube. It was incubated overnight at 37°C with shaking at 250 rpm. When the culture was ready, it was decanted in 1.5mL micro centrifuge tubes and centrifuged at full speed for two minutes to harvest the cells. The supernatant was discarded and the pellet was re-suspended in 100μL Resuspension solution (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 μg/mL RNase A) using a vortex. 200μL Lysis solution (0.2 M NaOH, 1% [w/v] SDS) was added and mixed gently. 200μL Neutralization solution (3.0M Potassium Acetate, 5.0M glacial acetic acid, [pH 5.5]) was added, mixed thoroughly and centrifuged at 13,200 rpm for 10 minutes. The supernatant was transferred to a new micro centrifuge tube. Two volume (approximately 1mL) chilled absolute ethanol was added to precipitate the DNA and centrifuged for 10 minutes to pellet the DNA. DNA pellet was washed with 70% (v/v) ethanol; air dried and dissolved in sterile distilled water (SDW). For DNA sequencing, the plasmid was isolated by Gene JET Plasmid Miniprep Kit (Fermentas). The culture of E. coli was decanted into 1.5 mL micro centrifuge tube and centrifuged for 2 minutes. The pellet was re-suspended in 250μL resuspension Solution and cells were lysed with 250μL Lysis Solution. 350μL Neutralization Solution was added mixed thoroughly and the tube was centrifuged at 13,200 for five minutes. A mini-column provided with the kit was inserted into the collection tube and the supernatant was transferred to the column. The column was centrifuged for one minute to bind the DNA to the matrix and the flow through in the collection tube was discarded. The matrix was washed twice, first with 700μL Column Wash Solution was added and incubated at room temperature for one minute and then centrifuged for one minute. The Wash solution was removed from the column and the column washed with 500μL Column Wash solution. Subsequently the column was centrifuged for one minute with an empty collection tube to remove residual ethanol. Finally the column was inserted into a fresh micro centrifuge tube. DNA in the column was dissolved in 50μL SDW, incubated at room temperature and recovered by centrifugation.
3.7 Digestion of plasmid DNA

Digestion of plasmids and PCR products was done using restriction endonucleases and their corresponding buffers in accordance with the supplier's (Fermentas) guidelines. A total reaction volume of 10µl was used when screening plasmid preparations for the expected insert, 20µl for digestions incubated overnight for cloning.

3.8 DNA Analysis

3.8.1 Agarose-gel electrophoresis

DNA was mixed with 5X loading dye and electrophoresed in 1% (w/v) agarose gels containing ethidium bromide (0.5µg/mL). Gels were prepared in a minigel apparatus (12 x 9 cm) or midigel apparatus (18 x 15 cm), containing either 1X TBE (890mM Tris [pH 8.3], 890mM boric acid, 20mM EDTA) or 1X TAE (40mM Tris-acetate [pH 8.4], 1mM EDTA) buffer. TBE gels were electrophoresed at approximately 50V and TAE gels at 110V. The DNA was viewed using a short wavelength ultraviolet (UV) transilluminator (BioRed) and fragment length estimated by comparison with a co-electrophoresed 1 kbp DNA ladder (Fermentas).

3.8.2 Southern Blot analysis

- 10µg genomic DNA per well was loaded on a 0.8% (w/v) agarose gel and run at 80V in TBE buffer for 4 to 5 h.
- Gel was stained with 0.5µg/mL ethidium bromide and DNA image was obtained under UV light in gel documentation apparatus (JRMEO,).
- After electrophoresis the gel was treated with depurination solution (0.25M HCl) for 15 minutes, denaturation solution (1.5M NaCl and 0.5M NaOH) for 30 minutes and neutralization solution (1M Tris [pH 7.4], 1.5MNaCl) for 30 minutes.
- The gel was rinsed briefly with distilled water between treatments and shaken moderately on platform shaker during each treatment.
- DNA in the gel was transferred to a nylon membrane (brand name) in 10X SSC and sometimes in 5X SSC (1.5M NaCl and 150mM sodium citrate) by capillary action.
The DNA on the nylon membrane was cross-linked by UV irradiation (CL-1000, UVP) at 120mJ/cm² energy.

The membrane was then rinsed in a solution containing 0.1X SSC, 0.5% (w/v) SDS at 65°C for 45 minutes to remove residual agarose.

Before hybridization the membrane was treated with 0.2 ml/cm² pre-hybridization solution (6X SSC, 5X Denhard’s solution (0.1% (w/v) each of bovine serum albumin, 0.5% (w/v) Ficoll (Mol. Wt. ~400,000) and PVP (Mol. Wt. ~40,000)), 50% (w/v) SDS) and 5mg/mL sheared and denatured salmon sperm DNA at 42°C for 24 hours in a hybridizer (model), to block non-specific binding sites.

