DEVELOPMENT OF GENERIC RESISTANCE STRATEGY AGAINST PaLCuV INFECTION BASED ON VIRAL GENOMIC VARIABILITY

THESIS

SUBMITTED TO

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2018

This thesis is

Dedicated to My family for their unconditional love, encouragement and constant support…..

DECLARATION

I, **Priyanka Varun** hereby declare that the thesis entitled "**DEVELOPMENT OF GENERIC RESISTANCE STRATEGY AGAINST PaLCuV INFECTION BASED ON VIRAL GENOMIC VARIABILITY**" is an authentic research work carried out by me under the guidance of Dr. Sangeeta Saxena, Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow. The research work is original, and no part of this work has been submitted for any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis titled "**DEVELOPMENT OF GENERIC RESISTANCE STRATEGY AGAINST PaLCuV INFECTION BASED ON VIRAL GENOMIC VARIABILITY**" submitted by **Ms. Priyanka Varun** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulation-1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Sangeela 20/12/2018

Supervisor

Date: 20/12/18

20/12/18

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Priyanka

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Abbreviations and Acronyms

%	Percent
ºC	Degree Celsius
BC1	Movement protein gene
BH	Bottom half region of begomovirus DNA-A component
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BV1	Nuclear shuttle protein gene
CP	Coat protein gene
CR	Geminivirus common region
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DW	Distilled water
EDS	Energy dispersive spectroscopy
EDTA	Ethylene diamine tetra acetic acid
FAOSTAT	Food and Agriculture Organization of United Nations Statistics Division
g	Gram
hrs	Hours
ha	Hectare
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase pair
L	Litre
LA	Luria Agar
LB	Luria-Bertani broth
M	Molar
mg	Milligram
min	Minute/s
miRNA	Micro RNA
ml	Milliliter
mM	Millimolar
MP	Movement protein gene
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NHB	National Horticulture Board
NLS	Nuclear localization signal
nm	Nanometer
NSP	Nuclear shuttle protein
NW	New World
OD	Optical density
ORF	Open reading frame
ori	Origin of replication
OW	Old World
PaLCuD	Papaya leaf curl disease
PaLCuV	Papaya leaf curl virus

PaSLCuV	Papaya severe leaf curl virus
PCR	Polymerase chain reaction
PDR	Pathogen-derived resistance
PSB	Priyanka Sangeeta Biotechnology (no. code assigned to the collected samples)
PSBB	Priyanka Sangeeta Biotechnology Betasatellite (isolate name assigned to betasatellite sequences)
psi	Pounds per square inch pressure unit
PTGS	Post-transcriptional gene silencing
RCA	Rolling circle amplification
RCR	Rolling circle replication
RDP	Recombination detection programme
REn	Replicase enhancer gene
Rep	Replicase gene
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
SDT	Sequence Demarcation tool
sec	Second
SEM	Scanning electron microscopy
siRNA	Small interfering RNA
ssDNA	Single stranded DNA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TrAP	Transcriptional activator gene
U	Unit
UH	Upper half region of begomovirus DNA-A component
UV	Ultraviolet
V	Volt
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
β	Beta
µg	Microgram
µl	Microlitre
µM	Micromolar

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Papaya (*Carica papaya* L.) is a commercial crop cultivated worldwide especially in tropical and subtropical countries. Papaya is originated from tropical America and southern Mexico (Morton J, 1987). India is the largest papaya producing country contributing 44.4% of world's total papaya production. According to Agricultural statistics at a glance (2016), 1,26,000 ha land area is covered under papaya cultivation in India with a total papaya production of 55,08,000 tonnes during 2014-15. In spite of contributing largest area under papaya production, productivity of papaya in India (43.7 tonne/ha) is very less as compared to other countries and listed after seven countries for productivity ranking, whereas, Dominican Republic has highest productivity (312.7 tonne/ha) among all papaya producing countries (FAOSTAT, 2014; Indian Horticulture Database, 2015). Among total papaya production, India exports 11,484 tonnes papaya to other countries and contributes rupees 3,826 lakh in the economy of India (Agricultural statistics at a glance, 2016). Papaya is a very common fruit plant which can be normally observed in every kitchen garden. Although India is the nation of different agro-climatic regions but papaya is cultivated throughout India and various states from north to south all are contributing in gross papaya production. Papaya has many health benefits with high nutritional and medicinal value. Papaya fruits are a good source of calcium and have high content of vitamin A and C. Milky latex of green, unripe fruit is rich in papain enzyme, a commercial valuable proteolytic enzyme (Dunne and Horgan, 1992). Papaya leaves are also known to be an effective therapeutics for the dengue fever (Ahmad *et al*., 2011).

There are many biotic and abiotic factors that affect the total crop production. Bacteria, fungi and viruses are the main pathogens and among them viruses heavily affect the papaya production throughout the world. There are many viruses reported that infect papaya plants and cause disease. Viruses are the main plant pathogens causing diseases like leaf curl, mosaic, ringspot, lethal yellowing and leaf distortion mosaic that harm papaya production and play a major role in crop losses (Mishra *et al*., 2007; Varun *et al*., 2017). Among above mentioned viral diseases, leaf curl disease of papaya caused by papaya leaf curl virus (PaLCuV) affects papaya production, yield and economy hugely and became an economically important disease (Saxena *et al*., 1998a). Papaya plants affected with leaf curl disease showing symptoms like downward or upward leaf curling,

crumpling and mottling of leaves, thickening of midrib and veins, yellowing of leaves, stunted plant growth etc. Papaya leaf curl disease (PaLCuD) has appeared as an important constraint for papaya cultivation. The disease was first described by Thomas and Krishnaswamy in 1939 in India and further, Saxena *et al*., in 1998 has studied and confirmed that papaya leaf curl disease is caused via a geminivirus named as papaya leaf curl virus (PaLCuV). Molecular evidence for disease causing begomovirus was also given by Saxena *et al*., 1998 b & c.

Begomoviruses are considered as plant pathogens that belong to the genera begomovirus which is the member of family *geminiviridae*. Begomoviruses are the emergent pathogens of tropical and subtropical countries, transmitted through insect vector whiteflies (*Bemisia tabaci*) thus, also known as whitefly transmitted geminiviruses (Markham *et al*., 1994; Hanley-Bowdoin *et al*., 1999). Family *geminiviridae* is classified into nine genera (Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus, Turncurtovirus) on the basis of vector transmission, genome organization of virus molecules and their possible hosts (https:// talk.ictvonline.org/ictv-reports/ictv_online_report/ssdna-viruses/w/geminiviridae).

Begomovirus contain more than 320 species and comprises as the largest genera in the *geminiviridae* family (Zerbini *et al*., 2017). Begomoviruses have quasi- isometric (geminate) shaped virus particle having single stranded DNA genome. On the basis of genome component in two types i.e. bipartite and monopartite begomoviruses exists in nature. Begomoviruses with two genomic components encapsidated separately and referred as DNA-A and DNA-B components are termed as bipartite begomoviruses whereas, begomoviruses with only one genomic component known as monopartite that contain genome like DNA-A segment of bipartite begomoviruses (Harrison BD, 1985; Stanley *et al*., 2005).

DNA-A component (~2.7 kb) of begomoviruses have six genes that transcribed in two different reading frames. AV2 and AV1 open reading fames (ORFs) present in virion sense strand while, four ORFs present in complementary strand named as AC1, AC2, AC3 and AC4 (Navot *et al*., 1991; Rojas *et al*., 2005). AV2 ORF encodes for pre-coat protein and AV1 ORF encodes for coat protein. Function of AV2 is also documented as

host silencing suppressor (Glick *et al*., 2008). ORFs in anti-sense strand/ complement strands are known as replicases and comprises of AC1, AC2, AC3, AC4 ORFs. These ORFs encode for various significantly functional proteins like replication initiator protein (Rep), transcription activator protein (TrAP) and replication enhancer protein (REn) respectively. Function of AC4 is unclear but reported as suppressor of host silencing machinery (Chellappan *et al*., 2005).

DNA-B component (~2.7 kb) encodes only two ORFs (BV1 and BC1). BV1 ORF present on virion sense and acts as movement protein whereas, BC1 ORF present on complementary strand and functions as nuclear shuttle protein (Noueiry *et al*., 1994; Ward *et al*., 1997; Hanley-Bowdoin *et al*., 2013). It also contributes in inter as well as intra-cellular movement of virus particle inside the plant host (Stanley J, 1983). Association of satellite molecules popularly known as betasatellites is very frequent with majority of monopartite begomoviruses (Briddon *et al.*, 2003). Betasatellite molecules have half genome size (~1.35 kb) as compared to their helper begomovirus (DNA-A component) and having only one ORF in complement strand i.e. βC1 that are responsible for producing severe disease symptoms. Symptom induction of begomoviral infection is dependent on their virulence and associated betasatellites increase the symptom severity of disease. DNA-B component as well as betasatellites lacks their own replication machinery and are dependent on DNA-A component for their replication and encapsidation but do not identified with extensive nucleotide sequence identity with their helper viruses for proliferation (Briddon *et al.*, 2008).

There are numerous reports on infection of different begomoviruses causing leaf curl disease on papaya plants (Raj *et al*., 2008; Singh-pant *et al*., 2012; Shahid *et al*., 2013; Varun and Saxena, 2018), on the other hand papaya leaf curl virus has also been observed on other plants causing infection and produce various symptoms. Infection of multiple begomoviruses and betasatellites on a single crop shows the wide spread nature of begomoviruses including papaya leaf curl virus to expand their unpredictable host range. So, for this reason present work was done for an inclusive study on the presence of all possible begomoviruses and betasatellite molecules infecting papaya.

There are many initiatives to combat papaya ringspot disease like successful commercial cultivation of transgenic papaya resistant against papaya ringspot virus (PRSV) (Gonsalves D, 1998) but no effective management is available for papaya leaf curl disease. High degree of evolution rate, mixed infection of various viral species and also the emergence of new species or strains (Padidam *et al*., 1999) are a big challenge in developing resistance against this disease. The genetic diversity studies of begomoviruses as well as identification of species or strain should necessarily be determined before developing any resistant strategy. Viruses belong to the different families have different suppressors that act upon several steps of host silencing pathway. RNA interference (RNAi) is a natural defense mechanism in which suppression of gene expression occurred during post transcriptional level and also known as RNA silencing/ post transcriptional gene silencing (PTGS) (Napoli *et al*., 1990). RNAi acts in sequence specific manner and degrade the targeted gene to provide resistance. Pathogen derived resistance (PDR) is used as a RNA silencing based approach against many begomoviruses (Sanford and Johnston 1985; Zrachya *et al*., 2007). Some viral genes are known for the suppression of host silencing machinery so the silencing of these suppressors is a good strategy to develop resistance against viral disease. Presence of viral suppressors supports the evidence for RNA silencing as an adaptive defense (Ding *et al*., 2004). Begomoviruses adapt different hosts and cause mixed infections by varying their suppressor genes that may regulate functioning of host plant regulatory system to combat resistance against viruses through RNA interference (Bisaro DM, 2006). This provides an understanding for RNA silencing mechanism and the relationships between virus and host silencing pathways.

Saxena *et al*., 2011 have suggested a generic resistance strategy based on RNA silencing to deal with papaya leaf curl disease by targeting conserved region of AV1 and AC1 genes of PaLCuV and tomato leaf curl virus (TLCV). Consequently, Saxena *et al*., 2013 studied AC2, AC4 and AV2 genes of begomoviruses because of their host silencing suppressor activity and designed siRNA against highly conserved region among different viral suppressors to develop resistance against begomoviruses infecting papaya crop and suggested these genes as putative target of thus designed siRNA. Similarly, Sharma *et al*., 2015 have also identified siRNA generating hotspot on viral suppressor genes and

concluded that 100- 150 bp in suppressors are a good source for generating siRNA molecules to develop transgenic plants against multiple virus infections. Zhang *et al*., 2011a have expressed short hairpin construct in soybean to develop resistance against mixed viral infections using RNAi based strategy.

Adaptation of begomoviruses to different/ wider host range is becoming a main concern for papaya production. There is an immediate requirement for the management of begomovirus infection to reduce crop losses. Begomoviruses are known for their rapid evolution, high genetic variations and adaptation to new hosts in different geographical conditions. Molecular variability and diversity of begomoviruses infeting papaya crop provides a better understanding about variation in begomoviral genome, in different climatic conditions. To overcome the highly variable nature of begomoviruses, RNAi is the best approach to combat resistance against these viruses. siRNA designed against conserved region of begomoviral genes after studying their molecular variability provides better and sustainable management against papaya leaf curl disease.

In this study molecular variability studies of all papaya leaf curl causing begomoviruses (PaLCuV isolates) as well as betasatellites associated with them were detected by comparison of isolates at genome organization level. All the coding regions of all isolates were studied to check the availability of conserved regions within them and searched for the best target to design siRNA. Keeping in mind the economic losses due to disease, expanding host range of begomoviruses and emergence of new viral species, studies were conducted for developing successful disease management approach against begomoviral infections with the help of following objectives-

OBJECTIVES

- To survey and collect samples of papaya leaf tissue exhibiting symptoms of leaf curl virus (PaLCuV) infection.
- Amplification and sequencing of key genes of PaLCuV isolates.
- Checking of genomic variability and identifying the conserved sequences among the different PaLCuV isolates.
- *In-silico* designing of siRNA based on the conserved region for generic resistance against all PaLCuV infection.

Papaya is a very important crop growing in tropical and subtropical countries of the world. This fruit bearing plant is growing commercially in all over the world is originated from Southern Mexico and Central America. Botanical name of papaya is *Carica papaya* L. belongs to the family *Caricaceae*. Papaya is a dicotyledonous plant which bears spirally arranged leaves at top of the stem. Papaya is considered as an economically important crop due to its nutritive and medicinal values (Eno *et al.*, 2000; Krishna *et al*., 2008). Papaya fruits are rich in proteins, carbohydrates and have high level of calcium, iron, vitamin B_2, vitamin C, β-carotene and niacin (Samson JA, 1986). Fruits as well as leaves of papaya has many therapeutic properties and used in the treatment of cancer, diabetes, dengue fever, malaria and sickle cell anemia (Nguyen *et al*., 2013; Sarala and Paknikar, 2014; Thadani *et al*., 2018). Papaya is mainly popular for its latex producing cysteine protease enzyme extensively used for protein digestion. Papain is a cysteine protease enzyme and also named as papaya proteinase-I that acts as a proteolytic enzyme present in leaf and raw fruit of papaya and uses as meat tenderizer (Amri and Mamboya, 2012).

2.1 Papaya production and distribution

India, Brazil, Indonesia, Dominican Republic, Nigeria and Mexico are the major countries that contribute in total papaya production. India leads in the total papaya production with a production of 55,08,000 tonnes (44.4% of global papaya production) during the year of 2014-15. Total area covered under papaya cultivation in India is 1,26,000 ha with 43.7 tonne/ha productivity during 2014-15 (Agricultural statistics at a glance, 2016). Total area used under papaya cultivation in India covers 1.8% area and contributing 6.3% of total fruit production during the year 2013-14 (Indian Horticulture Database 2015). Andhra Pradesh, Gujarat, Karnataka, Madhya Pradesh, West Bengal, Chhattisgarh and Telangana states are contributing in large in papaya production in Indian. India exports approximately 11,484 tonnes of papaya to different countries mainly Unite Arab Emirates, Saudi Arabia, Nepal, Netherland, Bahrain, Qatar, Kuwait, Oman, Germany and Bangladesh. Contribution in Indian economy through papaya export during 2014-15 was rupees 3,826 lakhs (Agricultural statistics at a glance, 2016). Although India is contributing highest in area and production of papaya in all over the world but productivity is comparatively very low. India stands at 7^{th} position among all

papaya producing country on account of productivity of papaya. However, Dominican Republic has the highest productivity (312.7 tonne/ha) of papaya even after very less area under papaya cultivation (FAOSTAT, 2014).

2.2 Diseases of papaya

Papaya is a very important horticultural crop not only due to its medicinal value but also because of its commercial use. Based on FAOSTAT, 2014 and Agricultural statistics at a glance, 2016 reports it is clear that production trend of papaya is increasing with increase in area under papaya cultivation but productivity is not up to the mark. This indicates the losses in papaya production due to biotic and abiotic stresses. Abiotic stresses include drought, flood, salinity, wind etc. that affect plant growth, fruit production, productivity and fruit quality of papaya. Many diseases like papaya ringspot disease, papaya leaf curl disease, mildew, Anthoceros, root rot and nematodes are the diseases due to living organisms (biotic stresses) like bacteria, fungi, nematodes and viruses that affect papaya cultivation in large. Biotic stresses affect the economic growth as well as practical development of crops. Among all diseases, viral diseases are the major concern for papaya cultivation. Many viral diseases are observed and reported on papaya like papaya ringspot, papaya mosaic disease, papaya lethal yellowing disease, papaya leaf curl disease, papaya leaf distortion mosaic and papaya sticky disease causing serious loss in papaya production. Causal organisms of these viral diseases belong to different virus families are listed in table 2.1.

Table 2.1: Details of viral diseases, their transmitting insect vectors and causal virus reported on papaya

Viral diseases	Transmitting vector	Causal virus	Virus genome	Genus,Family of causal virus	References
Papaya ringspot	Aphid	Papaya ringspot virus (PRSV)	ssRNA	Genus-Potyvirus Family-*Potyviridae*	Jenson DD, 1949
Papaya leaf curl disease	Whitefly	Papaya leaf curl virus (PaLCuV)	ssDNA	Genus-begomovirus Family-*Geminiviridae*	Saxena *et al*., 1998a
Papaya mosaic disease	Unknown	Papaya mosaic virus (PapMV)	ssRNA	Genus-Potexvirus Family-*Alphaflexiviridae*	Conover 1962
Papaya lethal yellowing disease	Not confirmed	Papaya lethal yellowing virus (PLYV)	ssRNA	Genus-Sobemovirus	Silva *et al*., 1997
Papaya leaf distortion mosaic	Aphid	Papaya leaf distortion mosaic virus (PLDMV)	ssRNA	Genus-Potyvirus Family-*Potyviridae*	Kawano and Yonaha, 1992
Papaya sticky disease	Unknown	Papaya meleira virus (PMeV)	dsRNA	Mycoviruses Family-*Totiviridae* (not approved by ICTV)	Rodrigues *et al*., 1989

2.3 Papaya leaf curl disease (PaLCuD) - historical review

Many viruses infecting papaya plant, among them viruses of family *geminiviridae* and *potyviridea* are very common and produce a great impact on its economy. Begomovirus, the largest genera in the family *geminiviridae*, is the group of most destructive plant pathogen producing a severe threat for crops grown in tropical and subtropical regions and their economy. Thomas and Krishnaswamy have first time observed papaya leaf curl disease (PaLCuD) in 1939 and Nariani TK in 1956 in India. Further, PaLCuD was extensively studied, provided molecular evidence of causal organism and concluded that whitefly transmitted geminivirus, named it as papaya leaf curl virus (PaLCuV) caused the leaf curl disease of papaya (Saxena *et al*., 1998 a, b, c). PaLCuD is reported in many other countries like Africa (Taylor DR, 2001), China (Wang *et al*., 2004), Korea (Byun *et al*., 2016), Nepal (Shahid *et al*., 2013), Pakistan (Nadeem *et al*., 1997) and Taiwan (Chang *et al*., 2003) that indicates the wider distribution of disease throughout the globe.

Considering PaLCuD as a preventive measure in majority of papaya growing areas, many surveys were conducted to record the disease incidence and loss to growers. Singh *et al*., 2008 conducted a systematic survey of different places in Bihar state during 2000- 2008 and observed 12-27 % incidence of leaf curl disease in papaya. Similarly, some districts of Uttar Pradesh were surveyed to observe the leaf curl disease of papaya and recorded 20- 32% disease incidence in order to increasing with time (Singh A, 2006; Singh SK, 2013) .

A host specific begomovirus i.e. papaya leaf curl virus (PaLCuV) causing leaf curl disease of papaya is a main concern for papaya growers. Initially the disease was reported to be caused by PaLCuV (a begomovirus) but with the expanding host range of begomoviruses many other begomoviruses are also reported to infect papaya plant and became a cause for leaf curl disease of papaya. Presence of chili leaf curl virus, cotton leaf curl Multan virus, papaya leaf crumple virus, tomato leaf curl New Delhi virus, tomato leaf curl Gujarat virus has already recorded on papaya in India (Singh-Pant *et al*., 2012; Sinha *et al*., 2013; Varun and Saxena, 2018).

2.4 Transmission of papaya leaf curl virus

There are many insects that sheltered on papaya like fruit flies, fruit spotting bugs, thrips, mites, aphids and whiteflies. Transmission of papaya leaf curl virus from one plant host to another is mediated only through whiteflies (*Bemisia tabaci*) (Saxena *et al*., 1998a). Transmission of virus can possible through grafting but not via mechanical means (Nariani TK, 1956). Whiteflies are sap sucking insects and being sap transmissible, PaLCuV has transferred to phloem sap during insect feeding on plants. Czosnek *et al*., 2002 has described that *B. tabaci* adults are the only vectors that transmit virus in a persistent circulative manner. Transmission of virus is dependent on availability of viruliferous whiteflies around the fields, virus inoculums and susceptibility of the host variety (Varun and Saxena, 2017a). PaLCuV showing tripartite relationship with insect vector and plant host (figure 2.1) that leads to the development of disease symptom and their survival. Specific interactions exist between viral isolates, their vector and host plants that facilitate their adaptation to new host in different geographical regions.

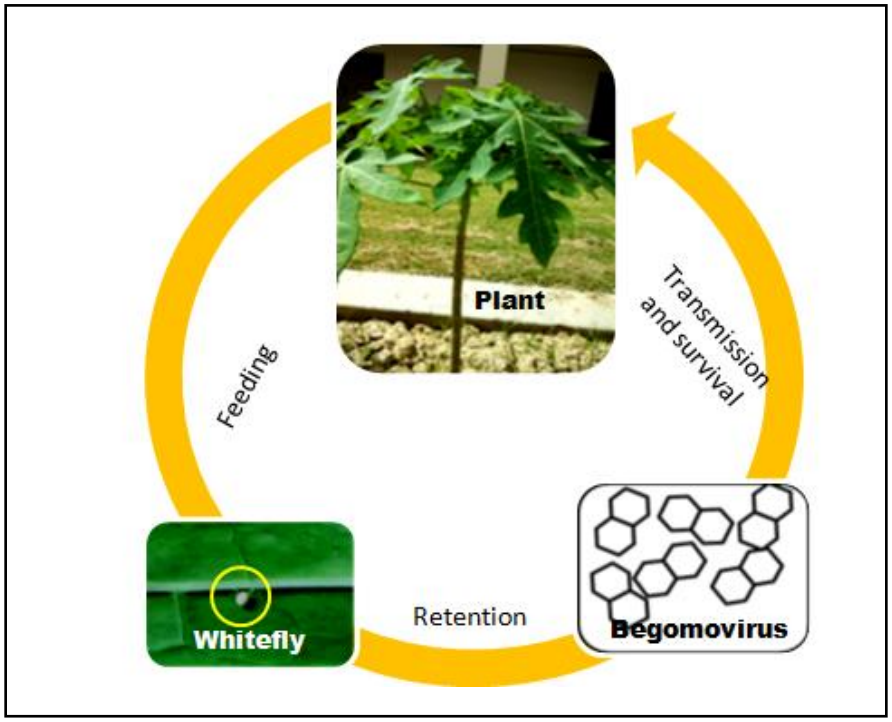


Figure 2.1: Plant mediated relationship between begomoviruses infecting papaya plants and their insect vector whitefly (*B. tabaci*).

2.5 Disease symptoms at cellular level: Scanning Electron Microscopy (SEM)

Plant diseases caused by distinct pathogens create difficulties in agricultural practices and pose actual economic pressure. Co-infection of different pathogens result in distinct function, so accurate diagnosis of disease is mandatory for early disease management. Investigation of plant surface using scanning electron microscopy (SEM) is an advance technique to visualize samples at high resolutions. SEM is an approach to detect, quantify and study viral infection based on histological and morphological analysis of plant tissues (Evert RF, 2006) that facilitate the discrimination between healthy and infected plants. SEM is an ideal technique for screening of native structure of leaf samples and also to observe the internal structure of phloem tissues (Pathan *et al*., 2010). Energy dispersive spectroscopy (EDS) analysis on the SEM produce X-ray spectrum from the area subjected for scan and display presence and distribution of elements of different atomic numbers. EDS allows the identification of elements and their relative proportions of atomic percentage (http://www.charfac.umn.edu/instruments/eds_on_sem_primer.pdf).

Begomoviruses are phloem bound viruses that produce symptoms like severe leaf curling, crumpling, mottling, swelling of midrib and vein thickening. SEM can be used for early detection of disease and also as important technique for diagnosis of emerging diseases prior to molecular detection of disease. SEM was used successfully to detect phytoplasmas and other bacterial phloem inhabiting pathogens associated with some emerging diseases of agricultural crops (Lebsky *et al*., 2010; Lebsky and Poghosyan, 2014). Quantification of histological features is a good parameter to examine the anatomy of infected tissue and can effectively discriminate between healthy and infected samples (Guines *et al*., 2003; Guillemin *et al*., 2011). Similarly, infection of bacterial diseases can easily be detected through SEM (Saldana *et al*., 2011). SEM has been also found effective during investigation of physical properties of leaf midrib and latex of papaya plants infected with sticky disease on papaya (Magana-Alvarez *et al*., 2016). Analysis of histology and morphology of papaya plant leaves were found effective to evaluate the viral infection in plant tissues (Varun *et al*., 2017; García-Viera *et al*., 2018).

2.6 Geminivirus taxonomy

Classification of viruses in categories of order, family, and genus was accorded in 1991 by the International Committee on Taxonomy of Viruses (ICTV) for worldwide virus classification. Van Regenmortel MHV, 1990 has defined the virus species as a polythetic class of viruses that occupy a particular ecological niche and constitute a replicating lineage. The term isolate was introduced that specified a virus species of an isolated virus that can further classify into a species or strain. Strain is a term that discriminates between different species and specifies the viruses of same species with stable and heritable traits like: biological, serological and molecular behavior.

Taxonomy and nomenclature of viruses are the scientific system that made virus studies easier in regard to their description and improved by the years. ICTV has given the guidelines for nomenclature of virus species (Van Regenmortel *et al*., 1997). A revised proposal for geminivirus nomenclature was consigned and after its acceptance by *geminiviridae* study group, DNA-A sequences of many begomoviruses were studied and the best species demarcation threshold (89% nucleotide sequence identity) was determined to discriminate between different species of geminiviruses (Fauquet *et al*., 2000). Virus nomenclature was improved with the addition of some descriptors like strain and variants that make virus identification easy even after rapidly increasing number of begomoviruses (Fauquet *et al*., 2003). Following the guidelines of ICTV, Fauquet *et al*., 2003 have published a detailed list of begomovirus species in the family *geminiviridae*. However, increasing recombination frequency between different begomoviral species is a major constraint in taxonomic status of geminiviruses which is immensely improved by geminivirologists through regular revising begomoviral taxonomy in some time intervals (Fauquet and Stanley, 2005; Brown *et al*., 2015) and decided to calculate pairwise identity scores with a species demarcation threshold of 91% pairwise identity to minimize taxonomic misplacements (Brown *et al*., 2015).

2.6.1 *Geminiviridae* family

Family *geminiviridae* was initially divided into three genera (Mastrevirus, Curtovirus and Begomovirus) based on biological properties and genome organization of geminiviruses (Rybicki EP, 1994) and one more genera (Topocuvirus) was added very soon in 1999

with the approval of ICTV (Pringle CR, 1999). Varsani *et al*., 2014 introduced three new genera (Becurtovirus, Eragrovirus and Turncurtovirus) to the family following the criteria include host range, transmitting vector, genome organization and genome-wide pairwise identities and further, Varsani *et al*., 2017 established the presence of two more genera (Capulavirus, Grablovirus) in the family *geminiviridae*. The current taxonomic status and increasing number of geminivirus species explains the diversity and evolution of geminiviruses due to their highly variable nature. Geminivirus infection on cultivated as well as non-cultivated crops with a varied no. of species is a major concern for geminivirologists and study of molecular variability and host range studies of geminiviruses are necessary to know the epidemiology of viruses and disease. Family *geminiviridae* includes total nine genera with more than 360 species among them begomoviruses contributing in large (Zerbini *et al*., 2017). Details of geminiviral taxonomy including genus in the family, their transmitting vectors, type species of genera and total no. of species present in the genera are discussed in table 2.2 and addition of genera in *geminiviridae* family after revision of geminivirus taxonomy is shown in figure 2.2.

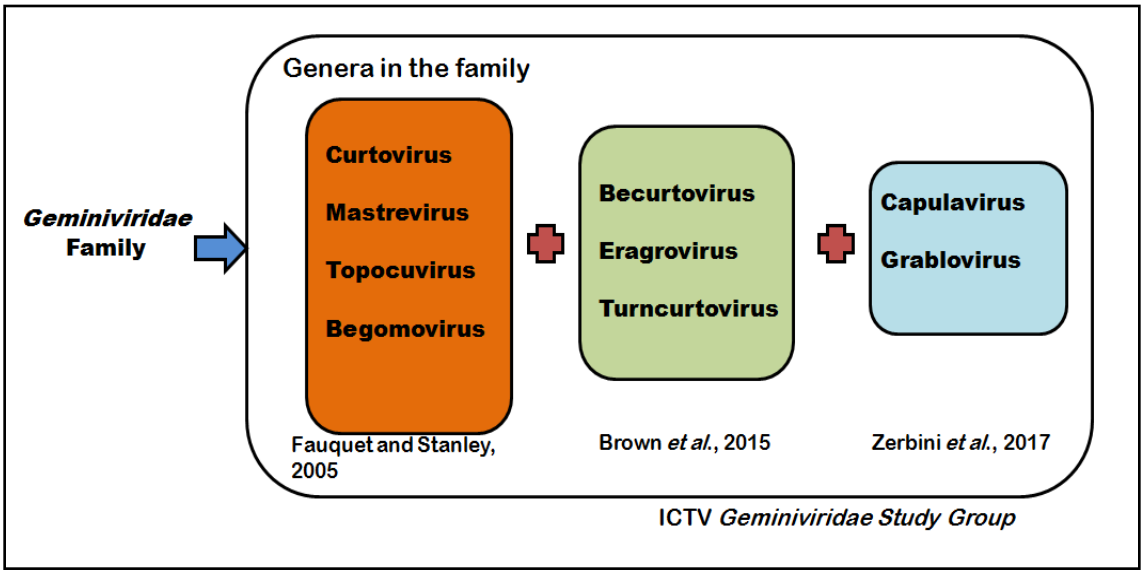


Figure 2.2: Taxonomic classification and establishment of different genera in the family *geminiviridae* during revision of geminivirus taxonomy.

Table 2.2: Details about all the nine genera in the *geminiviridae* family and their characteristics

Genus	Genome organisation	Transmitting vector	Type species	No. of species	References
Mastrevirus	Monopartite	Leafhopper	*Maize streak virus*	>30 species	Brown *et al*., 2015
Curtovirus	Monopartite	Leafhopper to dicot plants	*Beet curly top virus*	Three species	Chen *et al*., 2010
Topocuvirus	Monopartite	Treehopper to dicot plants	*Tomato pseudo-curly top virus*	One species	Briddon *et al*., 1996
Begomovirus	Mono/ Bipartite	Whitefly *(Bemisia tabaci)* to dicot plants	*Bean golden yellow mosaic virus*	>320 species	Zerbini *et al*., 2017
Becurtovirus	Monopartite	Leafhoppers to dicot plants	*Beet curly top Iran virus*	Two species	Varsani *et al*., 2014
Eragrovirus	Monopartite	Not-known to monocot plants	*Eragrostis curvula streak virus*	One species	Varsani *et al*., 2014
Turncurtovirus	Monopartite	Leafhopper to dicot plants	*Turnip curly top virus*	One species	Varsani *et al*., 2014
Capulavirus	Monopartite	Aphid species (*Aphis craccivora*)	*Alfalfa leaf curl virus*	Four species	Varsani *et al*., 2017
Grablovirus	Monopartite	Treehopper species (*Spissistilus festinus)*	*Grapevine red blotch virus*	One species	Varsani *et al*., 2017

2.7 Begomoviruses

All the viruses of family *geminiviridae* are known as geminiviruses and have ssDNA genome. Presence of unique envelope (capsid) of icosahedral morphology distinguishes them from other viruses. These are known to cause severe crop losses (up to 100%) to food, fiber, horticultural and ornamental crops (Moffat AS, 1999; Fargette *et al.*, 2006). These are phloem bound parasites, spread systemically in plant cells and known to adapt different plant host rapidly (Bedford *et al.*, 1994). Small genome, rapid mutation and diverse host range have made virus particles extremely dynamic and diverse. These properties formulate them as a potential threat for crop cultivation. Begomoviruses depend completely on their plant host for their replication and use cellular machinery of plant to replicate inside the host (Padidam *et al*., 1995).

2.7.1 Genome organization of begomoviruses

Begomovirus is the largest genus in the *geminiviridae* family which is named on its type species *Bean golden mosaic virus* (Padidam *et al*., 1996). Begomoviruses have single stranded DNA genome and known as whitefly transmitted geminiviruses that affect dicotyledonous plants such as tomato, cucurbits, papaya, peppers etc. Different members of the genus begomovirus can exist on same plant and cause mixed infection. During co-existence of several begomoviruses together in same host plant, they exchange their genetic material through recombination and pseudo-recombination events.

On the basis of genome arrangement and place of origin, begomoviruses are divided into two groups: new world (NW) viruses (Western Hemisphere, The Americas) and old world (OW) viruses (Eastern Hemisphere, Europe, Africa and Australasia) (Padidam *et al*., 1999). Begomoviruses of NW origin are bipartite whereas, OW viruses are either bipartite or monopartite. DNA-A genome of NW viruses do not have the AV2 ORF (Stanley *et al*., 2005). There are two transcription units present in both the genomic components that transcribed in opposite directions and separated by a highly conserved noncoding intergenic region (IR) which has a highly conserved region (CR) of approximately 200 nucleotides (Lazarowitz *et al*., 1992; Hanley-Bowdoin *et al*., 1999). Conserved region possess a stem loop structure that have a nonanucleotide sequence

(TAATATTAC), an origin of replication (ori) and specific virus recognition sequences (iterons) (Fontes *et al*., 1994).

DNA-A component of bipartite begomoviruses have six open reading frames (ORFs) two in sense strand (AV1, AV2) and four in complement strand (AC1, AC, AC3, AC4) and DNA-B component possess two ORFs BC1 (complement strand) and BV1 (sense strand) (figure 2.3). Monopartite begomoviruses are having only one genomic component i.e. similar to DNA-A component of bipartite begomoviruses.

2.7.2 Functions of proteins encoded by begomoviral DNA-A component

2.7.2.1 Coat protein (ORF AV1)

Coat protein (CP) is the most abundant protein encoded by AV1 ORF of begomoviruses. CP gene is a key player of viral infection cycle that participates in whitefly mediated transmission, capsid formation and virus movement inside the host (Azzam *et al*., 1994; Hohnle *et al*., 2001). C-terminal of coat protein is highly conserved while centre region contains both variable and conserved portions. ICTV has approved that only highly conserved coat protein sequence can be used to preliminarily identify the begomovirus (Mayo and Pringle, 1998).

2.7.2.2 Pre-coat protein (ORF AV2)

AV2 gene is a characteristic feature of begomoviruses native to old world while begomoviruses belong to new world lack this gene. Pre-coat protein is involved in virus movement inside the plant, known as pathogenicity determinant and involved in silencing suppression (Rojas *et al*., 2005; Zrachya *et al.*, 2007; Yadava *et al*., 2010).

2.7.2.3 Replication-associated protein (Rep) (ORF AC1)

Rep is a multifunctional protein of geminiviruses involved in various important functions including: recognizes the ori to facilitate replication and transcription of viral DNA (Laufs *et al.*, 1995) and interacts with host factors associated with replication machinery of host plants. Rep has ability to bind and hydrolyse ATP to initiate the replication of viral DNA (Heyraud-Nitschke *et al.*, 1995) and represses its own expression at transcription level (Sunter *et al*., 1993; Eagle *et al*., 1994). Rep recognizes the ori to bind with dsDNA template to perform replication and transcription of viral DNA. Rep initiates

the replication of virion strand by producing a nick at T_7-A_8 position of nucleotides present in nonanucleotide sequence to perform replication through rolling circle replication method and re-circularized into circular ssDNA after completion of replication of viral molecule. During infection process, begomoviruses are dependent on host factors for their replication and Rep is known to interact with host factors associated with replication machinery of host plants. Rep has several enzymatic activities like ATpase/endonuclease/helicase/ligase/oligomerase that act in sequence and strand specific manner to synthesize circular ssDNA monomers of viral DNA from double stranded replicative intermediate through rolling circle replication (RCR) method (Hanley-Bowdoin *et al*., 1999, 2004; Pasumarthy *et al*., 2010).

2.7.2.4 Transcription activator protein (TrAP) (ORF AC2)

AC2 gene of geminiviruses also known as L2 in new world viruses and C2 in monopartite begomoviruses encode transcriptional activator proteins (TrAP) which is required for transcription of CP gene and BV1 gene present in virion sense strand (Sunter *et al*., 1990). Further, they have identified that TrAP function as transcription activator of CP gene in mesophyll cells while repressed it in phloem cells (Sunter and Bisaro, 1997). TrAP is a potential silencing suppressor that plays a vital role in virus host interaction. TrAP protein may found in nucleus or cytoplasm of virus infected plant cells and suppress silencing machinery in various manner. Trinks *et al*., 2005 suggested AC2 gene as a silencing suppressor by studying expression profile of a cellular protein i.e. Werner exonuclease-like 1 (WEL1). C2 protein disrupt the ubiquitin E3 ligase complex (Lozano-Duran and Bejarano, 2011), inhibit jasmonate- mediated defense (Rosas-Díaz *et al*., 2016), inhibit methylation (Wang *et al*., 2005; Castillo *et al*., 2015; Tu *et al*., 2017), suppress the post transcriptional gene silencing (PTGS) (Kumar *et al*., 2015a) and transcriptional gene silencing (TGS) (Zhang *et al*., 2011b; Castillo *et al*., 2015).

2.7.2.5 Replication enhancer protein (REn) (AC3 ORF)

Protein encoded from AC3 gene is necessary for viral DNA accumulation in infected plants (Hanley-Bowdoin *et al*., 1999; Pasumarthy *et al*., 2011). Interaction of Rep and AC3 protein defined the replication of viral genes. Mutation in AC3 ORF is correlated with the reduced level of viral DNAs and responsible for delay in symptom development

(Morris *et al*., 1991; Settlage *et al*., 1996). Interaction of REn with some cell cycle regulators like maize retinoblastoma homolog (pRBR1) and PCNA of *Arabidopsis thaliana* and tomato facilitate successful virus replication (Settlage *et al.*, 2001; Castillo *et al.*, 2003).

2.7.2.6 AC4 protein (ORF AC4)

AC4 is the highly variable protein of begomoviruses and involved in many viral functions such as suppressor of host silencing machinery, symptom development and viral movement. Function of AC4 in systemic viral movement was first time identified through mutation analysis (Jupin *et al*., 1994) and cell to cell movement of viral DNA through plasmodesmata was suggested by Rojas *et al*., 2001. Role of C4 protein is also involved in suppression of post transcriptional gene silencing (PTGS) and symptom expression (Vanitharani *et al*., 2004; Chellappan *et al*., 2005).

2.7.3 DNA-B component of begomoviruses

DNA-B component of bipartite begomoviruses are known to involve in virus movement and symptom development have two open reading frames (ORFs) i.e. BV1 and BC1. BV1 ORF located in virion sense strand encodes for nuclear shuttle protein (NSP) and BC1 ORF present in complement strand and encodes for movement protein (Lazarowitz and Beachy, 1999).

2.7.3.1 Nuclear shuttle protein (NSP) (BV1 ORF)

Movement of viral DNA inside and out of the nucleus during virus transport within the plant is mediated through NSP but during initial infection nuclear import of viral DNA is facilitated through CP (Gafni and Epel, 2002).

2.7.3.2 Movement protein (MP) (BC1 ORF)

BC1 is known as movement protein that induces the formation of tubular structure for virus translocation from one cell to another adjacent cell (Ward *et al*., 1997). Gafni and Epel, 2002 has demonstrated that BV1 and viral DNA complex interacts with BC1 in cytoplasm and move to the plasmodesmata to facilitate cell-to-cell movement. BC1 is also responsible for symptom induction during viral infection. Involvement of BC1 in pathogenicity was demonstrated by working on transgenic tomato and tobacco plants and

found the proof that BC1 gene is responsible for pathogenicity (Saunders *et al*., 2001). Wege and Pohl, 2007 has determined the activity of DNA-B encoded proteins in the activation of silenced DNA-B component after the infection of cognate DNA-A component of virus.

2.8 Betasatellite molecules associated with begomoviruses

Betasatellite molecules are identified in association with monopartite begomoviruses having a single stranded circular DNA genome. Betasatellites having half genome size (~1.3 kb) of their helper begomoviruses with a single ORF in complementary-sense strand (βC1), a satellite conserved region (SCR) and an adenine rich (A-rich) region (figure 2.3). SCR in betasatellite having a stem loop structure and share the similar nonanucleotide sequences as present in ori of begomoviruses (Briddon *et al.*, 2001). Dry *et al*., 1997 first time identified the presence of a novel subviral agent (DNA satellite) of 682 nucleotides without open reading frame associated with tomato leaf curl virus (ToLCV). Ageratum yellow vein virus (AYVV) was observed to be associated with full length betasatellite (1347 nucleotide long) with a single ORF in *Agaratum conyzoides* (Saunders *et al*., 2000) and afterwards betasatellites have frequently found to be associated with begomoviruses. This begomovirus-betasatellite complex have become responsible for many economically diseases in various crops in Asia and Africa. Betasatellite molecules do not have their own replication machinery and dependent on their helper begomoviruses for their encapsidation, replication and survival.

2.8.1 Function of βC1 ORF

Betasatellites are of about 1350 nucleotide long genome carrying only one potential ORF (βC1) and require helper virus for their successful encapsidation and replication (Mansoor *et al.*, 2003c; Briddon and Mansoor, 2008). βC1 ORF is involved in viral movement, host determination, viral symptom induction, accumulation of both helper virus and satellite molecule and function as suppressor of post-transcriptional gene silencing (PTGS) (Briddon *et al.*, 2001, 2003; Zhou *et al.*, 2003; Qian and Zhou, 2005; Saeed *et al*., 2007). Betasatellites associated with some begomovirus infections affect the replication of their helper begomoviruses to induce disease symptoms in host plants (Saunders *et al*., 2000; Briddon *et al*., 2001; Zhou *et al.*, 2003). Evidences for its function

in symptom development by inducing abnormal cell division (Cui *et al*., 2005; Guan and Zhou, 2006), by multimerization (Cheng *et al*., 2011) and down-regulation of chlorophyll synthesis related genes rather than chloroplast structural genes (Bhattacharya *et al*., 2015).

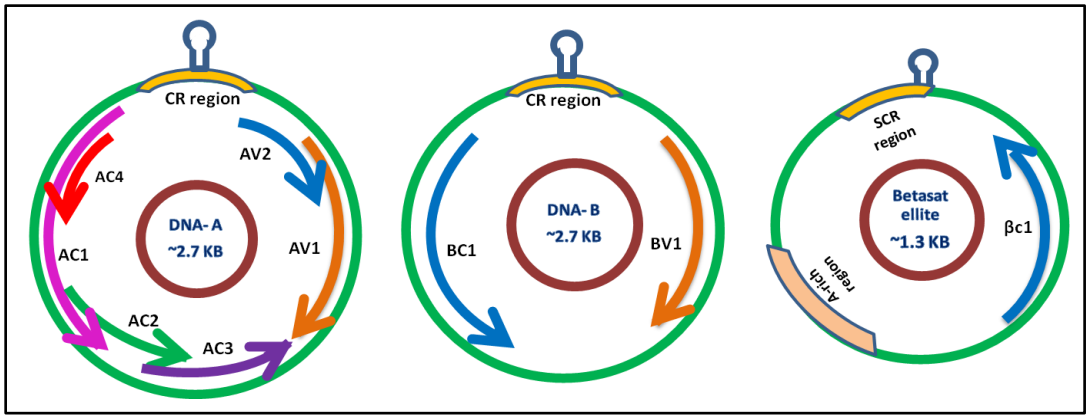


Figure 2.3: Genome organization of different genomic components of begomoviruses i.e. DNA-A and DNA-B and associated betasatellite molecule.

2.9 Expanding host range of begomoviruses and associated betasatellite molecules

Papaya is cultivated in different geographical locations throughout the year. Many other crops grown in nearby locations or presence of weeds/ non-cultivated crops around the papaya growing area were found as virus reservoir and affect other crops through insect transmission. So, it indicates that the virus is acquired from alternate hosts and then transmitted to papaya or vice versa results in a complex distribution and occurrence of viruses infecting papaya (Singh *et al*., 1978; Crill *et al*., 2000; Elena *et al*., 2009).

Papaya is the natural host of papaya leaf curl virus (PaLCuV) but many studies revealed the association of this virus with different crops (Chowda-Reddy *et al*., 2005). PaLCuV has a wide host range including cultivated crops and weeds. List of different crops/ weed hosts affected with leaf curl virus are listed in table 2.3. In the same manner different begomoviruses choose papaya as their alternate host during off-season and becoming an alternate choice for many begomovirus species (table 2.4). Similarly, infection of many betasatellite molecules on papaya is reported (table 2.5). This increasing host range of

begomoviruses and betasatellite is a major concern for viral disease management and necessary to determine the different begomoviral infection on same crop during disease control.

Table 2.3: Papaya leaf curl virus causing infection in other crop plants

Viruses (Acronym)	Host	Region: Country	Accession no.	Genome size(bp)	References
Papaya leaf curl virus (PaLCuV)	Cotton	Pakistan	AJ436992	2753	Mansoor *et al*., 2003b
Papaya leaf curl China virus (PaLCuCNV)	*Corchoropsis timentosa*	Nanning:China Guangxi: China	AJ876548	2735	Haung and Zhou, 2006
Papaya leaf curl virus(PaLCuV)	Tobacco	Bihar: India	GQ139516	850	Kumar *et al*., 2009
Papaya leaf curl virus (PaLCuV)	*R.capitata*	Mianwali: Pakistan	FM955602	2756	Ilyas *et al*., 2010
Papaya leaf curl China virus (PaLCuCNV)	*Solanum lycopersicum*	Henan:China Guangxi: China	AJ558116, AJ558117, AJ704604, FN256260, FN297834	2738–2751	Zhang *et al*., 2010
Papaya leaf curl China virus (PaLCuCNV)	*Siegesbeckia orientalis*	Fujian: China	JF682837	2754	Yang *et al*., 2011
Papaya leaf curl virus (PaLCuV)	*Kalimeris indica*	Lucknow: India	JQ954859	2746	Srivastava *et al*., 2013
Papaya leaf curl virus (PaLCuV)	*Amaranthus cruentus*	Lucknow: India	JN135233	2746	Srivastava *et al*., 2015

Table 2.4: List of different begomoviruses infecting papaya plants

Virus (Acronyms)	Host	Place /location	Accession no.	Genome Size(bp)	Reference
Papaya leaf curl virus (PaLCuV)	Papaya	Lucknow	Y15934	2746	Saxena *et al*., 1998c
Papaya leaf curl China virus (PaLCuCNV)	Papaya	Guangxi province: Chinna	AJ558122	2742	Wang *et al*., 2004
Papaya leaf curl Guangdong virus (PaLCuGDV)	Papaya	Guangdong Province: China	AJ558123	2740	Wang *et al*., 2004
Tomato leaf curl New Delhi virus (ToLCNDV)	Papaya	Lucknow	DQ431846	770	Raj *et al*., 2008
		New Delhi	DQ989325	2735	Singh-Pant *et al*., 2012
Papaya leaf crumple virus(PaLCrV)	Papaya	New Delhi	HM140367 HM140368 HM140369	2736	Singh-Pant *et al*., 2012
Chilli leaf curl virus (ChiLCuV)	Papaya	New Delhi, Panipat, Noida	HM140371 HM140370 HM140364 HM140365 HM140366 DQ989326	2763	Singh-Pant *et al*., 2012
Croton yellow vein mosaic virus (CroYVMV)	Papaya	New Delhi	EU126823	771	Singh-Pant *et al*., 2012
Pedilanthus leaf curl virus (PedLCV)	Papaya	New Delhi	HM134222	771	Singh-Pant *et al*., 2012
Tomato leaf curl virus (ToLCuV)	Papaya	New Delhi	HM134234	771	Singh-Pant *et al*., 2012
Ageratum yellow vein virus (AYVV)	Papaya	Nepal	KC282641	2753	Shahid *et al*., 2013
Tomato leaf curl Gujarat virus (ToLCuGuV)	Papaya	Lucknow	MG757245	2760	Varun and Saxena, 2018

Table 2.5: List of different betasatellite molecules infecting papaya plants

Betasatellite	Host	Place	Accession no.	Genome Size (bp)	Reference
Tomato leaf curl betasatellite (ToLCB)	Papaya	Panipat: India NewDelhi: India	EU126826 EU126825 HM143910 HM143909 HM143911 HM143901 HM143902 HM143905 HM143907	1365-1379	Singh-Pant *et al*., 2012
Croton yellow vein mosaic betasatellite (CroYMB)	Papaya	Panipat:India	HM143903 HM143908	1349 1358	Singh-Pant *et al*., 2012
Papaya leaf curl betasatellite (PaLCuB)	Papaya	Panipat:India	HM143906	1333	Singh-Pant *et al*., 2012
Chilli leaf curl betasatelite (ChiLCuB)	Papaya	Panipat:India	HM143904	1369	Singh-Pant *et al*., 2012
Tomato leaf curl java betasatellite (ToLCuJB)	Papaya	Nepal	KC282642	1356	Shahid *et al*., 2013
Tomato leaf curl Bangladesh betasatellite (ToLCBB)	Papaya	Lucknow: India	MG478451	1372	Varun and Saxena, 2018

2.10 Factors responsible for molecular variability of begomoviruses

Molecular variability study of an organism enlighten about evolution, host adaptation and taxonomy of viruses. Recombination studies revealed the diversification as well as adaptive evolution. Molecular variability studies facilitate the development of disease management strategies. Diversity study is an important way to know the epidemiology of plant virus population. Diversity studies provide some important information about alterations in genetic makeup with respect to adaptation to different geographical areas, hosts, specific interactions with their vector and hosts etc. There are several factors that became responsible for genomic variability. Mutation and recombination are the basic evolutionary driving forces that generate and modulate the plant virus populations. Selection and genetic drift are the evolutionary forces that determine the genetic diversity of plant viruses (Seal *et al*., 2006).

2.11 Diversity of begomoviruses and associated betasatellites

Papaya leaf curl disease is a severely affecting the papaya production globally. Disease is caused by whitefly transmitted geminiviruses (WTGs) which became a serious threat for crop cultivation. Nature of increasing begomovirus diversity increases the chances of their survival. Genetic variations are the reason for diversity at strain level and turn into a factor to drive evolution which is one of the major setbacks for disease management. Diversity among different begomoviral species/ strains existing in different geographical locations is a major challenge for developing management against the disease. Molecular genetic studies have provided detailed information on the genomic organization of viruses. Complexity in genome organization enlightens the rapid genetic diversity during evolution. Begomoviruses have overlapping regions like AC4 and AC1 genes that undergo through positive selection and indicated the independent evolution and cause infection (Yang *et al*., 2013).

Sanz *et al*., 1999 studied the genetic variability of begomoviruses and observed frequent recombinations during begomoviral evolution. Ambrozevicius *et al*., 2002 studied the begomoviruses infecting tomato plants and weeds that showed the close relationship between begomoviruses identified from tomatoes and weeds and suggested the transfer of begomoviruses from wild hosts to tomatoes. Similarly, Ha *et al*., 2008 identified some

indigenous geminiviruses and associated betasatellites infecting crops and weeds in Vietnam that were already present in old world and suggested that recombination, and sequence variability in step-loop sequences were the reason of this virus diversity. They have also advised the South-East Asia and Vietnam as an origin of begomovirus diversity. Likewise, Sohrab *et al*., 2016 has also studied begomoviruses causing tomato yellow leaf curl disease occurred on tomato crops in Saudi Arabia and genetic diversity as well as recombination studies showed that identified virus is a recombinant variant of already reported tomato yellow leaf curl virus. García-Andrés *et al*., 2006 has provided some support for the ecological adaptation of tomato infecting begomoviruses to the invaded area and *Solanum nigrum* as a natural host for begomovirus diversity. They have identified a novel viral isolate (tomato yellow leaf curl axarquia virus) infecting tomato plants in southern Spain originate from recombination events that cause tomato yellow leaf curl disease. Similarly, Sahu *et al*., 2015 showed the geographical diversity of begomoviruses and betasatellites identified from different crops in India and found that CP gene as well as betasatellite sequences revealed close relation between begomoviruses infecting diverse crops. These findings showed the extensive host range of begomoviruses and betasatellites in India. Wang *et al*., 2004 identified two distinct begomoviruses infecting papaya crops that caused significant loss to papaya growers in China. Similarly, Barboza *et al*., 2016 studied the diversity and distribution of begomoviruses infecting tomato and sweet pepper plants from different geographical locations in Costa Rica and observed the infection of different mono (tomato yellow leaf curl virus*)* and bipartite (tomato yellow mottle virus, tomato leaf curl sinaloa virus and pepper golden mosaic virus) begomoviruses. They have also suggested that agricultural system, climate and geography may affect the distribution of begomoviruses. Recombination is an important evolutionary process that influences begomoviral evolution and exchange of genetic material between different species and spread through human movement (Bruyn *et al*., 2012).

Betasatellites are considered as adapted from pre-existing begomoviral DNA but their evolutionary origin is unclear (Mansoor *et al*., 2006). Southeast Asia is regarded as centre of origin of betasatellites due to having highest diversity among betasatellite molecules (Nawaz-ul-Rehman and Fauquet, 2009; Venkataravanappa *et al*., 2011). Recombination

plays important role during evolution of betasatellites and involvement of host plants in betasatellite evolution has also been documented (Stanley J, 2004). Begomoviruses and associated betasatellites co-evolved during evolution and their genetic changes produce an impact on disease (Mansoor *et al*., 2003a,b; Zhou *et al*., 2003). Further, Briddon *et al*., 2014 demonstrated the effect of genetic changes in begomovirus-betasatellite complex that result in resistance breaking of cotton in South Asia.

Climatic changes, increasing whiteflies population, growing rate of virus spread and disease incidence, frequent introduction of various begomovirus species in the same crop plant etc. are the major factors that result in genetic variability of begomoviruses. Variation in crop production system, cropping system, use of susceptible and tolerant cultivars, transportation of infected plant materials are also contributing in genetic variation of begomoviruses. In addition to above mentioned factors high mutation and recombination rate, introduction of satellite DNA molecules, rapid rate of emergence and spread of new begomovirus species and their extended host range are also the reason of begomovirus diversity that facilitate virus adaptation in different climatic condition in various plant hosts (Seal *et al*., 2006). So, the information related to virus genome organization, occurrences of new virus species/strain, evolutionary background of begomoviruses and their interaction with the plant hosts provides a better understanding to develop sustainable disease management against viruses (Garcia-Andrés *et al*., 2007).

2.12 Management of begomoviral diseases

Several efforts have been taken for the management of viral diseases occurring on papaya. Description of some control practices are described below:

2.12.1 Conventional methods

Use of yellow traps and insecticides for vector control, uprooting of diseased plants, crop rotation, use of virus free propagules, cross protection and chemical control are the main conventional approaches are very common practices for regulating viral infection (Sastry and Zitter, 2014). Transmission of begomoviruses is naturally mediated through insect vector (whitefly) so, the use of some chemicals and insecticides are the main control measure for leaf curl disease. Long-term and frequent use of these insecticides became harmful for beneficial insects, produced several environmental hazards, human and

livestock concerns that produce economic load to the farmers (Kliot and Ghanim, 2012). Integrated management programmes for disease control are not so effective due to highly evolving nature of viruses. So, strategies based on viral genome, virus evolution and emergence of new viruses is the good practices for successful disease management (Varun and Saxena, 2017b).

2.12.2 Pathogen-derived resistance (PDR)

The understanding about viruses, their infection cycle, viral genome, plant-host interaction, and transmission increased the platform for viral management using recombinant DNA technology and antisense approaches. These approaches are based on the expression of virus derived genes or genomic fragments that provide protection against viral infection inside the host plant. Sanford and Johnston, 1985 introduced the concept of pathogen derived resistance for the cross protection of host plants. Since, many transgenic plants were developed using PDR approach (Baulcombe DC, 1994) that provide successful disease management.

Coat protein mediated PDR was successfully employed against many plant viruses (Abel *et al*., 1986; Beachy RN, 1997). Development of CP mediated resistant transgenic papaya (Rainbow and Sunup variety) against papaya ringspot virus was the most successful disease management (Gonsalves D, 1998, 2006). Replication associated protein (Rep) was also a popular gene for developing virus resistant variety. Rep became successful against African cassava mosaic virus (ACMV), Tomato yellow leaf curl Sardinia virus (TYLCSV), cotton leaf curl virus (Hong and Stanley, 1996; Brunetti *et al*., 1997; Asad *et al*., 2003). Initially antisense approach was used to develop resistance against geminiviruses (Shepherd *et al*., 2009) and later on, small RNAs offer better resistance against different viruses and antiviral strategies using siRNAs provide sustainable disease resistance against diverse virus species.

2.12.3 Ribozyme technology

Ribozyme is also a popular technique in which naturally occurring RNA molecules (ribozymes) catalytically cleave the target mRNAs at specific site (Lilly DMJ, 2003) to manage begomoviral diseases. Use of ribozymes in treatment of hepatitis C virus, HIV and cancer has been studied (Usman and Blatt 2000; Zinen *et al*., 2002; Weng *et al*.,

2005; Macpherson *et al*., 2005) similarly, activity of ribozymes targeting rep-mRNA has also been investigated against mungbean yellow mosaic India virus (MYMIV) (Mishra *et al*., 2014). Thus, ribozymes can also used as an antiviral agent against papaya leaf curl virus infection.

2.12.4 Peptide aptamers

There are several reports on the use of peptide aptamers that bind with viral proteins and provide resistance against different viral species. Development of transgenic against MYMIV, tomato yellow leaf curl virus (TYLCV) and tomato mottle virus through expression of peptide aptamers against different begomoviral proteins (Sunitha *et al*., 2011; Reyes *et al*., 2013). Aptamers can also be used against different viruses to provide better resistance against viral diseases.

2.12.5 CRISPR/Cas9 technology

The recent advancement of management practice is emerged as CRISPR/Cas9 technology in which host plant genome has been engineered to provide protection against viruses. CRISPR technology has been used successfully used for developing transgenic plants resistant against a specific or mixed viral infection (Ali *et al*. 2015; Baltes *et al*., 2015; Ji *et al*., 2015; Zaidi *et al*., 2016). Knowledge of complete papaya genome (Ming *et al*., 2008, 2012) may turn into an ideal for developing resistance against begomoviruses infecting papaya crop through CRISPR technology.

2.12.6 RNAi mediated resistance

RNA interference (RNAi) is a gene regulatory mechanism involves RNA silencing that provides adaptive defense against invading organisms, transgenes and viruses (Yu and Kumar, 2003). RNAi system act either at transcriptional level or at post-transcriptional level and provide transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS). TGS act in transcription reduction while PTGS functions in mRNA degradation in a sequence specific manner to regulate endogenous genes (Herr AJ, 2005). RNA silencing is a multistep process which uses dsRNA to target homologous mRNA for degradation or inhibiting its transcription or translation (Das *et al*., 2011). RNAi technology works in highly sequence specific manner, thus employed majorly in crop

improvement against plant viruses. Waterhouse *et al*., 1998 demonstrated RNAi mediated virus resistance against potato virus Y (PVY) in transgenic tobacco plants. PTGS is mainly siRNA based gene silencing (Bisaro DM, 2006) in which small interfering RNAs (siRNAs) of 21-24 nucleotides were generated from a long dsRNA precursor (Hamilton and Baulcombe, 1999) and lead to degradation of cognate mRNA. RNA silencing mechanism is start with the processing of dsRNA precursor with the help of RNA dependent RNA polymerases (RDR). dsRNA precursors binds with Dicers (RNase III endonuclease) that cleave it into small dsRNA fragments (siRNA) in an ATP dependent manner (Zou and Yoder, 2005). These small RNA duplexes then incorporated into an active multiprotein RNA induced silencing complex (RISC) with the help of Argonaut protein (AGO). Consequently, a helicase unwinds the siRNA duplex inside the RISC and one strand of duplex degraded (sense strand) and another strand (antisense strand) bind with messenger RNA (mRNA) in highly sequence specific manner **(**Stevenson M, 2004). After this mRNA-siRNA complex formation, an RNase (slicer) proceed the degradation of target mRNA (Elbashir *et al*., 2001). The transgenic common bean (*Phaseolus vulgaris*) was developed to use RNA silencing via the expression of an intron-containing hairpin RNA corresponding to a portion of the Rep (AC1) gene of Bean golden mosaic virus (BGMV) (Bonfim *et al*., 2007).

siRNAs are 20-24 bp long short dsRNAs (sense and antisense strand) generated from long dsRNAs having two nucleotide overhangs at 3'end (Bernstein *et al*., 2001). Sense strand of siRNA duplex mimics the sequence of target gene while antisense strand bind with mRNA. siRNA-induced silencing complex (siRISC) machinery degrade the sense strand and antisense strand bind with the mRNA sequence of target gene in highly homology dependent manner to degrade the target mRNA and offers an effective management against disease and may be used as significant solution for papaya leaf curl disease management. Saxena *et al*., 2011 have studied the diversity among some begomoviral genes and designed siRNAs against conserved regions to and further, Saxena *et al*., 2013 targeted the suppressors of host silencing machinery and concluded that siRNA based disease management is a sustainable approach that provide the generic resistance against a range of begomoviruses infecting papaya crop. Begomoviral genes (AC1, AC2, AC4, AV2) act as suppressor of host RNA silencing and proliferate virus

inside host and cause disease symptoms. AC4 interacts with AGO4 protein involved in RISC machinery and directed cytosine methylation of viral DNA to suppress the host silencing machinery and regulate PTGS (Wang *et al*., 2016; Vinutha *et al*., 2018).

First gene silencing tool to design highly potent siRNA was developed in 2002 by Dharmacon Inc. but nowadays, there are many online tools implemented with different parameters are available to design effective and thermodynamically stable siRNAs against most suitable (/effective) regions for siRNA based gene silencing. pssRNAit is a programme for siRNA designing that produce/ design siRNAs against most effective regions with an advance filter of off-targets. siRNAs designed from this tool were used for developing broad spectrum resistance against multiple viruses with high efficacy and performance (Kohnehrouz and Nayeri, 2015; Sharma *et al*., 2015; Hameed *et al*., 2017).

There is an immediate requirement of generic resistance approach against wide range of begomoviruses infecting papaya crop. Strategy based on RNA silencing using siRNAs at post transcriptional level is a most reliable method to counter the infection of invading begomoviruses. So, this approach can be useful in identifying the function of viral genes as well as in developing crop varieties with enhanced disease resistance trait against begomoviruses.

This chapter describes the details of experimental design and methodology employed during this study such as survey and sample collection of papaya leaves showing typical leaf curl symptoms from different geographical locations, detection of begomovirus infection on symptomatic papaya leaves through polymerase chain reaction, cloning, transformation and sequencing of complete DNA-A and betasatellite molecules. Diversity study of begomoviruses, betasatellite and designing of siRNAs against different begomoviral genes for the successful management against papaya leaf curl disease.

3.1 Materials

3.1.1 Plant material

Papaya leaves showing typical symptoms of leaf curl disease like leaf curling and crumpling, thickening of leaf veins and midrib, etc. were collected from different Indian states during 2014-2016.

3.1.2 Chemicals for molecular biology experiments

Chemicals required for DNA isolation were purchased from Himedia, Bangalore Genei and Thermo Fischer Scientific.

3.1.3 PCR components

10x Buffer, $MgCl_2$, dNTP mix and Taq DNA polymerase were procured from Thermo Scientific. Primers were synthesized from IDT solutions

3.1.4 Molecular Biology Kits

Gel extraction and PCR cleanup kit was purchased from invitrogen.

TA cloning kit was purchased from Promega (USA).

Rolling circle amplification (RCA) kit was purchased from GE Healthcare Life Sciences.

3.1.5 DNA Ladders

100bp DNA ladder and Low range ruler were obtained from Qiagen and 1kb plus DNA ladder from Thermo Scientific.

Restriction enzymes were purchased from Thermo Scientific.

3.2 Methods

3.2.1 Sample collection

Typical symptoms of papaya leaf curl disease like inward and outward curling, crumpling, vein thickening, yellowing etc. were observed on symptomatic plants during survey of different geographical locations of India during 2014-2016. Some districts of Assam (Guahati, Jorhat), Gujarat (Ahmedabad, Jamnagar), Haryana (Karnal), Jharkhand (Ranchi), Karnataka (Bangalore, Coorg), Madhya Pradesh (Bhopal, Guna, Gwalior, Jabalpur and Shivpuri), New Delhi, Punjab (Amritsar), Telangana (Hyderabad, Warangal) and Uttar Pradesh (Agra, Azamgarh, Deoria, Faizabad, Gorakhpur, Lalitpur, Lucknow, Mahoba, Mathura and Shahjahanpur) were surveyed. A detailed list of collected samples and variety of papaya plants which were found growing in above geographical locations is discussed in table 3.1. Samples were collected from papaya growing agricultural fields, nurseries, kitchen gardens and roadsides on the basis of apparent symptoms like downward, upward curling of leaves, crumpling, mottling, vein thickening etc. Leaf samples were collected in ziplock bags with silica gel pouches and stored at -20ºC in aluminum foil for further use. Healthy papaya leaf sample was taken from greenhouse for the study as a control throughout the study. Samples from different geographical locations of India as mentioned above were collected to assess the diversity study of begomoviruses infecting papaya plants.

3.2.2 DNA extraction from plant leaf tissue

Total plant DNA was isolated from collected leaf tissues following the CTAB method with some minor modifications (Murray and Thompson, 1980). 1 gm of plant leaf tissues were grounded in liquid nitrogen to make fine powder. Powder was then transferred to 2ml eppendorf tubes and equal volume of pre-heated 2% CTAB buffer was added to each tube and mixed well by inverting the tubes and incubated at 65°C for 45 min in water bath. Chilled chloroform: isoamyl alcohol (24:1) in equal volumes was added after incubation, gently mixed and centrifuged for 5 min at 13,000 rpm. Upper aqueous phase obtained after centrifugation was transferred to a fresh eppendorf tube and 1/5th volume of 5% CTAB solution was added, mixed gently by inversion and centrifuged the tubes for 5 min at 13,000 rpm. Resulting aqueous layer was then transferred to a fresh eppendorf

and then equal volume of CTAB precipitation buffer was added. The tubes were mixed well and centrifuged for 1 min at 10,000 rpm. DNA pellet obtained after centrifugation was dissolved in 200 µl of high salt TE buffer by gentle tapping the tube. The tube was then centrifuged at 13,000 rpm for 5 min and the supernatant was transferred in a fresh eppendorf tube. 2.5 volume of chilled ethanol (95%) was added to the solution and mixed gently by inversion. The tubes were centrifuged for 10 min at 13,000 rpm at 4ºC to precipitate DNA. After centrifugation, supernatant was discarded and equal volume of chilled ethanol (70%) was added to the DNA pellet and centrifuged for 5 min at 10,000 rpm at 4ºC. Supernatant was discarded and after air drying the pellet was dissolved in 50 µl of 0.1x TE buffer. Isolated DNA was further treated with RNase enzyme (10mg/ml) to remove the impurities of RNA molecules. RNase was added to the DNA sample and incubated at 37 ºC for 30 min. Further, nucleic acid was treated with phenol: chloroform: isoamyl alcohol (25:24:1) and further 95% ethanol (chilled) was added for the precipitation and extracted DNA was recovered by centrifugation at 13,000rpm for 10 min. Washing of DNA pellet was performed by adding 70% ethanol and after drying pellet was re-suspended in sterile distilled water and the DNA was stored at -20ºC for further use.

2% CTAB buffer: 2% CTAB, 100mM Tris, 20mM EDTA, 1.4M NaCl and 1% PVP
5% CTAB solution: 5% CTAB, 0.35M NaCl
CTAB precipitation buffer: 1% CTAB, 50mMTris, 10mM EDTA
High Salt TE buffer: 10mM Tris, 1.0 mM EDTA, 1M NaCl
0.1x TE buffer: 1mM Tris, 0.1mM EDTA

Table 3.1: List of papaya leaf samples collected from different geographical locations of India

State	Place	Date of sampling	No. of samples	No. code assigned	Location	Symptoms	Name of grown variety
Assam	Guwahati	6/6/2016	[2]	PSB 79, PSB 80	Nursery	Downward leaf curl, and yellowing	Red Lady
	Jorhat	8/6/2016	[2]	PSB 81, PSB 82	Roadside	Vein thickening, leaf curl and yellowing	Red Lady
Gujarat	Jamnagar	7/02/2015	[3]	PSB 33, PSB 34, PSB 35	Jamnagar Ayurved university	Downward curling, vein thickening	Red Lady
	Ahmedabad	9/02/2015	[2]	PSB 36, PSB 37	Papaya field	Downward curling, crumpling, leaf deformation	Red Lady
Haryana	Karnal	23/11/2016	[2]	PSB77, PSB 78	Roadside	Shoestring, upward curling	Solo, Washington
Jharkhand	Ranchi	1/11/2014	[3]	PSB 38, PSB 39, PSB 40	Kitchen garden	Curling, crumpling, yellowing, motteling	Pusa Delicious
Karnataka	Coorg	25/06/2016	[2]	PSB 55, PSB 56	Roadside	Leaf roll with crumpling	Coorg Honey Dew
	Bangalore	27/6/2016	[3]	PSB 57, PSB 58, PSB 59	Kitchen garden	Leaf curl, yellowing, crumpling	Honey Dew
Madhya Pradesh	Shivpuri	4/10/2014	[7]	PSB 25 to PSB 31	Dubey nursery	Leaf curl, vein thickening, crumpling and mixed infection	Red Lady
	Guna	5/10/2014	[2]	PSB 68, PSB 69	Papaya Field	Curling, vein thickening	Red Lady
	Gwalior	18/11/2016	[2]	PSB 64, PSB 65	Jiwaji University	Leaf roll, downward curling,	Solo
	Bhopal	9/04/2016	[2]	PSB 66, PSB 67	Tirupati Nursery	Downward curling, crumpling, yellowing, stunted growth	Red Lady
	Jabalpur	24/06/2016	[1]	PSB 32	Geeta Ashram	Upward curling, mosaic, ringspot	CO-7
New Delhi	Delhi University	9/11/2016	[2]	PSB 60, PSB 61	Kitchen garden	Severe curling of leaves, crumpling	Red Lady

	Satya Niketan	10/11/2016	[2]	PSB 62, PSB 63		Downward leaf roll with stunted growth	Red Lady
Punjab	Amritsar	22/11/2016	[2]	PSB 75, PSB 76	Nursery	Shoestring, leaf deformation, upward curling	Pusa Delicious
Telangana	Hyderabad	5/03/2016	[2]	PSB 51, PSB 52	University Campus	Severe downward curling, stunted plant growth,	Coorg Honey Dew
	Warangal	6/03/2016	[2]	PSB 53, PSB 54	Papaya Field	Mottling, crumpling	CO-1
Uttar Pradesh	Agra	20/11/2016	[2]	PSB 73, PSB 74	Udyan bhawan	Leaf curl, crumpling, vein thickening,	Pusa Delicious
	Azamgarh	6/11/2014	[1]	PSB 19	Road side	Downward curl and yellowing of leaves	Pusa Giant
	Faizabad	16/11/2014	[3]	PSB 44, PSB 45, PSB 46	Agricultural Univ. campus	Downward curling, vein thickening, crumpling	Pusa Majesty
	Gorakhpur	15/12/2014	[2]	PSB 49, PSB 50	Roadside	Leaf curl, vein thickening	Pusa Dwarf
	Khushinagar	20/12/2014	[1]	PSB 70	Road side	Leaf curl and crumpling	Pusa Dwarf
	Lalitpur	23/12/2015	[2]	PSB47, PSB 48	Roadside	Leaf curling, crumpling, defoliation of plant leaves, vein thickning	Pusa Dwarf
	Lucknow	10/7/2014 to 14/7/2014 15/11/2014	[16]	PSB 1 to PSB 15, PSB43	Different locations	Leaf curl, vein thickening, leaf deformation, symptoms of mixed infection	Pusa Delicious
	Mahoba	6/12/2014	[2]	PSB 41, PSB 42	Kitchen garden	Leaf curling, mosaic and yellowing of leaves	Pusa Nanha
	Malihabad	5/11/2014	[3]	PSB 16 to PSB 18	CISH	Crumpling, mottling, and curling	Pusa Delicious
	Mathura	21/11/2016	[2]	PSB 71, PSB 72	Nursery	Leaf curl, vein thickening	Pusa Majesty
	Shahjahanpur	26/10/2014	[5]	PSB 20 to PSB 24	Kitchen garden	Curling and yellowing of leaves	Pusa Majesty

3.2.2.1 Estimation of DNA

Quality and quantity of the isolated DNA was checked by agarose gel electrophoresis and nanodrop. Concentration of nucleic acids was determined by nanodrop (Thermo Scientific NANODROP 1000). Total DNA was measured at 260/280 nm and 260/230 nm to check the quantity of isolated DNA. DNA quality was estimated on 0.8% agarose gel electrophoresis. Gel was prepared in 0.5x TBE (Tris-Borate EDTA pH 8.0) buffer and used ethedium bromide (10 mg/ml) dye for staining. Bromophenol blue (6x loading dye) was used for loading of samples and run at 70V for 2 hrs. After electrophoresis gel was visualized under gel documentation system.

10x TBE (1000 ml): 108g Tris base, 55g boric acid, 9.3g EDTA

0.5x TBE (1000 ml): Dissolve 50 ml of 10x TBE in 950ml distilled water (DW).

3.2.3 Detection of begomoviral infection by polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) to amplify various viral DNA segments from total DNA was used to detect the presence of begomovirus DNA. Different sets of forward and reverse primers were used as listed in table 3.2 and concentrations of PCR components used for amplification are listed in table 3.3.

3.2.3.1 PCR Reaction

Polymerase chain reaction was used to amplify various viral DNA segments using different sets of reverse and forward primers. PCR reaction mixture containing all PCR components (10x assay buffer, MgCl2, primers, dNTPs, taq DNA polymerase enzyme and sterile distilled water) was used. PCR reactions were performed in 25µl reaction mixture containing 1x PCR assay buffer, 1.5mM $MgCl_2$, 200µM dNTP mix, 300nM of each primer and 1U Taq DNA polymerase. PCR consists of three basic steps that constitute a single step i.e. denaturation, annealing and extension for appropriate time with 30-35 cycles and all the PCR amplifications were carried out in an Applied Biosciences thermal Cycler. Total DNA isolated from plant leaves was used as template DNA to screen for the presence of begomoviral infection by using degenerate primer pairs for different begomoviral genes and full length viral genome. Primers specific for

DNA-B component and betasatellites were also used for screening. A detailed list of all the degenerate primers and PCR conditions for each amplification reaction used in this study are listed in table 3.2.

Table 3.2 List of primers, PCR profiles used in this study

Primer Name	Primer Sequence (5'-3')	PCR Profile	Product Size	Reference
PAL1v1978	5'GATTTCTGCAGTTDATRTTY TCRTCCAA3'	94 ºC-60 sec 58 ºC-45 sec	~1.6kb [Upper half	Rojas *et al*., 1993
PAR1c715	5'GCATCTGCAGGCCCACATYG TCTTYCCN GT3'	72ºC-110 sec	(UH) region]	
PAL1c1960	5`ACNGGNAARACNATGTGGG C 3`	94 ºC-50 sec 52 ºC-45 sec	~1.2 kb [Bottom	Rojas *et al*., 1993
PAR1v722	5`GGNAARATHTGGATGGA 3 `	72 ºC-90 sec	half (BH) region]	
AC1048	5'GCCYATRTAYAGRAAGCCM AG 3'	92 ºC-60 sec 60 ºC-20 sec	~560bp (Core coat	Wyatt and Brown, 1996
AV494	5'GGRTTDGARGCATGHGTAC ATG 3'	72 ºC-30 sec	region)	
PBL1v2040	5'GCCTCTGCACARTGRTCKAT CTTCATACA3'	92 ºC-60 sec 54 ºC-20 sec	~650bp (DNA-B)	Rojas *et al*., 1993
PCRc1	5'CTAGCTGCAGCATATTTACR ARWATGCCA3'	72 ºC-30 sec		
Beta01	5'GGTACCACTACGCTACGCAG CAGCC 3'	94 ºC-60 sec 60 ºC-45 sec	~1.3 kb (Betasatelli	Briddon *et al*., 2002
Beta02	5'GGTACCTACCCTCCCAGGGG TACAC 3'	72 ºC-90 sec	te)	
PSBP-F	5'GTCARTATGCAKCNAAGGA RCA3'	94 ºC-60 sec 58 ºC-30 sec	~2.7 kb (Complete	Abutting primers Present study
PSBP-R	5'CCTGTCAYAGTTGCGTMCCA C3'	72 ºC-90 sec	DNA-A)	

F: Forward primer, R: Reverse primer.
Degeneracy on nucleotide sequences are represented by IUPAC code: M = A/C, R =A/G, W = A/T, Y = C/T, S = C/G, K=G/T, H = A/C/T, V =A/C/G, D = A/G/T, B = C/G/T, N = A/C/G/T

Table 3.3: Components of PCR, their concentration and volume to set-up PCR master-mix

Components	Stock concentration	Working concentration	Reaction volume (25 µl)
PCR reaction buffer	10x	1x	2.5µl
MgCl2	15mM	1.5mM	1.5 µl
dNTPs	20mM	200µM	0.6 µl
Forward primer	100µM	300 nM	0.75 µl
Reverse primer	100µM	300 nM	0.75 µl
Taq DNA polymerase	3U/µl	1U/µl	0.3 µl
DNA template	----	150ng	1 µl
Sterile Distilled Water (SDW)	----	-----	17.6µl

3.2.3.2 PCR condition

General PCR profiling used to amplify different begomoviral genes/ genome is mentioned below-

Initial denaturation	94ºC	5min	
Denaturation	94ºC	1min	35 cycles[#]
Annealing[*]	52-60ºC	30sec	
Extension	72ºC	90sec	
Final extension	72ºC	10 min	
Store	4ºC	∞	

[*]Annealing temperature is dependent on primer pair and change with the primers.
[#] Increase in number of PCR cycle exponentially increases the amount of target DNA synthesized.

3.2.4 Gel extraction of desired amplified product of viral genes and sequencing

Amplified fragments were excised from the agarose gel under ultraviolet trans-illuminator and purified through PureLink® Quick Gel Extraction Kit (Invitrogen™ by life technologies). Excised gel slices were weighed transferred to an eppendorf and gel solubilization buffer (L3) was added in the ratio of 3 (buffer):1(gel slice). Tube containing gel pieces were incubated at 50ºC for 15 min in a heat block with occasional mixing to completely dissolve the gel slice. After complete melting the buffer containing gel pieces was transferred to a quick gel extraction column inside a wash tube. The column was centrifuged at 12000 rpm for 1 min to bind the DNA fragments with column and further, the column was washed with 500µl wash buffer (W1) by centrifugation at

12000 rpm for 1 min. To remove ethanol traces column was again centrifuged for 1 min at 12000 rpm. The flow-through was discarded and the column was transferred into a recovery tube. DNA bound with the column was further eluted with elution buffer (E5)/ sterile distilled water by centrifugation at 12000 rpm to collect the purified DNA which was stored at -20ºC and directly sequenced from Chromous Biotech Pvt. Ltd.

3.2.5 Scanning electron microscopic analysis

Differences in the leaf morphology of healthy and leaf curl infected papaya plants were visualized through scanning electron microscopy (SEM). Leaf tissues of healthy and leaf curl infected papaya plants of same age were collected and leaf edge, midrib and leaf vein portions from same position of both the leaves were cut into 2-4 mm size using sterile blade to prepare specimen for SEM analysis. The first step in sample preparation was fixation of sample in 2.5% gluteraldehyde for 2- 6 hrs at 4 ºC. After fixation samples were washed in 0.1M phosphate buffer for 15 min in three changes repeats. After washing specimens were dehydrated in acetone by keeping the sample in different concentration of acetone for 30 min at 4ºC. Stepwise graded series of increasing acetone concentration from 30%, 50%, 70%, 90% and 100% was used to remove all the traces of water (dehydration) from specimens. After dehydration process, specimens were dried by critical point drying (critical Point i.e. 31.5°C at 1100psi). Dried specimens were mounted on Aluminum stubs with carbon tape. After mounting, specimens were subjected for paladium (Pd) coating using sputter coater to make the specimen conductive for SEM visualization. Specimens were examined through scanning electron microscope model no. JSM 6490 LV (JEOL Japan). Energy dispersive spectroscopy (EDS) feature inbuilt in SEM was also used to examine the elements present in healthy and infected papaya leaves.

2.5% gluteraldehyde: 25% gluteraldehyde (100 ml), 0.2 M phosphate buffer (500 ml), distilled water (400 ml)

0.2 M phosphate buffer: Solution A. 5.93 g sodium dihydrogen orthophosphate dissolved in 190 ml distilled water Solution B. 23 g disodium hydrogen phosphate anhydrous dissolved in 810 ml distilled water.

Add Solution A and B to make 1000ml 0.2 M phosphate buffer.

0.1 M phosphate buffer (1000 ml): 500 ml 0.2 M phosphate buffer, 500 ml distilled water

3.2.6 Designing of abutting primers to amplify full-length viral genome

Abutting primers are ~20 mer oligonucleotide sequences designed from non-overlapping but adjacent position of a gene (Briddon *et al*., 1993). Abutting primers were used to produce a linear product from circular geminivirus genomic template. Amplified products of core region of coat protein (CP) gene and upper half (UH) region of begomovirus isolates through degenerate begomovirus primers were eluted from the gel, purified and sequenced. Sequencing data thus obtained was assembled, analyzed and further used for designing of abutting primer pairs to amplify complete DNA-A component of begomovirus isolates. All the sequences of CP gene and UH regions were aligned through clustal-w available in MEGA version 6.0 software (mega v6.0). The highly conserved regions within the aligned sequences were selected and primer pairs were manually designed and used for the amplification of complete DNA-A genome of begomovirus isolates used in the study. Primers are listed in table 3.2.

3.2.7 Rolling circle amplification (RCA) of begomoviral genome

Rolling circle amplification (RCA) is an isothermal amplification method which is used for enrichment of circular genomic DNA of viruses using bacteriophage ϕ29 DNA polymerase enzyme. This method exponentially amplifies single or double stranded circular DNAs and produces microgram quantities of DNAs from very small amount of starting material in a primer independent manner (Haible *et al*., 2006). Random hexamer primers anneal to the circular DNA template at multiple sites and extend with the help of ϕ29 DNA polymerase to produce high molecular weight double stranded concatamers of circular template DNAs. ϕ29 DNA polymerase ensures high fidelity of amplification due to proof reading activity.

Begomoviral DNA was amplified with RCA method using illustra™ TempliPhi DNA amplification kit (GE Healthcare, life sciences). 1µl of DNA template (100ng) was added to 5µl of sample buffer and heated at 95ºC for 3 min to denature the DNA. The sample was immediately kept on ice for cooling to prevent renaturation of separated strands. After that reaction solution prepared by adding 5µl of reaction buffer and 0.2 µl of enzyme mix was added to the sample with proper mixing to make final reaction and incubated at 30ºC for 18 hrs in a thermal cycler. Once the incubation was completed, the

enzyme was inactivated by heating at 65ºC for 10 min. Inactivation step ensures the degradation of DNA polymerase along with its exonuclease proof reading activity which permit samples for a long time storage without any further degradation. Amplified RCA products were then diluted in 40µl sterile distilled water and stored at -20ºC to conduct further experiments.

Components of Templiphi amplification kit

Sample buffer (contains random hexamers), Reaction buffer (salts and deoxynucleotides) and enzyme mix (ϕ29 DNA polymerase and random hexamers in 50% glycerol).

Kit also supplies 2 ng/µl pUC19 that works as positive control in the reaction.

3.2.8 PCR amplification of complete DNA-A component of begomoviruses and associated betasatellite molecules

All the begomovirus isolates were amplified using abutting primers designed during this study as listed in table3.2. The PCR amplified fragments of complete DNA-A component of viral isolates by using abutting primers and betasatellite molecules using betasatellite specific primers were eluted from gel, purified using invitrogen quick gel extraction kit. Purified products of betasatellites were directly sequenced from Chromous Biotech Pvt. Ltd. (Bangalore) and complete DNA-A components were cloned for further studies.

3.2.9 Cloning of complete DNA-A component of begomoviruses

Purified PCR products of complete DNA-A component were cloned into the pGEM-T easy vector using pGEM-T easy vector system cloning kit Promega (USA). The Ligation reaction of 10 µl was set up in 0.5ml tubes. All the components (2x rapid ligation buffer (5µl), pGEM-T easy vector (1 µl), eluted PCR product (3 µl), T4 DNA ligase (1µl) were mixed and incubated overnight at 4ºC for ligation. This ligated product was further used for transformation experiments.

3.2.10 Preparation of competent cells

Competent cells are the flexible cells that can infuse the plasmids during bacterial transformation experiments. Competent cells are acting as host for multiplication of

plasmids during transformation experiments. DH5α strains of *E. coli* bacteria were used for preparing competent cells in this study. Preparation of highly efficient competent cells was done through Sambrook and Russel, 2001 method. Desired strain of bacteria was streaked on Luria broth agar (LBA) plate and allowed to grow by incubating overnight at 37ºC. A single colony of bacterial strain was transferred into a 2ml Luria-Bertani broth (LB) containing tubes and incubated overnight at 37 ºC in a shaker incubator at 150-200rpm. After overnight incubation, 1 ml grown bacterial culture was inoculated in 50 ml LB and again incubated at 37 ºC in a shaker incubator at 200rpm until OD_{600} reached at 0.4- 0.6. Bacterial culture obtained at desired OD was then centrifuged in SS34 tubes at 7000 rpm at 4 ºC for 5 min. 20 ml chilled $CaCl_2$ (0.1M) was added in pellet and incubated on ice for 10 min and again centrifuged at 7000 rpm for 5 min at 4 ºC. Finally the pellet obtained after centrifuge was re-suspended in 4 ml of chilled $CaCl_2$ (0.1M) containing 100% glycerol and kept on ice for 5 min. 100 µl aliquots of competent cells were made in 1.5 ml eppendorf tubes and stored at -80 ºC for further use.

3.2.11 Transformation of ligated products

Tubes containing competent cells (DH5α) were kept on ice until just thawed. 5µl of ligated product was transferred to a sterile 1.5 ml centrifuge tube on ice. 50µl of competent cells were added with ligation mixture and mixed gently by tapping. Further, tubes were incubated on ice for 30-60 min with occasional mixing. The cells were given heat shock treatment for 2 min in a water bath maintained at 42 ºC and immediately returned on ice for at least 10 min. 950µl of sterile LB broth was added into each tube and allowed to grow the cells at 37 ºC for 1.5 hrs in a shaking incubator at ~150 rpm. After incubation, cells were evenly spread on Luria agar (LA) plates containing ampicillin, X-gal, IPTG using sterile glass spreader. The plates were incubated at 37 ºC overnight in an incubator for the growth of bacterial colonies.

Luria broth (LB)

Casein enzyme hydrolysate 10g/l, Yeast extract 5g/l, NaCl 10g/l, pH 7.5

Luria broth agar (LA)

Casein enzyme hydrolysate 10g/l, Yeast extract 5g/l, NaCl 10g/l, Agar 15g/l, pH 7.5

3.2.11.1 Screening of transformed colonies through colony PCR

Transformed colonies were selected based on blue and white plaques. The suspected transformants which appeared as white coloured colonies (recombinant colonies) were randomly picked from the plate using sterile tooth pick and sub cultured on the fresh LB agar plate containing ampicillin (100 µg/ml) while, blue coloured colonies were left out. The properly labeled plates were incubated overnight at 37ºC. Incubated white colonies were dissolved in 2 µl sterile distilled water in PCR tubes to perform colony PCR. Colony PCR was carried out by using primers used for PCR amplification of insert. Amplification results after colony PCR were analyzed on 1% agarose gel for the presence of desired DNA insert in plasmid. Positive colonies were then grown in 2 ml LB broth containing ampicillin (100µg/ml) at 37ºC in a shaker incubator at 120 rpm overnight to isolate plasmid DNA.

Ampicillin stock solution (100 mg/ml): 1g of ampicillin was dissolved in 10 ml of sterile distilled water. Solution was filter sterilized using Whattman poly ethersulfone membrane (0.2 µm pore size), stored in aliquots at -20°C.

X-Gal stock solution (20 mg/ml): 200 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D Galactopyranoside) dissolved in 10 ml of N, N dimethyl formamide. The solution was stored at -20°C in an amber colour bottle.

IPTG stock solution (100 mM): 240 mg of IPTG (Isopropyl-β-Dthiogalactopyranoside) dissolved in 10 ml of sterile distilled water. The solution was filter sterilized and stored in aliquots at 4°C.

Plates containing LB, Ampicillin, X-gal, IPTG: Required volume of ampicillin stock solution was added to the medium to reach the final concentration of 100 µg/ml. The medium was gently mixed and poured on 90-mm size petri plates. The plates were allowed to solidify and dried open under laminar for 30 min. 40 µl of X-Gal stock solution (20mg/ml) and 40 µl of 100 mM IPTG stock solution were spread evenly over each plate with sterile glass spreaders.