DNA probes were prepared using a Biotin Deca Label DNA Labelling kit (Fermentas) according to the manufacturer’s instructions.

Briefly, in a 1.5mL microcentrifuge tube 44 µL reaction mixtures was prepared by adding 50-200ng DNA template (purified PCR product), 10µL deoxynucleotide in 5X reaction buffer and nuclease free water.

The reaction mixture was vortexed briefly, centrifuged briefly in a microfuge to collect the contents at the bottom of the tube and incubated in boiling water (100°C) bath for 5-10 min.

After incubation the tube was cooled on ice, briefly centrifuged and the contents of the tube mixed with 5µL biotin labelling mixture and 1µL Klenow fragment exonuclease (5units) and incubated at 37°C for 1 to 2 hour.

To prepare hybridization solution, the biotin labelled probe was denatured at 100°C for 5 minutes, chilled on ice and mixed with pre-hybridization solution (25-100ng/ml).

After 2-4h treatment, the pre-hybridization solution was discarded and the hybridization solution was added to the membrane (60µL/cm²) and incubated
overnight in a hybridizer at 42°C.

The following day the membrane was washed twice with 2X SSC/0.1% (w/v) SDS at room temperature for 10 minutes.

The membrane was washed with 0.1X SSC/0.1% (w/v) SDS twice at 65°C for 20 minutes.

To detect the biotin-labelled DNA the membrane was washed in 30mL Blocking/Washing Buffer (provided by the manufacturer) at room temperature.

After 5 minutes the membrane was treated with 30mL Blocking Solution for 30 minutes to block non-specific binding sites on the membrane.

Streptavidin-AP conjugate was diluted in 20mL Blocking Solution and the membrane was incubated for 30 minutes.

The membrane was washed twice in 60mL Blocking/Washing buffer for 15 minutes and incubated with 20mL Detection Buffer for 10 minutes.

Finally the membrane was treated with 10mL freshly prepared Substrate Solution at room temperature in the dark until blue-purple precipitate became visible.

To stop the reaction, the substrate solution was discarded and the membrane was rinsed with water.

The blot was immediately photographed and the membrane was then air dried and stored.
3.9 Purification of DNA

3.9.1 Gel extraction and PCR product purification

DNA was run on 1% (w/v) agarose gels and the desired fragments were cut out from the gel using a scalpel under UV light. DNA from the gel was isolated using a Wizard SV Gel and PCR Clean-Up System (Promega) by the method described by the manufacturer. The excised gel slice was weighed and placed in a 1.5mL microcentrifuge tube to which was added 10µL Membrane Binding Solution per 10mg of gel slice. The tube was vortexed and incubated at 55-65°C until the gel slice was completely dissolved. An equal volume of Membrane Binding Solution was mixed with the dissolved gel mixture and then transferred to the Minicolumn assembly, incubated at room temperature for 1 minute and centrifuged at 16,000×g for 1 minute. The flow-through was discarded and the Minicolumn was reinserted into the collection tube. 700µL of Membrane Wash Solution was added and the tube with column centrifuged at 16,000×g for 1 min. Again the flow-through was discarded and 500µL Membrane Wash Solution was added in the column. After 5 min of centrifugation at 16,000×g the collection tube was emptied and the Minicolumn, with empty collection tube, was centrifuged for 1 min with the lid open to allow evaporation of any residual ethanol. Finally the Minicolumn was transferred to a clean microcentrifuge tube, 50µL nuclease-free water was added to the Minicolumn, incubated at room temperature for 1 min and centrifuged at 16,000×g for 1 min. The Minicolumn was discarded and purified DNA was stored at -20°C.

3.9.2 Phenol-chloroform treatment of DNA

To remove proteins from, phenol: chloroform (1:1) extraction method was used. Equal volume of phenol: chloroform was mixed with the DNA solution and vortexed until the mixture turned milky. It was centrifuged at 13,200 rpm for 10 minutes and upper aqueous phase was collected in such a way that the layer between upper aqueous phase and phenol: chloroform was not disturbed. 1/10 volume 3M sodium acetate [pH 5.4] and 2.5 volume chilled absolute ethanol was mixed into supernatant and placed at -20°C in a freezer for one hour. To pellet the DNA, it was centrifuged at maximum speed. DNA pellet was washed with 70% (v/v) ethanol, air dried and dissolved in appropriated amount of SDW.
3.10 Microbiological Techniques