3.2.12 Preparation of plasmid DNA using alkaline lysis method

Overnight grown cultures of positive bacterial colonies were centrifuged at 10,000 rpm for 1 min in a 1.5 ml microfuge tube. Supernatant was discarded and the pellet was suspended in 100 µl ice cold GTE buffer (solution I) by vortexing and added 200 µl of

freshly prepared alkaline-SDS (solution II) by gentle inverting the tubes several times for cell lysis. After complete lysis, 150 µl of chilled potassium acetate solution (solution III) was added to neutralize the solution and kept on ice for 15-20 min. After incubation tubes were centrifuged at 13,000 rpm for 15 min at 4ºC. the resulting supernatant was transferred to a fresh microfuge tube and extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), and further extracted twice with chloroform: isoamyl alcohol (24:1). Aqueous phase containing nucleic acid was precipitated with 2 volumes of absolute ethanol by incubating at 20ºC for 20 min and centrifuged at 13,000 rpm for 15 min at 4ºC. The DNA pellet was washed with 70% ethanol and dried in air. Pellet was dissolved in 50 µl TE buffer and stored at -20ºC.

Solution I (GTE buffer): 0.05 M Glucose, 0.025 M Tris Cl, pH 8.0 and 0.01 M EDTA, pH- 8.0)

Solution II (alkaline-SDS): 1% SDS and 0.2 N NaOH

Solution III (potassium acetate solution): 3 M potassium and 5 M acetate

3.2.12.1 Restriction digestion of plasmid DNA

Plasmid DNA isolated from bacterial colonies were further subjected for restriction enzyme mediated digestion using fast digest type II restriction enzymes; *Bam*H1, *Eco*RI and *Kpn*I. For restriction analysis of plasmid DNA total 20µl reaction was prepared that contain 2µl of 10x buffer, 1 µl restriction enzyme, 1µl of plasmid DNA and reaction was makeup 20µl by adding 16µl of nuclease free water. All the components were mixed well and incubated at 37ºC in hot water bath for 15 min. All the unit length plasmid DNA and viral DNA inserts were released. Restriction pattern of digested plasmid DNAs were visualized on 1% agarose gel and banding pattern showed the confirmation of recombinants.

3.2.12.2 Confirmation of recombinant clones

Quality of isolated plasmid DNA was assessed through agarose gel electrophoresis. The plasmids were again subjected to PCR and restriction digestion as described in 3.2.3.1 and 3.2.12.1 respectively for reconfirmation of the presence of expected insert and positive clones. Isolated plasmid DNAs were digested with those restriction enzymes

that have their sites at the flanking ends of the insert (in order to release the insert from the vector). Amplified as well as digested products were electrophoresed and analyzed in 1% agarose gel. Positive clones were selected for DNA sequencing.

3.2.13 *Insilico* analysis of complete DNA-A sequence and betasatellite sequence

3.2.13.1 Compilation of sequencing data

Sequencing data obtained from sequencing was compiled manually with the help of BLAST online tool. Both the strands (forward and reverse sequences) were analyzed and aligned to each other to determine the complete gene (/genome). Hence, a complete sequence of each component was developed by their multiple sequence alignment using clustal-w algorithm implemented in mega v6.0 (Tamura *et al*., 2013). After compilation of complete DNA-A/ betasatellite sequences, similarity search was performed using BLASTn (http://www.ncbi.nlm.nih.gov/cgi) to know the presence of begomovirus infecting papaya. Open reading frames (ORFs) present in begomoviral DNA-A component as well as in betasatellites were identified through ORF finder (http://www.ncbi.nlm.nih.gov/orffinder/). Complete DNA-A and betasatellite sequences obtained in this study were submitted in GenBank.

3.2.13.2 Retrieval of begomoviral/ genome sequence

Begomoviral DNA-A genome sequences and betasatellite sequences were retrieved from National Centre for Biotechnology (NCBI) (www.ncbi.nlm.nih.gov) database. Complete DNA-A sequences as well as betasatellite sequences of papaya infecting begomoviruses and isolates closely related with identified isolates were retrieved in FASTA format. The sequences of different begomoviral genes were derived from complete DNA-A sequences of begomovirus isolates.

3.2.13.3 Multiple sequence alignment (MSA)

To obtain highly accurate alignment of DNA-A genome as well as betasatellite sequences retrieved from database along with sequences obtained during this study MUSCLE algorithm (Edgar RC, 2004) implemented in MEGA version 6.0 software (mega v6.0) was used (Tamura *et al*., 2013). Default parameters were selected to get accurate and refined alignment information.

3.2.13.4 Pairwise identity calculations and distribution plots through sequence demarcation tool (SDT) analysis

International Committee on Taxonomy of Viruses (ICTV) has recommended sequence demarcation tool (SDT) (a highly automated tool) to classify DNA viruses. SDT version 1.2 (Muhaire *et al*., 2014) was used for the taxonomic classification of all begomovirus isolates and associated betasatellites identified in present study. This tool calculates the pairwise identity of datasets used for the analysis and displays pairwise identity scores in the form of a colour coded matrix that group the isolates based on close percentage proximity. FASTA files of DNA-A and betasatellite sequences used in the study were analyzed in SDT v 1.2 to access the overall relationship between them. Order of sequences along the axes of the colour-coded matrix obtained after analysis reflects the pairwise identities between the sequences. Colour of the cells in the matrix indicates the close relationships of novel identified sequences with the species, isolates or strains of begomoviruses and provide help in the classification of begomoviral sequences.

SDT also provides the facility to generate pairwise identity score frequency distribution plot that helps in establishment of species demarcation criteria for begomovirus taxonomy. Peaks of these plots showed the pairwise identity thresholds (maximum) while troughs show the pairwise identity threshold (minimum). The coloured matrix and frequency distribution plot were saved in enhanced metafile format (EMF)/ bitmap images (BMP) format for further display. Begomoviruses and betasatellites identified in present study were classified based on this matrix to fulfill the ICTV guidelines and submitted in GenBank database.

3.2.13.5 Phylogenetic analysis

Phylogenetic analysis was performed to know the evolutionary relationships among DNA-A component, different begomoviral genes and betasatellite molecules associated with viral isolates of the study along with some selected begomoviral genes/genomes retrieved from the GenBank database. All the genome sequences selected for the study were aligned using muscle algorithm implemented in mega v6.0 (Tamura *et al*., 2013). The multiple alignment file is further used for phylogenetic relationships among all the begomoviral and betasatellite isolates. Maximum likelihood (ML) method was used to

infer phylogenetic relevance because it is a best platform to study relationships among distantly related organisms. Maximum likelihood method available in mega v6.0 was employed with default parameters with 1000 bootstrap replications to study the statistically relevant evolutionary background and genetic variability among all begomoviral isolates.

3.2.13.6 Recombination analysis

Recombination analysis of viral DNA-A component and associated betasatellites was performed to check the contribution of recombinations in begomoviral evolution and genetic variability. Recombination detection programme version 4.4.39 (RDP4) implemented with RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMERA, SISSCAN and 3 SEQ detection methods (Martin *et al*., 2015) was used. All the begomovirus sequences used in phylogenetic analysis were used to predict the possible recombination events. Alignment file in FASTA format was subjected to RDP4 and their recombination analysis was performed through various detection methods like automated RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMERA, SISSCAN and 3 SEQ. All recombination analysis were conducted for complete viral DNA-A and associated betasatellite molecules with highest acceptable *p*-values at default for every programme. Recombination breakpoint polishing was selected with required topological evidence setting to generate phylogenetically significant results.

3.2.14 *Insilico* designing of siRNA

All the open reading frames available in DNA-A component of begomoviruses were deduced from the complete genome and multiple sequence alignment of all genes were performed as mentioned in section 3.2.13.3 and different begomoviral genes were further checked for the presence of highly conserved region to know the conserved region among various begomoviral species. Further, every begomoviral gene was subjected to pssRNAit programme for the prediction of siRNAs against the desired begomoviral gene (http://plantgrn.noble.org/pssRNAit/). Begomoviral isolates used for the phylogenetic study were investigated for the presence of conserved regions in complete DNA-A genome. The conserved regions were analyzed with pssRNAit softwares that provide the platform for siRNA prediction. pssRNAit web server predict highly efficient siRNAs due

to having many features like off-target prediction against query plant genome, mRNA-siRNA binding energy, homology and siRNA accessibility to RISC.

3.2.14.1 Parameters for *insilico* siRNA designing

Various parameters were selected to design efficient and specific siRNAs against highly conserved sequences of different begomoviral genes to develop generic resistance strategy for the successful sustainable disease management. These parameters included RISC binding score for antisense and sense strand of siRNA, GC content range (30% - 57%) and off-target filtering. RISC binding score of antisense and sense strand of siRNA ensures their binding to the RISC and GC content of siRNA showed their stronger efficiency for RNAi. Generated siRNAs through pssRNAit with a BLAST search with papaya to filter siRNA having off target.

3.2.14.2 Identification of target regions

Multiple sequence alignment (MSA) of all begomoviral genes was performed in mega v6.0 using muscle algorithm to get accurate alignment of sequences. Target sites of siRNAs designed from all the genes were examined in alignment file of every begomoviral gene generated through muscle alignment in mega v6.0 software and selected those siRNAs that target maximum number of begomoviral isolates taken into consideration.

3.2.14.3 Prediction of target site accessibility of siRNAs

Interaction of siRNA with the target mRNA is a major step during RNA silencing that governs the siRNA efficiency. Formation of secondary structure of mRNA hinders the siRNA-mRNA binding during RNA silencing (Bohula *et al*., 2003). siRNA-mRNA hybridization plot was generated using RNAup online programme (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAup.cgi) to ensure the efficacy of silencing caused by designed siRNAs. siRNAs designed against different genes were subjected to online available RNAup web server for the prediction of target accessibility. Binding of siRNAs with same conserved region chosen as putative target of suggested siRNAs, region of mRNA with minimum free energy value showed the specificity and stability of designed siRNAs. The plot of target accessibility of selected siRNAs

represented by specific energy (ΔGi) required for interaction of antisense siRNA with target mRNA. Energy plot showed that the energy required to open secondary structrue of mRNA around the target site was much lower than energy required for antisense siRNA interaction. This energy plot reflects the probability of stable siRNA-mRNA duplex formation and ensures the effective RNA silencing of targeted gene.

In this study we have surveyed different geographical locations in India and collected papaya leaf samples showing leaf curl symptoms. Present study includes the association of different begomoviral species and betasatellite components with the leaf curl disease of papaya and their diversity study from different geographic locations of India which may lead to design an RNAi strategy for generic resistance based on molecular variability of papaya leaf curl causing begomoviruses (PaLCuV isolates) infecting *Carica papaya* L.

4.1 Sample collection

During the year 2014 to 2016 many places were surveyed to collect samples from different geographic locations of India (figure 4.1).

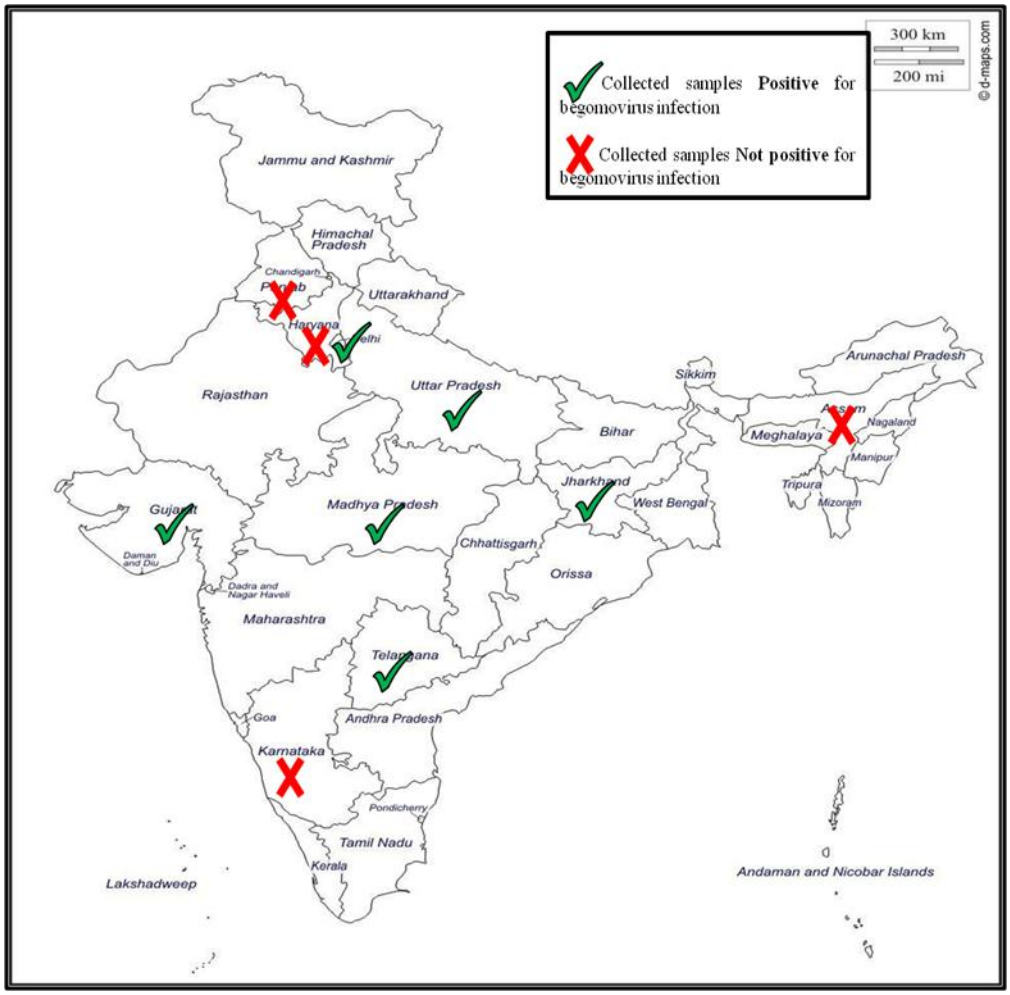


Figure 4.1: Survey of different geographical locations of India during sample collection and confirmation of begomoviral infections based on PCR based screening.

Begomoviruses infecting papaya plants have very high mutation and recombination rate this supports them to adapt in different environmental conditions very easily by genetic variation. Papaya plants are frequently found to be infected with leaf curl causing begomoviruses that produce typical leaf curl symptoms like downward/ upward curling of leaf margins, crumpling, severe curling in the apical region, yellowing of leaves, leaf deformation, vein thickening, vein clearing and stunted plant with reduced fruit size. Above mentioned symptoms were observed on papaya plants during surveys and leaf samples were collected from kitchen gardens, papaya farms, nurseries and roadsides. Papaya farms and fields surveyed during sample collection are shown in figure 4.2. Papaya leaves exhibiting leaf curl symptoms as well as healthy/ non symptomatic were observed and collected from various locations of ten different Indian states (Assam, Gujarat, Haryana, Jharkhand, Karnataka, Madhya Pradesh, New Delhi, Punjab, Telangana and Uttar Pradesh). Number codes were assigned to all collected samples (table 3.1). Symptom variations in plant leaves collected during surveys from different places are shown in figure 4.3. Leaves of healthy papaya plant were taken from greenhouse and used as healthy and negative control during screening.

Figure 4.2: Papaya fields visited during survey and sample collection.

Viruses are pathogens co-evolved with their plant hosts and utilize host machinery to replicate and spread. Begomoviruses are small sized plant pathogens depend completely on their hosts for their existence. Co-existence of different viruses and hosts produce various characteristics symptom development of disease and this symptoms diversity in

same host plant indicates the differential infection of begomoviruses on same plant (Lefeuvre *et al*., 2011; Lozano-Duran R, 2016; Barboza *et al*., 2016). This confers an idea to study infection of all possible begomoviruses infecting papaya in various geographical places in India. Singh-pant *et al*., 2012 studied begomoviral diversity of papayas grown in three states (Haryana, New Delhi and Uttar Pradesh) of the country. Present study carried out survey of 10 different states across India to conduct molecular characterization and diversity of begomoviruses infecting papayas to develop generic resistance strategy against papaya leaf curl disease (PaLCuD).

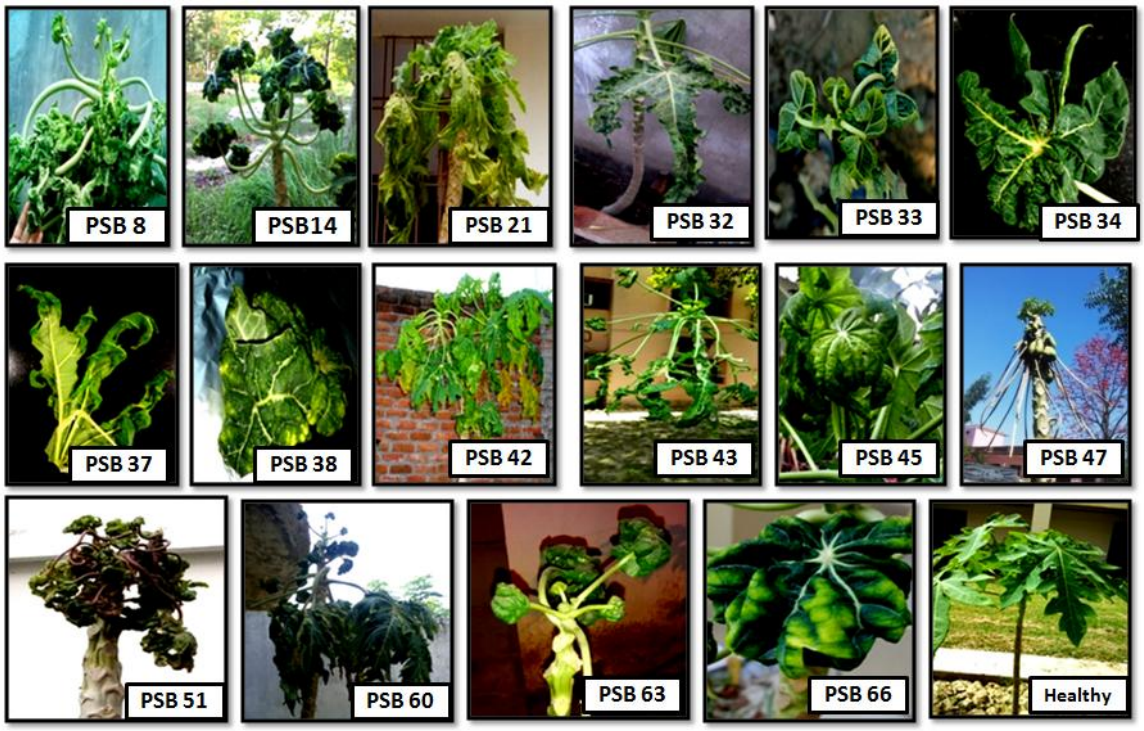


Figure 4.3: Papaya leaf samples showing leaf curl disease symptoms observed in different locations during survey and sample collection. Sample codes have been assigned to each leaf sample collected from various places and listed in table 3.1.

4.2 Genomic DNA isolation

Isolation of genomic DNA is an essential process in molecular biology experiments. Total genomic DNA of collected samples and healthy sample was extracted using Murray and Thompson, 1980 DNA isolation protocol. Qualities of extracted DNA samples were analyzed through 0.8% agarose gel electrophoresis. Concentrations of all the DNA

samples were found approximately 800- 1000 ng/µl (figure 4.4). Dilutions of 150ng/µl of all samples were used for PCR based screening.

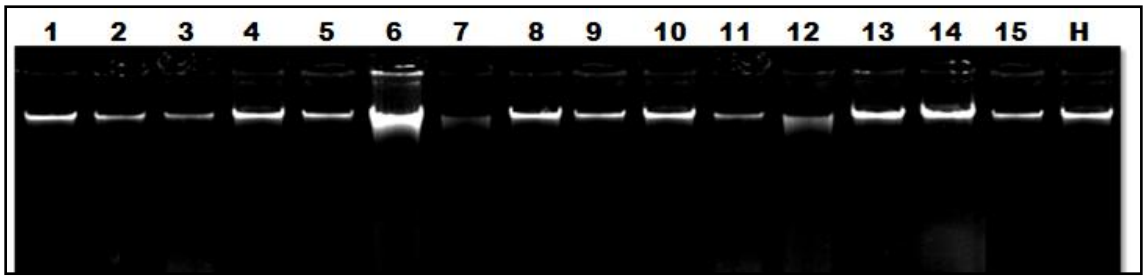


Figure 4.4: Gel image of total genomic DNA isolated from papaya leaves collected from different places.

4.3 Detection of begomovirus infection through polymerase chain reaction (PCR)

Papaya leaf curl disease symptoms occurred mainly due to begomoviral infection on plant but many other physiological disorders like leaf roll, deficiency of Mg^{++}, phosphorus (phosphate) and mixed viral infections mimic the leaf curl disease symptoms. Nowadays mixed viral infection and viral host switching is a very common problem in plants. Many begomoviral species infect the same crop; on the contrary, single begomovirus can infect different crop plants and causing leaf curl symptoms. Infection of several begomoviruses on papaya has become very frequent and cause major challenge for papaya growers. PaLCuD is mainly caused by whitefly transmitted begomoviruses i.e. papaya leaf curl virus (Saxena *et al*., 1998a) but many other begomoviruses on papaya have also been reported (Singh-Pant *et al*., 2012; Raj *et al*., 2008; Varun and Saxena, 2018). Diagnosis of begomoviral infection on papaya plants was necessary for further studies and apart from symptom characterization PCR was found to be the best way to detect begomovirus infection. Begomoviruses have a very complex genome organization with both monopartite as well as bipartite genomes. Begomoviruses with bipartite genome consist of two DNA components (DNA-A and DNA- B) while monopartite genome has only one DNA component. All the collected samples were screened for the presence of both the DNA components of begomoviruses. To know the infection of possible begomoviruses in symptomatic papaya plants, all the collected samples were screened for begomovirus infection by using degenerate primers designed for begomovirus detection.

Total plant DNA isolated from all collected symptomatic papaya leaves was subjected to PCR to detect the begomovirus infection. Many universal degenerate primer pairs reported for begomovirus detection were used to confirm the virus infection in collected samples. Degenerate primer pairs PAL1v1978 & PAR1c715; PAL1v1960 & PAR1v722 (Rojas *et al*., 1993) and AC1048 & AV494 (Wyatt and Brown, 1996) were used to detect the presence of DNA-A component of begomoviruses. Screening of all samples for DNA B component was done through combination of PCRc1 & PBL1v2040 primers (Rojas *et al*., 1993). PAL1v1978 & PAR1c715 primers used to detect upper half (UH) region of DNA-A component of begomoviruses and amplified approximately 1.6 kb (figure 4.5), PAL1v1960 & PAR1v722 primer pair amplified approximately 1.2 kb fragment of bottom half (BH) region of DNA-A component (Figure 4.6) and AC1048 & AV494 primers amplified approximately 560 bp fragments of core region of coat protein gene of begomoviruses (figure 4.7). Amplified PCR products were observed on 1.2% agarose gel and the desired amplicon size through amplification confirmed the infection of begomovirus in corresponding collected samples. No amplification from any sample was observed during DNA-B screening using PBL1v2040 & PCRc1 primer pair designed specifically for DNA-B component of begomoviruses. DNA isolated from healthy papaya sample did not show amplification from any primers.

Total 82 samples collected from different places during this study (2013- 2016) and 25 samples were found positive for begomovirus infections (DNA-A) based on PCR mediated screening. Samples collected from Uttar Pradesh, Madhya Pradesh New Delhi, Telangana, Gujarat and Jharkhand states were showed the presence of begomovirus through PCR whereas, samples collected from Karnataka, Assam, Punjab and Haryana during this study did not show amplification from any primer pair (table 4.1). Hence, supposed to be uninfected with begomoviral infection. No amplification was observed after repetitive attempts during the screening of all collected samples for the presence of DNA-B component of begomoviruses.

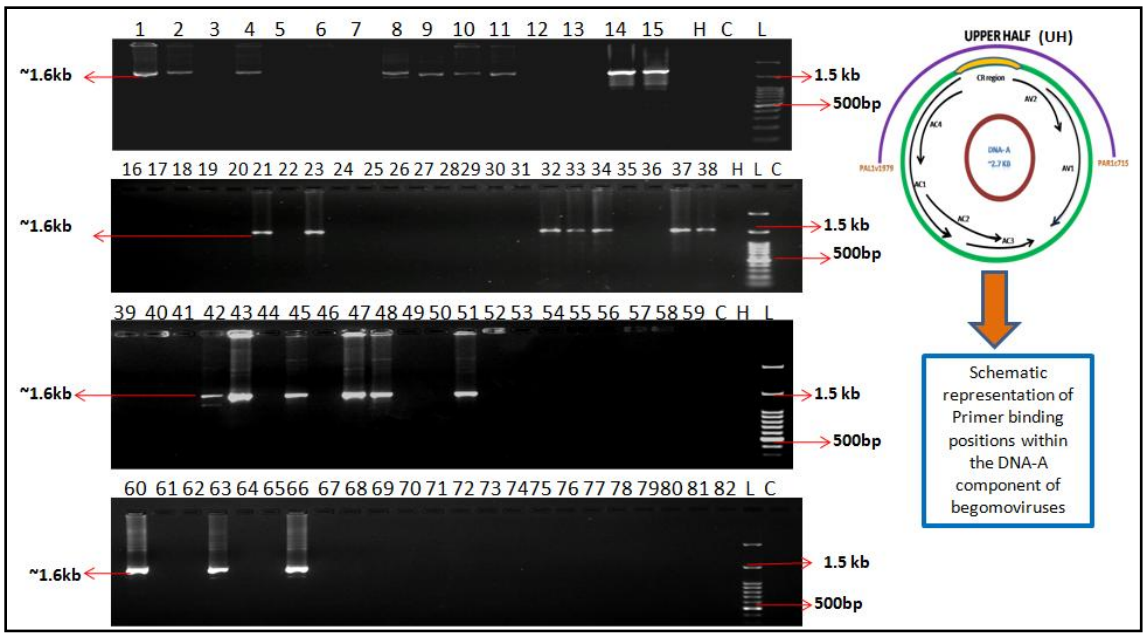


Figure 4.5: Screening of all the collected samples to detect begomovirus infection by using universal degenerate primers (Rojas *et al*., 1993) designed for upper half region of begomoviral DNA-A component. Numbering to the lanes represents the sample code no., H: healthy sample, C: control (without template DNA), L: 100 bp DNA ladder Qiagen.

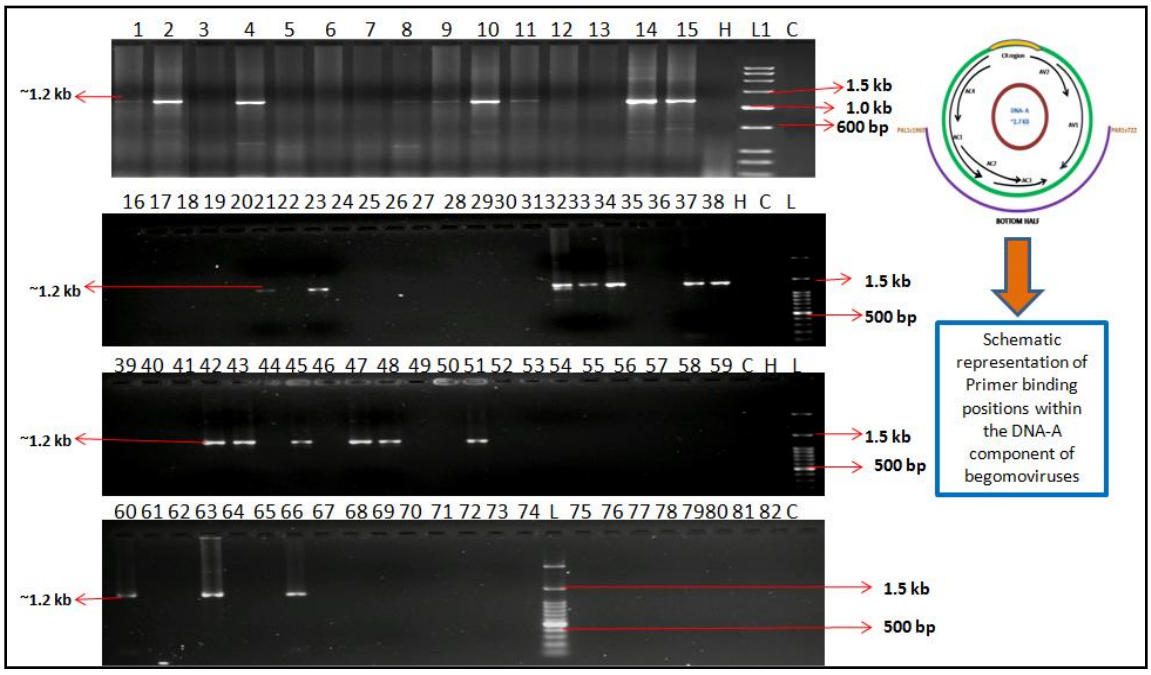


Figure 4.6: Screening of all collected samples for the presence of begomoviruses using universal degenerate primers (Rojas *et al*., 1993) designed for bottom half region of begomoviral DNA-A component. Numbering to the lanes represents the sample code no., H: healthy sample, C: control (without template DNA), L1: low range ruler (Qiagen), L: 100 bp DNA ladder Qiagen.

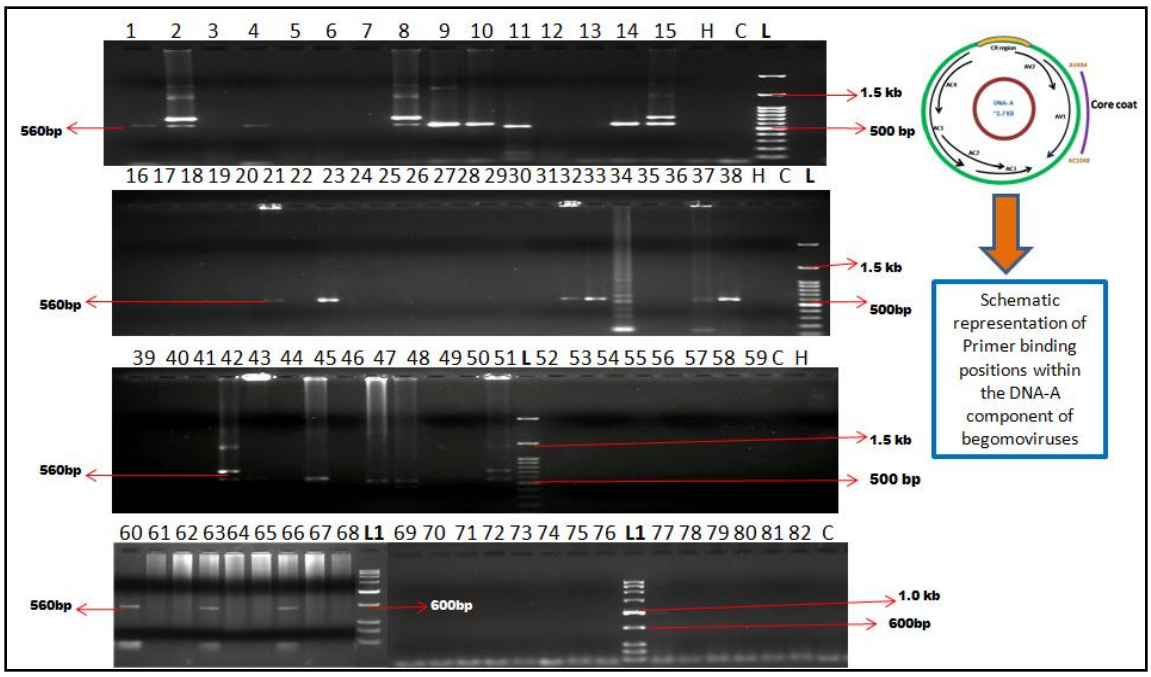


Figure 4.7: Screening of samples by using degenerate primers designed to amplify core region of coat protein gene of begomovirusses (Wyatt and Brown, 1996). Numbering to the lanes represents the sample code no., H: healthy sample, C: control (without template DNA), L1: low range ruler (Qiagen), L: 100 bp DNA ladder Qiagen.

Table 4.1: List of samples found positive for begomoviral infection and betasatellite association in present study

Place of collection	Positive samples (DNA-A)	Positive for Betasatellite	Samples not positive for begomoviral infection
Agra (U.P.)	-	-	PSB 73, 74
Ahmedabad (Gujarat)	PSB 37	-	PSB 36
Amritsar (Punjab)	-	-	PSB 75, 76
Azamgarh (U.P.)	-	-	PSB 19
Bangalore (Karnataka)	-	-	PSB 57, 58, 59
Bhopal (M.P.)	PSB 66	PSB66	PSB 67
Coorg (Karnataka)	-	-	PSB 55, 56
Faizabad (U.P.)	PSB 45	-	PSB 44, 46
Gorakhpur (U.P.)	-	-	PSB 49, 50
Guna (M.P.)	-	-	PSB 68, 69
Guwahati (Assam)	-	-	PSB 79, 80
Gwalior (M.P.)	-	-	PSB 64, 65
Hyderabad (Telangana)	PSB 51	PSB51	PSB 52
Jabalpur (M.P.)	PSB 32	-	-
Jamnagar (Gujarat)	PSB 33, PSB 34	PSB 34	PSB 35
Jorhat (Assam)	-	-	PSB 81, 82
Kushi Nagar (U.P.)	-	-	PSB 70
Karnal (Haryana)	-	-	PSB 77, 78
Lalitpur (U.P.)	PSB 47, PSB 48	PSB47	-
Lucknow (U.P.)	PSB 1, 2, 4, 8, 9, 10, 11, 14, 15,43	PSB 14, PSB 43	PSB PSB 3,5,6,7, 12, PSB 13
Mahoba (U. P.)	PSB 42	-	PSB 41
Malihabad (U.P.)	-	-	PSB 16, 17, 18
Mathura (U.P.)			PSB 71, 72
New Delhi	PSB 60, PSB 63	PSB60, PSB 63	PSB 61, 62
Ranchi (Jharkhand)	PSB 38	PSB 38	PSB 39, 40
Shahjanpur (U.P.)	PSB 21, PSB 23	PSB 21	PSB 20, 22, 24
Shivpuri (M.P.)	-	-	PSB 25- 31
Warangal (Telangana)	-	-	PSB 53, 54

Identification of disease through symptoms is not reliable due to mixed infection of different viruses, other plant pathogens or environmental stress conditions. Initially, dot- and squash-blot hybridization tests, enzyme linked immunosorbent assay (ELISA) and western blot were used to detect geminivirus infection (Gilbertson *et al*., 1991). Molecular biology techniques provide better approaches for the detection of causal

organism and PCR is a quick, specific, precise and sensitive method for molecular detection of begomoviruses. However, application of polymerase chain reaction (PCR) technology, often together with DNA sequencing, that revolutionized the detection and characterization of geminiviruses (Rojas *et al*., 1993). Furthermore, sequencing of the PCR amplified geminiviral DNA fragments allows for precise identification of the virus. Degenerate primer pairs (Rojas *et al*., 1993) designed to detect all begomoviruses infection has been largely applied for the primary detection of begomovirus infection. These primer pairs were used for detection of DNA-A and DNA-B component of many whitefly transmitted geminiviruses (WTGs) from various crop plants in different geographical locations (Zhou *et al*., 1997; Farag *et al*., 2005; Bela-ong and Bajet, 2007; Kumar *et al*., 2009; Fernandes *et al*., 2009).

Wide distribution and expanding host range of begomoviruses is a major concern for papaya growers and detection of some specific (known) begomoviruses may not identify the infection of uncharacterized/ novel begomovirus species infecting papaya in different locations. Begomoviruses have the ability to evade the plant defense response which is mainly due to interactions between plant and virus during infection. So study of different begomoviral species on papaya provides information of occurrence and distribution of different begomoviruses on papaya, as well as a better platform to develop a generic resistance approach against papaya leaf curl disease.

4.4 Enrichment of viral genomic DNA by rolling circle amplification (RCA) method

RCA (Rolling circle amplification) technique has been used as detection method as well as for exponential amplification of all circular viral DNAs. We used this technique to amplify complete viral genomes of all positive samples to get full length genome of DNA-A, betasatellite and DNA-B (if any). This method helps not only in virus detection in samples but also to get larger amount of viral templates for further analysis. High molecular weight concatameric viral DNAs were obtained through RCA (figure 4.8) which were further used for amplification of viral genes for further studies.

Begomoviruses are predominantly replicated through rolling circle replication (RCR) method. To identify uncharacterized different types of single stranded circular genome of begomoviruses through rolling circle amplification method is also a reliable diagnostics.

Inoue- Nagata *et al*., 2004 used RCA amplification technique for the first time to detect and clone bipartite geminiviruses infecting tomato plants in Brazil. RCA is the most recent technique to detect, amplify, clone and characterize geminiviruses infecting different crops with more accuracy in sequences (Kushwaha *et al*., 2010).

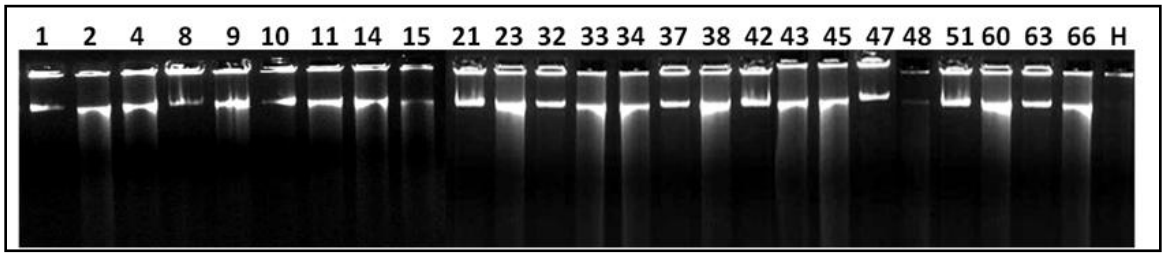


Figure 4.8: Screening of RCA product of virus isolates showing high molecular weight DNA obtained from infected samples. Healthy sample (H) did not show amplified fragment indicates the absence of viral infection. Numbers codes represent the collected sample; H: healthy sample.

4.5 Detection of betasatellite molecules associated with begomoviral positive samples

All the 25 positive samples for begomoviral infection were screened for the presence of associated betasatellite molecules by using universal degenerate primers beta 01 & beta 02 (Briddon *et al*., 2002). Amplified product of ~1.3 Kb on 1.2% agarose gel indicated the presence of betasatellite molecule associated with the corresponding DNA-A component of begomovirus (Figure 4.9).

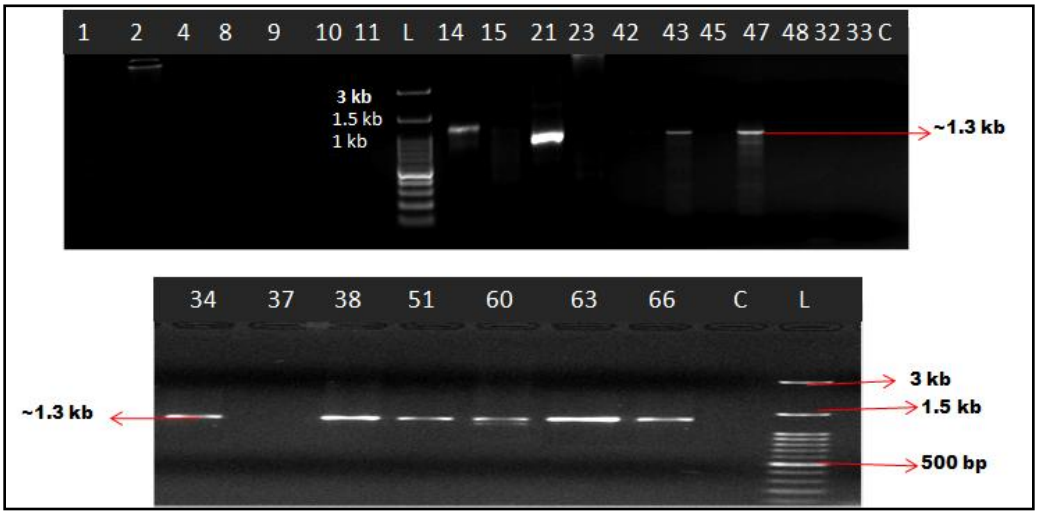


Figure 4.9: PCR mediated screening of betasatellite component in samples positive for begomoviral infection. ~1.3kb amplified fragment using degenerate primers (β01 and β02; Briddon *et al*., 2002) indicates the presence of betasatellite molecule. Number codes represent the sample code; C: control without template; L: 100bp DNA ladder (Qiagen).

Among all 25 begomoviral positive isolates, 10 begomovirus isolates were found to be positive for betasatellite association and this association with collected samples on the basis of PCR amplification has been summarized in table 4.1. Among all positive samples collected from Uttar Pradesh, two viral isolates of Lucknow (PSB 14, PSB 43), one isolate from Shahjahanpur (PSB 21) and one isolate from Lalitpur (PSB 47) were found to be associated with betasatellite molecule. Isolates PSB 66 of Bhopal, PSB 34 (Jamnagar), PSB 38 (Ranchi), PSB 51 (Hyderabad) and both the isolates collected from New Delhi (PSB 60, PSB 63) were found positive for betasatellite association. Amplified products of betasatellite from begomoviral positive samples were further sent for sequencing to confirm the betasatellite association.

Betasatellites are ssDNA molecules having approximately 1.3 kb genome (half genome size of begomovirus DNA-A genome i.e. helper begomovirus) and found frequently associated with monopartite begomoviruses. Betasatellites are dependent on helper begomoviruses for their replication and helps in the development of severe disease symptoms. In case of monopartite begomoviruses, DNA-A component of begomoviruses could cause infection alone and able to induce symptoms and systemic spread of disease but satellite molecules help begomoviruses in developing severe symptoms of disease on infected plants. There are many reports on association of betasatellite molecules with begomoviruses infecting papaya (Singh-Pant *et al*., 2012; Shahid *et al*., 2013, Varun and Saxena, 2018).

4.6 Sequence analysis of partial DNA-A component

Samples found positive for begomovirus infection were selected for further sequence analysis of core region of coat protein (CP) and upper half (UH) region of DNA-A component of begomoviruses infecting papaya plants. As we wanted to amplify conserved region of the begomovirus genome, our preferred choice was core region of coat protein gene which is considered to be conserved across the genera. Degenerate primers AC1048 & AV494 (Wyatt and Brown., 1996) were used to amplify and further sequence the same in order to identify the existing begomovirus species on collected papaya samples. CP genes of all the positive samples were amplified and desired amplified fragments were eluted from gel, purified by using gel extraction kit and

sequenced from Chromous Biotech. Pvt. Ltd. Similarly, UH region of begomoviruses were also amplified using primer pair PAL1v1978 & PAR1c715 (Rojas *et al*., 1993) and sequenced. Sequence information of amplified fragments was necessary to confirm the PCR positive samples.

Obtained sequencing data from both forward and reverse orientations were combined in mega v6.0 and analyzed through BLAST online tool (http://blast.ncbi.nlm.nih.gov/Blast. cgi) to detect the sequence identity of begomoviral isolates of this study with begomoviruses available in GenBank database. Percent identity of viral isolates with begomoviruses available in database has provided the clue regarding begomovirus species/strains associated with collected samples from different geographical locations. ORF finder search tool available at NCBI was used to identify open reading frames (ORFs). These comparisons revealed the highest identity percentages of particular begomoviral isolate available in NCBI database. According to earlier studies CP gene and conserved region (CR) is the highly conserved region within the whole viral genome and supposed to be representative sequences of whole begomoviral genome (Padidam *et al*., 1995). So that, sequence comparisons of CP genes and UH regions (covering CR region) of all PCR positive samples were used for preliminary detection of begomovirus infection.

4.7 Morphological analysis of healthy and infected papaya leaf by scanning electron microscopy (SEM)

Healthy and PCR positive infected leaves of papaya were examined through scanning electron microscopy (SEM) to observe the differences in leaf morphology of papaya plants. In order to examine plant leaf surfaces, specimens were prepared from leaf edge, midrib and vein portions of both healthy and infected leaves to evaluate the changes occurred in leaf morphology after begomoviral infection. SEM micrographs of abaxial surface of papaya leaf at 500x resolution revealed the clear leaf distortion, compactness and roughness in infected leaf tissues whereas; a smooth epidermal layer with some waxy deposition could be clearly seen in healthy leaf sample (figure 4.10a,b). Further, for the clear visualization of stomata micrographs were taken at 1500x resolution and it was observed that in healthy papaya leaf sample crescent shaped stomata with guard cells

were clearly visible while in infected papaya leaf sample stomata were sunken (figure 4.10 c, d). SEM micrographs predominantly showed the thickenings of veins (at 500x resolution) as well as midrib (at 100x resolution) of infected leaf as compared to healthy leaf sample (figure 4.10 e- h).

Veins of plant leaves have been originated from midrib and further branched to finer veins that transport essential nutrients to leaf tissues and distribute the photosynthetic products from leaves to the other plant parts. Thickened midrib and veins of diseased leaf in SEM micrographs have pointed out the obstruction in nutrients transport to leaf cells as well as photosynthetic products from leaf to other plant parts. SEM analysis of different parts of infected and healthy papaya leaves (figure 4.10) showed major differences that clearly indicate the changes in leaf morphology after viral infection.

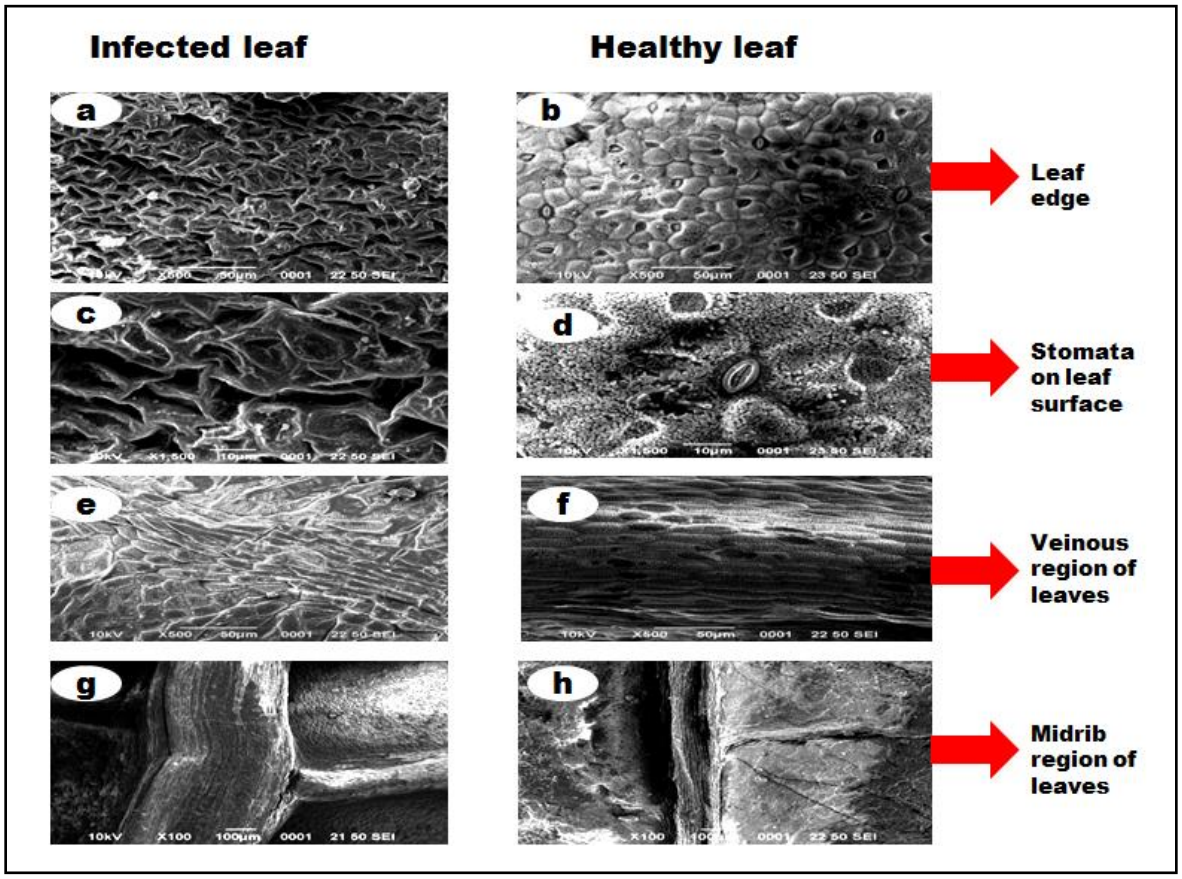


Figure 4.10: Scanning electron micrographs of abaxial surface of infected and healthy papaya leaf. a-d shows the ultra structure of leaf edge, e & f shows the morphology of veinous region and g & h shows the midrib region of papaya plant leaf.

Shai *et al*., 1986 studied the structure of both adaxial and abaxial surfaces of papaya leaf and found crescent shaped stomata surrounded by several epidermal cells present only on abaxial side of leaf surface while a thick cuticular layer that protect cells present on both abaxial and adaxial leaf surfaces. Interestingly, our SEM results showed changes in leaf morphology after begomoviral infection in papaya plant. Pathan *et al*., 2010 discussed that analysis of leaf tissue morphology is the preliminary feature to detect plant health and suggested that SEM is an ideal technique to visualize the native structure of leaves at high resolution and many researchers have evaluated the anatomy of plant leaves, fruit (Shai *et al*., 1986; Nwofia *et al*., 2012; Baez-Parra *et al*., 2018) and described the changes in leaf tissues of plants after viral infections (Magana-Alvarez *et al*., 2016, Gracia-Viera *et al*., 2018) through microscopy techniques. Our results have also demonstrated the similar findings and able to differentiate between morphology of infected leaf samples to healthy leaf samples.

Energy dispersive spectroscopy (EDS) feature is an advance feature of SEM which detects photons and analyzes their energy which is used to study the chemical elements present in samples. This feature mainly facilitates the description of nano-particles present in samples. EDS analysis of specimens produced an energy spectrum along with quantitative data of chemical elements and their relative proportions (atomic percentages) present in samples. Spectrum of healthy leaf (figure 4.11a) clearly indicates the electron emission from atomic cells (K, L and M cells) while in spectrum of infected leaf (figure 4.11b), electron emission occurs only from K cell. Elemental analysis of plant leaf specimens disclosed the presence of a range of elements within the leaves. On the basis of EDS data of leaf samples, proportions of carbon (C) and oxygen (O) elements were found in both healthy as well as infected leaf samples with highest atomic percentages. Some heavy elements were detected in healthy plants from secondary emission with reference to L and M cells while no such elements were detected in infected leaf samples (table 4.2).

SEM micrographs and EDS analysis of healthy and infected papaya leaf samples extended the information on leaf characteristics and clearly defined the differences between leaf midrib, vein, stomata and leaf epidermis. Presence of potential compounds

in healthy and infected plant leaves indicate plant activities during stress conditions. Thus, identified differences between healthy and infected plant leaves at cellular level.

Table 4.2: Energy dispersive spectroscopy (EDS) spectrum analysis of healthy and infected leaf in SEM

Elements	Standards	Healthy leaf sample		Infected leaf sample	
		Weight %	Atomic %	Weight %	Atomic %
C K	CaCO3	51.08	65.30	69.05	75.47
O K	SiO2	27.98	26.85	28.22	23.15
Mg K	MgO	0.87	0.55	-	-
K K	MAD-10 Feldspar	0.42	0.16	-	-
Ca K	Wollastonite	17.71	6.78	-	-
SiK	SiO2	-	-	0.21	0.10
P K	GaP	-	-	0.79	0.33
S K	FeS2	-	-	0.26	0.11
Na K	Albite	-	-	1.48	0.84
Zr L	Zr	2.19	0.37	-	-
Pt M	Pt	-0.24	-0.02	-	-

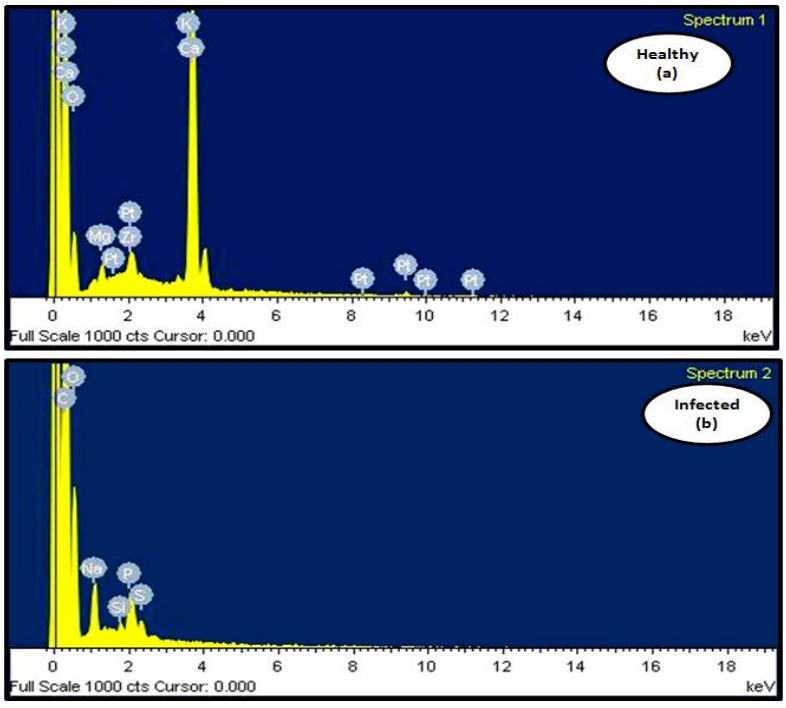


Figure 4.11: Spectrum of healthy (a) and infected (b) papaya leaf generated through energy dispersive spectroscopy (EDS) analysis inbuilt in scanning electron micrograph (SEM).

4.8 Amplification of full length DNA-A component of begomovirus isolates

Partial sequencing results of DNA- A component of virus isolates of present study were aligned using clustal-w implemented in mega v6.0. Highly conserved regions within alignments were used for the designing of abutting primers to amplify complete DNA-A component of begomoviruses. Abutting primers were designed from adjacent position in coat protein and pre-coat gene. Some degeneracy was used in primers to cover nucleotide differences among begomoviral isolates. Several abbuting primers were designed to amplify the complete DNA-A genome (~2.7 kb fragment). Primer pair PSBP-F & PSBP-R was found effective for amplification of complete DNA-A component (~2.7kb) of all viral isolates studied during present study (figure 4.12) and amplification products were further used for cloning and sequencing of full length DNA-A genome of begomoviruses.

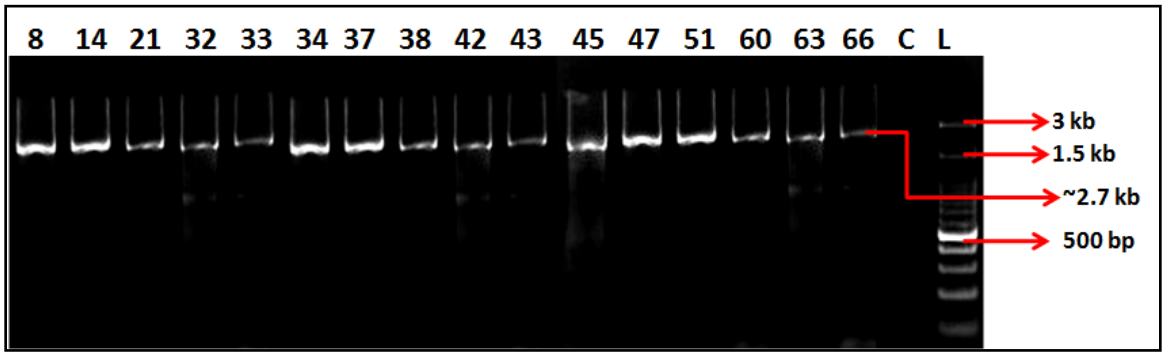


Figure 4.12: Amplification of full length DNA-A component of begomovirus isolates using abutting primer pair (PSBP-F & PSBP-R). L: 100 bp DNA ladder, C: negative control without DNA template.

4.9 Cloning and sequencing of complete DNA-A genome of begomoviruses

Sequences of CP gene and UH region were used for the preliminary detection of begomovirus infection on all 25 begomoviral positive samples. Sequences obtained from different isolates from same place were showing 100% similarity with each other, so we have excluded the identical samples and proceed with samples showing diversity with each other for further studies. 16 viral isolates were selected for sequence analysis of complete DNA-A genome of begomoviruses associated with collected samples. PCR products (~2.7 kb) amplified through abutting primers (PSBP-F & PSBP-R) designed during this study (figure 4.12) were extracted from the gel and further DNA was eluted

and purified using kit (invitrogen quick gel extraction kit). In order to do further cloning pGEM-T easy vector system from Promega, USA was used and amplified fragments were ligated to the vector. Ligated mixture was transformed into DH5α strain of *E. coli* and transformants were checked through blue and white selection. Positive colonies were confirmed for inserts through colony PCR and selected colonies were cultured in Luria broth (LB) overnight. Cloned plasmids were isolated from bacterial culture and sent for sequencing. Sequences were generated from overlapping regions and multiple sequences obtained were assembled and analyzed.

4.10 Sequence analysis of complete DNA-A component of begomoviruses

4.10.1 BLAST analysis of complete DNA-A sequences of begomoviruses

After sequencing of full length DNA-A component of begomoviral isolates were analyzed through BLAST online tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis of complete DNA-A component of begomoviral isolates collected from different locations of Lucknow was done. Maximum identity of 87% was observed in case of PSB 14 (BBAU) isolate with papaya leaf curl virus (PaLCuV) isolate reported from Pakistan, while PSB 8 (Aliganj) revealed 90% sequence identity with pepper leaf curl virus (PepLCV) (Bangladesh) infecting papaya in Lucknow, India. Whereas, PSB 43 isolate of Gomti Nagar was documented as an isolate of tomato leaf curl Gujarat virus (ToLCuGuV) with 91% sequence identity. During the study of DNA-A component of isolates collected from other districts of Uttar Pradesh e.g. isolates PSB 21 (Shahjahanpur), PSB 42 (Mahoba) and PSB 45 (Faizabad) were supposed to be infected with different isolates of chilli leaf curl virus (ChiLCV) showing 93- 94 % identity and interestingly, the isolate PSB 47 (Lalitpur) revealed 94 % identity with papaya leaf crumple virus (PaLCrV) isolate reported from Lalitpur itself. Isolates from Jabalpur and Bhopal districts of Madhya Pradesh (PSB 32, PSB 66) were showing 95% identity with the isolates of PaLCrV whereas, isolates PSB 33, PSB 34 from Jamnagar and PSB 37 from Ahmedabad (Gujarat) were showing highest identity (92- 93%) with tomato leaf curl virus (ToLCV), PaLCuV and pedilanthus leaf curl virus (PedLCuV) respectively. Isolate of Ranchi (PSB 38) was found 94% identical with croton yellow vein mosaic virus (CroYVMV) while Hyderabad viral isolate (PSB 51) showed maximum 95%

identity with papaya yellow leaf curl virus (PaYLCV). Interestingly, isolate PSB 60 from New Delhi showed 97% identity with previously reported PaLCrV isolate of New Delhi and isolate PSB 63 from New Delhi was showing 93% identity with duranta leaf curl virus (DuLCV) isolate reported in Pakistan. BLAST percent identity of all isolates of this study is listed in table 4.3.

Table 4.3: BLAST percent identity of DNA-A component of begomovirus isolates of this study with other begomovirus isolates available in database

Isolate name	Location	Highest identity with isolate (Accession no.)	Percent (%) Identity
PSB 8	Lucknow	Pepper leaf curl Bangladesh virus segment A (AF314531.1)	90%
PSB 14	Lucknow	Papaya leaf curl virus (FM955601.1)	87%
PSB 21	Shahjahanpur	Chilli leaf curl India virus segment A, complete genome (FM877858.1)	93%
PSB 32	Jabalpur	Papaya leaf crumple virus isolate Mohali (KR052159.1)	96%
PSB 33	Jamnagar	Tomato leaf curl virus (FJ514798.1)	93%
PSB 34	Jamnagar	Papaya leaf curl virus isolate India-Jamnagar1-Cluster bean-2015 (KT253644.1)	92%
PSB 37	Ahmedabad	Pedilanthus leaf curl virus (KX168427.1)	93%
PSB 38	Ranchi	Croton yellow vein mosaic virus segment A (FN645898.1)	94%
PSB 42	Mahoba	Chilli leaf curl India virus segment A, complete genome (FM877858.1)	94%
PSB 43	Lucknow	Tomato leaf curl Gujarat virus isolate TC51 segment DNA-A (KP164863.1)	91%
PSB 45	Faizabad	Chilli leaf curl India virus isolate 58SA segment DNA-A (KT948070.1)	94%
PSB 47	Lalitpur	Papaya leaf crumple virus isolate MJS2 (KR071789.1)	94%
PSB 51	Hyderabad	Papaya yellow leaf curl virus isolate DP2 (KX353622.2)	92%
PSB 60	New Delhi	Papaya leaf crumple virus-Nirulas (HM140368.1)	97%
PSB 63	New Delhi	Duranta leaf curl virus isolate 57SA segment DNA-A (KT948069.1)	93%
PSB 66	Bhopal	Papaya leaf crumple virus isolate Kolkata (KX302711.1)	95%

These results indicate that the infection of multiple begomovirus species was observed on papaya plant in different geographic locations of India or papaya is also a host of these viruses or these are papaya viruses which also go on tomato, chilli and identified from those hosts and named accordingly.

Majority of samples are found to be infected with the isolates of papaya leaf curl virus (PaLCuV), papaya leaf crumple virus (PaLCrV), chilli leaf curl virus (ChiLCV) and tomato leaf curl virus (ToLCV). In addition to above discussed isolates, some isolates showed identity with begomovirus species reported on different weeds like croton, pedilanthus and duranta. Based on begomovirus taxonomy report of Brown *et al*., 2015, species demarcation criteria for begomovirus classification is ≥ 91% pairwise sequence identity with already reported begomovirus species. Interestingly, two begomoviral isolates (PSB 8 & PSB 14) of Lucknow origin showed 87- 90% sequence identity and supposed to be a new species of begomovirus infecting papaya. International committee on taxonomy of viruses (ICTV) has recommended the study of sequences by pairwise comparison to classify them as a new species so; sequences were further studied through sequence demarcation tool (SDT) for the proper classification of begomovirus species identified on papaya during this study.

4.10.2 Open reading frames (ORFs) analysis of begomoviral isolates

DNA-A sequences of begomovirus isolates of this study were 2736- 2764 base pair long. Arrangement of genes within the sequences obtained after sequencing was investigated using ORF Finder (NCBI; http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Presence of potential open reading frames (ORFs) having an arrangement typically like monopartite (DNA-A component) begomoviruses originated from old world were analyzed in all 16 begomoviral isolates, and these results confirmed the presence of particular begomovirus species with leaf curl disease of papaya. DNA- A component of begomoviruses have mainly six ORFs namely AV2, AV1, AC1, AC2, AC3, AC4 within the viral genome. Two ORFs are found in sense orientation (AV2, AV1) while four ORFs are found in antisense orientation (AC1, AC2, AC3, AC4). ORF locations of all predicted genes within all 16 begomoviral isolates identified in present study are listed in table 4.4. Presence of all six ORFs in DNA-A component including AV2 ORF (encodes pre-coat

protein) determines that all the viral isolates have typical genome organization of begomoviruses and belong to old world as suggested by Stanley *et al*., 2005.

4.10.3 Pairwise genetic identity calculations by sequence demarcation tool (SDT)

Pairwise sequence identity is the accurate and consistent tool for classification of viruses. ICTV has recommended the pairwise sequence identity criteria to determine the begomovirus classification. To fulfill the criteria for characterization of begomoviral species/ strain or novel begomoviruses, sequence demarcation tool (SDT) was used. According to the ICTV taxonomy report, species demarcation threshold for begomovirus species is ≥91% and use of SDT has now become necessary for species classification.

To study the taxonomic status and species classification of begomovirus isolates identified in present study along with some begomoviruses available in database were examined through SDT. The pairwise identity scores of isolates obtained through SDT v1.2 (Muhire *et al*., 2014) were displayed in a colour-coded matrix (figure 4.13) that showed the overall relationship between sequences. Plot of percentage identity values for pairwise comparisons are shown in figure 4.14, which indicate the threshold for the classification of begomoviruses.

Colour-coded matrix obtained from sequence input in SDT, isolates PSB 32, PSB 47, PSB 60, PSB 66 were showing highest pairwise identity with different isolates of PaLCrV whereas, isolates PSB 8, PSB 21, PSB 42, PSB 45 were showing identity with isolates of PepLCV and ChiLCV. Isolate PSB 33 and PSB 43 were found closely related with ToLCV and ToLCuGuV isolates respectively. Isolate PSB 14, PSB 34, PSB 51 and showed highest identity with different papaya leaf curl virus. Isolates (PSB 37, PSB 38 and PSB 63) showed identity with leaf curl causing begomoviruses reported on weeds like pedilanthus, croton and duranta respectively.

Table 4.4: Open reading frames (ORFs) within the DNA-A genome of begomovirus isolates identified in this study

Isolate no. (Accession no.)	AV2 ORF	AV1 ORF coat protein (CP)	AC2 ORF transcription activator protein (TrAP)	AC3 ORF replication enhancer protein (REn)	AC4 ORF	AC5 ORF	AC1 ORF replication associated protein (Rep)	AC6 ORF	Complete length (bp)
PSB 8 (MH988457)	146-502	306-1076	1218- 1622	1073- 1477	2196- 2453	284- 574	1525- 2610	531- 821	2754
PSB 14 (MH98845)	58-504	308-1078	1220-1624	1075-1479	2198-2584	338-973	1527-2612	--	2754
PSB 21 (MH765693)	145- 510	305-1075	1217- 1624	1072-1476	2198-2455	283 – 573	1554 - 2612	--	2755
PSB 32 (MH674437)	121-459	281-1051	1193 - 1600	1048-1452	2129- 2431	259 – 549	1524 - 2588	--	2738
PSB 33 (MH765694)	144-509	304-1074	1216-1620	1071-1475	2158-2451	282-560	1523- 2608	529-969	2760
PSB 34 (MH807205)	147-503	307-1077	1225-1629	1080-1484	2203- 2586	298-615	1532-2668	--	2757
PSB 37 (MH765695)	97-501	305-1075	1217-1621	1072- 1476	2150-2452	--	1524- 2696	--	2764
PSB 38 (MH765696)	145-501	305-1075	1223-1627	1078-1482	2202- 2459	Ac5a 740-1144 AC5b 296-613	1735- 2616	--	2760
PSB 42 (MH765697)	147-512	307-1077	1219-1626	1074-1589	2200-2457	480-575	1556-2614	--	2758
PSB 43 (MG757245)	144-491	304-1074	1216-1620	1071-1475	2158-2451	295-969	1523 - 2608	--	2760
PSB 45 (MH765698)	145-510	305-1075	1217-1624	1072-1476	2198-2455	283-561	1554-2612	--	2754
PSB 47 (MH807200)	120-458	280-1050	1192-1599	1047-1451	2128-2469	252-548	1529- 2593	--	2736
PSB 51 (MH807204)	145- 501	305-1075	1217- 1621	1072- 1476	2159- 2452	530- 820	1524- 2606	--	2760
PSB 60 (MH807201)	120-485	280-1050	1192-1599	1047-1451	2128-2436	258- 548	1523- 2587	--	2736
PSB 63 (MH807202)	147- 512	307- 1077	1219- 1626	1074- 1478	2173- 2457	285- 452	1550- 2614	--	2760
PSB 66 (MH807203)	120-458	280 -1050	1192 - 1599	1047 -1451	2128- 2430	258 – 425	1523 – 2587	--	2736

BLAST identity scores cannot be translated into genome-wide pairwise identity scores. So, the pairwise identity scores are the best way to classify any viral genome and also for describing relatedness of different viral isolates. Different begomoviruses available in database were used for the comparison/ classification of isolates identified in this study. This tool gives the accurate relatedness and taxonomic place of all viral isolates identified in this study with the begomoviral species or strain of already existing in database. After comparison of DNA-A sequences of all begomovius isolates through SDT were submitted in GenBank database for getting accessions against all identified begomoviruses during this study. Details of isolates and accession no. assigned to begomoviruses identified in present study are listed in table 4.5.

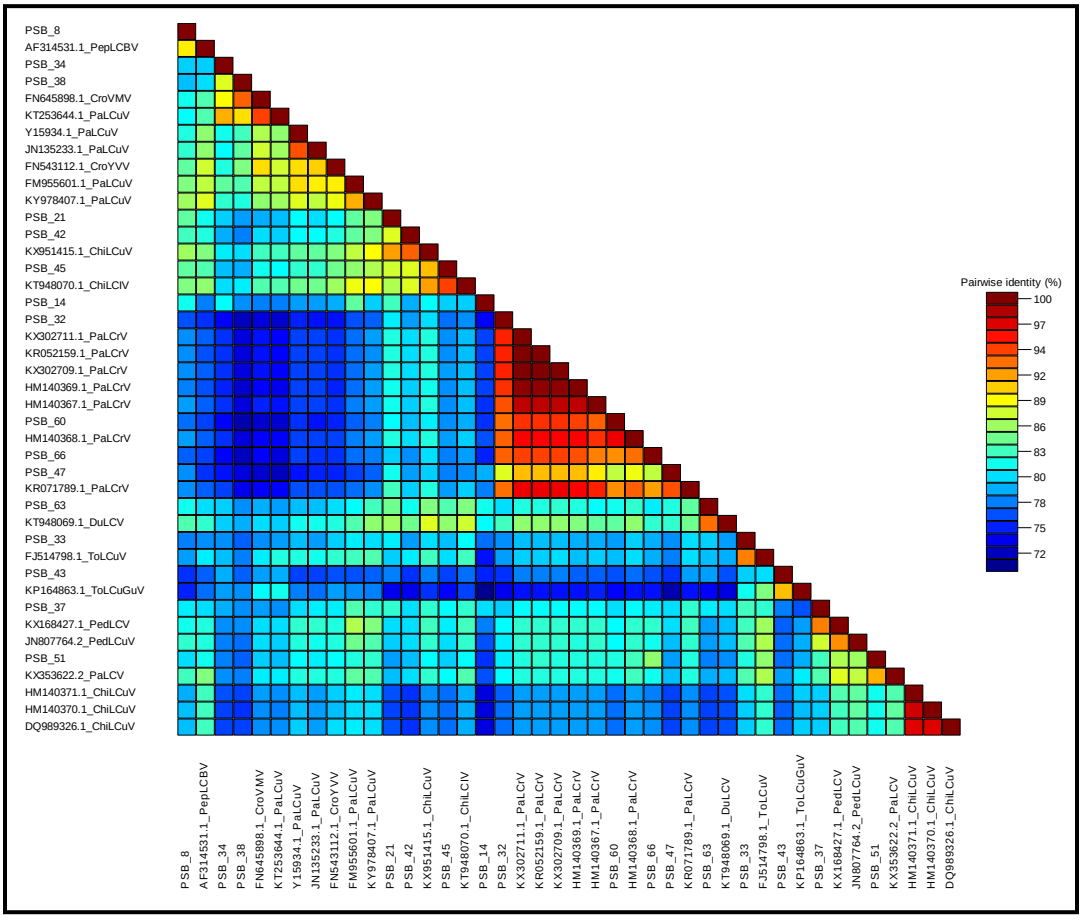


Figure 4.13: Colour-coded matrix of complete DNA-A component of 42 begomovirus isolates generated through SDT v1.2. Every coloured cell indicates the pairwise percent identity of two sequences. Colour bar represents the association between colours showed in matrix and pairwise percentage identity.

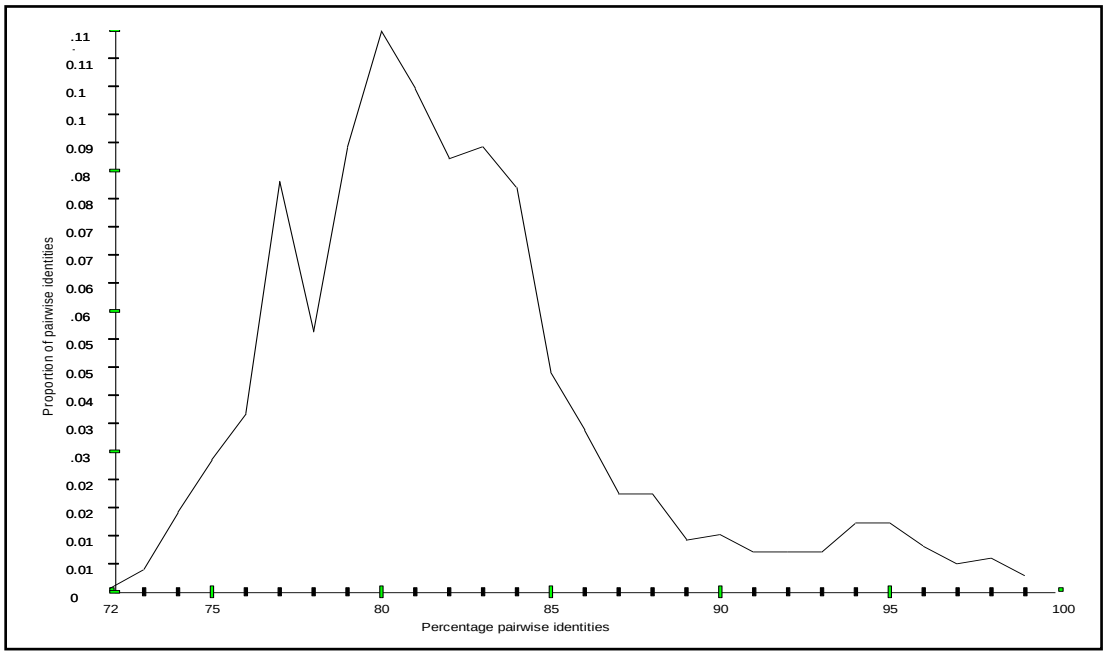


Figure 4.14: Pairwise identity frequency distribution plot of DNA-A component of begomoviruses. The horizontal axis and vertical axis indicates percentage pairwise identities and proportions of these identities within the distribution respectively. Peaks in the graph show maximum threshold values of pairwise sequence identity and troughs represent thresholds for least ambiguous classifications.

Table 4.5: List of accession numbers of DNA-A component of begomoviruses isolated in present study

Isolate	Location	Begomovirus species	Accession no.	Size (bp)
PSB 8	Lucknow	Papaya severe leaf curl virus	MH988457	2754
PSB 14	Lucknow	Papaya severe leaf curl virus	MH988458	2754
PSB 21	Shahjahanpur	Chilli leaf curl virus	MH765693	2755
PSB 32	Jabalpur	Papaya leaf crumple virus	MH674437	2738
PSB 33	Jamnagar	Tomato leaf curl virus	MH765694	2760
PSB 34	Jamnagar	Papaya leaf curl virus	MH807205	2757
PSB 37	Ahmedabad	Pedilanthus leaf curl virus	MH765695	2764
PSB 38	Ranchi	Croton yellow vein mosaic virus	MH765696	2760
PSB 42	Mahoba	Chilli leaf curl virus	MH765697	2758
PSB 43	Lucknow	Tomato leaf curl Gujarat virus	MG757245	2760
PSB 45	Faizabad	Chilli leaf curl virus	MH765698	2754
PSB 47	Lalitpur	Papaya leaf crumple virus	MH807200	2736
PSB 51	Hyderabad	Papaya yellow leaf curl virus	MH807204	2760
PSB 60	New Delhi	Papaya leaf crumple virus	MH807201	2736
PSB 63	New Delhi	Duranta leaf curl virus	MH807202	2760
PSB 66	Bhopal	Papaya leaf crumple virus	MH807203	2736

4.10.4 Phylogenetic relevance of DNA-A component of begomoviral isolates

Analysis of begomoviral sequences of this study through BLAST and SDT provides the evidence of being begomovirus infection on papaya in different regions of India. Distribution of begomoviruses on papaya was assessed through molecular variability and phylogenetic studies. Infection of several begomovirus species on papaya plants is evident from sequencing results and these begomovirus species provides an evidence for their variable host range. Evolutionary background of viral isolates identified in present study and their phylogenetic relevance was determined through mega v6.0. Phylogenetic analysis based on maximum-likelihood tree generated after 1000 bootstrap replications showed two major clusters (cluster-I and Cluster-II) with separate outgroup used in the study (figure 4.15). Cluster-I clearly shows that papaya leaf crumple virus isolates (PSB 32, PSB 47, PSB 60, PSB 66) grouped together with other papaya leaf crumple virus isolates. Similarly, chilli leaf curl virus isolates of this study (PSB 21, PSB 42 and PSB 45) were grouped with other chilli leaf curl viruses. Grouping of papaya leaf curl virus isolate (PSB 34) and isolate PSB 14 with papaya leaf curl virus isolates showed their close relationship with papaya leaf curl virus, while isolate PSB 38 and PSB 63 showed relationship with croton yellow mosaic virus (CroYVMV) and duranta leaf curl virus (DuLCV) respectively with clear separate groups. Cluster-II generated in tree showed the clear grouping of ToLCV, papaya yellow leaf curl virus (PaYLCV) and pedilanthus leaf curl virus (PedLCV) isolates. Clustered phylogenetic analysis of begomovirus isolates used in this study was inferred through the software clearly relate the emergence of begomovirus species/ strains with their respective and already exiting begomoviruses. On the basis of sequencing results of begomoviral isolates confined that leaf curl disease of papaya is caused by the complex of different begomoviruses. Occurrence of different begomoviruses on papaya from different geographical locations reflects their rapid adaptation to variable environmental conditions hence, the highly variable nature of begomoviruses. These begomoviral complexes on papaya also explain the property of their variable host range.

Analysis of complete DNA-A component during this study revealed the presence of isolates of nine different already existing begomovirus species and one new begomovirus species causing infection on papaya. This study identified the infection of two

begomovirus species (duranta leaf curl virus, tomato leaf curl Gujarat virus) that were neither expected to infect papaya crop nor previously been identified in papaya alongwith a novel species i.e. papaya severe leaf curl virus (PaSLCuV) from two different locations of Lucknow. These findings provide a support for papaya as a new host for these begomoviruses as well as expansion of their hosts. Our results also explain that leaf curl disease of papaya is caused by several begomoviruses and support the existence of papaya leaf curl disease complexes. Therefore, we suggest for extensive surveys of papaya growing regions, continuous monitoring of weeds and other plant reservoirs, to understand the etiology of papaya leaf curl causing begomoviruses.

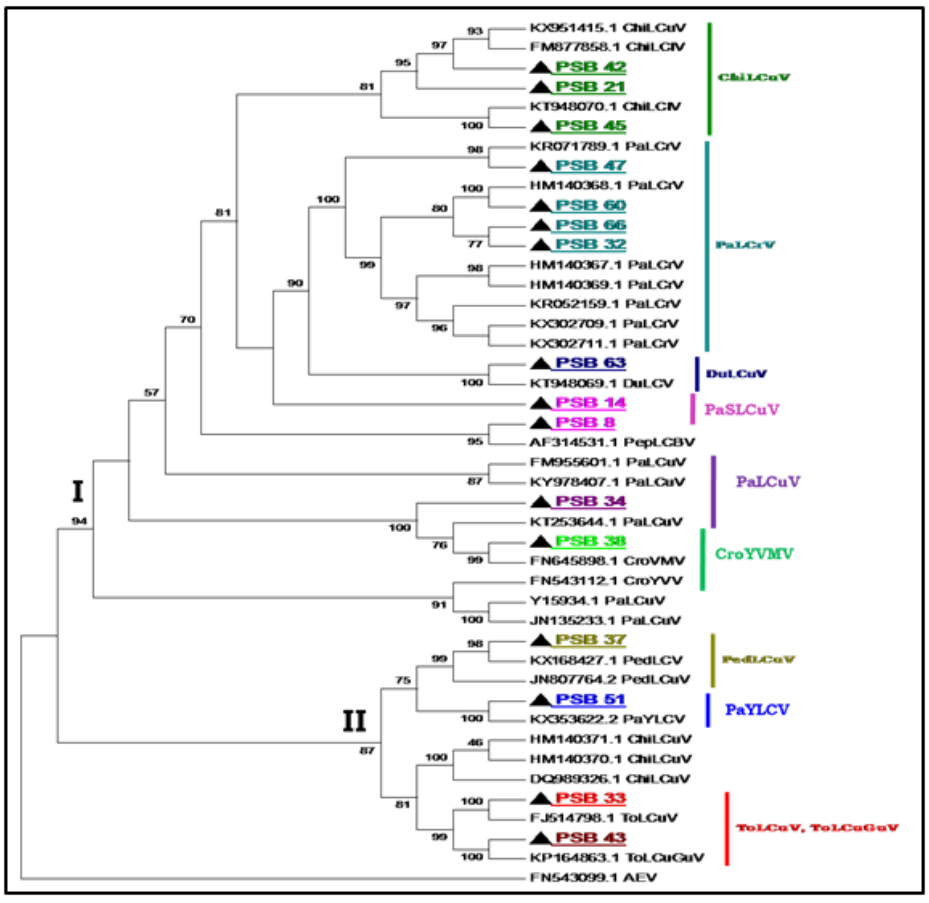


Figure 4.15: Molecular phylogenetic analysis of complete DNA-A component of begomovirus isolates by maximum likelihood method conducted in mega v6.0. Isolates identified in this study are marked with symbol (▲).

4.10.5 Detection of recombination events within DNA-A component

Despite of well defined genome organization, begomoviruses and satellite molecules both may go through several rounds of recombination and pseudo recombination in nature which results in development of new recombinants. Such changes in nucleotide sequences can lead to new viral strains with more virulence, which may break the well designed resistance strategy or result in severe symptoms. Recombination detection programme, RDP v 4.4.39 (RDP4) was used to detect likely parents and the possible recombination sites in DNA-A of begomovirus isolates of papaya. Different begomovirus species e.g. papaya leaf curl virus, papaya leaf crumple virus, chilli leaf curl virus, pepper leaf curl virus, tomato leaf curl virus, tomato leaf curl Gujarat virus were used for recombination analysis. Sequences were aligned in mega v6.0 and exported to the RDP4 programme to detect putative recombinations. RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN and 3SEQ programmes implemented in RDP4 were applied to analyze recombinants. In this study we have attempted to study the possible recombination events among viral isolates of present study with the same selected isolates used during SDT analysis. We considered the recombination events which were detected by more than three or even three methods employed in this study. Recombinants resulting from major and minor parents were considered on the basis of several parameters e.g. site and probability of recombination. Detailed information of recombinants obtained during study are listed in table 4.6 and graphically showed in figure 4.16.

Croton yellow mosaic virus (PSB 38), duranta leaf curl virus (KT948069.1) and pedilanthus leaf curl virus (KX168427.1) isolates or their unknown parents were majorly playing role as major parents while, chilli leaf curl India virus (KT948070.1), chilli leaf curl virus (KX951415.1) and papaya leaf curl virus (KY978407.1) or their unknown parents were found as minor parents in majority of recombination events that are responsible for the evolution of new virus species/ strains of this study. There was no any significant recombination was observed in papaya leaf curl virus isolate PSB 34 (Jamnagar) however, this isolate was observed as major parent of PSB 14, PSB 37 and minor parent for PSB 21 isolates of present study. Recombination break points during

recombination are showing the site of recombination during evolution. Although recombinations are observed throughout the genome but coat protein gene and replication initiator protein are found as major hot-spot for recombination events. Recombination study of all begomoviral species has shown their emergence through recombination between existing begomoviruses. Begomoviral evolution is predominantly affected through interspecies recombination which is a major reason for the emergence of new begomoviral species (Garcia-Andre´s *et al*., 2007; Varsani *et al*., 2008). A major reason behind evolution and newly emerging begomoviruses is recombination occur during their replication (Lefeuvre and Moriones, 2015). Expansion in begomoviral host range, adaptation of different environmental conditions and emergence of new begomoviruses are driven through nucleotide diversity and recombinations in begomoviral genome (Padidam *et al*., 1999). Results of this study showed the significance of diversity and recombinations in begomoviral evolution.

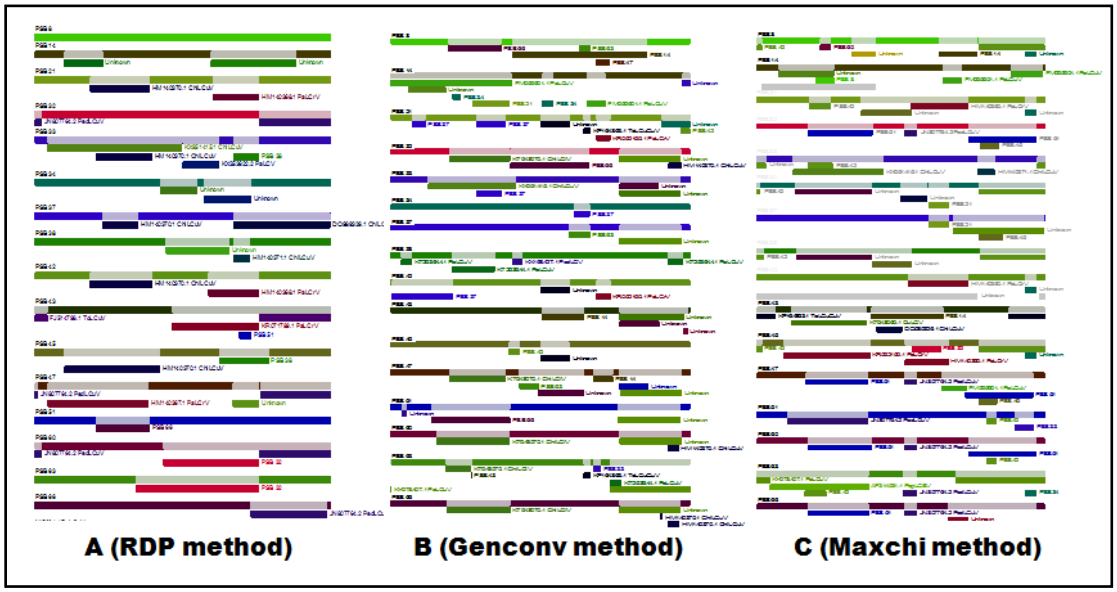


Figure 4.16: Graphical representation of recombination events among complete DNA-A components of begomoviruses detected by RDP (A), Genconv (B) and maxchi (C) methods occurred during evolution of begomovirus isolates.