3.10.1 Preparation of heat shock competent *Escherichia coli* cells

A single colony from a freshly grown plate of *E. coli* was inoculated into 20mL LB medium in a 50mL flask and incubated at 37°C overnight with vigorous shaking. The following day 2mL of the overnight culture was taken and diluted to 250mL in 1L flask and shaken vigorously at 37°C until an OD600 of 0.5-1 was achieved. The culture was chilled on ice for 30 minutes, transferred aseptically to sterile disposable 50mL propylene tubes and centrifuged at 4000 rpm at 4°C for 5 minutes to pellet the cells. The pellet was re-suspended in 20mL of 0.1M MgCl2 and centrifuged again. Pallet was re-suspended in 20mL of 0.1 M CaCl2, incubated on ice for 30 minutes and centrifuged at 400 rpm. Finally the pellet was re-suspended in appropriate amount of 0.1M CaCl2 and filter-sterile cold 30% (v/v) glycerol. The cells were stored in aliquots of 200µl at −80°C.

3.10.2 Preparation of electro competent *Agrobacterium tumefaciens* cells

Before preparing the electro-competent cells, the *Agrobacterium tumefaciens* strain GV3101 was transformed with pSoup plasmid and grown in LB media containing tetracycline (50 µg/ml) as the pGO029 can’t replicate in *Agrobacterium* without the pSoup being co-resident (Hellens, Mullineaux, and Klee, 2000). A single colony from a freshly grown plate of *A. tumefaciens* was transferred using a sterile tooth pick and inoculated into 20mL LB liquid medium with 25µg/mL rifampicin in a 50mL autoclaved flask and incubated with vigorous shaking (140 rotations per minute) at 28°C for 48 hours. 5mL of the culture was re-inoculated into a 1L flask containing 250mL-350mL of the LB medium with 25µg/mL rifampicin and put on shaking at 28°C until the OD600 of the cells was 0.5-1. When the desired OD600 of the cells was achieved the flask was cooled on ice for half an hour before starting the next step. Set the centrifuge temperature of about 4°C before starting the cells making. The cells were transferred aseptically to ice cold 50mL propylene tubes, centrifuged at 4000 rpm for 10 minutes at 4°C. The pellet was re-suspended in 50mL of cold SDW and centrifuged again under the same conditions. Cells were again re-suspended in cold double distilled deionized water and the wash was repeated. Now the cells were re-suspended in 10mL cold SDW containing filter sterilized 10% (v/v) glycerol and centrifuged at 4000 rpm. This step was repeated. Finally the cells were re-suspended in 2ml of filter sterilized cold 10% (v/v) glycerol, aliquoted in 1.5mL microcentrifuge tubes and stored at −80°C.
3.10.3 Agrobacterium-mediated inoculation

Clones in the binary vector pGreen0029 were transformed to Agrobacterium strain GV3101. For agro-inoculation, glycerol stocks of Agrobacterium strains GV3101 with required clones were streaked on solid LB plates containing 12.5µg/mL rifampicin and 50µg/mL kanamycin and incubated at 28°C for 48h. A single colony of bacterial cells was transferred with a sterile wire loop and inoculated in 50mL liquid media containing antibiotics and put on shaking (160 rotations per minute) at 28°C until the O.D600 of the culture was 1. The cells were harvested by centrifugation at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10mM magnesium chloride containing 100µM acetosyringone. For agro-inoculation to N. benthamiana plants at 4 to 5 leaves stage were not watered for 24 hours before inoculation. N. benthamiana seedlings were inoculated infiltrating the bacterial suspension under pressure into the leaf stab using a sterile disposable 5ml syringe without a needle.

3.11 Plant growth conditions

All plants were grown in controlled conditions in growth rooms at 25°C with 16h dark period/8h light period and 65% humidity in small 5 inch diameter plastic pots containing clay, silt, sand and compost in equal proportions. Cotton plants were grown at 28°C. All plants were watered daily.
Chapter 4: RESULTS

Results:

4.1 Symptomatology:
Since 2011 and 2012 intensive sampling of *Eclipta prostrata* infected plants was done from distant and different districts of Punjab as is show on the map below (Fig 4.2). This weed was found almost at every place of sampling around the cultivated crops especially sugarcane, cotton and rice and also besides the water courses. *Eclipta prostrata* infected plants at every place were showing almost the same vein yellowing symptoms. These symptoms of vein yellowing on infected plants were so prominent that noninfected plants show clear distinction. (Fig 4.1).

![Eclipta prostrata plant A) Symptomatic plant showing vein yellowing symptoms; B) asymptomatic plant](image)

Fig. 4.1: *Eclipta prostrata* plant A) Symptomatic plant showing vein yellowing symptoms; B) asymptomatic plant
4.2: Detection of Geminiviruses:

4.2.1: Detection of components of Begomovirus disease complex through PCR

For the detection of geminiviruses form the collected samples of infectious plants, especially for confirmation of begomoviruses diagnostic PCR was performed using diagnostic Primer pair designed to the conserved sequence of begomoviruses CP. An amplification of ~ 0.8 kb was expected if the begomoviruses were present in the infected samples. For detection of DNA B component of begomoviruses primer pair BC1F/BC1R was used (Hussain et al., 2004). These primes were designed at the MP gene sequence of ToLCNDV and an amplification of 850 bp was expected in the presence of DNA B component of begomoviruses.
4.3: Detection of satellites in the Begomovirus disease complex through PCR:

4.3.1: Detection of Alphasatellites:

For the detection of alphasatellites associated with the disease complex of begomoviruses, universal primer pair α01 and α02 was used (Ref). This primer pair yielded an amplification of ~ 1.4 kb showing the presence of the alphasatellites in the infected samples. This PCR of the alphasatellites proved the association of alphasatellites with the begomovirus disease complex of *E. prostrata*. Genetic features of the begomovirus and satellite infecting *E. prostrata* are given in the (Table 4.1).

4.3.2: Detection of Betasatellite:

Detection of betasatellites associated with the begomovirus disease complex was done by using universal primer pair Beta01/Beta02 (Briddon, Bull et al. 2002). An amplification of ~ 1.4kb was expected in this case but in spite of multiple efforts using infected samples from different regions no amplification of betasatellite was obtained.
Table 4.1: Features of Begomovirus isolated from Eclipta prostrata.

<table>
<thead>
<tr>
<th>Begomovirus</th>
<th>ORFs</th>
<th>Start codon (nucleotide coordinates)</th>
<th>Stop codon (nucleotide coordinates)</th>
<th>Predicted size of ORFs (bp)</th>
<th>Predicted size (no. of amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP</td>
<td>314 1084</td>
<td>771</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rep</td>
<td>2618 1533</td>
<td>1086</td>
<td>361</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TrAP</td>
<td>1630 1226</td>
<td>405</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>REn</td>
<td>1485 1081</td>
<td>405</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C4</td>
<td>2461 2171</td>
<td>290</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

4.4: Detection of components of begomovirus disease complex using RCA

Detection of full length ssDNA molecules of begomoviruses and associated satellite from the infected samples was also done by using \( \phi 29 \) DNA polymerase enzyme, which has the potential to amplify all circular molecules (Johne, Müller et al. 2009). All the amplifications were subjected to restriction analysis using different single restriction enzymes like BamH1, EcoR1, HindIII, Xba1 and Sal1, and both 2.8kb and 1.4kb fragments produced by restriction of RCA product were cloned (Fig 4.3).
4.5: Cloning and sequencing of full length viral molecules

Amplified products of both RCA and PCR were cloned in pTZ57R/T. Confirmation was done by restriction analysis using suitable restriction enzymes. The confirm molecules of both 2.8kb and 1.4kb were prepared for sequencing. Sequences obtained were used for BLAST analysis.

Fig. 4.3: Amplification and cloning of viral molecules associated with vein yellowing disease of Eclipta prostrata; A) Amplified RCA product and restriction of RCA product yielding 2.8kb and 1.4kb fragments. B) Confirmation of cloning of virus 2.8 kb in pTZ57R/T vector. C) Confirmation of cloning of satellite molecules i.e., 1.4kb in pTZ57R/T vector
Fig. 4.4: Phylogenetic dendrogram based upon selected complete sequences of AlYVV. Begomovirus sequences used for comparison are downloaded from databank. The database accession number in each case is given. The sequences associated with Alternanthera yellow vein disease are indicated by a red color in each case.
4.6: Sequence and Phylogenetic analysis

4.6.1: Sequence and Phylogenetic analysis of Alternanthera yellow vein virus

From all collected infected samples from different and distant place of Punjab province, around 75 full length (2.8kb) viral molecules were cloned; which revealed higher level of sequence similarity with *Alternanthera Yellow Vein Virus* (AIYVV). Then 15 molecules were completely sequenced. These complete sequences of isolated viruses revealed the presence of AIYVVonly. For the confirmation of DNA-B the PCR was performed by using diagnostic primers but found no DNA-B molecule. All isolated viruses from Eclipta prostrata showed higher level of sequence similarity with AIYVV reported earlier from china. Although samples were taken from the infected plants from the distant places of Punjab province. In the phylogenetic tree it was evident that sequences of the AIYVV had a high level of sequence similarity with the viruses isolated from Vietnam and China. For example the AIYVV isolated from Vietnam had a 91% similarity index with the AIYVV isolated from Pakistan.