Table 4.6: Recombination detection program (RDP) v 4.4.39 based detection of recombination breakpoints with seven different methods in the DNA-A genome of begomoviral isolates identified from papaya in this study

Recombinant isolate	Recombination break point	Major parent	Minor parent	Av. *p*-value (highest)	Recombination detection methods
PSB 8	508- 1008	PSB 38	PSB 66	3.479×10^{-18}	R, G, **B**, M, C, S, 3S
	1108- 2378	KT 948070.1	PSB 14	6.480×10^{-5}	**G**, S, 3S
	1896- 2024	PSB 51	PSB 47	1.630×10^{-11}	**G**, B, M, C, S, 3S
PSB 14	1641- 2456	KT 948070.1	PSB 38*	1.492×10^{-11}	**R**, M, C, S, 3S
	138- 486	PSB 37	KX 951415.1	7.072×10^{-20}	G, M, C, **S**, 3S
	733- 1083	PSB 34	PSB 21	3.872×10^{-26}	G, B, M, C, S, **3S**
	1810- 1987	JN 135233.1*	FM 955601.1	7.953×10^{-8}	G, M, C, **S**
PSB 21	158- 295	KX168427.1*	PSB 37	2.528×10^{-7}	G, M, C, S, **3S**
	771- 1056	PSB 45	PSB 37	7.021×10^{-4}	G, B, M, C, **3S**
	1373- 1653	KX 168427.1	KP 164863.1*	2.605×10^{-14}	G, M, C, S, **3S**
	1902- 2034	KT 948070.1	KR 052159.1	3.933×10^{-12}	G, M, C, S, **3S**
	2520- 2755	PSB 14	PSB 34*	7.135×10^{-6}	R, G, M, C, **S**, 3S
	481- 692	PSB 37	PSB 42	3.492×10^{-3}	**M**, S, 3S
PSB 32	495- 1064	PSB 38*	KT 948070.1	4.0799×10^{-20}	G, B, M, C, **S**, 3S
	2091- 2654	KT 948070.1	KY 978407.1*	1.3997×10^{-39}	R, **G**, M, C, S, 3S
	442- 1075	PSB 43*	PSB 51	2.696×10^{-21}	G, B, M, C, **S**, 3S
PSB 33	90- 1364	JN 807764.2	KX 951415.1	2.617×10^{-15}	**R**, G, B, M, C, S, 3S
	317- 1145	KP 164863.1	KX 951415.1	4.544×10^{-76}	R, G, M, C, S, **3S**
	770- 1019	PSB 45	PSB 37	7.021×10^{-14}	G, B, M, C, **3S**
	2011- 2674	KY 978407.1	KT 948069.1*	1.66×10^{-32}	**G**, M, C, S, 3S
	2122- 2291	KX 353622.2	HM 14037.1	4.870×10^{-6}	G, B, M, **C,** 3S
PSB 34	-	-	-	-	-
PSB 37	609- 953	PSB 34*	HM 140370.1	2.001×10^{-9}	R, **G**, B, M, C, **S**, 3S
	1857- 2757	PSB 14	DQ 989326.1	6.425×10^{-16}	R, M, C, **S**, 3S
	2112- 2684	KT 948069.1	KY 978407.1	1.538×10^{-41}	**G**, M, C, S, 3S
PSB 38	1216- 1826	KT 253644.1	FM 955601.1*	7.107×10^{-20}	R, M, **S**
	69- 159	KX 951415.1	KT 253644.1	2.939×10^{-16}	G, M, C, **S**, 3S
	537- 951	FN 543112.1	KT 253644.1	2.722×10^{-26}	G, M, C, **S**, 3S
	1120- 1212	PSB 43	KX 168427.1	1.263×10^{-10}	G, **S**, 3S
	2576- 2695	FN543112.1	KT 253644.1	4.801×10^{-8}	**G**, M, C, 3S
	347- 1094	PSB 51	PSB 66*	2.567×10^{-33}	R, G, B, M, C, **S**, 3S
PSB 42	5- 555	KX168427.1*	PSB 37	2.528×10^{-7}	G, M, C, S, **3S**
	1378- 1656	KX 168427.1	KP 164863.1	2.605×10^{-14}	G, M, C, S, **3S**
	1910- 2037	KT 948070.1	KR 052159.1	3.933×10^{-12}	G, M, C, S, **3S**
PSB 43	1391- 1781	DQ 989363.1	PSB 14	1.837×10^{-16}	R, G, **B**, M, C, S, 3S
	2111- 2705	KY 978407.1	KT 948069.1	1.668×10^{-32}	**G**, M, C, S, 3S
	301- 1049	KX 353622.2	KT 948069.1	4.371×10^{-23}	G, M, **S**, 3S
	1080- 1781	HM 14037.1	PSB 14	5.143×10^{-15}	R, G, **B**, M, C, S, 3S
PSB 45	1729- 2197	PSB 21	PSB 38	7.309×10^{-10}	R, B, M, C, **S**, 3S
	1376- 1654	KX 168427.1	KP 164863.1*	2.605×10^{-14}	G, M, C, S, **3S**
	1415- 1840	FN 645898.1	HM 140369.1	3.968×10^{-10}	G, B, M, C, **S**, 3S
PSB 47	68- 1029	PSB 60*	HM 140367.1	9.598×10^{-14}	R, **G**, C, S, 3S
	494- 1026	PSB 38*	KT 948070.1	4.079×10^{-20}	G, B, M, C, S, **3S**
	2107- 2670	KT 948069.1	KY978407.1*	1.397×10^{-39}	R, **G**, M, C, S, 3S
	425- 1032	PSB 43*	PSB 51	2.696×10^{-21}	G, B, M, C, **S**, 3S
	1977- 2634	PSB 21	PSB 51	3.590×10^{-21}	R, G, B, M, C, **S**, 3S

PSB 51	546- 1064	PSB 47*	PSB 66	6.58 x10^{-12}	R, G, B, M, C, S, **3S**
	347- 1088	PSB 38	PSB 66	3.479 x10^{-18}	R, G, **B**, M, C, S, 3S
	2101- 2680	KT 948069.1	KY 98407.1*	1.608 x10^{-39}	**G**, M, C, S, 3S
	262- 1058	AF 314531.1	JN 807764.2	2.460 x 10^{-5}	M, **C**, S, 3S
	2211- 2311	HM 140371.1	PSB 32	3.861 x 10^{-16}	M, C, **S**
PSB 60	368- 1057	PSB 38*	KT 948070.1	4.079 x 10^{-20}	G, B, M, C, **S**, 3S
	2090- 2670	KT 948069.1	KY 978407.1	1.397 x 10^{-39}	R, **G**, M, C, S, 3S
	438- 1063	PSB 43*	PSB 51	2.696 x 10^{-21}	G, B, M, C, **S**, 3S
	2009- 2658	PSB 21	PSB 51	3.590 x 10^{-21}	R, G, B, M, C, **S**, 3S
PSB 63	489- 724	PSB 38*	KT 948070.1	4.079 x 10^{-20}	G, B, M, C, **S**, 3S
	1994- 2700	KT 948069.1	JN 807764.2	3.036 x10^{-30}	**R**, G, M, C, S, 3S
	468- 1063	PSB 38*	KT 948070.1	4.079 x 10^{-20}	G, B, M, C, **S**, 3S
PSB 66	2090- 2652	KT 948069.1	KY 978407.1	1.397 x 10^{-39}	R, **G**, M, C, S, 3S
	438- 1037	PSB 43*	PSB 51	2.696 x10^{-21}	G, B, M, C, **S**, 3S
	2005- 2659	PSB 21	PSB 51	3.590 x10^{-21}	R, G, B, M, C, **S**, 3S

*unknown parent detected during recombination analysis but closely related to the inferred betasatellite molecules in this analysis. R=RDP, G=GENCONV, B=BOOTSCAN, M=MAXCHI, C=CHIMAERA, S=SISCAN, 3S=3SEQ; Highlighted recombination method denotes the maximum av. *p*-value.
Highest *p*-value among all possible detection method is given.

Emergence of begomoviruses on crop supports the fact of their prior existence on weeds and wild relative of crop plants in that area and goes through nucleotide change to adapt new plant hosts (Nawaz-ul-Rehman and Fauquet, 2009). These new begomovirus species and their strains have appeared through recombination and assortment during evolution (Mubin *et al.*, 2010; Nawaz-ul-Rehman *et al*., 2012). Thus, recombination acts as an ultimate strength for diversification of begomoviruses. Recombination events detected during present study show inter/ intra species recombinations and their relationship. So, the strategy to develop generic resistance against begomoviruses will be a sustainable approach against all papaya infecting begomoviruses.

4.11 Sequence analysis of associated betasatellite molecules

4.11.1 BLAST analysis of betasatellite sequences

Betasatellite molecules belong to highly diverse group of ssDNA molecule with ~1.3 kb genome and known to induce symptom severity on diseased plants. Betasatellites are frequently found to be associated with monopartite begomoviruses. All begomovirus positive isolates (25 in no.) were screened for the presence of betasatellites and among them ten samples were found positive for betasatellite association based on PCR amplification results using betasatellite primers. PCR amplified products of all ten betasatellite molecules were sequenced and studied to know the possible association of betasatellites with leaf curl disease of papaya. Sequences obtained from forward and

reverse orientation of amplified fragments were combined and compared through BLAST online tool for the confirmation of betasatellite molecules. Sequencing results obtained during this study revealed 89% - 99% sequence identity with different betasatellite sequences already reported in NCBI database (Table 4.7).

Table 4.7: BLAST percent identity of betasatellite molecules obtained in present study with betasatellite isolates available in NCBI database

Isolate	Location	Highest identity with isolate	Percent (%) Identity
PSBB 14	Lucknow (U.P.)	Croton yellow vein mosaic betasatellite isolate Lucknow (EU604296.2)	96%
PSBB 21	Shahjahanpur (U.P.)	Papaya leaf curl beta - [India:Chinthapalli:2005] (DQ118862.1)	98%
PSBB 34	Jam nagar (Guarat)	Luffa leaf distortion betasatellite (JX315326.1)	94%
PSBB 38	Ranchi (Jharkhand)	Croton yellow vein mosaic betasatellite isolate WOK47 (KT390489.1)	95%
PSBB 43	Lucknow (U.P.)	Tomato leaf curl Bangladesh betasatellite isolate India/Bijnour/chilli (KF188707.1)	89%
PSBB 47	Lalitpur (U.P.)	Cotton leaf curl virus betasatellite isolate Lucknow (GU440581.1)	92%
PSBB 51	Hyderabad (Telangana)	Tobacco leaf curl Patna betasatellite isolate Pusa-Bihar (HQ180394.1)	97%
PSBB 60	New Delhi	Chilli leaf curl virus satellite DNA beta C1 gene (AM279663.1)	94%
PSBB 63	New Delhi	Tomato leaf curl betasatellite-Naj 2 (HM143911.1)	93%
PSBB 66	Bhopal (M. P.)	Tomato leaf curl betasatellite isolate ToLCB-[IN:Bih6:10], complete sequence (GU732206.1)	99%

4.11.2 Pairwise sequence identity calculations of betasatellite molecules using sequence demarcation tool (SDT)

Pairwise identity calculations of betasatellite molecules identified in present study with some selected betasatellite molecules available in NCBI database were studied through SDT v1.2. The colour-coded matrix was obtained as a result of pairwise comparisons that showed the separate groups of closely related betasatellites in different colours. Isolates with highest similarity occupied the place next to the similar betasatellite isolate (figure

4.17) and order of sequences in matrix reflects their evolutionary background. Frequency distribution plot generated through pairwise identity calculations illustrated the threshold cut-off values of analysis (figure 4.18).

Matrix showed the close relationship of betasatellite isolates PSBB 63 & PSBB 66 with tomato leaf curl betasatellite (ToLCB) isolates Betasatellite isolates PSBB 60 and PSBB 21 had highest pairwise percent identities with chilli leaf curl betasatellite (ChiLCB) and papaya leaf curl betasatellite (PaLCuB) molecule respectively. Isolate PSBB 14, PSBB38 showed close relationship with croton yellow vein mosaic betasatellite (CroYVMB). Betasatellite molecules PSBB 34, PSBB 43, PSBB 47 and PSBB 51 were showing highest pairwise percentage identity with luffa leaf distortion betasatellite (LuLDB), tomato leaf curl Bangladesh betasatellite (ToLCBB), cotton leaf curl betasatellite (CLCuB) and tobacco leaf curl betasatellite (TbLCB) respectively. Interestingly, these results are the novel identifications of betasatellite associations with leaf curl disease of papaya. Although betasatellite sequences of PSBB47 and PSBB63 isolates belong to different places but they share highest pairwise identity among all betasatellite molecules identified on papaya during this study. Betasatellite molecules identified in association with begomoviruses infecting papaya during this study showed 89-99% sequence identity with already reported betasatellites. Sequence information of all the betasatellites have the typical characteristics of betasatellite DNA like a single gene in complementary sense strand i.e. βC1, an adenine rich region (A-rich region) and a sequence conserved region (SCR) (Briddon *et al*., 2003). The complete nucleotide sequences of 10 betasatellite molecules identified in this study were submitted in GenBank nucleotide sequence database. Details of coding region and accession numbers of all betasatellite sequences identified in present study are listed in table 4.8.

BLAST and SDT analysis of betasatellite sequences obtained during present study revealed their percent sequence identity and pairwise identity respectively, which supported the classification criteria of betasatellite molecules. As par betasatellite classification 78% sequence identity is the threshold in order to be classified as new betasatellite molecule (Briddon *et al*., 2008). Thus, following species demarcation criteria for betasatellite molecules, eight different betasatellite molecules were identified from papaya in different locations while, no any novel betasatellite was identified during this

study. Results of this study showed the infection of papaya leaf curl betasatellite (PSBB 21), cotton leaf curl betasatellite (PSBB 47), chilli leaf curl betasatellite (PSBB 60), tomato leaf curl betasatellite (PSBB 63, PSBB 66), tobacco leaf curl betasatellite (PSBB 51), tomato leaf curl Bangladesh betasatellite (PSBB 43), croton yellow vein mosaic betasatellite (PSBB 14, PSBB 38) and luffa leaf distortion betasatellite (PSBB 34) molecules in association with different begomovirus species infecting papaya in India.

Table 4.8: Details of open reading frames (ORFs) within betasatellite components identified in present study and their accession numbers as deposited in GenBank

Betasatellite isolate no.	Location	Betasatellite name	Accession no.	BetaC1 (βC1) ORF	Size (bp)
PSBB 14	Lucknow	Croton yellow vein mosaic betasatellite (CroYMB)	MH825683	220- 576	1348
PSBB 21	Shahjahanpur	Papaya leaf curl betasatellite (PaLCuB)	MH825684	189- 557	1368
PSBB 34	Jamnagar	Luffa leaf distortion betasatellite (LuLDB)	MH825685	205- 654	1354
PSBB 38	Ranchi	Croton yellow vein mosaic betasatellite (CroYMB)	MH825686	223- 579	1368
PSBB 43	Lucknow	Tomato leaf curl Bangladesh betasatellite (ToLCBB)	MG478451	200- 556	1372
PSBB 47	Lalitpur	Cotton leaf curl betasatellite (CoLCB)	MH825687	201- 563	1366
PSBB 51	Hyderabad	Tobacco leaf curl betasatellite (TbLCB)	MH825688	193- 549	1341
PSBB 60	New Delhi	Chilli leaf curl betasatellite (ChiLCB)	MH825689	201- 563	1369
PSBB 63	New Delhi	Tomato leaf curl betasatellite (ToLCB)	MH825690	202- 564	1372
PSBB 66	Bhopal	Tomato leaf curl betasatellite (ToLCB)	MH825691	191- 571	1355

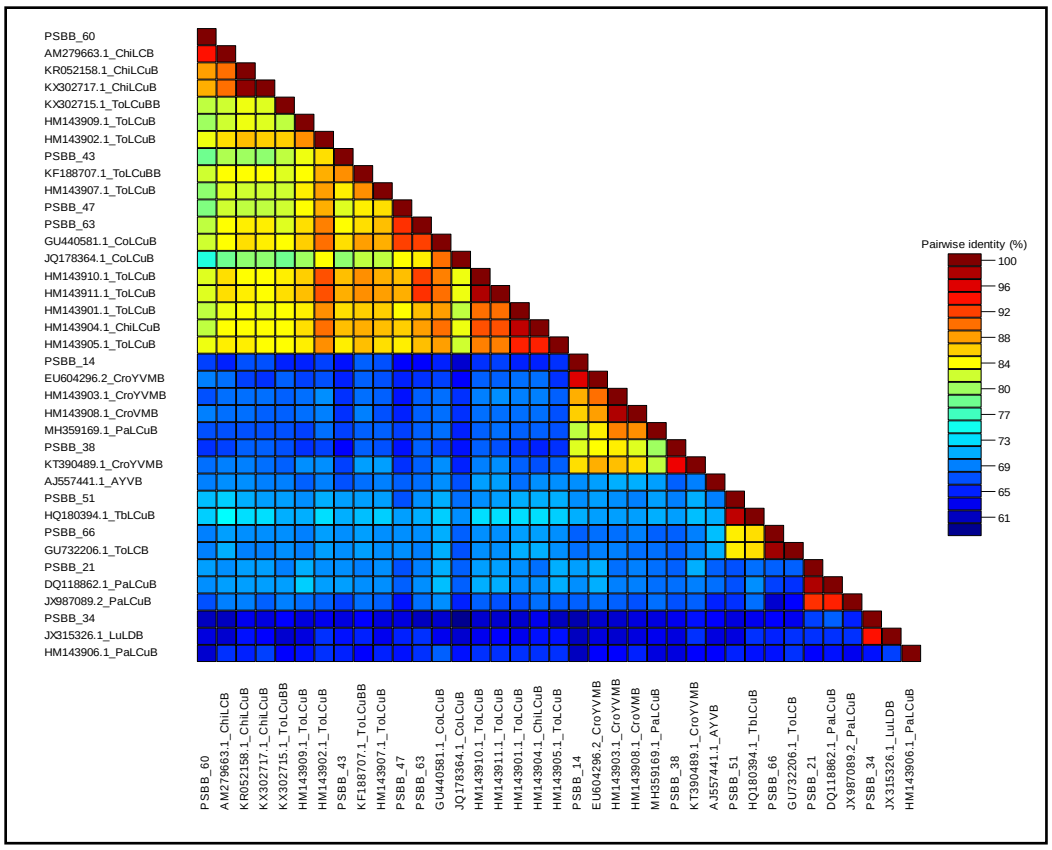


Figure 4.17: Colour-coded matrix representing the pairwise identities among betasatellite sequences generated through sequence demarcation tool (SDT) analysis. Every coloured cell indicates the pairwise percent identity of two sequences. Colour bar represents the association between colours showed in matrix and pairwise percentage identity.

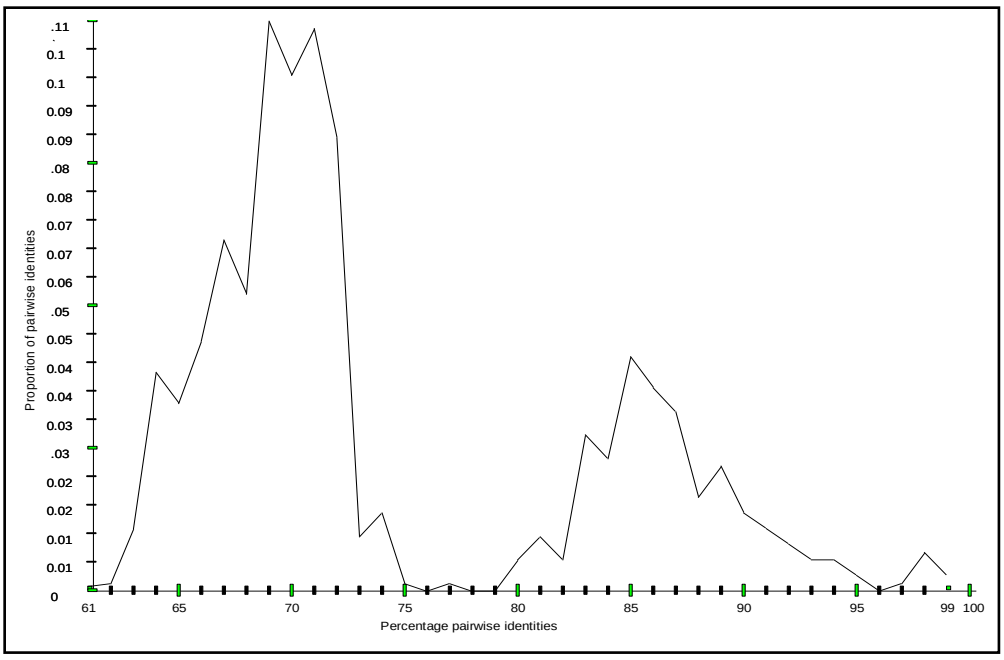


Figure 4.18: Pairwise identity frequency distribution plot of betasatellite sequence analysis through sequence demarcation tool (SDT). The horizontal axis and vertical axis indicates percentage pairwise identities and proportions of these identities within the distribution respectively. Peaks in the graph show threshold values of pairwise sequence identity and troughs represent thresholds for least ambiguous classifications.

4.11.3 Co-occurrence of begomovirus and betasatellite molecules on papaya

Analysis of DNA-A component of begomoviral isolates and betasatellite sequences obtained during this study illustrated the occurrence of different begomovirus-betasatellite complex. Papaya severe leaf curl virus isolate of Lucknow (PSB 14), a new begomovirus species identified during this work showed the association with croton yellow vein betasatellite (PSBB 14) while, tomato leaf curl Gujarat virus isolate (PSB 43) was in association with tomato leaf curl Bangladesh betasatellite (PSBB 43). Presence of tomato leaf curl betasatellites PSBB 63 and PSBB 66 were observed with duranta leaf curl virus (PSB 60) and papaya leaf crumple virus (PSB 66) isolates reported from New Delhi and Bhopal respectively. Similarly, papaya leaf crumple virus isolate (PSB 60) was found to be associated with chilli leaf curl virus betasatellite (PSBB 60). It is interesting to note that croton yellow vein mosaic virus isolate (PSB 38) identified from Ranchi was found to be associated with croton yellow vein mosaic betasatellite that indicated the gene transfer from weeds present around the crops as their original host. Whereas,

betasatellite isolate of New Delhi (PSBB 63) was found similar to satellite molecule already reported on papaya in New Delhi i.e. tomato leaf curl betasatellite. Papaya yellow leaf curl virus isolate (PSB 51) from Hyderabad origin was co-existing with that of tobacco leaf curl betasatellite (PSBB 51) whereas; isolate PSB 47 (papaya leaf crumple virus) identified from Lalitpur showed infection with cotton leaf curl betasatellite (PSBB 47). Papaya leaf curl virus isolate (PSB 34) identified in this study from Jamnagar showed infection with luffa leaf distortion betasatellite (PSBB 34) while chilli leaf curl virus isolate of Shahjahanpur (PSB 21) showed association of papaya leaf curl betasatellite molecule (PSBB 21). Diversity study of betasatellite molecules associated with monopartite begomoviruses showed their close relationship with their host and geographic origin (Saunders *et al*., 2000; Briddon *et al*., 2001, 2003) and can coexist with distinct begomoviruses naturally (Zhang *et al*., 2016; Tahir *et al*., 2017). Mansoor *et al*., 2003b worked on begomoviruses and betasatellites complex associated with cotton leaf curl disease and suggested that a single class of betasatellites has the capability to be recruited by diverse begomoviruses. Likewise, Senanayake *et al*., 2013 demonstrated that various begomovirus species affect the production of chilli in Indian subcontinent and reported a new begomovirus and betasatellite complex in chilli in Sri Lanka. Recently, Shakir *et al*., 2018 identified a new complex of begomovirus and betasatellite causing leaf curl disease of Petunia. Thus, it can be concluded that betasatellites can easily co-exist with several different begomovirus species and can adapt new hosts efficiently. Our analysis has also identified the occurrence of various begomoviuses and betasatellites on papaya plants and documented their co-existence; these results uncovered the association and co-existing nature of betasatellites with diverse begomovirus species.

4.11.4 Recombination analysis among betasatellite molecules

To know the possible recombination events occurred during evolution, betasatellite molecules of this study alongwith some other betasatellite sequences were studied using RDP v4.4.39 (RDP4). All the parameters used for analysis were same as used for DNA-A component analysis. Details of recombination break points detected within the recombinant betasatellite molecules obtained during present study with their major and minor parents are listed in table 4.9 and the graphical pattern of recombination is displayed in figure 4.19.

Among all the putative recombination events, it was observed that majority of recombination break points were detected between 900-1100bp region of betasatellites i.e. A-rich region and isolate KX302715.1 (tomato leaf curl Bangladesh betasatellite) was recognized as major parent in most of the recombination events during analysis. Only two isolates i.e. tomato leaf curl Bangladesh betasatellite (PSBB43) and chilli leaf curl betasatellite (PSBB 60) showed recombination within its coding region whereas, remaining recombination events were detected mainly in their A- rich region.

A-rich region is a highly variable segment of betasatellite genome and required for trans-replication of betasatellite DNA by helper begomovirus (Shahid *et al*., 2007). Therefore, this region is very prone to mutation/ recombination during process (Amin *et al*., 2006; Akhtar *et al*., 2014). Betasatellite molecules can replicate by using ori sequence of diverse begomoviruses and can vary their sequences through recombination for their existence. Hence, betasatellite molecules can easily exist with numerous begomoviruses and cause diversity. Recombination study of different betasatellites molecules from various locations showed that recombination is one of the major driving forces during evolution of betasatellites and originitated from their closely related betasatellites.

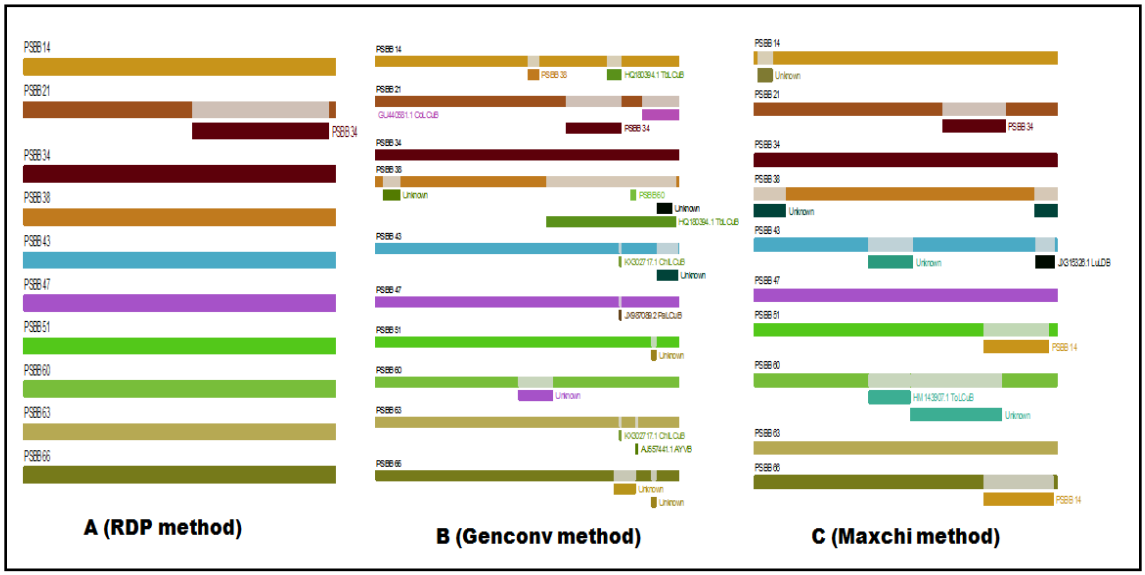


Figure 4.19: Graphical representation of recombination analysis of betasatellite molecules identified in present study. A= RDP method, B=GENCONV method, C=MAXCHI method.

Table 4.9: Details of recombination events detected through RDP v4.4.39 and probable major and minor parents of betasatellite isolates identified in present study

Recombinant	Recombination break point	Major parent	Minor parent	Av. *p*-value	Recombination detection methods
	1050- 1109	JQ178364.1*	PSBB 63	$3.680x10^{-4}$	**G**, M,C, S
PSBB 14	828-1334	JX9870889.2	HQ180394.1	$4.470x10^{-12}$	B,M,C,**S**
	745-782	HM143908.1	PSBB 38	$6.014x10^{-6}$	G, **B**, S, 3S
	992- 1070	KX302715.1	HQ180394.1	$3.945x10^{-9}$	G,B,M,**S**
	1131-1322	JX9870889.2	PSBB66	$4.000x10^{-4}$	G,**B**,M,S
	990- 1152	KX302715.1	HM143906.1	$6.854x10^{-4}$	B, M, C, **S**, 3S
PSBB 21	767-1335	PSBB 51	PSBB 34	$2.888x10^{-4}$	R,M,**C**,S
	904- 1092	KX302715.1	PSBB 34	$9.376x10^{-6}$	G, B, M,**C**
	823-1123	KX302715.1	PSBB 34	$1.507x10^{-9}$	G,B,M,C,**S**
	876- 1112	KX302715.1	PSBB 34	$7.769x\ 10^{-16}$	M,C,**S**
PSBB 34	183- 493	HM143904.1	JX987089.2	$5.333x10^{-15}$	M,C,**S**
PSBB 38	1057- 1159	JQ178364.1*	PSBB63	$3.680x10^{-4}$	**G**, M, C, S
	803-1350	KX302715.1	HQ180394.1	$3.945x10^{-9}$	G,B,M,**S**
PSBB 43	559- 686	PSBB 47	KX302717.1*	$8.667x10^{-5}$	G, **M**, C, S, 3S
	454-751	HM143911.1	KX302715.1*	$6.713x10^{-9}$	B,M,C,**S**
	1083-1094	JX9870889.2	KX302717.1	$1.007x10^{-7}$	G,M,**S**
	558-757	HM143907.1	KX302715.1*	4.886x10-6	G,M,**C**,3S
PSBB 47	678- 791	KF188707.1	HM143909.1	$3.612x10^{-7}$	G,B,M,C,S, **3S**
	1082-1093	KX302717.1	JX9870889.2	$1.007x10^{-7}$	G,M,**S**
	397-963	PSBB 63	GU440581.1	$4.035x10^{-5}$	**M**,C, S, 3S
	731-1098	KX302715.1*	AM279663.1	$1.039x10^{-8}$	G,B,M,**C**
	1070- 1106	PSBB 21	HM143904.1	$8.855x10^{-3}$	G, **M**, S
PSBB 51	819-1322	KX302715.1	KT390489.1	$1.987x10^{-8}$	B, M, C, **S**
	977-1303	HM143909.1	PSBB14	$4.530x10^{-3}$	B,M,**C**
	1043- 1102	KX302715.1	DQ118862.1	$6.861x10^{-3}$	R, G, **M**, C
PSBB 60	486- 722	HM143907.1*	HM143902.1	$3.924x10^{-5}$	G, M, **C**, S
	699-856	KX302715.1	PSBB 47*	$2.610x10^{-4}$	**G**,B, 3S
	750-1106	PSBB 47	HM143907*	$5.5882x10^{-10}$	M,C, **S**
	707-1074	JQ178364.1	HM143902.1*	$2.739x10^{-10}$	G, M, C, **S**, 3S
	694- 1079	KX302715.1	JQ178364.1*	$8.446x10^{-5}$	G, M, C, **S**, 3S
PSBB 63	679- 801	KF188707.1	HM143909.1	$3.612x10^{-7}$	G, B,M,C,S, **3S**
	1086-1097	JX9870889.2	KX302717.1	$1.007x10^{-7}$	G,M,**S**
PSBB 66	972- 1113	KX302715.1	DQ118862.1	$6.861x10^{-3}$	R, G, **M**, C
	984-1334	HM143909.1	PSBB14	$4.530x10^{-3}$	B,M,**C**

*unknown parent detected during recombination analysis but closely related to the inferred betasatellite molecules in this analysis. R=RDP, G=GENCONV, B=BOOTSCAN, M=MAXCHI, C=CHIMAERA, S=SISCAN, 3S=3SEQ; Highlighted recombination method denotes the maximum av. *p*-value.

4.11.5 Phylogenetic relevance of betasatellite molecules associated with leaf curl disease of papaya

Betasatellite molecules identified in present study were studied through maximum likelihood (ML) method implemented in mega v6.0 to infer their evolutionary history. Tree generated after ML analysis by giving 1000 bootstrap replications was broadly divided in two clusters in which PaLCuB (PSBB 21), LuLDB (PSBB 34) and CroYVMB (PSBB 14, PSBB 38) were placed in cluster-I and ToLCuBB (PSBB43), ToLCuB (63, 66), TbLCuB (51), ChiLCB (PSBB60), CoLCB (PSBB47) isolates were placed in another separated cluster (cluster-II) with other closely related betasatellite molecules. Cluster II has divided into sub-clusters that are separated with their closely related betasatellites. Different ToLCuB isolates were placed at three different sites in cluster II, among them isolate PSBB 66 occupied quite distant position with its closely related tomato leaf curl betasatellite isolate (figure 4.20). Major deviation was observed in branching order while studying phylogenetic analysis of betasatellite sequences. Position of betasatellite isolates along with their recombinant parents explain their evolution from their putative recombinant ancestors originated from their original plant hosts. These findings support the fact regarding betasatellite movement across plant species. Diversity and distribution of betasatellites is based on geographic locations and available plant hosts and their co-adoption with different helper begomoviruses.

Briddon *et al*., 2003 studied the diversity of betasatellite molecules associated with some monopartite begomoviruses and suggested that betasatellites co-evolved with their respective begomoviruses and were subsequently distributed based on their host and topography. Complex association between begomovirus and betasatellite molecules occurring in chilli leaf curl disease was studied by Kumar *et al*., 2015b and demonstrated the role of betasatellites during symptom development in chilli plants. Similarly, Sivalingam *et al*., 2010 also studied the diversity of betasatellite associated with leaf curl disease of tomato. Studies on host range and diversity of betasatellite molecules is increasing through mutations and recombination occurred during the course of time (Leke *et al*., 2012; Kumar *et al*., 2015b). Present study has also provided the information regarding the expanding host range and complex distribution of begomoviruses and

betasatellites on papaya and papaya as a reservoir of different begomovirus-betasatellite complexes in nature.

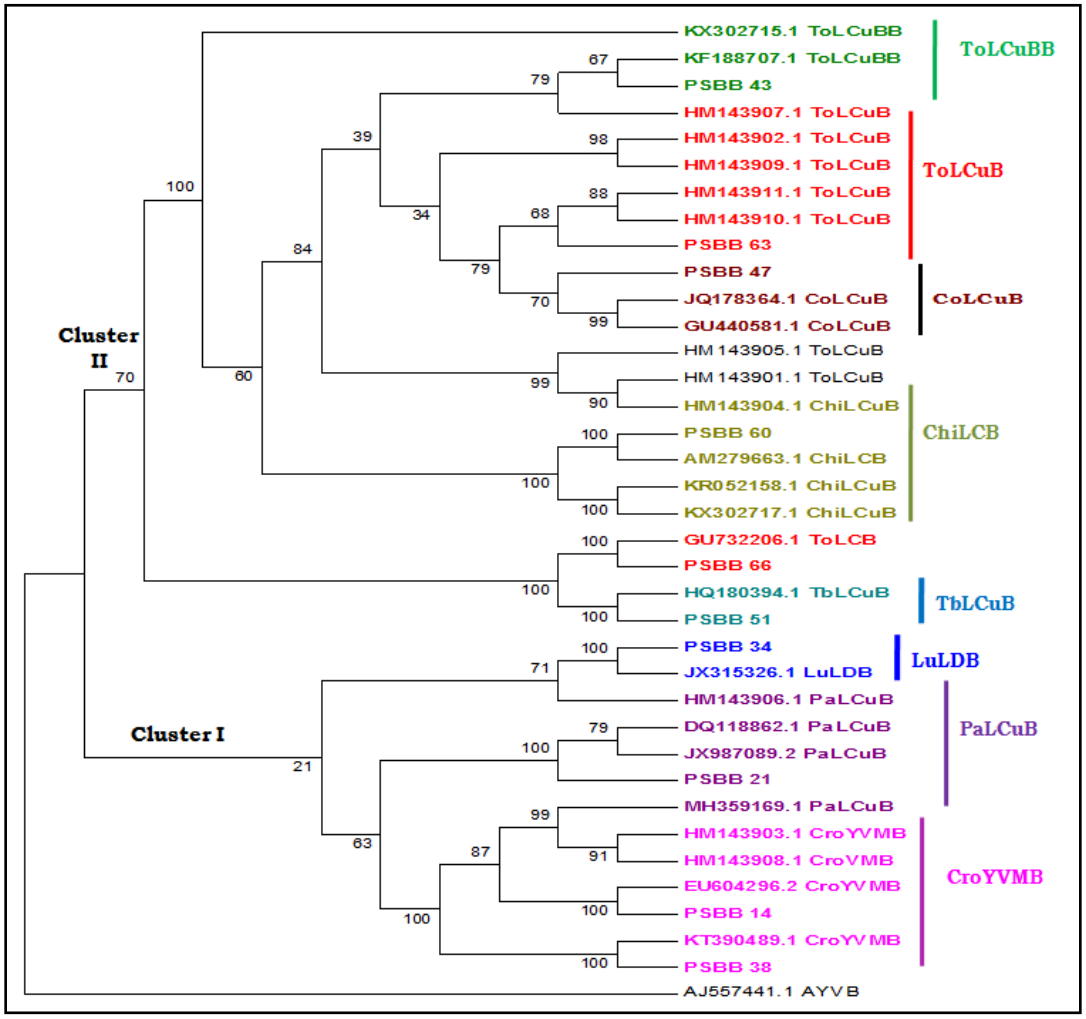


Figure 4.20: Phylogenetic tree of selected betasatellite molecules generated through mega v6.0, values at nodes indicates bootstrap percentages. All the isolates identified in present study are represented as their isolate names and other selected betasatellite sequences are represented with their accession no. and acronyms.

4.12 Phylogenetic analysis of different begomoviral genes

AV1 gene of begomoviruses encodes for coat protein that mediate their transmission through whitefly and considered as the most conserved gene among begomoviruses. Phylogenetic analysis of CP gene of begomoviruses was done and it was found that similar begomovirus species were closely associated and form a subcluster based on their geographic origins. (figure 4.21a). AV2 gene is known for translocation of virus particles inside the plants and showed phylogenetic relatedness with similar begomoviral species (figure 4.21b). Similarly, AC1 of begomoviruses encodes protein responsible for replication initiation of begomoviruses and phylogenetic study of AC1 genes of different begomoviruses showed close relationship with their closely related species/ strain (figure 4.22a). Although AC4 gene is the highly variable gene of begomovirus genome but AC4 gene of isolates belong to same species showed similarity and grouped together in phylogenetic tree (figure 4.22b). Interestingly, AC2 gene of begomovirus isolates identified in this study found closely related with begomoviruses identified on papaya (figure 4.23a). Phylogenetic analysis of AC3 gene of begomoviruses showed that isolates of same species clustered together and showed close relationship with isolates identified on same plant host (figure 4.23b). CP gene is highly conserved across the genera and showing variations with the change in geographic locations. Phylogenetic study of AC2 and AC3 gene of begomoviruses were found similar during evolution and altered according to their host plants. Although, AC4 is considered as highly variable gene of begomoviruses but showing similar evolutionary pattern as AC1gene showed. So, these results suggested that distribution of begomoviral genes is dependent on the infecting begomovirus, geographic locations and plant host.

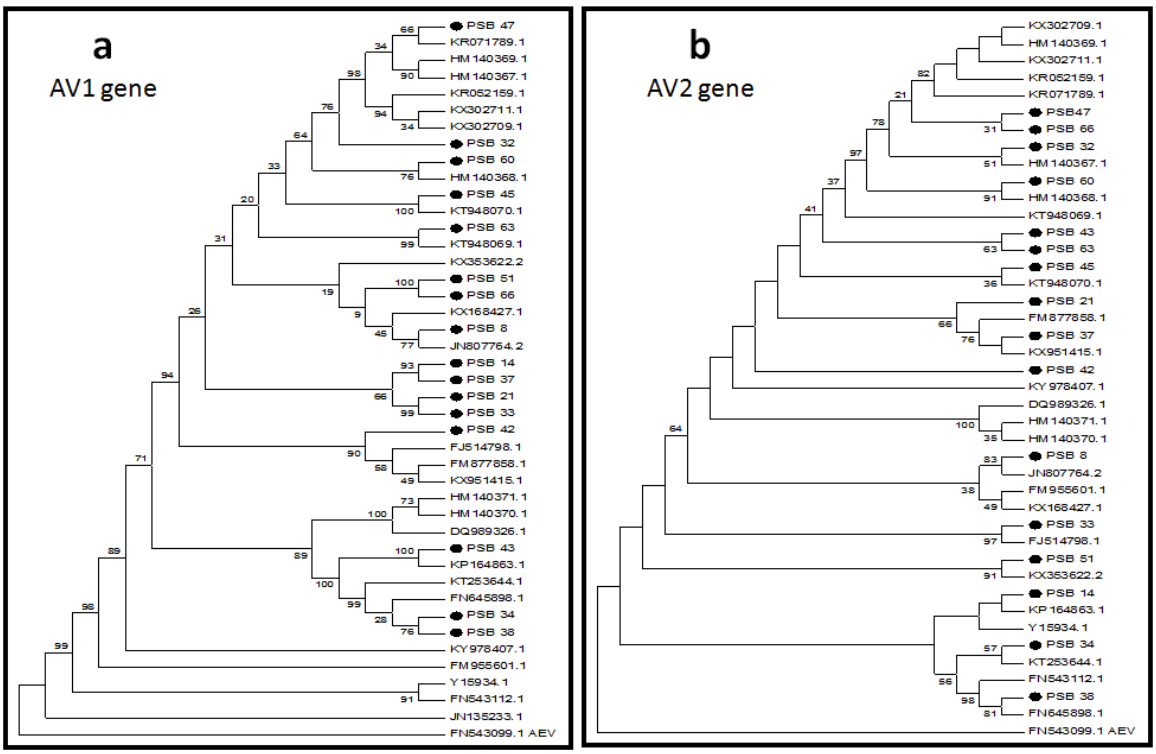


Figure 4.21: A maximum likelihood tree generated using mega v6.0 by giving 1000 bootstrap replication showing evolutionary relationship among coat protein gene (AV1) (a) and pre-coat (AV2) gene (b) of begomoviruses identified during this study with their closely related species. Isolate Ageratum Enation Virus (AEV) was taken as out-group for this analysis. Isolates of present study are marked with symbol (●).

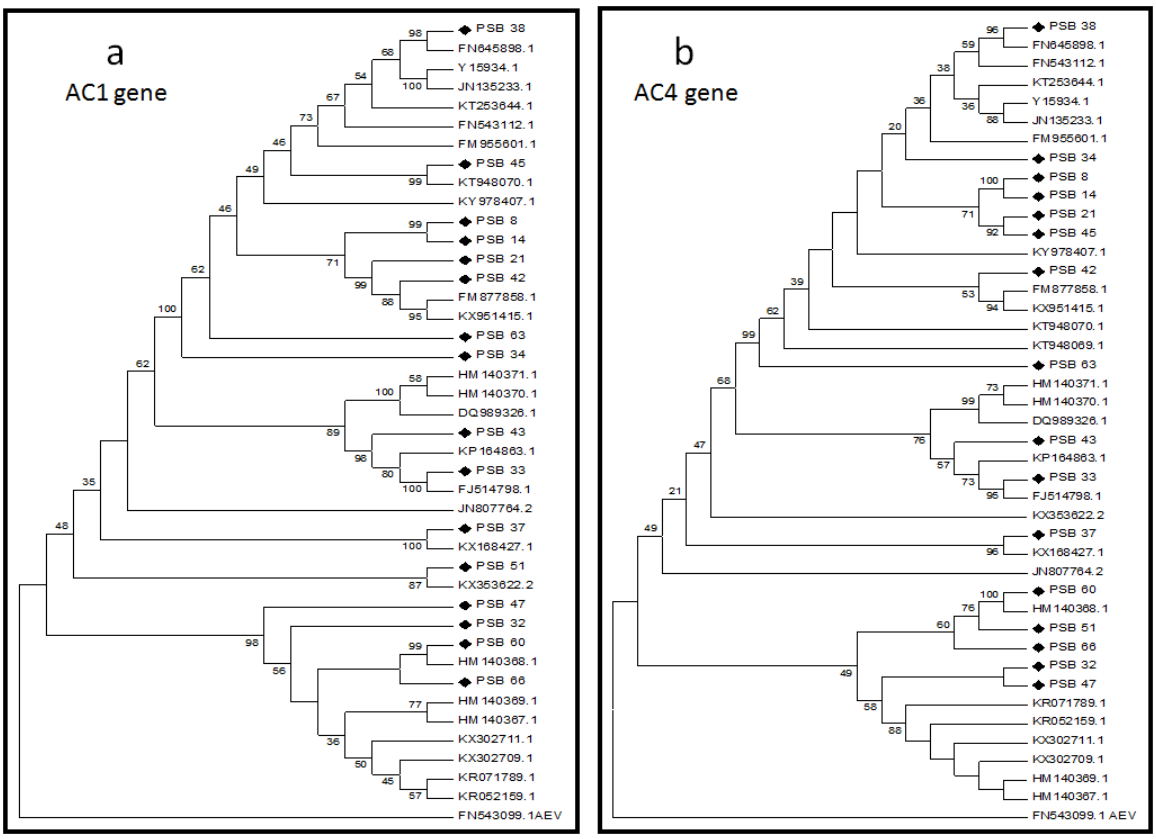


Figure 4.22: A maximum likelihood tree generated using mega v6.0 by giving 1000 bootstrap replication showing evolutionary relationship among replication gene AC1 (a) and AC4 gene (b) of begomoviruses identified during this study with their closely related species. Isolate Ageratum Enation Virus (AEV) was taken as out-group for this analysis. Isolates of present study are marked with symbol (◆).

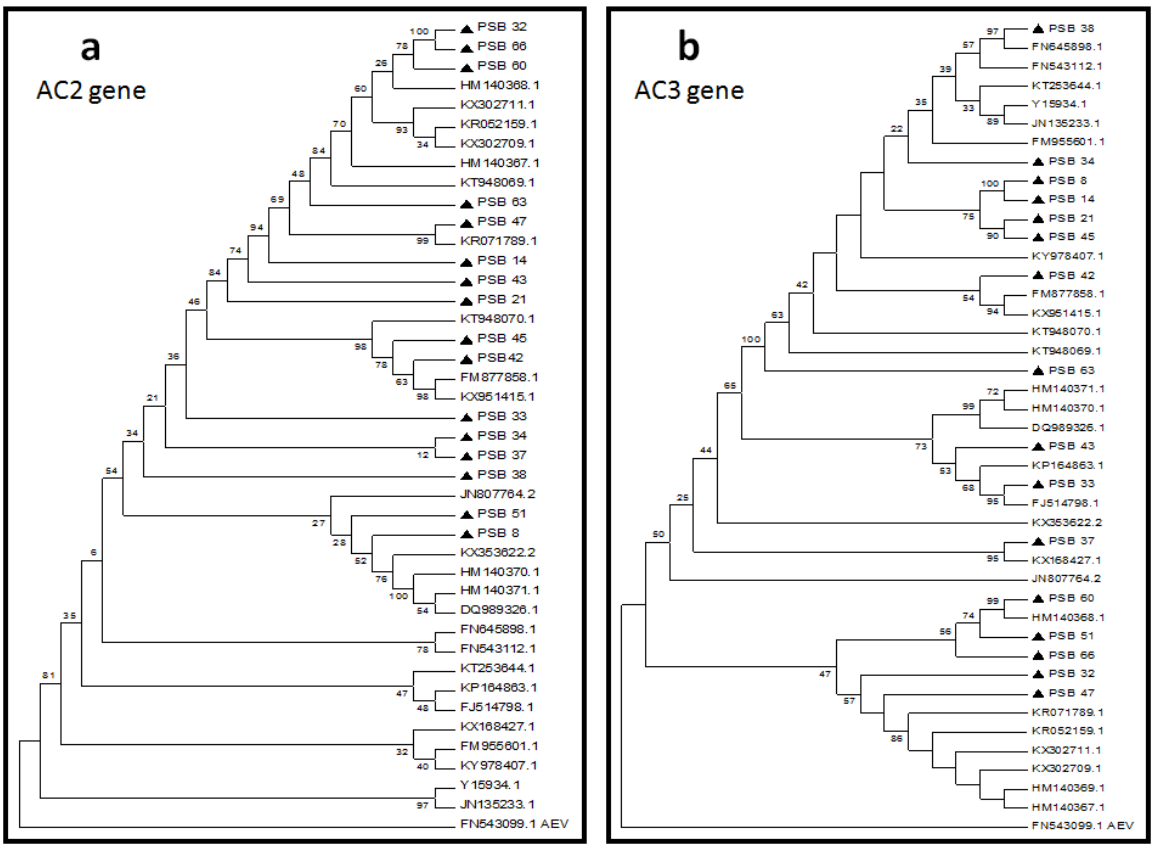


Figure 4.23: A maximum likelihood tree generated using mega v6.0 by giving 1000 bootstrap replication showing evolutionary relationship among replication gene AC2 (a) and AC3 gene (b) of begomoviruses identified during this study with their closely related species. Isolate Ageratum Enation Virus (AEV) was taken as out-group for this analysis. Isolates of present study are marked with symbol (▲).

4.13 *Insilico* designing of highly efficient siRNAs

RNA interference (RNAi) using small interfering RNA (siRNA) a technique which is used to down regulate the target gene expression in a highly sequence-specific manner. siRNA is gaining popularity as an advance molecular biology technique because as per recent reports it works efficiently to develop disease resistance by specifically targeting the genes involved in disease pathway/ pathogenesis. Another important application of siRNA is in functional genomics as it gives the information about the function of genes by means of "loss of function". siRNAs recognize and bind with target genes with complete homology and silence their expression by cleaving its mRNA. One of the drawback in the case of siRNA is that sometimes it can lead to off-target gene silencing if the sequences of siRNAs bear complementarity to the untargeted gene. However, siRNAs generated from same target may have different efficiencies to trigger RNAi mechanism (Yan *et al*., 2012).

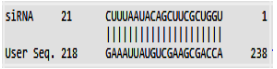
After studying molecular variability among begomoviral genome, different genes of begomoviruses were used as target for *insilico* siRNA designing to combat resistance against begomoviruses in papaya. AV1, AV2, AC1 and AC2 genes were showing some conserved regions in the multiple sequence alignment of begomoviruses even after molecular variability whereas, AC3 and AC4 genes were found highly variable within begomoviral DNA-A genome. All the begomoviral genes were used for siRNA prediction for developing effective management approach. Nucleotide sequences of all the genes were separately analyzed for the siRNA designing specifically against those genes. An advance siRNA prediction tool, pssRNAit (http://plantgrn.noble.org/pssRNAit/) was used to design highly efficient and thermodynamically stable siRNAs with an advance filter of off-targets. Two siRNAs generated from every begomoviral gene were selected based on siRNA efficiency to target nearly all begomovirus species, RISC binding capacity of sense and anti-sense strand of siRNA, GC content and off-target filtering (table 4.10). BLASTn analysis of designed siRNAs was also performed to validate the off-targets prediction. Target sites of designed siRNAs against all the genes were analyzed on multiple sequence alignment (MSA) of all begomoviruses taken for analysis and further subjected to RNAup web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAup.cgi) to check the accessibility of target site for siRNA

binding. Complementarities of designed siRNAs with target genes within alignment were evaluated to verify the targeted gene prediction.

Two siRNAs designed against different position of AC1 gene were analyzed found that the siRNAs target most of the begomovirus isolates and showed the best binding with that particular region on AC1 gene mRNA (figure 4.24), similarly siRNAs designed against AC2 gene were also found effective (figure 4.25). siRNAs designed against both the genes AV1 (figure 4.26) and AV2 (figure 4.27) were found more accurate to target almost all the begomoviruses taken for analysis. Among both siRNAs designed against AC3 gene, one was found more appropriate for silencing studies (figure 4.28a) as compare to other (figure 4.28b). While, siRNAs designed against AC4 gene were not targeting majority of begomoviruses during analysis (figure 4.29) so cannot be used for generic resistance management. Since, we were looking for broad resistance against all the begomoviral species found on papaya, multiple sequence alignment was done and those siRNAs which were targeting the most conserved region among all isolates taken into consideration were selected.

Table 4.10: siRNA sequences designed online through pssRNAit (http://plantgrn.noble.org/pssRNAit/server) and their target gene in DNA-A component of begomoviruses infecting papaya and binding of siRNA with predicted begomoviral target sequence

S. No.	siRNA antisense	siRNA sense	Gene	Efficiency	RISC binder antisense score	RISC binder sense score	Target accessibility	No. of off targets	Alignment
1.	AAAGUGCUUUAG GUAGUGGGG	CCACUACCUAAAG CACUUUAA	AC1	8.3	2.26	-1.45	17.138	19	siRNA 21 GGGGUGAUGGAUUUCGUGAAA 1 User Seq. 880 CCCCACUACCUAAAGCACUUU 900
2.	UUAGGUCUCAAUG GCCGCGCA	CGCGGCCAUUGAG ACCUAAUA	AC1	8.57	2.03	-2.37	20.131	7	siRNA 21 ACGCGCCGGUAACUCUGGAUU 1 User Seq. 624 UGCGCGGCCAUUGAGACCUAA 644
3.	UCAUUGGCUGACU GUUGUCCC	GACAACAGUCAGC CAAUGAUG	AC4	7.75	-0.06	-1.27	19.135	19	siRNA 21 CCCUGUUGUCAGUCGGUUACU 1 User Seq. 218 GGGACAACAGUCAGCCAAUGA 238
4.	UUAGCUCCCUGAA UGUUCGGA	CGAACAUUCAGGG AGCUAAGU	AC4	9.33	1.4	-1.57	15.414	16	siRNA 21 AGGCUUGUAAGUCCCUCGAUU 1 User Seq. 107 UCCGAACAUUCAGGGAGCUAA 127
5.	GUGAUGAGUUCCC CUGUGCGU	GCACAGGGGAACU CAUCACUG	AC2	7.16	0.65	-1.51	20.295	19	siRNA 21 UGCGUGUCCCCUUGAGUAGUG 1 User Seq. 154 ACGCACAGGGGAACUCAUCAC 174
6.	GUUGGACCUUGAU UGGUACCU	GUACCAAUCAAGG UCCAACAC	AC2	6.37	0.21	-0.72	15.106	20	siRNA 21 UCCAUGGUUAGUUCCAGGUUG 1 User Seq. 38 AGGUACCAAUCAAGGUCCAAC 58
7.	AGAAACGACCAGU CGGAGGCU	CCUCCGACUGGUC GUUUCUUA	AC3	7.87	1.5	-1.4	19.886	16	siRNA 21 UCGGAGGCUGACCAGCAAAGA 1 User Seq. 227 AGCCUCCGACUGGUCGUUUCU 247
8.	AAGAAACACUUCA UUAUCCCC	GGAUAAUGAAGU GUUUCUUGG	AC3	7.9	0.49	-0.47	24.32	20	siRNA 21 CCCCUAUUACUUCACAAAGAA 1 User Seq. 180 GGGGAUAAUGAAGUGUUUCUU 200
9.	AGAGCCUGCUCCU UCGAUGCA	CAUCGAAGGAGCA GGCUCUCG	AV1	7.37	1.09	-0.71	19.476	11	siRNA 21 ACGUAGCUUCCUCGUCCGAGA 1 User Seq. 573 UGCAUCGAAGGAGCAGGCUCU 593

10.	UUGCCUUUGUGACGCGGACAA	GUCCGCGUCACAAAGGCAAGA	AV1	8.41	0.07	-1.72	24.35	19	
11.	AUCUGCUGGUCGCUUCGACAU	GUCGAAGCGACCAGCAGAUAU	AV2	8.43	-0.13	-0.52	18.716	18	
12.	UGGUCGCUUCGACAUAAUUUC	AAUUAUGUCGAAGCGACCAGC	AV2	7.06	-0.29	-1.85	20.27	20	

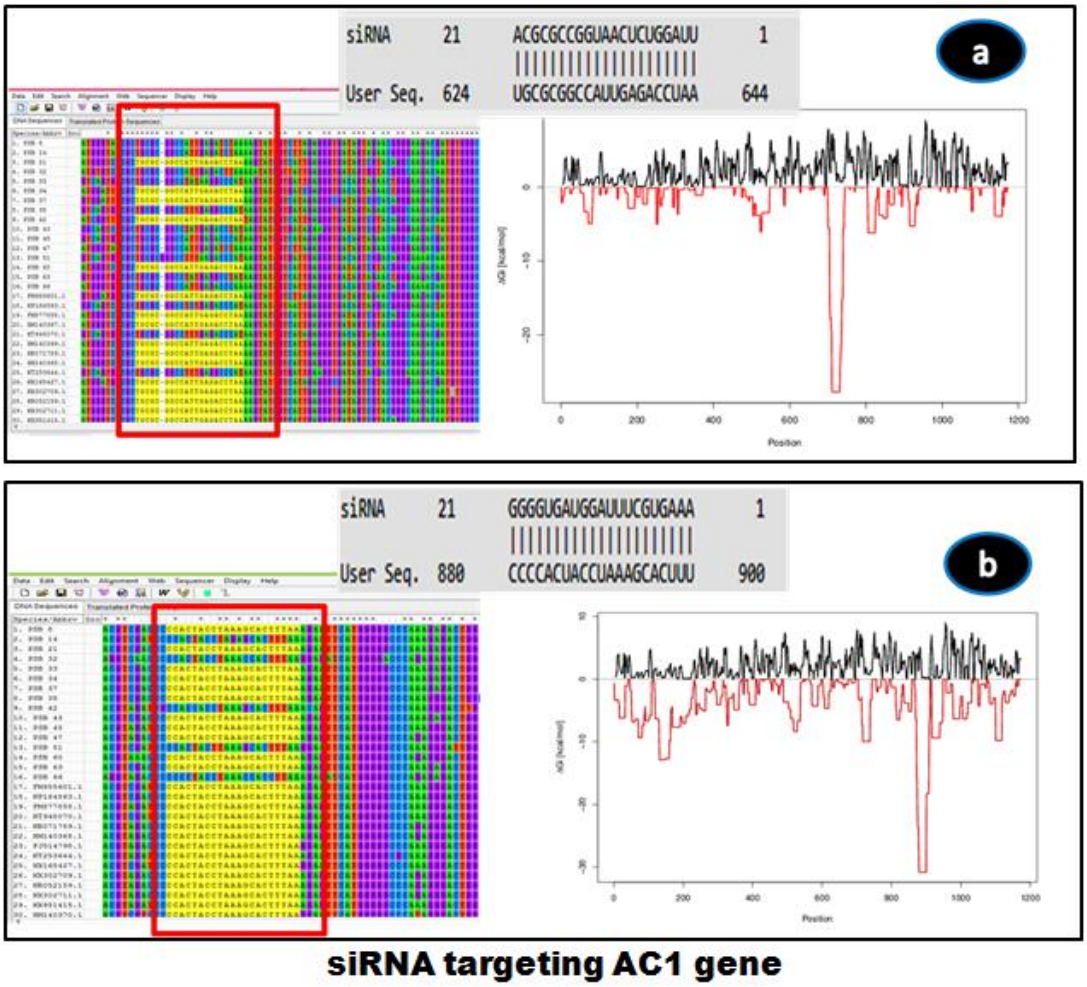


Figure 4.24: Target prediction of siRNAs designed against AC1 gene on multiple sequence alignment file of begomoviral AC1 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.

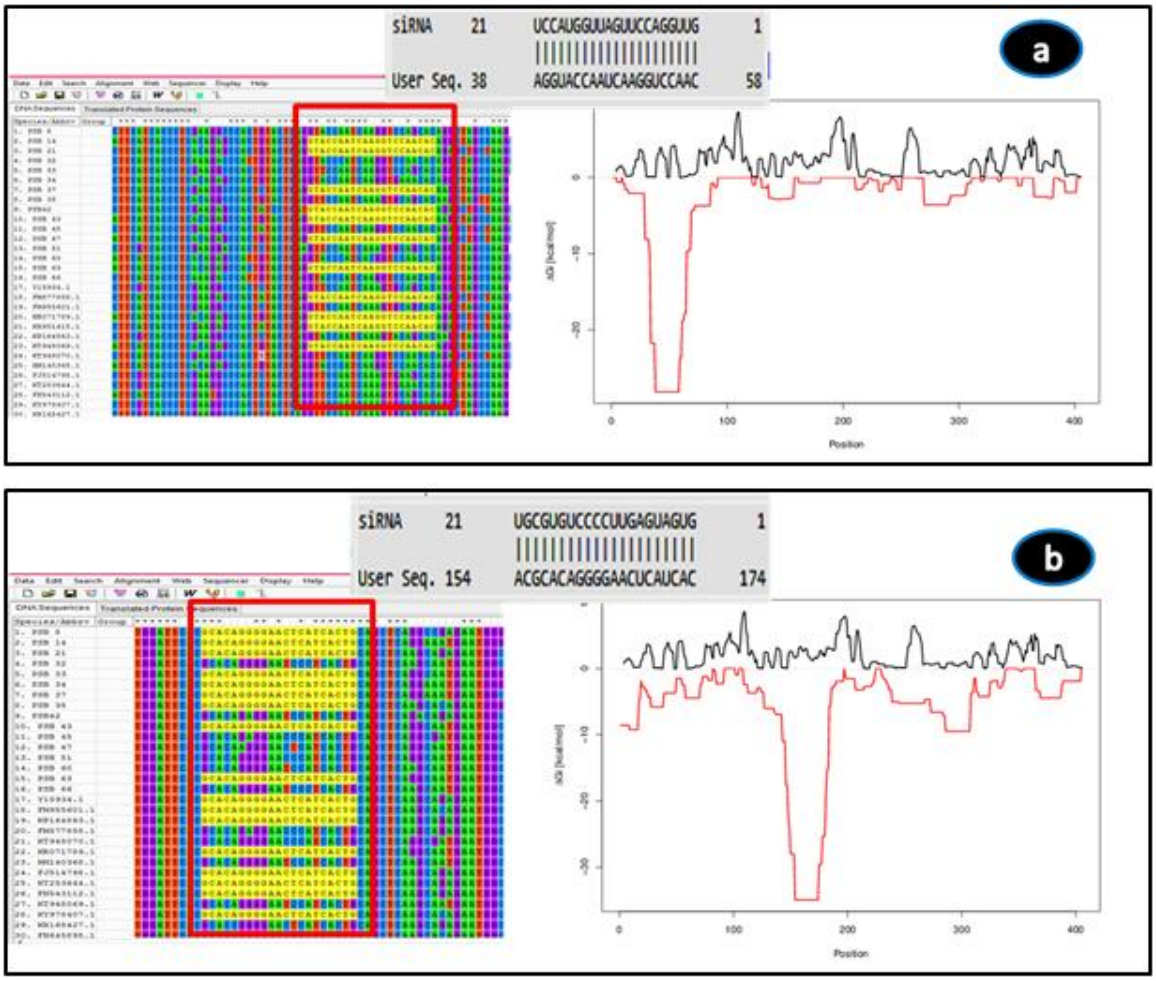
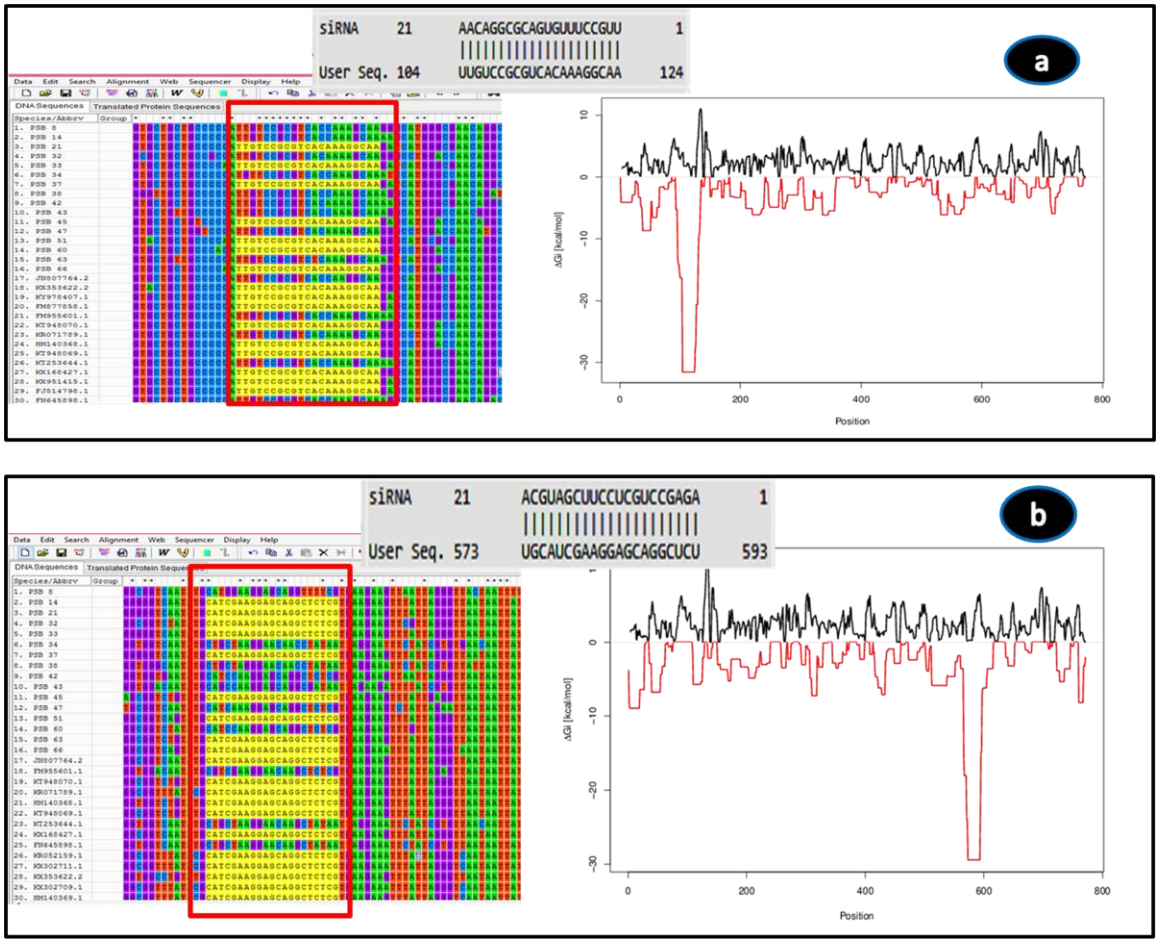
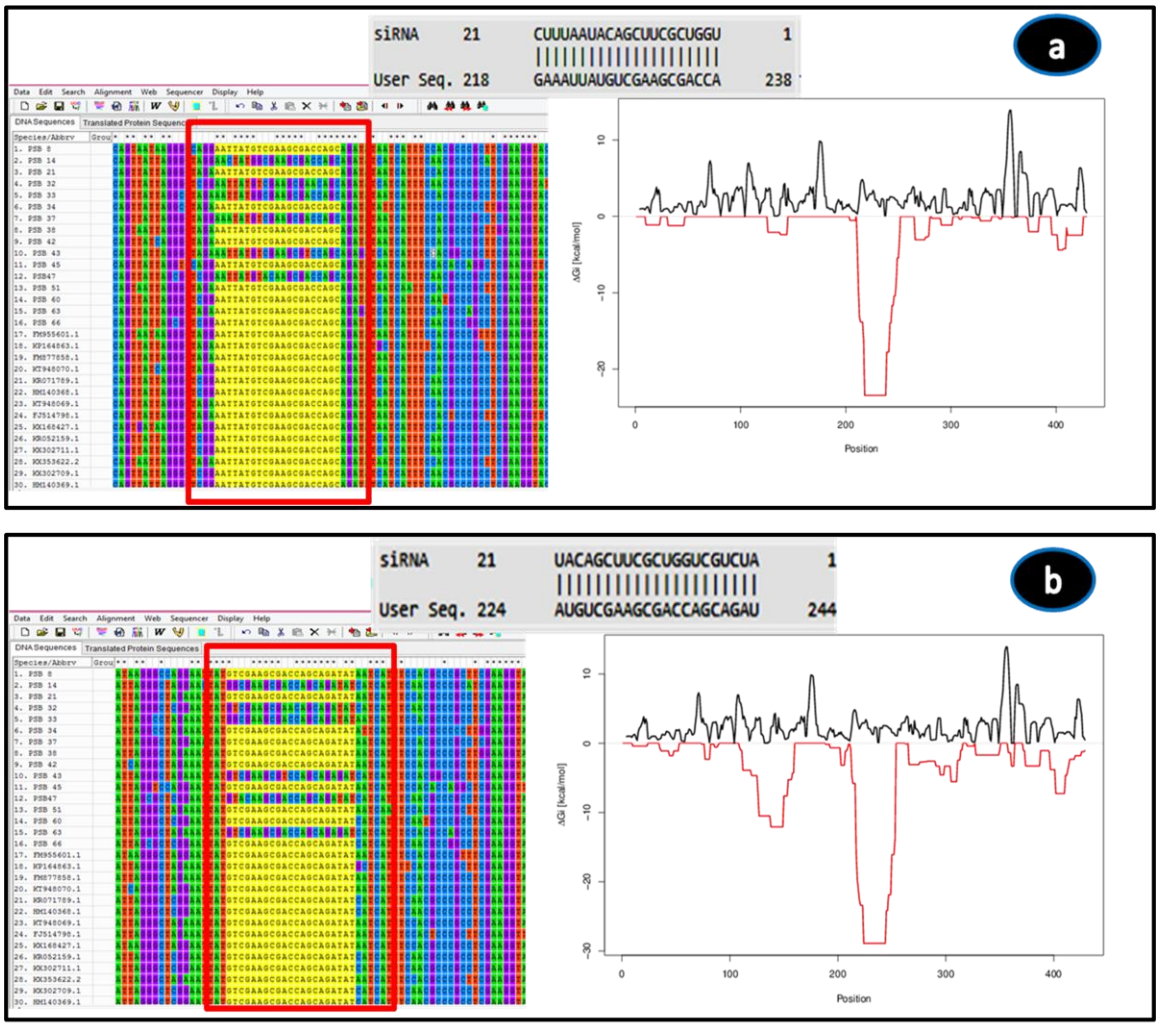


Figure 4.25: Target prediction of siRNAs designed against AC2 gene on multiple sequence alignment file of different begomoviral AC2 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.



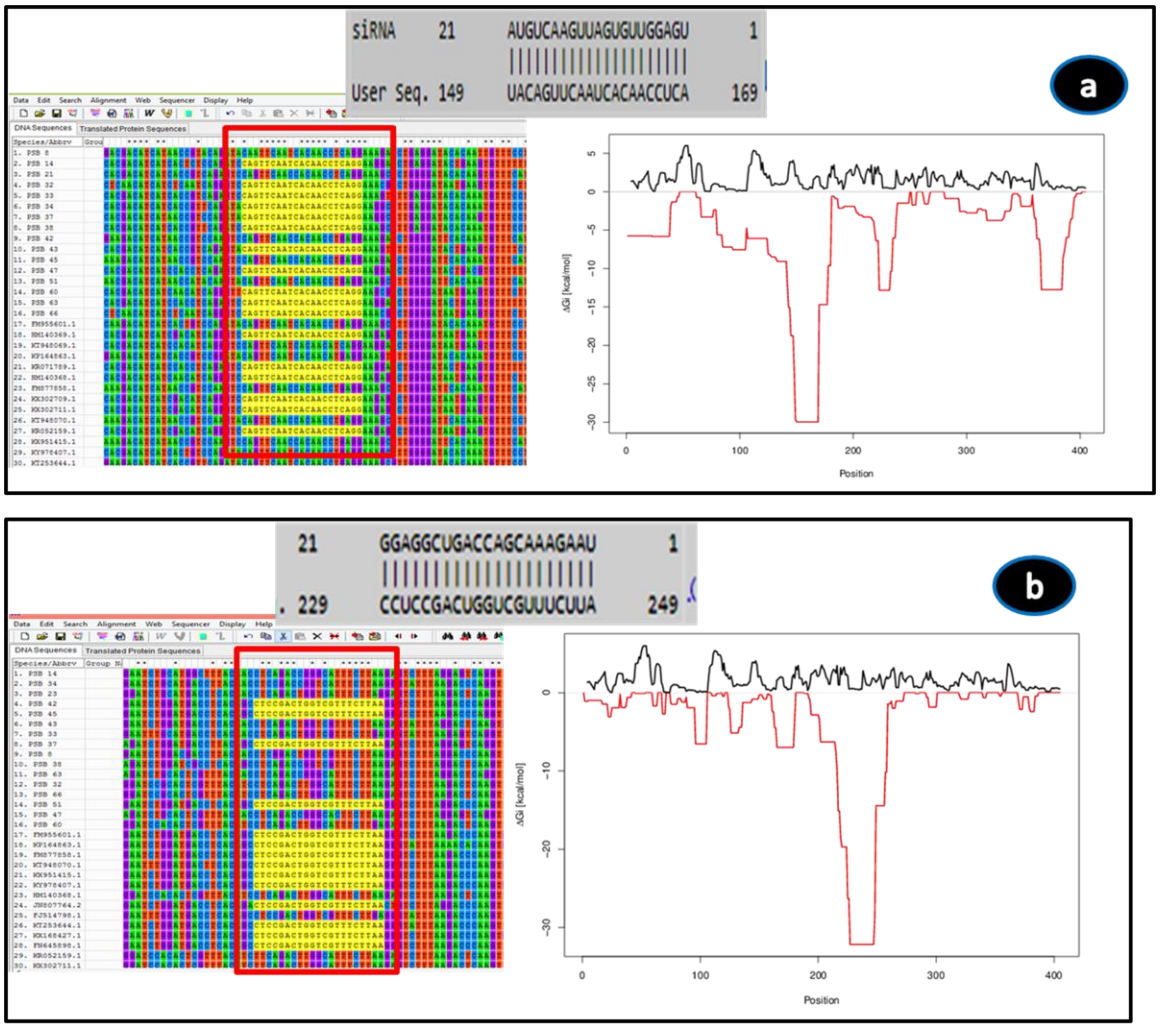
siRNA targeting AV1 gene

Figure 4.26: Target prediction of siRNAs designed against AV1 gene on multiple sequence alignment file of different begomoviral AV1 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.



siRNA targeting AV2 gene

Figure 4.27: Target prediction of siRNAs designed against AV2 gene on multiple sequence alignment file of different begomoviral AV2 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.



siRNA targeting AC3 gene

Figure 4.28: Target prediction of siRNAs designed against AC3 gene on multiple sequence alignment file of different begomoviral AC3 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.

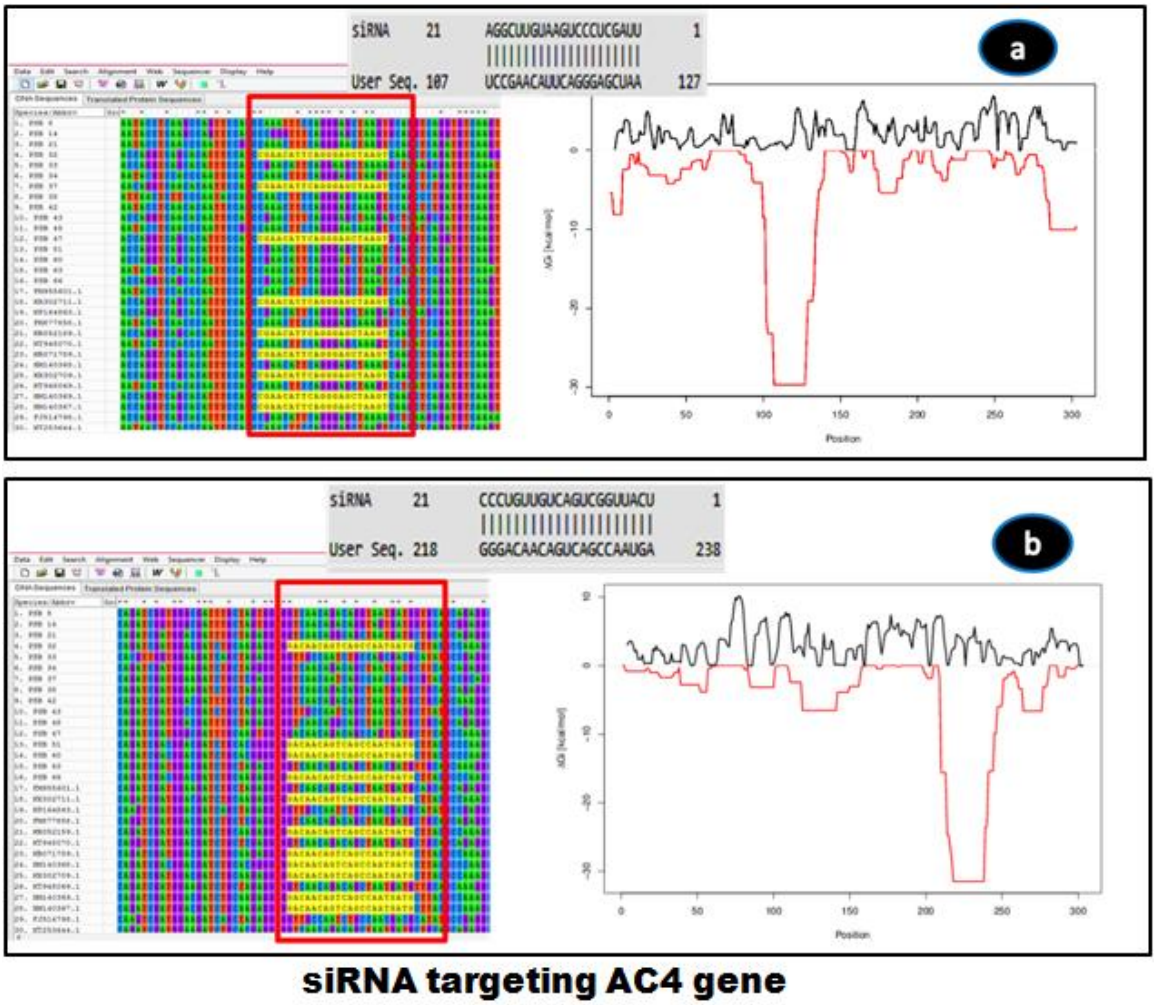


Figure 4.29: Target prediction of siRNAs designed against AC4 gene on multiple sequence alignment file of different begomoviral AC4 genes. Computational prediction of siRNA and corresponding mRNA hybridization plot for its target accessibility. a and b represent the analysis of both siRNAs designed during study.

siRNAs designed against AV1, AV2, AC1 and AC2 were targeting almost all the begomovirus species infecting papaya whereas, siRNAs designed against AC3 and AC4 did not target all the begomoviruses. In view of the fact that successful applications of post transcriptional gene silencing is depend on high target specificity and target efficiency, siRNAs designed against AV1, AV2, AC1 and AC2 genes were observed more effective against different begomoviruses studied during this work. AV1 and AC1 are known as most conserved genes of begomovirus genome and identified as a good target for siRNA mediated resistance. Similarly, siRNAs generated from AV2 and AC2 genes were also found effective against almost all the begomovirus species identified on papaya during this study. AC2 and AV2 genes are known as viral suppressors and also identified as good candidate for developing resistance against leaf curl causing begomoviruses. In such cases where complete conservedness in the genomic region of genes is not seen we can always introduce degeneracy in siRNA sequence or use a cocktail of siRNAs where nucleotide bases can be changed at few positions to target frequentl occurring isolates in nature. Hence, designing of stable as well as effective siRNAs during this study supports the concept of targeting begomoviral suppressors of different begomoviruses as an effective approach for broad spectrum /successful disease management.

siRNA mediated antiviral approach provides effective disease management against multiple virus species (Sharma *et al*., 2015; Wang *et al*., 2010). Earlier efforts towards successful management against papaya leaf curl disease were hypothesized through *insilico* siRNA search (Saxena *et al*., 2011, 2013) but did not considered the expanding begomovirus infection while studying. So, Present study involved the occurrence of several begomoviruses from different geographical regions and further, designed the siRNAs against different viral genes to develop generic resistance against papaya leaf curl disease.

siRNA based silencing was also employed for developing resistance against tomato leaf curl virus disease, golden mosaic disease (Asad *et al*., 2003; Abhary *et al*., 2006). AC1 gene has been already reported as an effective target for RNAi based approaches due to having 66-77% conservedness among geminiviruses so, Chellappan *et al*., 2004 used siRNA approach to silence AC1 gene and found it effective against gemeniviruses

infecting cassava and tomato. Similarly, Sanjaya *et al*., 2005 used a successful approach to target AC1 and AV2 gene to develop resistence against cassava mosaic disease. Conserved nature AV2 gene among begomoviruses is a best target for developing broad spectrum resistance, Mubin *et al*., 2007 has developed disease resistance against a bipartite begomovirus species by targeting AV2 gene using antisense technology. Recently, Tomar *et al*., 2018, identified RNAi approach targeting AC1 gene as a promising approach to develop resistance against tomato leaf curl New Delhi virus (ToLCNDV) infecting potato plants.

Present study also designed some effective siRNAs targeting AC1, AC2, AV1 and AV2 genes, so these siRNAs can be used to silence/ developing resistance against papaya infecting begomoviruses. siRNAs designed against different begomoviral genes can be used individually or in combination with others using a single ihpRNAi construct during developing transgenics to provide effective broad spectrum resistance against multiple begomoviruses infecting papaya.

This study provides the information on different begomoviral species and associated betasatellites infecting papaya crop in India. Results of this study have provided a base-line to identify the distribution of begomoviruses infecting papaya crop according to geographical locations. Papaya leaf curl samples collected from different places were studied and the infection of different begomoviral species as well as betasatellites on papaya was determined. Complete DNA-A analysis of 16 begomoviral isolates and their associated betasatellites was performed. Efforts were made towards development of generic resistance strategy through *insilico* designing of significant siRNAs (using pssRNAit software) based on genetic variability of begomoviral isolates. Thermodynamically stable siRNAs were designed from all the genes that target conserved regions among different begomoviral genes to develop successful management approach against different begomoviral species and their strains infecting papaya in India. Results of this study can conclude the following points:

- Different papaya leaf samples exhibiting typical leaf curl symptoms were collected from 10 different states of India including Assam, Gujarat, Haryana, Jharkhand, Karnataka, Madhya Pradesh, New Delhi, Punjab, Telangana and Uttar Pradesh.
- 25 samples were found positive for begomoviral infection based on PCR screening using degenerate primers specific for DNA-A genome of begomovirus detection.
- 10 samples were found to be associated with betasatellite molecules while none of the sample showed presence of DNA-B component during PCR based screening.
- Partial sequencing of all 25 begomoviral DNA-A (CP gene and UH region) was performed and identical isolates belong to same place were excluded and 16 representative isolates from every place were proceeded for the analysis of complete DNA-A sequences.
- Abutting primers were designed based on partial DNA-A sequences to amplify complete DNA-A component (~2.7kb) of begomoviruses infecting papaya plants in India.

- Analysis of all identified DNA- A components as well as betasatellites associated with begomoviruses was classified through sequence demarcation tool and submitted in GenBank database.
- Infections of different begomoviruses were identified on papaya eg. chilli leaf curl virus, croton yellow vein mosaic virus, duranta leaf curl virus, papaya leaf curl virus, papaya leaf crumple virus, papaya severe leaf curl virus, papaya yellow leaf curl virus, pedilanthus leaf curl virus, tomato leaf curl virus and tomato leaf curl Gujarat virus. These results provide the support for the papaya leaf curl disease complex.
- Different betasatellite molecules e.g. chilli leaf curl betasatellite, cotton leaf curl betasatellite, croton yellow vein betasatellite, luffa leaf distortion betasatellite, papaya leaf curl betasatellite, tobacco leaf curl betasatellite, tomato leaf curl Bangladesh betasatellite and tomato leaf curl betasatellite molecules were identified on papaya and showed their existence with different begomoviruses on papaya.
- Present study reports the presence of duranta leaf curl virus (PSB 63:MH807202) and tomato leaf curl Gujarat virus (PSB 43: MG757245) on papaya plants in India for the first time.
- This study identified the presence of two isolates (PSB8:MH988457 and PSB 14: MH988458) of a novel begomovirus species named as papaya severe leaf curl virus (PaSLCuV) on papaya in Lucknow.
- This study documented for the first time the occurrence of tomato leaf curl Bangladesh betasatellite (PSBB43:MG478451), luffa leaf distortion betasatellite (PSBB 34:MH825685) and cotton leaf curl betasatellite (PSBB47:MH825687) on papaya.
- Occurrence of several begomoviruses and betasatellites on papaya provide the evidence that papaya is a natural host for different begomoviral species and betasatellite molecules.
- Molecular diversity study and phylogenetic relationship of begomovirus species identified in present study show their evolutionary background and their widespread range to different geographic regions.

- Novel recombination events between different begomovirus species may play an important role in evolutionary mechanism and adaptation to different climatic conditions and hosts.
- Information on sequence diversity of different isolates of begomovirus found on papaya can result in developing an effective disease management strategy as an applied aspect, also understanding the origin and biology of the virus provides an insight in the basic subject domain.
- Our results suggested AV1, AV2, AC1and AC2 genes as best target for siRNA mediated gene silencing against papaya leaf curl disease.
- Thus designed siRNAs from present study will provide us an opportunity to explore the siRNA mediated generic resistance against papaya leaf curl disease.

Leaf curl disease of papaya is caused by papaya leaf curl virus. Frequent reports on occurrence of different begomoviruses on papaya are a major setback for papaya cultivation. In the present study survey of different geographic locations of India was conducted during 2014- 2016 to collect papaya leaf samples showing typical symptoms of leaf curl disease. Various locations of ten different Indian states were visited during sample collection and 82 samples were collected from several places of Assam, Gujarat, Haryana, Jharkhand, Karnataka, Madhya Pradesh, New Delhi, Punjab, Telangana and Uttar Pradesh states to access diversity among all collected papaya leaf curl virus isolates for developing a generic resistance strategy against papaya leaf curl disease. Number codes were assigned to all samples and work was initiated by preliminary screening of virus through PCR using different degenerate primers designed for begomovirus (coat protein (CP), DNA-A and DNA-B) detection. Among all collected samples, 25 samples were found positive for begomoviral DNA-A however, presence of DNA- B component was not detected in any sample during PCR screening. Association of betasatellites with begomoviruses on same plants is an additional potential threat for crops that increase the severity of disease symptoms. Among all 25 positive samples preliminary screening was done to detect betasatellite molecule using betasatellite specific primer pair (β01 & β02) in PCR. During PCR screening 10 samples showed desired amplification of expected size hence, were considered positive for presence of betasatellite on papaya infected with leaf curl disease.

As begomoviruses use rolling circle replication (RCR) method for their multiplication, rolling circle amplification (RCA) kit principally based on RCR method was used for detection as well as enrichment of all genomic components of begomoviruses in positive samples. These virus enriched amplified RCA products were used as template for further amplification of viral genes and full length viral DNAs as well as betasatellite molecules. After initial screening for presence of begomovirus, further confirmation of begomovirus on collected samples was done. For that, the coat protein (CP) gene and upper half (UH) region were selected for sequencing as they have conserved regions across the genera. PCR amplified product of CP gene and UH region (using same degenerate primers that were used for screening) were eluted from the gel, purified and directly sequenced from Chromous Biotech Pvt. Ltd. Obtained sequences were combined and analyzed through

BLAST online tool to identify the begomovirus infecting papaya plants during study. BLAST analysis of sequences showed the percent identity of all isolates with begomoviral species already available in NCBI database. These nucleotide percent identities provided a preliminary idea about infecting begomovirus isolates found on papaya crop in different locations.

According to International Committee on Taxonomy of Viruses (ICTV) report sequence information of complete DNA-A is necessary to characterize and classify the existing begomovirus species on collected samples (Brown *et al*., 2015). To get the complete DNA-A genome (~2.7 kb) of all viral isolates, abutting primers (PSBP-F & PSBP-R) were designed from sequences of CP and UH region. Amplified products of complete DNA-A were further cloned into pGEM-T easy vector system (Promega, USA) and transformed into DH5α strain of *E. coli* bacteria. Transformed positive clones were completely sequenced from Chromous Biotech Pvt. Ltd., Bangalore. The obtained sequences were used for the sequence based characterization of begomoviruses infecting papaya samples.

Analysis of leaf morphology is a preliminary feature to detect plant health during stress conditions. So, simultaneously, infected as well as healthy leaf samples were examined through scanning electron microscopy (SEM) to visualize leaf anatomy and also to analyze differences in morphology of healthy and infected leaf samples. Begomoviruses are phloem bound and affect mainly leaf edges, veins and midrib portion of plant leaves to produce symptoms, so specimens were prepared from all those regions to be examined under SEM. Analysis of healthy plant leaf showed typical anatomy of papaya leaves i.e. crescent shaped stomata surrounded with guard cells, wax deposition on abaxial leaf surface and evenly distributed veins. On the contrary, infected leaf sample were observed with distorted leaf, stomata were sunken and difficult to identify. Midrib and veins of infected plant leaf sample were found to be thickened and swollen while distorted veins were also observed that specify the changes in plant leaves at cellular level.

Leaf samples were also subjected for comparative assessment of existing elements through energy dispersive spectroscopy (EDS) feature of SEM that observed carbon and oxygen commonly in both healthy as well as infected leaves. Oxygen atomic percentage

was more in healthy leaf whereas infected leaf was observed with higher atomic percentages of carbon. Si, P, S, Na atoms were detected only in infected leaf while Mg, K, Ca elements were detected only in healthy samples. Presence of some nanoparticles like Zr and Pt were only observed in healthy leaf samples. SEM micrographs and EDS analysis revealed the differentiation in characteristics and elemental analysis of healthy and infected leaf samples.

Complete genome sequences of DNA-A component of 16 begomoviral isolates were analyzed through BLAST online tool to know their percent identity with previously reported begomoviral isolates. Some closely related begomoviral species alongwith previously reported begomoviruses on papaya were retrieved from NCBI database to conduct further studies. All DNA-A sequences taken for analysis were subjected to sequence demarcation tool (SDT) to calculate percent pairwise identities intended for the accurate classification of begomoviral isolates identified during present study. BLAST and SDT analysis of DNA-A sequences revealed the presence of different begomoviral species infecting papaya in India. Complete DNA-A sequences of all identified begomoviruses during this study were submitted in GenBank to get their accession numbers. Papaya leaf curl virus (PSB34:MH807205), papaya leaf crumple virus (PSB32:MH674437; PSB47:MH807200; PSB60:MH807201; PSB66:MH807203), papaya yellow leaf curl virus (PSB51:MH807204), tomato leaf curl virus (PSB33:MH765694), tomato leaf curl Gujarat virus (PSB43:MG757245), pedilanthus leaf curl virus (PSB37:MH765695), duranta leaf curl virus (PSB63:MH807202), croton yellow vein mosaic virus (PSB38:MH765696) and chilli leaf curl virus (PSB21:MH765693; PSB42:MH765697; PSB45:MH765698) were found to be existing on papaya and causing leaf curl disease of papaya in different geographic locations. Among all viral isolates, two isolates of Lucknow PSB8 and PSB14 showed 90% and 87 % sequence identity with already reported begomoviruses which is below than species demarcation threshold (≥91%) suggested by ICTV (Brown *et al*., 2015) hence, classified as a new begomoviral species and submitted in GenBank database as a distinct begomoviral species i.e. papaya severe leaf curl virus (PaSLCuV) (PSB8: MH988457; PSB14: MH988458).

Betasatellites are DNA molecules that are frequently identified on papaya; though apparently they do not appear to be common but are increasingly found on infected papaya plants. Amplified products of all the betasatellite were sequenced and analysis of betasatellite sequences showed the presence of different betasatellite molecules. All the betasatellite sequences were analyzed through BLAST online tool and further pairwise sequence comparison was calculated through SDT. Based on these comparison and following species demarcation threshold criteria for betasatellites (89% nucleotide sequence identity), all betasatellite sequences were categorized into eight distinct betasatellites and submitted in GenBank database as cotton leaf curl betasatellite (PSBB47:MH825687), croton yellow vein mosaic betasatellite (PSBB14:MH82583; PSBB38: MH825686), chilli leaf curl betasatellite (PSBB60:MH825689), tomato leaf curl betasatellite (PSBB63:MH825690; PSBB66:MH825691), tobacco leaf curl betasatellite (PSBB51:MH82568), luffa leaf curl betasatellite (PSBB34:MH82565), papaya leaf curl betasatellite (PSBB21:MH8256884) and tomato leaf curl Bangladesh betasatellite (PSBB43:MG478451) molecules. In the present study complex of different begomoviruses associated with betasatellites were identified on papaya in India. Data found from present study showed the presence of ten different begomovirus species and eight betasatellite molecules infection on papaya and provided the evidence for papaya developing as an alternate host for many begomovirus species / betasatellites in different environmental conditions.

Distribution, diversity and incidence of begomoviruses is increasing very rapidly due to increase in the population of transmitting vectors, climatic conditions of different geographical regions, presence of off-season plant reservoirs as an alternate host for begomoviruses, high mutation and recombination rate of begomovirus genome etc. (Rojas *et al.*, 2005; Nawaz-ul-Rehman and Fauquet, 2009). To understand the occurrence of different begomoviruses and betasatellite molecules, recombination as well as diversity study was conducted and it was observed that emergence of these new species and adaptations to different environmental conditions are mainly driven by recombination followed by nucleotide diversity. Phylogenetic study of isolates of this study showed close relationship with most likely related organism of similar geographic origins. Information of recombination events and branching order in phylogenetic tree provided

an evidence for gene flow amongst begomoviruses/ betasatellites from their closely related species, geographical locations as well as related alternative plant reservoirs around the area. Emergence of begomoviruses and betasatellites in papaya crop is a major concern and need to be monitored at early stages of infection. Expanding host range as well as mixed infection of different begomovirus species is also an important issue for managing the losses to papaya growers. To overcome these problems we have worked on an RNAi based strategy to provide sustainable resistance against papaya leaf curl disease.