The phylogenetic tree is also showed that this conservation in the sequence of AIYVV was not only consistent with respect to the geographical positions rather it was also consistent over the period of time. The sequence of the virus isolated from Pakistan in 2009 had 98% to 100% similarity level with each other. Similarly the full length AIYVV isolated from China in 2006 had least difference in the nucleotide sequence and had a higher level of homology with each other and these viruses were seemed to be the strains of each other without having considerable difference over a period of 8 to 10 years.

Higher level of sequence similarity also revealed that apparently no recombination of AIYVV occurred with any other begomovirus.
Fig.4.5: Phylogenetic dendrogram based upon selected complete sequences of alpharetellites molecules. Alphasatellite sequences used for comparison are downloaded from databank The database accession number in each case is given. The sequences associated with Alternanthera yellow vein disease are indicated by a magenta color in each case.
4.6.2: Sequence and Phylogenetic analysis of Alphasatellites i.e., ChLCuA and AIYVA

Total of about 200 molecules of 1.4kb size i.e., typical size of satellites associated with begomoviruses were cloned from samples collected from different geographical locations of Punjab. These molecules were completely sequenced and analyzed using BLASTone by one to determine the type of satellites found associated with the full length virus molecules in the disease complex.

For all the 200 1.4kb molecules it was found that two types of satellite molecules are associated with the full length AIYVV. Interestingly only Alphasatellites were found. There was no betasatellite associated with AIYVV. This was very unusual but we have sequenced 200 molecules so it might be possible that AIYVV was producing vein-yellowing symptoms with the help of Alphasatellites not betasatellite. One of the Alphasatellite molecules, which was associated with the AIYVV virus was Cotton leaf Curl Rajasthan Alphasatellite (CLCuRaA), which is normally associated with the Cotton leaf Curl Rajasthan virus (CLCuRaV).

The other Alphasatellite associated with AIYVV was a uncharacterized molecule. It was a new species and Alternanthera yellow vein Alphasatellite (AIYVA) was proposed. This new alphasatellite was found to have 93% sequence similarity with the Mesta Yellow Vein Alphasatellite (MeYVA).

Sequences analysis showed that unlike AIYVV frequent recombinations were found in the alphasatellite molecules. AIYVA molecule was found to be recombinant of two alphasatellite molecules. This recombinant alphasatellite derived its Rep from Chili leaf Curl Alphasatellite (ChLCuA) and a fragment downstream of origin of replication from Tobacco Curly Shoot alphasatellite (TbCSA) (Fig 4.4).

One of the interesting facts this research revealed was that the AIYVV was not found associated with a betasatellite. All the 1.4kb molecules, which were sent for the sequencing, were alphasatellite molecules no betasatellite molecules was found associated with the AIYVV from all the samples of all the districts of sample collection areas. In the virus disease complex betasatellites are important pathogenicity determinant but interestingly this AIYVV was found to be betasatellite independent virus.
Fig 4.6: Recombinant Alphasatellite AIVY. Red color segment shows the part of ChLCuA and Blue color shows a part from TbCSA.

4.7: Synthesis of viral infectious molecules:

In order to fulfill the Koch postulate the infectivity analysis was performed by inoculating the infectious molecules to the *Nicotiana benthamiana* plants. Infectious molecules were synthesized for both the satellite molecules and for the virus. Dimeric molecules (tandem repeats for the origin of replication) were first cloned in the pTZ57R/T. A schematic diagram below shows that how the infectious molecule was constructed (Fig 4.5).

For the synthesis of infectious molecule i.e., full dimer of DNA A of AIVVV, 2.8kb molecule was self ligated to make it circular. This circular molecule was subjected to RCA. This RCA product was then given a partial restriction and a fragment of 5.6kb was generated, which was cloned in pTZ first and after confirmation of full dimeric molecule it was shifted in pGreen0029 vector.
For synthesis of infectious molecule of alphasatellite, 1.4kb Chili leaf curl Multan
alphasatellite molecule originally cloned at HindIII was restricted with HindII and BglII
producing one with 200 bp and other with 1200bp. Fragment with 1200bp contain the origin
of replication as confirmed through sequencing. It was ligated with full-length viral molecule
already cloned in pTZ57R/T. After confirmation the infectious molecule was shifted to
pgreen0029 vector.

In case of Alternanthera yellow vein alphasatellite, which was originally cloned at HindIII.