Effective siRNA prediction is an important issue to knockdown/ silence the targeted viral gene. As RNA interference using siRNA is based on highly homology dependent manner, therefore we have focused on siRNA designing against conserved region present within the genes of begomoviruses. Efficient siRNAs against begomoviral genes were identified *insilico* using pssRNAit web server (http://plantgrn.noble.org/pssRNAit/). Further, siRNAs were designed by keeping in mind the thermodynamic stability of siRNA-mRNA interaction and off-target filtering. siRNAs designed against AC1, AV1, AC2 and AV2 were found to be more significant and target almost all the begomoviruses infecting papaya. These designed siRNAs can thus be used for successful disease management against papaya leaf curl disease.

This study provides the incidence of begomovirus species and betasatellite molecules occurring on papaya plants based on their geographical significance. Diversity study of begomoviruses and associated betasatellites infecting papaya can assist in developing resistance against begomoviruses. Improvement of some other variables such as growth conditions, cropping system, neighboring plants and weeds, vector population can also add to additional understanding of virus-host relationship. Studies of these parameters may provide more accurate approaches for begomoviral disease management and to develop effective, sustainable and robust disease management strategies.

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Complete DNA- A sequences of begomovirus isolates identified from this study

> MH988457 [Papaya Severe Leaf Curl Virus, Lucknow isolate PSB-8], Complete DNA-A sequence

ACCGGATGGCCGCGATTTTTTTCTAGTGGGCCCCACAACGCACGTGCTGACAAGGACAAACGG
ACCAATGAAATTGGCTCCTCGTTGCTTAATTATTTAATGGTCCCTCCTATAAACTTAGTGCGCA
AGTTGTGCACGCTTACCAATGTGGGATCCATTAGTAAACGAGTTTCCCGAAACCGTTCACGGT
TTTCGATGTATGCTAGCAGTTAAATATATGCAGCTCGTAGACAATACGTATTCTCCAGAGACT
TTGGGGTACGATTTAATTAGGGATTTAATATCAGTAATAAGGGCCAGGAATTATGTCGAAGCG
ACCAGCAGATATAATCATTTCCACGCCCGCTTCGAAGGTACGTCGCCGTCTCAACTTCGACAG
CCCATATGCGAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACCAAAGCAAGGGCATGGGCGAA
CAGGCCCATGAACAGGATGCCAAGGATGTACAGGATGTACAGAAGCCCAGATGTTCCTAGAG
GATGTGAAGGCCCATGTAAGGTCCAGTCATTTGAGTCTAGACATGATGTCCAGCACATTGGTA
AAGTCATGTGTGTTAGTGATGTTATTCGTGGAACTGGGTTGACTCCCCGAGTCGGCAAAAGGT
TTTGTGTGAAGTCCGTTTATGTTCTGGGCAAGATCTGGATGGATGAGAACATCAAGACCAAGA
ATCATACGAATAGTGTTATGTTTTTCCTTGTTAGGGATCGTAGGCCCGTTGATAAGCCCCAAG
ATTTTGGTGAGGTTTTTAATATGTTTGATAATGAGCCCAGTACGGGGACAGTGAAGAAGGTGC
ATCGTGATAGGTACCAGGTGCTCAGGAAGTGGCACGCCACTGTGACAGGCGGTCAATATGCA
TGGAAGGAGCAGGTTTTCGTGAAGAAGTTAATTAGGGTTACTAATTTTGTTGTGTATAACCAC
AAAGAGGCTGGCAAGTATGAGAACCATACTGAGAATGCATTGATGTTGTATATGGCATGTAC
CCCTGCCTCTAACCCTGTGTATGCTACCTTGAAGATACGGACCTATTTCTATGACTCACTAACA
AATTAATAAATATAGAATTTTATTATGTTTGATGGTCCTACATACATTGTTTGTGCTAATACAT
TCCATAATACATGATCAACTGCTCTAAGTACATTATTTATACTAATGACAGCTAAATTATCTAA
ATACTGCAGAACTTGGGTCCTAAAGACCCTTAAGAAACGACCAGTCCGAGGTTGTAAGGTCGT
CCAGATTCGGAAGGCTAGGAAACAATTGTGTATCCTCAGCTCTTTCCTGAGGTTGTGATTGAA
TTGTATCTGTACGGTTATGATGTCGTCCTTCATTAGGAATGGCCGGTTGTCGTGCTCTGTTATC
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GCAGTGTAGGTCAACGCTCCGACGCCTGATCCCCTTCTTGGCTAGCCTGTGCTGGACCTTGATT
GGTACCTGAGTACAGTGGGCCTTCGAGGGTGATGAAGGTCGCATTCTTTATAGCCCAATTTCT
GAGTGCCGAATTCTTCTCCTCATCCAAGTATTCTTTATAGCTGGAATTTGGTCCAGAATTGCAG
AGGAAGATAGTGGGAATACCACCTTTAATTTGAACTGGCTTCCCGTACTTGGTGTTGCTTTGCC
AGTCTCTTTGGGCCCCCATGAATTCTTTAAAGTGCTTTAGGTAGTGGGGGTCGACGTCATCAAT
GACGTTGTACCACTCGTCATAAATGCATACCTTAGGACTAAGGTCTAGATGACCACACAAATA
ATTGTGTGGACCCAGTGACCTGGCCCACATCGTCTTCCCCGTTCTACTATCACCCTCTAAGACA
ATACTTTTAGATCTCAATGGCCGCGCAGCGGTACCCATCACATTTTCAGCAGCCCATTCCTCTA
GGCCTCCGGCACCTTGATCGAATGAAGAAGAAGAAATGGGGGAAACATAAACCTCCACTGGA
GGTGTCCAAATCCTATCTAAATTATTTTTTAAATTATGATATTGAAAAATAAAATCTTCTGGGA
GCTTTTCCCTTATAATTGCTAATGCAGCTTCCGCTGAACCTGCATTTAAGGCCTCTGCTGCACC
ATCATTACCTGTCTGTTGACCTCCACTAGCAAATCGCCCACCGATCTGAAACTCACCCCAGTC
GATGTACTCTCCGCCCTTCTGGATGTAGGACTTGACACCTGACCTGCACTTAGCTCCCTGGAA
ATTTGGGTGGAACTGGCTTGAGGTATTAGGGCGAGCGACATCGAAATGTCTTGGGTTTCTGAA
CCGGGATTTCCCTTTGAATTGGATGAGCGCCTGGATACGCAAGCTCCCATCTTGGTGTTTTTCC
TGTGACACTCTGATAAATAATTTATCAGAAGGACAATTTAACGATTTCAGGATTTCGAGCATT
TGTTCTTTGGGTATTGGGCATTTTGGATAAGTAGGGAAGATATTTTTGGCTTTAACTTGGAATT
GATGAGTACGTGGCATATTGAATTGGGTGCTCTCCAAAACTCTACGGAATGGGGAGCTTTGGG
TGCCTATTTATAGCGAGCTCCCAAATGGCATTATCGTAATTTAGGAGAAATAATTCAAAATCT
TCACGCTCCTAAAAGCGGCCATCCGTATAATATT

>MH988458 [Papaya Severe Leaf Curl Virus, Lucknow isolate PSB-14], Complete DNA-A sequence

ACCGGATGGCCGCGATTTTTTTTTTGTGGCCCCAACAAAGCACTAACTGACAATGACAAGTTG
ACCAATGAGAATCGTTCCTCGTCGCCTAATTGTTTCGGGGTTCCCCCTATAAACTTAGTGCGCA
AGTTGTGTTTTCCCTTCACTATGTGGGATCCATTGTTAAACGAGTTTCCCGAAACCGTTCACGG
TTTTAGATGTATGTTAGCAGTTAAATATTTGCAGTTAGTAGAAAAGACTTATTCTCCCGATACA
TTAGGTTACGATTTAATTAGGGATTTAATTTCAGTTATTAGGGCTAGGAACTATGGCGAAGCG
ACCAGCAGATATCATCATTTCAACGCCCGCATCGAAGGTACGTCGCCGTCTCAACTTCGACAG
CCCATATGTGAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACCAAAGCAAAAGCATGGGCGAA
CAGGCCCATGAACAGAAAGCCCAGGATGTCCGGGATGTACAGAAGTCCAGATGTCCCTAGGG
GCTGGGAAGGTCCAGTTAAGGTCCAGTCCTTTGGATGCAGACATGATTTTGATCACATGGGTA
AGGCCATTTGTCTTTGTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGCGAAGAGGT
TTTGTGTGAAGTCTGTTTATGTGTTGGGTAAAATATGGATGGATGAAAATATAAAGACCAAGA
ATCACACGAACAATGTGATGTTCTTTCTCGTTCGTGACCGTAGACCAGTTGACAAGCCTCAGG
ATTTTGGTGAGGTTTTTAACATGTTTGATAATGAGCCCAGCACGGTGACGGTGAAAAATGTGC
ATCGGGATAGGTACCAGGTGCTCAGGAAGTGGCATGCAACTGTGACAGGGGGTCAATATGCA
TCGAAGGAGCAGGCTCTCGTGAAGAAGTTTATTAGGGTTAATAATTATGTTGTGTACAACCAA
CAAGAGGCTGGCAAATATGAGAATCATACTGAGAATGCGTTAATGTTGTATATGGCGTGTACG
CACGCCTCTAATCCCGTGTATGCTACATTGAAGATACGGATCTACTTCTATGACTCTGTATCGA
ATTAATAAATATAGAATTTTATTGAAGATGATTGGTCTAAATATACATCATGTTCTGATACATC
GTATAATACATGTTTAAAGGCCCTAACTACATTATTTATACTAATGACTCCTAGATTATCTAAA
TACTTTAAAACCTGACTCCTAAAGACTCTTAAGAAATGCCCGGTCTGAGGTTGTAAAGCCATG
CAGATTCTTAAGCCCAAAAAACACTTCAGTATCCTCAGTTCCTTCCTGAGGTTGTGATTGAACT
GGATCTGGAGAGTGATGATGTCGTGGTTCATGGAAAATGGCCTTTGGTCGTGGTCTGAGGACT
TGAAATATAGGGGATTTGGGACCTTCCAGATATACACCCCATTCATTTCCTGAGCTGCAGTGA
TGAGTTCCCCTGTGCGTGAATCCATGATTGTGGCAGGCTGATGGTACGTAGTAAGAGCAACCA
CCCTCGAGATCAACACTTCGCCGGATGGTCCCCTGCTTGGCTAGCCTGTGTTGGACCTTGATTG
GTACCTGAGTACAGTGGCTCTGTGAGGGTGATGAATTTTGCATTCTTTATAGCCCACGCTCTTA
GTGCCGAATTCTTGTCCTCGTCCAAGAACTCTTTATAGCTGGAAGTGGACCCAGGATTGCAGA
GGAAGATAGTGGGAATGCCCCCTTTAATTTGAACGGGCTTCCCGTACTTGGTGTTGCTTTGCC
AGTCTCTTTGGGCCCCCATGAATTCTTTAAAGTGCTCTAGGTAGTGGGGATCGACGTCATCAA
TGACGTTGTACCAGGCCTCGTTGCTGTAGACCTTTGGACTAAGGTCTAAATGACCACACAGAT
AATTGTGTGGACCCAGTGACCTGGCCCACATCGTCTTCCCCGTTCTACTATCACCCTCTAAGAC
AATACTTTTAGATCTCAATGGCCGCGCAGCGGTACCCATCACATTTTCAGCAGCCCATTCCTCT
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CATCACTACCTGTCTGTTGACCTCCACTAGCAAATCGCCCACCGATCTGAAACTCACCCCAGT
CGATGTACTCTCCGCCCTTCTGGATGTAGGACTTGACACCTGACCTGCACTTAGCTCCCTGAAA
CCCGGGGTGGAACTGGCTTGAGGTATTAGGGCGAGCGATTTCAAAATGTCTGTCTTCTCTGAA
CCGCAATTTCCCTTTGAATTGGATGAAACCCTGGATACGCAGAGACCAATCTTGGTGTTTTTCT
TTTGATACTCTGATAAACAATTTATCAAGAGGACAGGTAATGTTTTTAAATAGTTCGAGCATTT
GCTCTTTGCGTATTGGGCATTTGGGATAAGTAAGGAAGATATTTTCGGCATTTATACAGAAGG
AGTTATTACGACGCATATTGGATTGGAGACATTCAAAACTCTGAGGAATCCGGGACTCCCGGG
ACGCAATTATATGGTGTCCCCAAATGGCAATTTGGTAATTCAGAAAGAAATTTCAAAATCTCC
ACGCTCCAAACCGCGGCCATCCGTATAATATT

> MH765693 [Chilli leaf curl virus isolate PSB-21], DNA-A complete genome

ACCGGATGGCCGGGATTTTTTTTGTGGCCCCCACAACGCGCTAACTGACAATGACATGTGGAC
CAATGAGAATCGTCCCTCATGGTCTCAATGTTTTGTGGTCCCCCCTATAAATTAGTCCCCAAGT
ATTGGTCTTTATCCACAATGTGGGACCCGTTAGTAAACGAGTCTCCCGAAACCGTTCACGGTT
TTAGGTGTATGCTAGCAGTTAAATACCTGCGGCTAGTAGAAAATACGTATTCCCCAGACACTC
TGGGCTACGATTTAATTAGGGATTTGATTTCAGTTATTAGGGCTAGAAATTATGTCGAAGCGA
CCAGCAGATATAATCATTTCCACGCCCGCCTCGAAGGTACGCCGTCGTCTGAACTTCGACAGC
CCATATACCAGCCGTGCTGCTGCCCCCATTGTCCGCGTCACAAAGGCAAGATCATGGGCGAAC
AGGCCCATGAACCGAAAGCCCAAGATGTACCGGATATACAGAAGCCCAGATGTTCCGAGGGG
ATGTGAAGGCCCGTGTAAGGTCCAGTCATTTGAGTCCAGACCTGATATCCAGCACATTGGTAA
AGTCATGTGTGTTAGTGATGTTACTCGTGGTATTGGGCTGACTCCCAGGGTTGGCAAGAGGTT
CTGTGTGAAGTCCGTTTATGTTTTGGGCAAGATCTGGATGGACGAGAACATCAAGACTAAGAA
TCATACGAATAGTGTTATGTTTTTCCTTGTTAGGGATCGAAGGCCTGTTGATAAGCCTCAGGAT
TTTGGTGAGGTTTTTAACATGTTTGATAATGAGCCCAGCACGGTGACGGTGAAAAATGTGCAT
CGGGATAGGTACCAGGTGCTCAGGAAGTGGCATGCAACTGTGACAGGGGGTCAATATGCATC
GAAGGAGCAGGCTCTCGTGAAGAAGTTTATTAGGGTTAATAATTATGTTGTGTACAACCAACA
AGAGGCTGGCAAATATGAGAATCATACTGAGAATGCGTTAATGTTGTATATGGCGTGTACGCA
CGCCTCTAATCCCGTGTATGCTACATTGAAGATACGGATCTACTTCTATGACTCTGTATCGAAT
TAATAAAATTTAAATTTTATTGAATATGATGGGCCTACATACACAATGTGATGGAGTACATCG
TATAATACATGTTTAAAGGCCCTAACTACATTATTTATACTAATGACTCCTAATCTATCTAAAT
ACTTAATAACTTGAGTCTTAAAGACTCCTAAGAAATGACCAGTCTGAGGTTGTGAGGTCATGC
AGATCCGGAAGCCCATGAAACACTTGTGTATCCCCAGCGCTTTCCTGAGGTTGTGGTTGAACT
GGACTCTGACGGTGATGATGTCGTGGTTCATCAGGAATGGCCTGTTGTCGTGCTCTGTTACTTT
GAAATATAGGGAATTTGGGACCTTCCAGGTATACACGCCATTCTCTGCTTGAGCTGCAGTGAT
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ATAATTGTGTGGTCCCAAAGACCTGGCCCACATTGTCTTGCCTGTACGACTATCACCCTCAATG
ACGATACTTTTAGGTCTCAATGGCCGCGCAGCGGGACCCATCAGATTTTCAGAGGCCCATTCC
TCTATGGCCTCTGCAACCCGATCGAATGAAGAATAAAGAAATGGGGGAACATAACCCTCCAC
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AGTCGATGTAATCACCGTCCTTCTGGATGTAGGACTTGACATCTGAGCTGGACTTAGTTCCCTG
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CATTTGCTCTTTGGGTATTGCCCCTTTTGGAAAAACAAGGAAAATTTTTTTGGCTTTAATTTGG
CACTGATGAGCACGAGGCATTATGAATGGGGGGAAAATTCAAACTCCGTTCAAAAGGGCACC
CTGTGTACCTATTTATATCGAGCTCCCAAATGGCATTATCGTAATTTGGGGAAATAATTCAAA
ATCCCCACGCTCCAAAAAGCGGCCATCCGTATAATATT

> MH674437 [Papaya leaf crumple virus isolate PSB-32], DNA-A complete genome

ACCGGATGGCCGCGCAAATTTTTAGGTGGGCCCTCAACCAATGAAATTCACGCTACATGGCCT
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GACCCACTATTGAATGAGGTTCCAGAAACTGTTCATGGGTTTATGTGCATGTTGGCAGTGAAA
TACCTCCAGCTAGTAGAAAATATGTATTCCCCAGACACTCTGGGATACGATTTAATTAGGGAT
TTAATTTCAGTTATTAGGGCTCGGAATTATGTCGAAGCGAACAGCAGATATCATCATTTCAAC
GCCCGCCTCGAAGGTATGCCGCCGCCTCAACTTCGACAGCCCATATGCGAGCCGCGCTGCTGC
CGCCATTGTCCGCGTCACAAAAGCAAGGGCCTGGACCAACAGGCCCATGAACCGAAAGCCAA
GGATGTACAGGATGTATAGGAGCCCAGATGTTCCAAGGGCCTGTGAAGGCCAGTGTAAGGTC
CAGTCATTTGAGACCAGACATGATATCCAGCACATTGGTAAAGTCATGTGTGTTAGTGATGTT
ACTCGTGGTATTGGGCTGACCCACAGGGTTGGCAAGAAGTTCTGTGTGAAGTCCGTCTATGTA
TTGGGCAAGATTTGGATGGATGAGAACATCAAGACTAAGAATCATACCAATAGTGTTATGTTA
TCCCTTGTTAGGGATCGTAGGCCTGTTGACAAGCCTCAGGATTTTGGTGAGTCTTTCAACATGT
TCGATAATGAGCCCAGCACGGCTACTGTGAAGAATGTACATCGAGATAGGTACCAGGTTCTA
AGGAAGTGGCATGCAACTGTGACAGGCGGTCTATATGCATCGAAGGAGCAGGCTCTCGTGAA
GAAGTTCGTTAGGGTTAATAATTATGCCGTGTACAACCAGCAAGAGGCTGGCAAGTATGAGA
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TTGGTCAATCGGTACTCAGCATTAGTCCTACTGGCAATTGGTGATCAGTACTCAATATATAGT
GAGTACCAAATGGCAGAATTGTATTTTGGAAAAGAAAATTACTTTAATTCAAATCCCCATAAA
GCGGCCATCCGTATAATATT

> MH765694 [Tomato leaf curl virus isolate PSB-33], complete DNA-A genome

ACCGGATGGCCGCGAATTTTTATGTGTGCCCGTCAACGCACTAACGGACAAGGACATGCTATC
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ACTCTGACGGTGATGATGTCGTGGTTCATCAGGAATGGCCGGTTGTCGTGGTCTGTTATCTTGA
AATATAGGGGATTTTGAATCTCCCAGATAAACACCCCATTCATTGCCTGAGCTGCAGTGATGA
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CGATTGGAGGCCGCCATATTGGCTTGGTCAATCGGTGTCTCTCAACTATCTCTATGTATCGGTG
TACTGGAGTCCTATATATATAGAGACTCTAATAACATAATTGTAATAAAAGAACTTTAATTTG
AAATTCAAAATGAAAGGCTAAAGCGGCCATCCGTATAATATT

> MH807205 [Papaya Leaf Curl Virus isolate PSB-34], Complete DNA-A genome

ACCGGATGGCCGCGATTTTTTTTTGAGGGCCCGGCACCGCACTAACTGACAAGGACATGTCGG
TATCGATAAAAAGACCTCCTCAGAGCTTAATTGTTTCGTGGTCCCCTATTTAAACTTCGCCACC
AAGTAGTGCATTGCGCACTATGTGGGATCCATTGTTAAACGAGTTTCCCGAAACCGTTCACGG
TTATAGATGTATGTTAGCAGTTAAATATCTGCAGTTAGTAGAGAAGACTTATTCTCCTGAGAC
ATTAGCGCTCGATTTAATTAGGGATTTAATTTCAGTTATTAGGCCTAGAAATTATGTCGAAGC
GACCAGCAGATATATTCATTTCCCCGCCCCCTTGGAAGGTACGCCGTCGTCTCAACTTGGCCA
GCCCATATGTGACCCGTGCTGCTGCCCCCATGTTCCGCGTCACCAAAGCAAATCCATGGGCCA
ACAGGCCCATGTACCGGAAGCCCAGGAGTTACAGGATGTTCAGAAGCCCAGAGGTCCCTAGG
GGCTGGGAAGGTCCAGTTAAGGTCCAGTCTTTGGATGCGAAGAATGATTTTGATCACATGGGT
AAGGCCATTTGTCTTTGTGATGTTACTAGGGGTATTGGGCTGACCCATCGAGTAGCGAAACGG
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CTGCTGAAGCATCATTAGCTGTCTGTTGACCTCCCCTAGCAGATCTTCCATCGATCTGAAACTG
ACCCCAGTCGATGTAGTCACCGTCCTTCTGGATGTAGGACTTGACATCTGAGCTGGACTTAGC
TCCCTGAAAGTTTGGATGGAATTGGGTGGACGTATTAGGGTGAGCGACATCGAAAAGTCTAG
GGTTTCGGAACTTGTATTTACCTTCGAATTGGATGAGGACATGGATATGAAGAGACCCATCTT
GGTGTATTTCTTGGGACACTCTATATAATTTATCAGAAGGACAGGTAACGTTTTGTAGTAGTTC
GAGCATTTGCTCTTTCGGTATAGAGCATTTTGGATAAGTAAGGAAGATATTTTTGGCATTTATA
CAGAAAGAGTTAATACGAGGCATATTGGATTGGAGGACGCTCAAAACTCTGAGGAATGGGAG
ACTCCGGGGACGCATTTATATGGTGTCCCTAAATGGCATTTATGTAATTTGGTAAAGTAATTC
AAAATCCTAACGCTCCAAAAAGCGGCCATCCGTATAATATT

> MH765695 [Pedilanthus Leaf Curl Virus isolate PSB-37], Complete DNA-A sequence

ACCGGATGGCCGCGCTTTTTTTATGCCCCCCACAGAGCACTAACTGACAATGACATGTGGACC
AATGAGAATGGTTCCTCATAGCTTAATTAATTCATGGTCCCCCGTATAAACTTAGTGCGCGAG
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ACGCTTAGGAGGAGACATTGAATTGGTCAAATGGGTATCAATTGGGTTCTGGATGTTAATCAC
CTGTATATCGGTACTCAATATATAGTGTGTACCAAATGGCATAATTGTAATAAAAGAACTAAA
ATTTGAAATTCAAACGAAAAGGCTAAAGCGCCCATCCGTCTAATATT

> MH765696 [Croton yellow vein mosaic virus isolate PSB-38], Complete DNA-A genome

ACCGGATGGCCGCGAAAAAAAATGTGGACCCCACGGCGCACTAACTGACAAAGACATCACGA
CCAATGAAAAGAGCTCCTCAGAGCTAATTTTGTTTTGTGGTCCCCTATTTAAACTTCGCCACCA
AGTAGTACATTGCGCACTATGTGGGATCCATTAGTTAATGAGTTTCCGGAAACCGTTCACGGG
TTTAGATTTATGTTAGCAGTTAAATATCTGCAGTTAGTAGAGAAGAGTTATGCTCCTGACACA
TTGGGACACGATTTAATTAGGGATTTAATTTCAGTAATTAGGGCTAGAAATTATGTCGAAGCG
ACCAGCAGATATAATCATTTCCACGCCCGCTTGGAAGGTACGCCGTCTTCTCAACTTCGACAG
CCCATATTTGAACCGGGTTGCTGCCCCCATTGTCCGCGTCACCAAAGCAAAGGCATGGGCGAA
CAGATCCATGTCCCGGAAGCCAAGGATGTACAGGATGTACAGAAGCCAAGATGTCCCTAAGG
GATGGGAAGGTCAATGGAAGGTGCAGTCTTTGGATGCGAAGAATGATTTTGGTCTCATGGGTA
AGGCCATTTGTCTTTGTGATGTTACTAGGGGTATTGGGCTGACACATCGAGTAGCGAAACGGT
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GCCAGTCGATGTAGTCACTGTCCTTCTCGATGTTGCACTTGACATCAGGGCTGGACTTTGCTCC
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GCTGAAATTGGCTTTACCTTGGAACTGGATGAGGGCATGGATATGCAGAGACCCATCTTGGTT
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AGCATTTGCTCTTTGGGTATTGGGCATTTTGGATAAGTGAGGAAGATATTTTTGGCATTAACAC
AAAAAGAGATAATACGAGGCATATTGAATTGGGGACACTCAAAACTCTGAGGAATGGGGGAC
TCGCGGGACGCATTTATATGGTGTCCCCAAATGGCTTTTCCGTAATTACGAAAGAATTAATTC
AAAATTCTCACGCTCCCAAAAGCGGCCATCCGTATAATATT

> MH765697 [Chilli Leaf Curl Virus isolate PSB-42], Complete DNA-A genome

ACCGGATGGCCGCGATTTTTTGTGTGGGCCCCCACACACGCGCTAACTGACAATGACATGTGG
ACCAATGAGAATGGTGGTGCTTGGTCTCATTGTTTTGTGGTCCCCCCTATAAATTAGTCCCCAA
ATATTGGTCTTTATCCACAATGTGGGATCCGTTAGTGAACGAGTTTTCCGAAACCGTTCACGGT
TTTAGGTGTATGCTAGCAGTTAAATATCTGCAGCTAGTAGAAAATACGTATTCCCCAGATACT
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GATGTGAAGGCCCATGTAAGGTCCAGTCATTTGAGTCCAGACCTGATATCCAGCACATTGGTA
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CAAGAGGCTGGCAAGTATGAGAATCATACTGAGATTGAGTTGTTGTTGTATATGGCGTGTACC
CACGCCTCTAACCCTGTGTATGCTACATTGAAGATACGGATCTACTTCTATGATTCAGTATCGA
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CTTTGGTTGCTTATTATATATCGAGCTCCCAAATGGCATTATCGTAATTTGGGGAAATAATTCA
AAATCCCCACGCTCCAAAAAGCGGCCATCCGTATAATATT

>MG757245 [Tomato Leaf Curl Gujarat Virus Lucknow isolate PSB-43], DNA-A component, complete sequence

ACCGGATGGCCGCGATTTTTTTTTTTGGACCCCACAACGCGCTTACTGACAAAGACAAGTGGA
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AGGTGTATGCTAGCAGTTAAATACCTGCGGCTAGTAGACAATTCGTATTTCCCAGACCCTGTG
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GCAGAGATCATCATTTCCACGGCCGCTTCGAAGGTACGCCGGCGTCTCAACTTCGACACCCCA
TCGGTGAGCCGTGCTGTTGCCCCCATTGTCCGCGTCACCAAAGCAAAGGCATGGGCCAACGGG
CCCATGTACAGGAAGCCCAGGATCTACCGAATGTACAGGAGCCCTGATGTCCCTAAGGGCTGT
GAGGGCCCGTGTAAGGTCCAATCGTTCGATGCTAAGAACGACATTGGTCACATGGGTAAGGT
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GCGATTGGCGGCTGCCATGTTGACTTGGTCAATCGGTGTCTCTCAACTCTCTCTTTTGTATCGG
TGTATTGGAGTCCTATATATATGGAGGACTCTAATGGCATAAATGTAAATATTGAACTTTAAA
TCAAAACCCTTACGCTCCAAAAAGCGGCCATCCGTATAATATT

> MH765698 [Chilli Leaf Curl Virus isolate PSB-45], Complete DNA-A genome

ACCGGATGGCCGCGATTTTTTTTGTGGCCCCCACTGCGCACTAACTGACAATGACATATGTAC
CAATGAGGATCGTTCCTCATCGCTTCATTGTTTCGTGGTCCCCCTATAAACTTAGTGCGCAAGT
ATTGGTCTTTAGCCACAATGTGGGACCCATTAATAAACGAGTTTGCCGAAACCGTTCACGGGG
TTAGGTGTATGGTGGCAGTTAAATATCTGCAGCTAGTAGAAAATACGTATTCCCCAGATTCGG
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CCAGCAGATATAATCATTTCCACACCAGGCTCGAAGGTTCGACGTCGTCTGAACTTCGGCAGC
CCATATGCCAGCCGTGCTGCTGTCCCCATTGTCCGCGTCACAAAGGCAAGAGCATGGACCAAC
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> MH807200 [Papaya Leaf Crumple Virus isolate PSB-47], Complete DNA-A genome

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AGATGGAAGATACGGATCTACTTCCATGACTCTGTATCGAATTAATAAAATTTAAATTTTATA
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GAACTCTTTAAAGTGCTTTAGGTAGTGGGGGTCTACGTCATCAATGATGTTATACCATGCGTC
ATCTGACTTTATCTTGGGACTCAGGTCTAAATGACCACACAGATAATTGTGTGGACCCAGTGA
CCTGGCCCACATCGTCTTCCCCGTTCTACTATCACCCTCTAAGACAATACTTTTAGATCTCAAT
GGCCGCGCAGCGGTACCCATCACATTTTCAGCAGCCCATTCCTCTATGGCCTCTCGGCACCTG
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TAAATTATTTTTTAAATTATGATATTGTAAAATATAATCTTCTGGGGCTAACTCCCTAATAATT
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CTGGATATAGGACTTGACACCTGACCTGCACTTAGCTCCCTGAATGTTCGGATGGAAATGTGC
TGACCTGGTTGGGGATACCAGGTCGAAGAACCTGTTATTTTGGCACTTGTATTTTCCTTCGAAC
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CATGGTCAATCGGTACTCAGCACTAGTCCTATGGCAATTGGTGATCAGTACTCTATATATAAT
GAATACCAAATGGGAGAATTGTAATTTGAAGAAGAAAATTACTTTAATTCAAATTCCCATAAA
GCGGCCATCCGTATAATATT

> MH807204 [Papaya Yellow Leaf Curl Virus isolate PSB-51], complete DNA-A genome

ACCGGATGGCCGCGATTTTTAAATGGCCCGCGCAACGCAAAATCTGACAATGACATGTGGAC
CAATCACTTTGGCTGGTCGTGGGTTAATTTTTTTGTGGTGGCTTATTTAAAGTTGCTCACCAAG
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TTAAGATGTATGTTAGCAGTTAAATATCTGCAGTTAGTAGAGAAGACTTATTCTCCCGATACA
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ACCAGCAGATATAATCAATTCCACGCCCGCTTCGAAGGTACGCCGCCGTCTCAACTTCGACAG
CCTATATGCGAGCGGTACTGCTGCCCCAATTGTCCGCGTCACAAAGGCAAGGGCATGCGCGA
ACAGGCCAATGTACAGGAAGCCCAGGATGTACAGGCTGTACAGAAGTCCGGATGTTCCTAGG
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AAAGTTATGTGTGTTAGTGATGTTACTCGTGGTATTGGTTTGACCCACAGGGTTGGCAAGAGG
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CAAGTTCTTCTGGAACTTGATCGAAGGAAGATGAAGAAAAGGGGAAAACATAAACCTCCATT
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TCGAGGGTGTCTCCGTCCTTCTCCATATAGGACTTGACATCTGAGCTCGATTTAGCTCCCTGAA
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ACTTGAATTTTCCTTCGAATTGGATAAGCACGTGAAGATGAGGAGCCCCATCTTCGTGTATCT
CTCTGCAGATCTTGATGAATTTTTTGGATGGTTGGGTTTGGGTATTTAACATTTGGGAAAGTGC
CTCTTCCTTAGTAAGTGAGCACTTAGGATATGTGAAGAAATAATTTTTGGCATTTATTTGAAAC
CGCTTGGGGGTCATATTGACTTGGTCAGAGGATCGGGTATCTCAGACTGGACTCTATTATCGG
TGAATTGGTACTCAATATATAGTGAGACTCAAATGGCAATTTGGTCATTTTGAACAAATTATT
CAAGATCCTAAAGCCCCCATCAGCGGCCATCCGTATAATATT

> MH807201 [Papaya Leaf Crumple Virus isolate PSB-60], Complete DNA-A genome

ACCGGATGGCCGCGCAAATTTTTAGGTGGGCCCTCAACCAATGAAATTCACGCTACATGGCCT
ATTTAGGGCGGGGGAACCAATAAATAGACTTGCTCACCAATTTTGTATTGACAAACATGTGGG
ACCCACTATTGAATGAGTTTCCAGAAACTGTTCATGGGTTTAGGTGCATGCTAGCAGTGAAAT
ACCTGCAGCTAGTAGAAATTACGTATTCCCCAGACACTCTGGGATACGATCTAATTAGGGATA
TAATTTCAGTTATTAGGGCTCGGAATTATGTCGAAGCGACCAGCAGATATCATCATTTCAATG
CCCGCCTCGAAGGTACGCCGCCGTCTCAACTTCGACAGCCCATATGCGAGTCGTGCTGCTGCC
CACATTGTCCGCGTCACAAAGGCAAGGGCCTGGACCAACAGGCCCATGAAACGAAAGCCCAG
GATGTACAGGATGTACAGAAGCCTAGATGTTCCGAGGGCCTGTGAAGGCCCATGTAAGGTCC
AGTCATTTGAGTCCAGACATGATATCCAGCACATTGGTAAGGTCATGTGTGTTAGTGATGTTA
CTCGTGCTATTGGGCTGACCAACAAGGTTCCCAAGAGGTTCGGTGTGACGTCCGTTTATGGTT
TGGGCAAGATCTGGATTGATGAGAACATCAAGACTAAGACTCATACGAATAGTGTCATGGTTT
CCCTTGTTAGGGATCGTAGGCCCGTTGATAAGCCTCAAGATTTTGGTGAGGTTTTTAACATGTT
CGATAATGAGCCCAGCACGGCGACTGTGAAGAATGTGCATCGTGATAGGTACCAGGTTCTTA
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ATGATCCTCAATTACATTAATTGTGCCCTCAAGTACATCATATAATACATGTCTGAAAGCCTTA
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CGGATGGCTTTACGCTTGGCTAGCCTGTGTTGGACCGTGATGGGTACCTGAGTACAATGGCTC
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TTGGATAAGTGAGGAAGTAATTTTTGGCATATATTTGAAATTGTTTGGGAGGAGCCATTGACT
TGGTCAATCGGTCCCCAGCACTAGTCCTATGGCAATTGGTGAACGGTACCCTATATATAGTGG
GTAACAAATGGCAGAATGGTAATTTGGAAAAGAAAATTACTTAAATTCAAATTCCCATAAAG
CGGCCATCCGTATAATATT

> MH807202 [Duranta Leaf Curl Virus isolate PSB-63], Complete DNA-A genome

ACCGGATGGCCGCGATTTTTTTGTCCCCTCGTGGGTCCCACCAAGGTGGTCCATGGACAAATG
GCCCAATCAAAAACACTTCTTAAAGCTTTATATGATGTGTGGGCCCCATATATAATGACTTGC
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CTGTGGGATATGATTTTATTAGGGATTTGATTTCAGTTATTAGGGCTAGAAATTATGTCGAAGC
GACCAGCAGAGATCATCATTTCCACGCCAGCCTCGAAGGTACGCCGCCGTCTCAACTTCAACA
GCTCATATGCGAGCCGTGCTGTTGCCCCCATTGTCCGCGTCTCAAAGGCAAAGTCATGGGCCA
ACAGGCCCATGAATCGAAAGCCCAAGATGTACCGGATATACAGGAGCCCAGATGTTCCGAGG
GGATGTGAAGGCCCGTGTAAGGTCCAATCATTTGAGTCCAAACATGATATTCAGCACATTGGT
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TTCTGTGCGAAGTCCGTTGATGATCTGGGCAAGATCTGGATGGATGAGAACATCAAGACTAAG
AATCATACGAATAGTGTTATGTTTTTCCTTGTACGTGATAGGCGTTCTGTTGACAAGCCTCAAG
ATTTTGGTGATGTGTTCAACATGTTTGATAATGAGCCCAGCACGGCGAATGTGAAGAGTGTGC
ATCGTGATAGGTACCAGGTGCTTAGGAAGTGGCATGCAACGGTTACAGGCGGTCTGTATGCAT
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CAGTCGATGTAATCTCCGTCCTTCTCGATGTAGGATTTGACATCGGATGAGGACTTAGCTCCCT
GAATGTTTGGATGGAATTGTGTGGATGTATTTGGGTGAGTGAGGTCGAAATGTCTATTGTTTCT
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TTCCTGTGACACTCTGATAAATAATTTATTAGAAGAACAAGAAATATATTTAAGGAGTTCGAG
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AACTGATGAGTACGAGGCATATTGAATTGGGTGCTCTCTAAAACTCTGAGGAATGGAAATCTT
TGGGTGCCTATTATATGGAGCACCCAAATGGCCTTTTCGTAATTTTGACATAAAATACAAAAT
TCAAATTTCAAATCCCAAAAGCGGCCATCCGTATAATATT

> MH807203 [Papaya Leaf Crumple Virus isolate PSB-66], Complete DNA-A genome

ACCGGATGGCCGCGCAAATTTTTAGGTGGGCCCTCAACCAATGAAATTCACGCTACATGGCCT
ATGTAGTGCGGGGGGACCAATAAATAGACTTGCTCACCAATTTTGTATCTCCAAACATGTGGG
ACCCACTATTGAATGAGTTTCCCGAAACCGTTCACGGTCTTAGGTGCATGTTGGCAGTGAAAT
ACCTCCAGCTAGTAGAAAATACGTATTCCCGAGACACTCTGGGATACGATTTAATTAGGGATT
TAATTTCAGTTATTAGCGCTCGGAATTATGTCGAAGCGACCAGCAGATATCATCATTTCAACG
CCGGCCTCGAAGGTACGCCGCCGTCTCAACTTCGACAGCCCATATGCGAGTCGTGCTGCTGCC
CCAATTGTCCGCGTCACAAAGGCAAGGGCCTGGACCAACAGGCCCATGTACAGAAAGCCCAG
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AGTCATTTGAGTCCAGACATGATATACAGCATATTGGTAAAGTTATGTGTGTTAGTGATGTTA
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CATATAGGACTTGACATCTGAGCTCGATTTAGCTCCCTGAATGTTCGGATGGAAATGTGCTGA
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ATAAGCACGTGAAGATGAGGTTCCCCATCTTCGTGGATCTCTCTGCAGATTTTGATGTATTTTT
TATTTACTGGTGTACTTAGGTTTTGAAGTTGGGTGAGTGCCTCTTCCTTAGTAAGAGAGCATTT
AGGATAAGTCAGAAATAAATTTTTGGCATATATTTGAAAACGCTTGGGGGGAGCCATTGACTT
GGTCAATCGGTACTCAGCACTGGACCTATGGCAATCGGTGATCAGTACTCAATATATAGTGAG
TACCAAATGGCAATTTGTCATTTTGAAAAGAAAATTACTTTAAGTCAAATTCCCCATCAAGCG
GCCATCCGTATAATATT

Complete betasatellite sequences identified from this study

> MH825683 [Croton Yellow Vein Mosaic Betasatellite Lucknow isolate PSBB- 14], Complete Sequence

ACCGTGGGCGAGCGAAATTTTGGCCGAAAAAGTGGGCCCCACAAGAGTCTGTTGCATAGAAG
GATGTTCTGTAGCTATTGGGCTTTCTAATTAATTGGGCCTCGGACCGATTAATTTGCAGTTGGG
CCTGTTCGTAGAACATGCACCCATATAATGAAATATATTGGACTGGCTTGAAAATTGCAAGCA
ACATTTAATTTTGTTCAATAAAAATACAAATTACGCATTTACATATTTAGACACAACATATTCA
TCGTCAATATGAATATCGTGTACAGGACCTTCATGTATCATCAAAATATCTATGATTTCAGTCA
TATCTTCCTCCTTGGATTCATCGAATGATGCCATCCTATCCATGATGGCTAATATGGTTCTGAT
TCCCTCTTCCAAATGATTGGAATCAAACGGAAGGATTATCCCTTCGTGGCCATAAGGGATGAT
GAACTTGTCCTTGACAAGAACCGGAGATCTTGTAGAGAATAGCTGAAGTATAACATTGAATG
AGTTGTTACTCTTCAACTTCACATCGATAATGAATTTCAAACCCGTCTCATTCTGATATATGAT
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AATTATGGACATAGATGAAATACGTGTTCTTATAATATTCACATAAATGGAATCCGATAAATT
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GAGCCCCCAATTGAGTCCCCAATATATCCGGGTCTTGGAGGAGGAAATTAACCCCTGATTCCT
AAAATACCCCTGTATCTGTGTCTGGTAGGCGCGTGATAGTGGACTGAAAAAGTAGAGTTTCTC
TCTCCTAAAACTCCTCCGATCTCCGATTCTGGCACTTCCGGTCATCAATTTACGACACGCGCGG
CGGTGTGTACCCCTGGTAGGGTAGGTACCACTACGCTACGCAGCAGCCTTAGCTAGGCCGGAG
CTTACCTCGCCCACGTTCTAATATT

> MH825684 [Papaya Leaf Curl Betasatellite Shahjahanpur isolate PSBB-21], Complete Sequence

ACCGTGGGCGAGCGGTGCCTCTGGTGAACGCAGTGGGCCCCACATCAAACATGAGACTAAAA
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TCAATTACACACTCGCGCAGTTACATTAGTACATACACCATATTCATCAAATATCTGAATATC
AAATACTGGAGCTTCATGCATCATTAATATATCAATTATCTCCGTCATGTCTTCCTGCTTGAAC
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TGAAGTCGAAAGGAGGTATAATCCCATCATGCTTGTATGGAAGCATGAACTTGTTCTTCACAA
GAGCTGGAGATCTTGTGGATGTTAGCTGAATGTAAACCTTAATAGATTCATCACCTCTCAATG
TGACATTGATAGTATATACCAAACCCTTTTGATTAACGTATTTGATAGTCATATTGTTTATGAA
TGTTCTGAATTCATATGATCATTATATACTGAAAGGAAAATCAATATATGATCCATAACAACA
CACGTATTTCATTAATAACCACATGACTAGAGTTCCATATATATTTGATAGTGACACCACTATC
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AACGAAATTAAAAACTGGCTAGAAATGTGAGAAAAAACGAAATTAAAACTTCACGGTTTTGA
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> MH825685 [Luffa Leaf Distortion Betasatellite Jamnagar isolate PSBB- 34], Complete Sequence

ACCGTGGGCGAGCGGAGATCGAAGCTCAAAAGTGGCTCCCGCATGGTTGTGGACTGAAGAAG
AAGAACCATTATAATGGGCTTAAATCAGTAAATCCCAATAAGAAATGTTGGGCCTTGATATGG
CCTGTGTGATATTACGGCCCTGTATGGTGGTAAACGATTGTTTTCGTTAATTATGTCCTTGATC
GGCTGTGTTTGGAATTTAAACGGTGAACGTCTTATTGAACACGTACGGTTCGATTACATCCATT
CGCAAGATTTCTGGGTTTCCAAGTACAAGTATATCTAGTCTTTGAACTATGTCTTCTATCTCGA
TCTCTCCTATCTTTGCTCCGTTGTTTGCGAATAAGAAATTCGCAATGATATTGCCTTCTAATCC
GTTAAAGTCGAATGGAACGTGCAAGTCTCCGTACGTGTACTGGACGAACCCTTCATACTTGAT
GAGCGCTGCTGACATGTTGCATACTATCCGAATGTGAATGAATATTTTCTTGTTCTCCATGATG
CGAACGTCGACTGTGAACTTCACTCCTTGCTTGTTTGATGCGCTGGTGGTCATCTCCGCTTGTG
TGATGGAATTTATCATTATGCCTGCATTTATAGACCTAAACGTGAGATATTTGGGGTTGTGGTG
TGGTTGTGACTGATCCATTATTATGTGATTGTGGATGATTATGCTGAAGTGATGATGGAGATG
TATTACATGTGTTGTGATGCTGACTTGAAATCTTTATACACGGGTTGGTTACGTATATGCCTGT
ATATACGGATAGAAAAAGGATAAGAAAAATGTGGACTGAATTGAAAAGGAAAGAAGAAGAA
AAGAAGAAAAGAACTAATATCAAAGATAAAATGGGAGCGCAGCGGATCGAAACAAAGAACC
CTGGGAAATGAAGGAAATAAAAAGTATACAAAAAATAAATCTCGAAAACGTCATCGTTTGAG
ATGGAAGGGTAAAAAAAGAAAAAAGAAATGAGGAACCAAAAAAGGACACGTATGAAGGTGA
CTGGGTGGTTTTACCATTTACTGGGCGGTAAATGGTAAATGTGTTAAAAAGGGTTAAAAACAT
GAGACCCCGATAGGTAAATGTACCCCAATATATTGGGGTACAATTGGGGACTCAGAAATTAG
CTTTACTAAAATACCCCTGGTTTTGTTTCTTAGAGGCGCATCGGAGTGCGCCGATAAAGTTAA
CATTCTCTCTCCTATTTTAGGACCTAATGCAATTCCCGGTGATCGGAGTCGAATTCTCCGACAC
GCGCGGCGGTGTGTACCCCTGGTAGGGTAGGTACCACCACGCTACGCAGCAGCCTTAGCTACG
CCGGAGCCTAGCTCGCCCACGTTCTAATATT

> MH825686 [Croton Yellow Vein Mosaic Betasatellite Jharkhand isolate PSBB- 38], Complete Sequence

ACCGTGGGCGAGCGGAGGATTTGGTGTGCAAAAGTGGGTCCCGCATGGTGGTGGACTGAAGA
AGAAGACCATTATAGCTTTTGGGCTTACTTTTCATTAAAGCCTTGATGGGAAGTAACTTGCATT
AGGGTTCTGTGAAATTGAATTGATACTAATAATAAAAAAATTTTGGGCTGGATTGGAAATGCA
AACAAACTTTAATTTCTTCAATAAAAATACATCTTACACATTTACATATTTAGACACATCAGAT
TCATAATCAATATGGATATCATGTACAGGAGCTCCATGCACCATCCAAATATCTATGAGTTCA
ATCATATCCTCCTGCTTGAATTCATCGAATGATGCCATCTTATACATGATGGTTAATATGGGTT
TGATTGCTGTTTCCAAGTTATTAAAATCGAACGGAGGGATTATCCCTTCGTGGCCATAAGGGA
TGATGAACTTGTTCTTGACAAGAGCCGGAGATCTTGTGGAGAATAGCTGAATGATAACATTGA
AGGAGTTGTTACCCTTCAACTTCACATCAATAATGAATTTCAACCCCGTCTCATTCTGATATAT
GATCGTCATTTTAGATATGCAATCACTTAAACCAAAGATCAATTTATAACGAAGTACATAGAT
AATATATGGTCATGAATGAACTACGTGTGCTGACAATATTCACATAAATGGTATCCGATAAAT
CAAATGATAGTGACACCACTATCAAAATACCAATAATTAAAAAAAAAAAACCATAAATGACAA
TTACGTGAGTGATTGTCGTATCAGAAGCAAAAGAAAAAAAAAGAAAATTAATGAAATTGAAC
CAGTAAACCCATCTATTATCCTAAAATGGGAGCGCAGCTAAACAAACCACCGAAACTTGAAG
AAGGCAACTTAAAAACTCATACACCCACAAGCATTAAAACCTAAGAAGGAAGATCAGTGGAC
CCCACTGAATTAAAACGATTAGAAAAAAAAAATGACTTAATCCGTACACATGAGTTATTTACTG
TTTTACTGAATTAAAATTGTAAATGATATTTACCCAAGGGTAAATATCTGAGTCCCCAATAGG
TAAATGTGCCCCCAATTGAGTCCCCAATATATCGGGGACGCGGAGAGAGAAATAAATCCCGG
ATTCCCAAAATACCCCTGTCTCTGTGTCTGGTAGGCGCGTGTGAGTGGACTGAAAAAGGTGAC
CTTCTCTCTCCTAAAACCCCTCCGATCTCCGATTCAGGCACTTCCGGTCGACAATTTGGGACAA
GCGCGCTGGTGTGTACCCCTGGGACCGTAGGAACCGTCGATTAAGGTTACAGTACGCTACGCA
GCAGCGTTAGCTACGCCGGAGCTTAGCTCGCCCACGTTGTAATATT

> MG478451 [Tomato Leaf Curl Bangladesh Betasatellite isolate PSBB-43] Complete Sequence

ACCGTGGGCGAGCGGGGTTTTTGGCGTTCCAAGTGGGTCCCACATTATCCAAGGGAAGAATAT
TGGACTGGGTCAATGCAATTGGGCCGTAAATGAAATGGGCTTGGACCAGTAGATTCGAGACT
GGGCCAATAAAATTAAATAACAAATGGCCTCACAATCAAATCAAAGTTTTTATTCATGTCAAA
CACATTACACATCACACACACACATTCGTACATACATCATATTCATCCCCTATACGTATATCCA
TTAATGGTGCTTCATGCATCATCATTATATCAATAGCCTCTACCATTTCCTCCTGTCGAAACTC
CCAAATACGACGATCCCTGAACATGACCTTCAATATAATACGTATCCCTTCCTCCAAATTGAT
GAAATCCAAAGGTGGTATGATCCCATCATGGCCGGATGGTATCATGAAGGTCGTCTTTGCTAG
GGCTGGTGATCTCGTTGAACGCAATTCGATCGTAACAAGAACTGAATTGTCTTCGGTGATCTT
CACGTCGATGGTAAATTCCATACCCCTCTCGTTACTATATTTGATAGTCATTTGAATGGAGAGA
TACACATGAGTTACATCGTCTTATATAGGTGAGATACATCCACATATAGAGACATCAGTCATT
TACGTTGTTCAATAAGCATCATGACAAGTGGAGGTGATGCATATGGGGTTGACACCTACATGA
CTATCTGTCATTGACAAGAGAAAAGGAAGTGAAGAATACTTATTCATTAAAGGATTAAGGGA
GAAAAAAATAAAAGAAGAAATAAAAGGAAAAGAAAACTAAGAAAAAAATAAAAAAAGATG
TACAATAAAAATGTAAAAAAAACACGAAAGGAAAACACAAACATCAGCACAAACAGAAACC
TCTTGAGAAAAAATGGGAGCGCAGCGTGAAAACCAAGAAAAACAAACCAAGGAAGACACAT
GAGCAAAAAACAGAAAACACAAAACATAACTATGAGGGTCGTACATGAAATTTGAGAAAGTC
CGTACACAGTAATTAATCATTAACTACTGCGCAGTAAATGTGAATAAAATTAACCCAAAGGGT
TAATTTTGGAGACACCAATAGGTAAATGAGTCTCCAATTGAGGCACCGATATATCGGGGCCTC
GGAGAGAGAAATAATTCCCGGATCCCGAAAATACCCCTAAGTCTGTGTCTGGAAGGCGCGTG
GTAATGCGCGGAAAAAGATGACCTTCTCTCTCCTAAAAACTCACCGGAACCTCCAAACTGGCT
GCTGATCCCGGTGTCTATTTCCGACACACGCGGCGGTATGTACCCTTGGTAGGATAGGTACCA
CTACGCTATGCAGCAGCTTAGCTACGCCGAAGCTTAGCTCGCCTACGTTCTAATATT

> MH825687 [Cotton Leaf Curl Betasatellite Lalitpur isolate PSBB- 47], Complete Sequence

ACCGTGGGCGAGCGGGGTCTTTGGCGTTCCAAGTGGGTCCCACAATATCCAAAGGAAAAATA
GTGGACCGGGTCAATGCAATTGGGCCGTGAATTAAATGGGCCTGGACCAGTAGATTCGAGAC
TGGGCCAATATAACCAAATAACAAATGGGCTCATAATCAACTCATAGTCTTTAGTTATGTCAA
ATACGGTACACACTCACACACACACATTCGTACATACATCATATTCATGTGCTACACGTATAT
CAACAAACGGGGCCTCATGCATCATCACTATATCAATAGCCTCTACCTTGTCCTCCTGGCAAA
AGTGCCCAACATAACAATCCCTGTACATGGTCTTCAATATATTATGTATCCCTTCCTCCAAATT
GTTGAAGTGGCAAGGTAGTATGTTCCCATCATGGATGTATGTGATCATGAAGGTCTTCCTTGC
CAGAGCAGGTGATCTGGTTGAGCACAATTCAATCCGCACAAGGATTGAATTGTCCTCGTGGAT
CTTCACGTCGACGGTAATTACCATCCCCCTCTCGTTAGTATGCTTGATTGTCAGTTGCATGTAA
TTATGGACAACACATGAGATGATTCTTCTTAAATAGCGTCCATATATTTGGATATATCGACAT
AATGCATATACGTGGTGCAATAATTATCATATGAATATGAGTGGAGACACATATGATTGATAT
CTACGGGGCTATCAATCATCGTCAAGAAAAAAGGGATAAAAGAAAACTTAATCATGAAAGGA
TTAAGTAGGGAAAAATAAAAGAAGAAATAAAGGGAACCGAAAACCAACAATGAAATAATGA
AGCAAAAAACGAGAAAAAAAGAACACAAGAAAGGAAAACAGAAACATGAGCACAAACAGA
AACCTCTTGAGAAAATATGGGAGCGCAGCGGGAAAACCTAGAAAAACATACCAAGGAAGAC
ACATGAGCAAAAAGGGAAAACACAAACTAAAATAAGAGGGTCCTACATGAAATTTTGGAAA
GTCCGTACGCAGTAATTAATCATTAATTACTCCGCAGTAAATGTGAATAAAATTAACCCAATG
GGTTAATTTAGAGTCCCCAATAGGTAAATGAGTCCCCAATTGATCACCGTCTATATCGGTGCC
TCGGAGAGAGAAATAAACCCCGGATTCCAAAAATACCCTCTAGTCTGTGTCTGGAAGGCGCG
TGGTAATGCGCTTAAAAAGTGGACCTTCTCTCTCCTAAAACTCACCGGAACGGCCAAACTGGC
TGATTCCGGTGTCTATTCACGACACGCGCGGCGGTGTGTACCCCTGGGAGGGTAGAAAACTAC
GCTACGCAGCAGCCTTAGCTACGCGGGAGCTTAGCCCGCCCACGTTCTAATATT

> MH825688 [Tobacco Leaf Curl Betasatellite, Hyderabad isolate PSBB- 51], Complete Sequence

ACCGGGGGCGAGCGGTGTTTTTCGTGTTCGAGGTGGGTCCCACTTTCCGCGGAAGAGGAAAG
GTTGACTGGGTCAATGCAATTGGGCCTCAATTTTATGGGCTTTGGTTATTGGGCTTCGTTATTG
GACTCAAAAAAATATTAAAATTAAAATTTTATTAATATCTCATAGGAATACATTTCGTACACA
TATTTATACACTAGCATTAGTATACACATCGTACAAGTCGTGTACATTAATATCAACCACTGA
GGCTTCGTGCATCATCAGGATATCAATAGTCTCAACCATATCCTCCCGCCGGAATTCCCCAAT
GGTGGAGTCGTTGTACATGATCTTCAACAGATGACGTATCCCCTCCTCAATACTGTTGAAATC
AAAAGGACCTGTGAACCCATGATGGCCGTATGGGATCAAGAACTTGGCCTTGGCTAGTGCCG
GTGACCTTGTTAATATCAGATCCACCTGAACGATGATGGAATTATCGTTCCTCAACCTGACATT
GATAGTAAACTCCATTCCATTCTTATTATAATATTTGATCGTCATTTTATGTGTCTGGGTCTAA
ACATGAGTGTACCCCGTTAATTAGGCTTCATATATCTAGATATATGGACACAAATCAATTACG
TGGTCTTGTCTAGAACACTTGAGTTGTGTGGAGAATGTATGTTGGATAGTGATAATACTATCA
AATATCTAAAAAAAGAAAAAGAAAATAAAATCATAATTATAAATAATTATGACTAATAAAAA
AAGAAAATAACAAAATCTGATTGATAAAACACTAAACACAACCCTAAACAAACACTATCACA
ATCACAATATACATTAAAAAAAATAAAAGGAAGCGCAGCGACGAACCAAGCCAGAACTGAC
AACAACAAAAAAATGTGACAAAAAATAAATTAAAAAAATATAAAAATAATAAACATGAATA
ATTGGGCCCCACAATTATGAATAGTTAAATCCGTACACACAAGTCAGTTACTGTATTACTGAC
ACGGTAAAATAGTAAATCATATTTACCCCGAGGGTAAATATCTGAGTCCCCAATAGGTAAATT
TGACCCCGATATATCGGGGACTCAATTGGAGACAGGAAAGAAAATAATCCGGGATTCCAAAA
TTACCCTTATCTCTGTCTCTGGAAGGCGCGTGGGGGTGCGCTGCAAAAGTAGACTTTCTCTCTC
CTAAAACTCGTCGGGACTCCGATTCTGGCACTTAGGGTCACCAATTTGCGACACGCGCGGCGA
TGTGTACCCCTGGGAGGGTAGGTACCGCTACGCTACGCAGCAGCCTTAGCTACGCCGGAGCTT
AGCTCGCCCCCGTTTCTAATATT

> MH825689 [Chilli Leaf Curl Betasatellite New Delhi isolate PSBB-60], Complete Sequence

ACCGTGGGCGAGCGCGTTCTTTGCCGTTGAAGGTGGGCCCCACACTATGCATAGGAAGACTAA
TGGGCTTACTCAATGCATTTGGGCCATATCTGAAATGGGCTTGGACCAGTAGTTTCGTGACTG
GGCAAACCAAATAAGATAAGAATATTGGCTCACAAACAAAACAAAGGATTTATTTATTTCAA
ATACATACACAGTCACACACACACATTCGTACATACATCATATTCATCTCCTATACGTATATCA
ACTACAGCGGCCTCATCCATCATCAATATATCAATAGATTCTACCATGTCCTCCTGTCGAAACT
CCCGTATAACAGAATCTTTGTACATGATCTTCAATATATTATGTATCCCGTCCTCCAAATTTTT
GAAGTTGAAAGGTGGTATGATCCCATCATGGCCGTATGGGATCATGAAGGTCTTCTTTGCCAG
TGCTGGTGATCGTGTTGAGCACAAGTCAACACGGACAAGAAGTGAATTGTCCTCGTTATTCTT
CACGTTGATGCTAAACTCTATCCCCTTCTCGTTATTATATTTGAGCGTCATTTGCATGTAATTAT
GAACTATACATGAGTCTAGTCGTCTTAAATAGTGTCCATATATCTGGGTATATGGACATAATT
CATTTACGTGGTGCAATAATCATCATATGAATGGGAGTGGAGATAGATATGGTCGATAGTGAC
AAAACTATCAATCATGTGTTCCAGATCAAAAGAAAAATAAAACCTAATCATCATAATGATTAG
GAAAAGAGAAAAGGGAAAATAGAAATGAATTCAAAAGAATTCAGGAGATAAAATAAAAAAA
AAATGAAGAAAAATAAAACTCTAAAACTATGAACCAGAAACATGAGCAGAAACAAAAATCT
CATGAGAAAATATGGAAGCGCAGCGTACGAGATCCCAAAACAGAAAGAAACGGAGATCATG
AGGAAAAAAGAAAAAACACAAAACTATACTAAGAGGGTCGTACATGAAAATACACAAAGTC
CGTACACAGTAATTAATCATTAATTACTGTCCGGTAAAAGTCAAGACAATTAACCACCGGTTA
ATTGCTGAGTCTCCAATAGGTAAATGTGCCACCAATTGTGTCCCCCATACATTGGGGGCTCGG
AAAGAGAAATAAATCCCGGATTCCCAAAATACCCTTAAATATGTGTCTGGAAGGCGCGTGGT
AATGCGCTGAAAAAGGTGACCTTCTCTCTCCTAAAACTCACGGGAACCTCCAAACGGGCTGAT
TCCGGTGTCAATTTCGACGCGCGCGGCGGTGTGTACCCCTGGGAGGGTAGGTACCACTACGCT
ACGCAGCAGCCTTAGCTACGCCGGAGCTGAGCTCGCCCACGTTCTAATATT

> MH825690 [Tomato Leaf Curl Betasatellite, New Delhi isolate PSBB- 63], Complete Sequence

ACCGTGGGCGAGCGGGGTCTTTGGCGTTCCAAGTGGGTCCCACAATATCCAAAGGAAAAATA
GTGGACCGGGTCAATGCAATTGGGCCTGAATATGAAATGGGCCTGGACCAGTAGATTCGAGA
CTGGGCCAATATAACCAAATAACAAATGGGCTCATAATCAAATCATAGTCTTTAGTTATTCCA
AATACATTACACACTCACACACACACATTCGTACATACATCATATTCATCCCCTACACGTATAT
CAACTAATGGGGCCTCATGCATCATCACTATATCAATAGCCTCTACCATGTCCTCCTGTCTAAA
GTGCCCAACATGACAATCTCTGTACATGGTCTTCAATATATTATGTACGCCTTCCTCCAAATTG
TTGAACTCGAACGGTAGTATGATCCCATCATGGCCGTATGAGATCATGAAGGTTTTCTTTGCC
AGGGCTGGTGATCCTGTTGAGCACAATTCAATCCTGACAAGAACTGAATTGTCCTCGTTGATA
TTCACGTCGACGGTAAATACCATCCCCTTATCGTTATTATACTTGATCGTCATTTGCATGTAAT
TATGAACAAAACATGAGATGAATCTTCTTAAATAGCGTCCATATATCTGGATATATGGACATA
ATGCATATACGTGGTGCAATAATTATCATATGAATATGAGTGGAGACACATATGATTGATATC
TACGGGACTATCAATCATCGTCAAGAAAAAAGGAGATAAAAGAAAACTTAATCATGAAGGGA
TTAAGTAGGGAAAAATAAAAGAAGAAATAAAAGGAAAAGAAAACCAACAATGAAATAATGA
AGCAAGAAAAAATTAAAAAATAACACAAGAAAGGAAAACAGAAACATGAGCACAAACAGAA
ACCTCTTGAGAAAATATGGGAGCGCAGCGGGCAAACCTTGAAAAACAAACTAAGGAAGACAC
ATGAGCAAAAAAAGGAAATACAAAACTAAAATAAGAGGGCTTTACATGAAATTTTAGAAAGT
CCGTACGCAGTAATTAATCATTAATTACTCCGCAGTAAATTTGAATAAAATTAACCCAATGGG
TTAATTTTAGAGTCCCCAATAGGTAAATGAGTCCCCAATTGAGTCACCGTTATATCGGTGCCTC
GGAGAGAGAAATAAATCCCGGATTCCCAAAATACCCCTCTGTCTGTGTCTGGAAGGCGCGTG
GTAATGCGCTTAAAAAGGCGACCGTCTCTCTCCTAAAACTCACCGGAACCTCCAAACTGGCTG
ATGCCGGTGTCAATTCACGACACGCGCGGCGGTGTGTACCCCTGGGAGGGTAGAAACCACTA
CGCTACGCAGCAGCCTTAGCTACGCCGGAGCTTAGCTCGCCCACGTTCTAATATT

> MH825691 [Tomato Leaf Curl Betasatellite, Bhopal isolate PSBB- 66], Complete Sequence

ACCGGGGGCGAGCGATGTTTTTGGTGTTCGAGGTGGGTCCCACTTTCTTCTGAAGAAAAAAGG
TTGACTGGGTCAATGCAATTGGGCGGGCGATTTGATGGGCTTTGGTTAATGGGCTTCGTTATT
GGACTCTAAAATATTAAAATTAAAATTTTATTAATATCTCATACAAATACATCCGTACACGTA
TTCATACACTTGCACTTGTATACACACTAAATGAATCATGTACATTAATATCAATCACTGGAG
CCTCGTGCATCATCAGGATATCAATAGTCTCGACCATGTCCTCTTGCCGGAATTCTCCTGTGGG
TTTATCCTTGTACATGACCTTTAACAGCTGACATACCCTTTCTTCAAGACGGTTGAAGTCAAAT
GGACCTGTGAACCCATGATGGCCGTATGGGATCAAGAACTTGGTCTTGGCTAGTGCCGGTGAC
CGTGTGGATACCAAATCCACCTGAACGATGATGGAATAATTGTTGGTCAACCTCACATTCACA
GTGAACTCCATCCCTCTCTTATTATCATATTTGATCGTCATTGTTAATTCTGTGGGTCTAAACAT
GAGTTTAGTCCCTTTAAATAGGGTTCATATATCTGGATTATGGACCAAGTCAATTACGTGGTCT
GGTCTATGTCACATATATTATGTGAAGACCTTATGTTTGATAGTGATAACACTATCAAATATCT
AAAAAAATAAAAAGAAAAGAAAATCATAATTATAACATAATTAAGAACAATAAAAAAAGAA
AAAAAAAAACAAACACTAAATAACTTAAACAATAAACACTATCCAAAACCTAAACAAACACT
AACACAATCACAATATATATTAAGAAAAGAAAAGGGAGCGCAGCGACGAAACAAAAAAGAA
CTGACAACAACAAAAAAGAAATCATAAAAAATAAAAAGAAAAGAAAAAAACAAAATCATGA
ATAATTGGGCCCTACAATTATGAATGGTGAAATCCGTACACATGAGTCATTTACTGTTTTACTG
CACGGTAAAATAGTAAATTATGTTTACACCAAGGTAAATATTTGTACACCAATAGGTAAATTG
AACACCGATACACCGATACATCGGTGTTCAATCGGGTACTCATAAAAGAATAAGTCTTAGACC
CTAAAATACCCCTATCTCTGTGTCTGGAAGGCGCGTGGGAGTGCGCTGCAAAAGTAGAGTTTC
TCTCTCCTAAAACTCATCGGAACTCCGATTCCGGCACTTCCGGTCACCATTTTACGACACGCGC
GGCGGTGTGTAACCCTGGGAGGGTAGGTACCATGGTACACGCTACGCAGCAGCCTTAGCTAC
GCCGGAGCTCAGCTCGCCCCCGTTCTAATATT

Details of the genomic sequences used for phylogenetic studies of all begomoviral genomes (DNA-A as well as whole genome sequences) reported and as retrieved from the GenBank.

Accession No.	Begomovirus name	Host	Place	Year	Size (bp)
AF314531.1	Pepper leaf curl Bangladesh virus	Chilli	Bangladesh: Bogra	2003	2753
Y15934.1	Papaya leaf curl virus	Papaya	India :Lucknow	2005	2746
FM877858.1	Chilli leaf curl India virus	Capsicum sp.	India:North India	2010	2755
JN135233.1	Papaya leaf curl virus	Amaranthus cruentus L.	India:Lucknow	2011	2746
FN543112.1	Croton yellow vein virus	Croton glandulosus	Pakistan:Punjab	2011	2744
HM140367.1	Papaya leaf crumple virus	Carica papaya	India:Haryana	2012	2736
HM140368.1	Papaya leaf crumple virus	Carica papaya	India: New Delhi	2012	2736
HM140369.1	Papaya leaf crumple virus	Carica papaya	India: New Delhi	2012	2736
HM140370.1	Chilli leaf curl virus	Carica papaya	India: New Delhi	2012	2763
HM140371.1	Chilli leaf curl virus	Carica papaya	India: Uttar Pradesh	2012	2762
DQ989326.1	Chilli leaf curl virus	Papaya	India: New Delhi	2012	2764
FN645898.1	Croton yellow vein mosaic virus	Acalypha sp.	India: Haryana	2012	2760
JN807764.2	Pedilanthus leaf curl virus	Crape jasmine	India	2013	2764
FM955601.1	Papaya leaf curl virus	Rhynchosia capitata	Pakistan: Kundian, Mianwali	2013	2754
KP164863.1	Tomato leaf curl Gujarat virus	Tomato	India: Rahuri, Maharastra	2015	2758
KR071789.1	Papaya leaf crumple virus	Glycine max (soybean)	India: Lalitpur	2015	2736
KR052159.1	Papaya leaf crumple virus	Papaya	India:Mohali	2015	2736
KT948069.1	Duranta leaf curl virus	Duranta repens	Pakistan: Bhera	2016	2759
KT948070.1	Chilli leaf curl India virus	Duranta repens	Pakistan: Bhera	2016	2753
KT253644.1	Papaya leaf curl virus	Cluster bean	India: Gujarat, Jamnagar	2016	2756
KX951415.1	Chilli leaf curl virus	Mirabilis jalapa	India	2016	2755
KX168427.1	Pedilanthus leaf curl virus	Daucus carota (carrot)	India	2017	2764
KX302711.1	Papaya leaf crumple virus	Papaya	India:Kolkata	2017	2736
KX353622.2	Papaya yellow leaf curl virus	Papaya	India	2017	2759
KY978407.1	Papaya leaf curl virus isolate	Capsicum sp.	Pakistan: Islamabad	2017	2741
KX302709.1	Papaya leaf crumple virus	Papaya	India:Lucknow	2017	2736
FJ514798.1	Tomato leaf curl virus	Mentha spicata	India: Punjab	2018	2759
FN543099.1	Ageratum enation virus	Zinnia sp.	India:Kangra	2009	2753

Details of the genomic sequences used for phylogenetic studies of all betasatellite sequences reported and as retrieved from the GenBank.

Accession	Betasatellite name	Host	Place	Year	Size (bp)
DQ118862.1	Papaya leaf curl beta	Mungbean	India:Chinthapalli	2007	1367
AM279663.1	Chilli leaf curl virus satellite	Capsicum annum	Pakistan:Sialkot	2009	1370
GU440581.1	Cotton leaf curl virus betasatellite	cotton	India: Lucknow	2010	1366
EU604296.2	Croton yellow vein mosaic betasatellite	Jatropha gossypifolia	India: Lucknow	2011	1315
JX315326.1	Luffa leaf distortion betasatellite	Dolichos	India: Bihar, Samastipur	2012	1352
HM143910.1	Tomato leaf curl betasatellite	Papaya	India: New Delhi	2012	1370
HM143909.1	Tomato leaf curl betasatellite	Papaya	India: New Delhi	2012	1369
HM143911.1	Tomato leaf curl betasatellite	Papaya	India: New Delhi	2012	1370
HM143901.1	Tomato leaf curl betasatellite	Papaya	India: Haryana	2012	1369
HM143902.1	Tomato leaf curl betasatellite	Papaya	India: Haryana	2012	1370
HM143903.1	Croton yellow vein mosaic betasatellite	Papaya	India: Haryana	2012	1349
HM143904.1	Chilli leaf curl betasatellite	Papaya	India:Panipat: Haryana	2012	1369
HM143905.1	Tomato leaf curl betasatellite	Papaya	India:Panipat: Haryana	2012	1373
HM143906.1	Papaya leaf curl betasatellite	Papaya	India:Panipat: Haryana	2012	1333
HM143907.1	Tomato leaf curl betasatellite	Papaya	India:Panipat: Haryana	2012	1375
HM143908.1	Croton yellow vein mosaic betasatellite	Papaya	India:Panipat: Haryana	2012	1358
JQ178364.1	Cotton leaf curl virus betasatellite	Jatropha integerrima	India	2012	1366
KF188707.1	Tomato leaf curl Bangladesh betasatellite	Chilli	India	2013	1362
GU732206.1	Tomato leaf curl betasatellite	Tobacco	India: Bihar	2013	1357
KT390489.1	Croton yellow vein mosaic betasatellite	Hibiscus cannabinus	India: Uttar Pradesh, Mirzapur	2015	1367
JX987089.2	Papaya leaf curl betasatellite	Parthenium hysterophorus	India:Lucknow	2015	1367
KR052158.1	Chilli leaf curl betasatellite	Papaya	India:Mohali: Punjab	2015	1239
KX302715.1	Tomato leaf curl Bangladesh betasatellite	papaya	India:Kolakata	2017	1379
HQ180394.1	Tobacco leaf curl Patna betasatellite	Nicotiana tabacum	India:PUSA	2017	1338
KX302717.1	Chilli leaf curl betasatellite	Papaya	India:Mohali: Punjab	2017	1240
MH359169.1	Papaya leaf curl betasatellite	wild sunflower	India	2018	1364
AJ557441.1	Ageratum yellow vein India betasatellite	Ageratum conyzoides	India:Madurai	2018	1353

List of Publications

I. Research Papers

- **Varun P**, Ranade SA, Saxena S (2017) A molecular insight into papaya leaf curl—a severe viral disease. Protoplasma, 254(6):2055-2070, Springer (**IF-2.457**). https://doi.org/10. 1007/s00709-017-1126-8.

- **Varun P** and Saxena S (2018) Association of tomato leaf curl Gujarat virus and tomato leaf curl Bangladesh betasatellite on papaya showing typical leaf curl symptoms in North India. 3 Biotech, 8:243, Springer (**IF-1.497**) https://doi.org/10.1007/s13205-018-1254-7.

II. Book Chapters

- **Varun P**, Saxena S (2017) Transmission of Begomoviruses. Begomoviruses (Chapter:4). occurrence and management in Asia and Africa. Springer Nature, Singapore. https://doi.org/10.1007/978-981-10-5984.
- **Varun P**, Saxena S (2017) Leaf curl disease of *Carica papaya* (Chapter:7). Begomoviruses: occurrence and management in Asia and Africa. Springer Nature, Singapore. https ://doi.org/10.1007/978-981-10-5984.
- **Varun P**, Saxena S (2018) Role of women scientists in the development of science and society: past, present and future (Chapter:8). Reinventing the women past, present and future. APP Press, Lucknow. ISBN: 9788193260500

III. Abstracts in Conferences/ Smposia

- **Varun P**, Saxena S (2017) Association of different begomoviruses with leaf curl disease of papaya. 58th Annual Conference of Association of Microbiologists of India (AMI-2017) & International Symposium on "Microbes for Sustainable Development: Scope & Applications" (MSDSA-2017). BBAU, Lucknow (16-19 November, 2017).
- **Varun P**, Saxena S (2017) Genome characterization of papaya leaf curl virus (PaLCuV) isolates causing leaf curl disease in papaya. 4th Lucknow Science Congress (LUSCON), BBAU, Lucknow (3-4 March, 2017).
- **Varun P**, Ranade SA, Saxena S (2016) Studies on evolutionary relationship of papaya leaf curl betasatellite DNA, 8th International Geminivirus Symposium & 6th International ssDNA Comparative Virology Workshop", New Delhi, (7-10 November 2016).
- **Varun P**, Ranade SA, Saxena S (2016) PCR validation of putative PaLCuV in symptomatic diseased plants. XL All India Cell Biology Conference & International Symposium on Functional genomics and Epigenomics. Jiwaji University, Gwalior (17-19 November, 2016)
- **Varun P**, Saxena S (2014) Papaya leaf curl geminivirus infecting papaya crop cultivated in India: molecular characterization. 2nd Lucknow Science Congress (LUSCON), BBAU, Lucknow (27- 28 March, 2014).

Association of tomato leaf curl Gujarat virus and tomato leaf curl Bangladesh betasatellite on papaya showing typical leaf curl symptoms in North India

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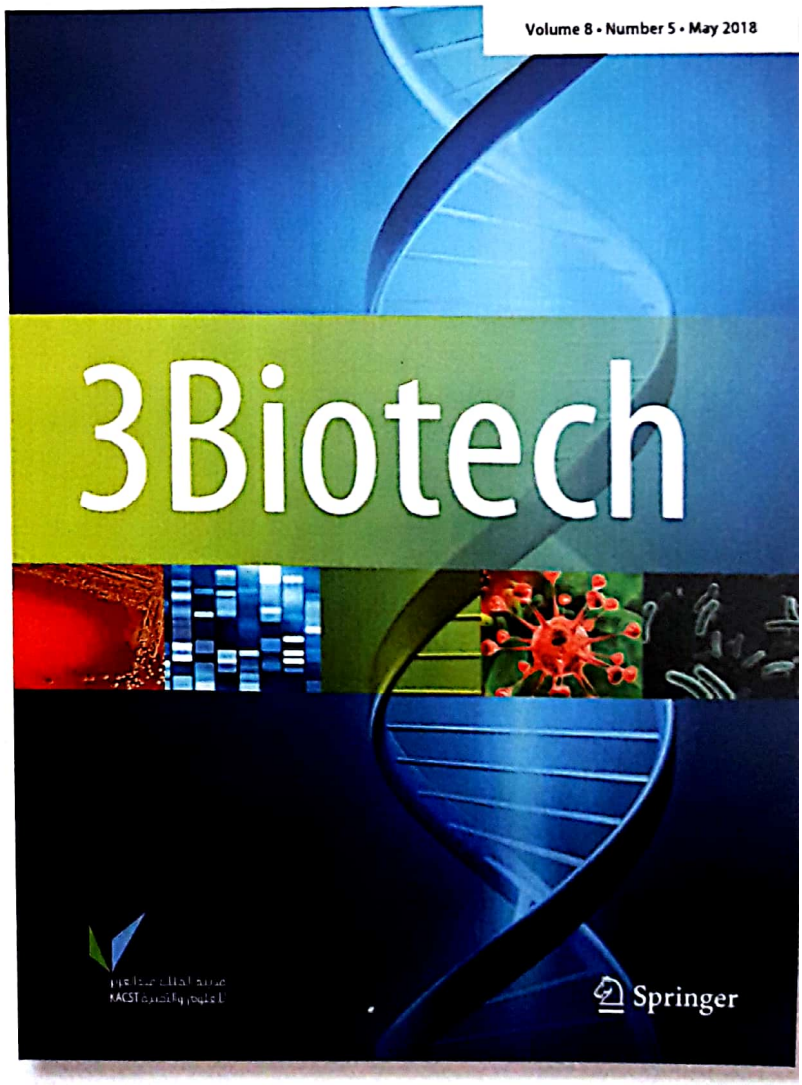

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GENOME REPORT

Association of tomato leaf curl Gujarat virus and tomato leaf curl Bangladesh betasatellite on papaya showing typical leaf curl symptoms in North India

Priyanka Varun[1] · Sangeeta Saxena[1]

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Abstract
Papaya leaf curl is an economically important disease occurring in papaya growing tropical and subtropical areas. Papaya leaf curl virus, a begomovirus, is the main causative agent for the disease, but many other begomoviruses as well as betasatellites have also been reported on papaya leaf curl disease. Rapidly evolving host range of begomoviruses is a major issue for developing successful resistance strategies against begomoviral infection considering their expanding host range and mixed infection. In our study, we have identified the presence of begomovirus and associated satellite molecule on papaya showing severe leaf curl symptoms in Lucknow, India. Analysis of complete DNA-A component of this isolate (MG757245) revealed the highest similarity (91%) with tomato leaf curl Gujarat virus (ToLCuGuV), while sequence data of betasatellite (MG478451) showed maximum (89%) identity with tomato leaf curl Bangladesh betasatellite (ToLCuBB). This is the first report on identification of ToLCuGuV and ToLCuBB coinfecting papaya plants in Lucknow, Uttar Pradesh (India).

Keywords Betasatellite · Coat protein gene · Geminiviruses · Papaya leaf curl disease · ToLCuGuV · ToLCuBB

Introduction

Papaya is a significant horticultural crop of tropical and subtropical areas in the world having a great nutritional and economical importance. India is the major papaya producer contributing 44.6% of global papaya production (http://nhb.gov.in/area-pro/horst_galance_2016.pdf). Leaf curl disease is one of the major threats for papaya cultivation, resulting in huge yield loss and marketability of fruits and is known to be caused by whitefly transmitted geminiviruses (Saxena et al. 1998a). Plant viruses are a group of plant pathogens that have enormous negative impact on horticultural crop production throughout the world. Begomovirus is the largest among all nine genera in the family geminiviridae comprising of whitefly transmitted geminate shaped virus particles having ~ 2.7 kb long circular single-stranded DNA genome (Zerbini et al. 2017). Begomoviruses may have monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genome. DNA-A component encodes the genes required for replication, encapsidation, and control of gene expression and transmission, whereas DNA-B component encode the genes responsible for virus movement. The majority of begomoviruses are frequently found with association of a satellite DNA molecule (i.e., betasatellite) that induces symptom severity in plants infected with begomoviruses. Although DNA-A component of begomoviruses is capable to cause infection, but associated satellites enhance their virulence and increase symptom development of disease on plants. Betasatellites are DNA molecules that depend on 'helper' begomoviruses for their replication and encapsidation. Betasatellites encode a single protein (i.e., βC1) that works as pathogenicity determinant and silencing suppressors (Briddon et al. 2008). Begomoviruses are known to adapt new host very easily in different geographic conditions for their multiplication and survival. As papaya leaf curl virus (PaLCuV), a leaf

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A molecular insight into papaya leaf curl—a severe viral disease

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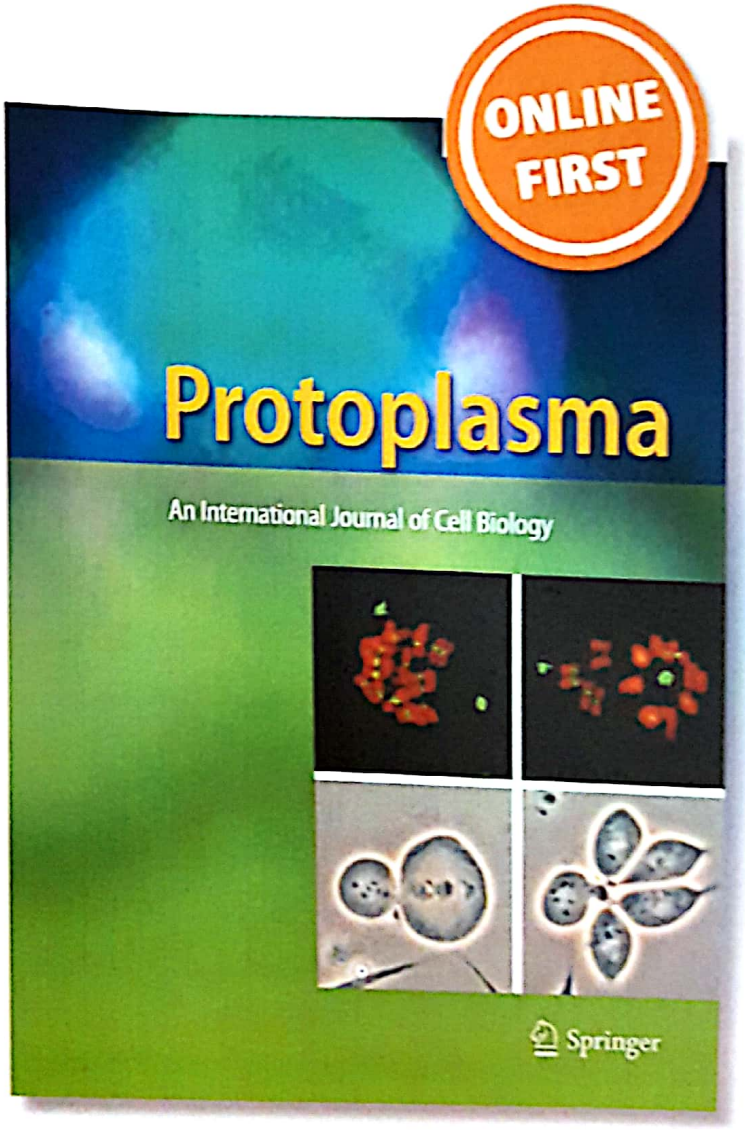

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REVIEW ARTICLE

A molecular insight into papaya leaf curl—a severe viral disease

Priyanka Varun[1] · S. A. Ranade[2] · Sangeeta Saxena[1]

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Abstract Papaya leaf curl disease (PaLCuD) caused by papaya leaf curl virus (PaLCuV) not only affects yield but also plant growth and fruit size and quality of papaya and is one of the most damaging and economically important disease. Management of PaLCuV is a challenging task due to diversity of viral strains, the alternate hosts, and the genomic complexities of the viruses. Several management strategies currently used by plant virologists to broadly control or eliminate the viruses have been discussed. In the absence of such strategies in the case of PaLCuV at present, the few available options to control the disease include methods like removal of affected plants from the field, insecticide treatments against the insect vector (*Bemisia tabaci*), and gene-specific control through transgenic constructs. This review presents the current understanding of papaya leaf curl disease, genomic components including satellite DNA associated with the virus, wide host and vector range, and management of the disease and suggests possible generic resistance strategies.

Keywords Beta-satellite · Geminiviruses · Papaya leaf curl disease · Papaya leaf curl virus · Resistance strategies · Viral genome

Priyanka Varun and Sangeeta Saxena, with a deep sense of gratitude and respect, would like to acknowledge the immense scientific input, able guidance, and contribution to the scientific world by the most beloved Dr. S. A. Ranade posthumously.

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Introduction

Papaya (*Carica papaya* L.; Family Caricaceae), is a widely grown, important tropical fruit crop with fresh ripe fruits being consumed as a dessert and the green unripe fruits being used in salads and as a meat tenderizer. Papaya is native to Central and Southern America and is believed to have originated in Southern Mexico and Costa Rica. Presently, it is naturalized in the Caribbean islands, Hawaii and Florida states of USA, South Africa, India, Philippines, Malaysia, Indonesia, and Australia. Papaya is now cultivated commercially and in kitchen gardens in every tropical and subtropical country. It is a fast-growing plant fruiting within 3 years. However, due to high frost sensitivity, its production is limited to tropical climates and altitudes lower than 1500 m. Globally, India leads in papaya production (reportedly 5.5 million tons during 2013–2014, i.e., 43.7% share of the world production) and is followed by Brazil (11.8%) and Indonesia (7%). Other papaya-producing countries account for the rest of the global papaya production (Papaya production statistics from Food and Agricultural Organization of United Nations 2016; Indian horticulture database 2015). Papaya production in India increased significantly in the last few years, mainly in the states of Andhra Pradesh, Assam, Gujarat, Maharashtra,

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Sangeeta Saxena · A. K. Tiwari *Editors*

Begomoviruses: Occurrence and Management in Asia and Africa

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Transmission of Begomoviruses

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Priyanka Varun and Sangeeta Saxena

Abstract

Transmission is the mechanism of pathogen transfer from an infected plant to another host. Begomoviruses are emerging and economically very important phloem-bound plant pathogens that choose the single species of whitefly, i.e. *B. tabaci*, as vector for their spread in many crops. Mouthparts of whiteflies are designed to detain begomoviruses while feeding on phloem sap of plants. An interaction between mouthparts and coat protein of virus confers *Begomovirus*-whitefly specificity. High-degree conservation of capsid protein of begomoviruses is the main reason for the choice of their vector. Once virus particle enters, it further moves along in the body of vector in a persistent circulative manner and is introduced back into the plant with salivary secretion during next feeding. There are many proteins present inside the vector that facilitate the efficient transmission of begomoviruses. Variations in the begomoviral coat protein can change their vector preferences. Viruses have the ability to manipulate the behaviour of their vector to enhance their transmission; as a result, begomoviruses negatively affect the longevity and fertility of their whitefly vector, whereas behaviour of whiteflies and their feeding habits can also affect the population genetics, behaviour and evolution of viruses. Whitefly-*Begomovirus* relationship is an example of co-evolution, and the studies on transmission mechanism, virus-vector interactions and proteins involved in virus translocation inside the vector can help in developing new virus management strategies.

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Leaf Curl Disease of *Carica papaya*

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Priyanka Varun and Sangeeta Saxena

Abstract

Papaya leaf curl disease is caused by *Papaya leaf curl virus* (PaLCuV), a begomovirus naturally transmitted through whitefly (*Bemisia tabaci*). Main symptoms of papaya leaf curl disease are inward/outward curling of plant leaves, vein thickening, and stunted plant growth with small distorted fruits or no fruits. *Papaya leaf curl virus* is a major threat for the crop production, and the virus has the capability to adapt new plant hosts very rapidly which helps in their host range extension that also has emerged as an evolving risk in papaya production. Whitefly management is the main method to control the spread of this virus so far. Several diagnostic techniques especially molecular techniques have been developed to detect the begomoviruses at early stages of infection to control the further spread of the begomovirus, but so far not much reports are available to control the begomoviral infection at later stage. This chapter provides the information about many aspects like causal pathogen, vector responsible for disease spread/transmission, host range and phylogenetic analysis of virus associated with the papaya leaf curl disease, and different resistance approaches for possible management of the disease.

7.1 Introduction

Papaya (*Carica papaya*) is a widely distributed agricultural crop in the tropical and subtropical regions and has been grown on a wide commercial scale throughout the globe. Papaya is very popular among kitchen gardeners, as packed delicious fruit

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Reinventing the Women

Past, Present and Future

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ole of Women Scientists in the Development of Science and Society: Past, Present and Future

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Vomen in general are born scientists and therefore, play a big role in many ways contributing to the ever development of human society in a scientific manner. Years of growing up as a g female, working hard for an accomplished career , experiencing motherhood, later on looking the family needs and nurturing them leads to women becoming a scientist by all means. and said "Scientist", it doesn't mean only in terms of university degrees rather in all practical t of our everyday life. There are several examples where women even if they haven't taken l college education show exemplary excellence in the area of physics, chemistry, biology, ulture, medicine and many others which she comes in touch with. On a lighter note, few very sting examples to prove the point from daily household routine of women. While fixing the meals as an experienced chemist a woman is able to check the color, smell, ionic strength of s, viscosity of daal, homogenization of chutney, permeability of soups and juices, strength and ntration of coffee all sans equipments and machines.

Now have a look at her as a microbiologist when she precisely adds yeast and lactobacillu ms to dough, batter and milk for fermentation and curdling of milk respectively. Not only tha t depend on cfu counts or gram+ve /-ve test to declare that water is contaminated an sumption.

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