Restriction with HindIII and SalI produced two fragments of 850 bp and 575 bp. The
fragment with 575bp contains origin of replication as confirmed through sequencing. This
fragment was ligated with full-length molecule in pTZ. After confirmation of infectious
molecule the whole construct was shifted to pGreen0029.
Fig. 4.7: Flow chart representing how to synthesize an infectious molecule.
Fig. 4.8: Confirmation of dimeric molecule of 1) AlYVV (DNA-A), M is 1kb Marker. 2) ChLCuA; A,B and C are the confirmation of ChLCuA infectious molecules by single restriction, D,E and F represent confirmation by double restriction. M is the 1kb Marker. 3) AlYVA; A, B and C are the confirmation of AlYVA infectious molecules by single restriction, D and E represent confirmation by double restriction. M is the 1kb Marker.
4.8: Infectivity assay:

Fresh cultures of *Agrobacterium tumefaciens* cells transformed with the binary vectors harboring infectious molecules for both the satellites and the DNA A were grown under standard conditions. Prepared inoculum from these cultures was used for inoculation of the *Nicotiana benthamiana* plants. Infected plants showed symptoms 12-14 dpi. Healthy *Nicotiana benthamiana* plants with 4 to 5 leaves grown under controlled conditions of 16/8 hours light period and 25C temperature were used for inoculation.

<table>
<thead>
<tr>
<th>No of Plants</th>
<th>Virus</th>
<th>Satellite-1</th>
<th>Satellite-2</th>
<th>Symptoms</th>
<th>Southern blot analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 plants+10 control</td>
<td>DNA-A (AlYVV)</td>
<td>none</td>
<td></td>
<td>25 plants showed symptoms</td>
<td>All Plants positive for DNA-A</td>
</tr>
<tr>
<td>60 plants+15 control</td>
<td>20 DNA-A (AlYVV)</td>
<td>20 AlYVV + <em>Mesta yellow vein Alphasatellite</em></td>
<td>20 AlYVV + Chili leaf Curl Multan Alphasatellite</td>
<td>16+17+16 showed symptoms</td>
<td>All Plants positive for DNA-A+ PCR positive for Alphasatellite</td>
</tr>
<tr>
<td>30+15 controls</td>
<td>10 DNA-A</td>
<td>10 AlYVV + Tobacco leaf curl betasatellite</td>
<td>10 AlYVV + Cotton leaf curl betasatellite</td>
<td>8+8+7 showed symptoms</td>
<td>All Plants positive for DNA-A+ positive for betasatellite</td>
</tr>
</tbody>
</table>

Table 4.2: Representing the inoculation of plants with combinations with alphasatellites and betasatellites
After inoculation of *Nicotiana benthamiana* plants with AIYVV only leaf curling symptoms produced after 12 dpi. Table 4.2 enlists the details of the experiment. There was observed upward curling of leaves and somewhat stunted growth. Southern hybridization was used for the confirmation of systemic spread and replication of viruses (Fig 4.7). For this purpose CP was used as a probe. Positive control is used in the form of PCR product of CP after purifying while for negative control simple *Agrobacterium tumefaciens* inoculated plant leaves were used.

In next experiment AIYVV was coinoculated with AIYVA. This time symptoms were produced after 14 dpi but the symptoms were comparable to the symptoms produced in case of DNA A alone (Fig 4.7). AIYVV was also coinoculated with the *Chili leaf Curl Alphasatellite* (ChLCuA). Again the symptoms appeared 14 dpi but the produced symptoms were delayed and milder as compared to those produced when plants were inoculated with the DNA A alone (Fig 4.7). Table 4.2 enlists the details of the experiment.

We checked the movement and presence of Alphasatellites through diagnostic PCR in those plants, which showed negative results in southern blot analysis. We were able to amplify AIYVA in 6 plants out of 10 plants and ChLCuA from 5 plants out of 10 plants (Fig 4.8). It means in our infectivity experiments AIYVV supported both Alphasatellites but titre was too low to be detected through southern blot analysis. All these experiments were repeated thrice to validate the results.
Fig. 4.9: A) Agroinfiltration of ALYVV and associated satellites on N. benthamiana plants; 1) plant infected with the ALYVV alone showing leaf curling. 2) Plant inoculated with ALYVV+ALYVA showing same symptoms as 1. 3) Plant inoculated with ALYVV+ChLCuA showing same symptoms as 1 and 2. (B) Southern blot analysis of Nicotiana benthamiana plants, agro infiltrated with of with infectious molecules of ALYVV and associated satellites ChLCuA and ALYVA. CP gene was used as probe. Purified PCR product of CP was used as positive control. 1 to 4 ALYVV alone. 5 to 8 ALYVV+alphasatellite, 9 to 12 ALYVV+betasatellite, second last from left is –ve control and last from left is +ve control.
Fig 4.10: PCR amplification from systemic leaves. 
A) Confirmation of alphasatellite AlYVA replication through PCR. 1 to 6, M is the Marker. 7 is –ve control while 8 is positive control. 
B) 1 to 5 ChLCuMuA, 6 is –ve control and 7 is +ve control.
Chapter 5: Discussion

Begomoviruses infecting crop plants are economically important. Begomoviruses are fatly spreading and emerging plant infecting viruses because of existence of alternative hosts and increasing population of insect vectors. These begomoviruses hibernate on alternate hosts when the main crop is not present in the field. These alternate hosts are usually weeds found naturally inside or around the crop fields. Higher diversity of economically important viruses is found in these weeds. But unfortunately these reservoirs are often neglected due to less/no economic importance. One of the major obstacles to implement efficient virus control strategies is our incomplete knowledge of genetic diversity and populations of begomoviruses within a distinct area in all crops and non-crop hosts. Pakistan is heaven for begomoviruses and there is a high disease incidence as well as high diversity of viruses infecting Ageratum conyzoides, Zinnia elegans and Solanum nigrum, (Haider et al., 2007), Duranta erecta (Iram et al., 2004), chili pepper, tomato (Hussain et al., 2004; Shih et al., 2003; Siddiqui et al., 1999; Mansoor, Khan, and Saeed, 1997) Croton bonplandianus (Amin et al., 2002) radish, okra and watermelon (Mansoor et al., 2000a; Mansoor et al., 2000b; Mansoor et al., 2000c), Vigna aconitifolia (Qazi et al., 2006), mungbean (Bashir, Ahmad, and Mansoor, 2006; Hameed and Robinson, 2004), Eclipta prostrata (Haider et al., 2005), papaya (Nadeem et al., 1997). (Mubin, 2009) isolated begomoviruses infecting field crops from a weed host Digera arvensis L. Sonchus arvensis and Xanthium strumarium.

OW begomoviruses infecting economically important field crops are believed to be originated from the weeds usually grow in or around the fields of these crops (Ndunguru, Legg et al. 2005), Eclipta prostrata is also harboring a complex of viral disease components, including begomovirus molecules and its associated satellite molecules. Resistance breaking begomoviruses originated from the weed hosts like Cotton Leaf Curl Burewala Virus (CLCuBuV), which is a recombinant molecule sharing genome segments from Cotton Leaf Curl Multan Virus (CLCuMuV) and Cotton leaf Curl Khokhran Virus (CLCuKhv) isolated by (Mubin, 2012 #317) from a weed known as Xanthium strumarium L. found inside and around the cotton fields.
In this study, the genetic biodiversity of begomoviruses in naturally infected *E. prostarata* plants was investigated. *E. prostarata* is widely grown in Asia as a natural weed. The viruses infecting *E. prostarata* are already known. However, the viruses infecting *E. prostarata* from Pakistan are unknown. Therefore, in this study we conducted a survey to isolate begomoviruses and their satellites from *E. prostarata* plant.

We selected the Punjab province of Pakistan for isolation of viruses. Although, Eclipta is famous to grow, where there is standing water for a longer period of time around the water channels. We found *E. prostarata* plants growing in the dry areas of Punjab as well. This shows its wide adoptability in different regions of Punjab. Throughout the Punjab province, there were only one kind of symptoms i.e., vein yellowing in the leaves.

Cloning of begomovirus and its associated satellites was done from all the samples collected from the different district of Punjab. Due to the fact that Phi29 DNA polymerase amplifies all kinds of circular molecules, therefore we used it as a tool. Indeed, a huge amount of nucleic acids was amplified, which upon restriction digestion produced several monomers from concatamers. Initially to avoid any loss of cloned molecules, we cloned and sequenced all the recombinant plasmid containing colonies. The viral DNA sequencing followed by BLAST analysis produced the inference that ALYVV viruses isolated from distant places were having least differences in their genome with higher level of sequence similarity (92 to 98%). The homology difference was observed as a point mutation throughout the genome as compared to recombination.

The sequencing of satellites sized molecules revealed the presence of alphasatellites only. Two different kinds of alphasatellites, one of them showing less than 80% sequence similarity with Mesta yellow vein alphasatellite was found from different samples. As this is a new species, we propose a new name Alternanthera yellow vein alphasatellite for this alphasatellite. This alphasatellite was recombinant of chili leaf curl alphasatellite (ChLCuA) and tobacco curly shoot virus alphasatellite TbCSA. ChLCuA donated RepA gene and TbCSA donated a region upstream of origin of replication. Other was 95% similar to chili leaf curl alphasatellite. The alphasatellites found in combination with ALYVV from *E. prostarata* were unique and have not been reported before.
Throughout our research not a single example was observed where betasatellite could be found along with ALYVV. Therefore, using all possible ways of PCR and RCA from the diluteions of infected plant samples no betasatellite was isolated. This showed that Alternanthera yellow vein virus is not associated with E. prostarata yellow vein disease in E. prostarata plants. Similar results were shown by (He, 2008), as they isolated the AIVVV from Zinnia elegante Eclipta prostrata and. Role of alphasatellites in the etiology of AlYVV is unclear. (Briddon, Bull et al. 2004)Postulated in series of experimental studies that the alphasatellite, being an autonomous in sense of replication but depends on helper virus for its encapsidation and spread through insect to other plants and even within the plant while it has, apparently no role in the symptom modulation or it have no role in the systemic disease spread. In our study when we had the comparison of the plants inoculated alone with the AIVVV and AIYVV in combination. He et al., were also unable to isolate betasatellite from E. prostarata plants. However, they found betasatellite in combination with ALYVV from the Zinnia elegante. This gives an indication that for AIVVV to interact with betasatellite, there is a need of suitable hosts. Or in other words E. prostarata plants do not support betasatellites replication.

Previous information from China, Vietnam, India and Pakistan about the E. prostrata showed that it harbor begomovirus complex which includes a monopartite begomovirus and its associated satellite molecules. Mubin, et al., isolated E.prostarata infecting Alternanthera yellow vein virus (AIYVV) from another weed plant known as Sonchus arvensis L. which include AIYVV, alphasatellites and betasatellites as well (Mubin et al., 2010). Sequence analysis of these molecules was found to be 95-98% similar to the AIYVV isolated from China.

Infectivity of dimeric molecules for AIYVV alone and in combination with two different alphasatellites showed that perhaps alphasatellites have no role in disease symptoms development in N.benthamiana plants. The attempts to inoculate E. prostarata plants were failed by agrobacterium. Indeed, the agrobacterium produced sever necrosis on the patch of inoculation, but no systemic movement or symptoms could be reproduced in the inoculated plants.
Interestingly in Pakistan’s geographical location begomoviruses are usually found associated with betasatellites but in our study not a single example form this large set of samples was observed. Another interesting fact is that even though samples were collected from very distant places but there was least differences among the sequences of all isolated full length viruses of ALYVV, with no observation of any recombination event. The association of different alphasatellites with same helper monopartite begomovirus was discussed. Infectious molecules were made and infectivity analysis was performed. Regarding these discoveries the future directions are to search for pathogenicity determinant responsible for vein yellowing in this disease complex. The aim of this study was to understand the begomovirus complex responsible for the disease in *E. prostrata*. What are the possibilities of emergence of new disease complexes were so that a strategy can be developed before the emergence of disease complexes. Findings of these studies were very interesting and novel in this regard that such extensive work on *E. prostrata* has not been reported so far. In this study *E. prostrata* proved to be very important reservoir for begomoviruses. So there is always a chance that whitefly feed on different weed hosts and mix up these components. Crop plants showed specificity to specific viruses and components and thus only specific viruses and their components propagated in crop plants. In this sense weeds act as reservoir of these viral components. Weeds also act as recombination vessels where when all the components are present together there would be a greater chance of recombination.
Chapter 6: SUMMARY

*Eclipta prostrata* is an annual weed that grows along the water channels, inside or around the crop fields of cotton, sugarcane, rice and vegetable crops. *E. prostrata* is infected by a begomovirus and produces typical symptoms of begomovirus infection. On cloning and further nucleic acid sequencing analysis it was found that a monopartite begomovirus known as *Alternanthera Yellow Vein Virus* (AlYVV), which was first isolated from China was infecting *E. prostrata*. Typical symptoms of vein yellowing indicated the presence of some other pathogenicity determinant like betasatellite. But after intensive cloning from infected plants of different region only alphasatellites were isolated, which were found to be associated with this monopartite begomovirus. Despite of intensive efforts and biodiversity studies no other pathogenicity determinant i.e., betasatellite was found associated with this monopartite begomovirus. Replication studies of AlYVV were conducted in the model plant *Nicotiana benthamiana* and AlYVV replication and movement was confirmed by Southern blotting analysis. Interaction studies of AlYVV were conducted by inoculation of AlYVV with both alphasatellites i.e., Alternanthera yellow vein alphasatellite and chili leaf curl alphasatellite, which has been found associated with it in infected plants over different locations of distant areas. Symptoms were produced after co-inoculation but the symptoms were mild as compared to the symptoms produced by AlYVV alone. Replication of alphasatellites with the AlYVV was confirmed by PCR. All this information revealed that alphasatellites are found naturally associated with AlYVV and it produces infection by its own and it is not dependent on any other pathogenicity determinant and hence AlYVV is a betasatellite-free virus.
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