Cloning and Partial Characterization of Begomovirus(es) from

Duranta repens

By

Neelam Urooj

MS Industrial Biotechnology

Registration No: 00000205172

Supervisor

Dr. Najam us Sahar Sadaf Zaidi

Atta- ur- Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST),

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In

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Declaration

I certify that this research work titled "**Cloning and Partial Characterization of Begomovirus(es) from *Duranta repens***" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Neelam Urooj

Dedication

To my lovely Parents who always encouraged me to work hard and supported me to achieve goals.

To my Supervisor, without her inspiration, coaching and enthusiasm none of this would have been possible.

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All praises to **Almighty ALLAH** the most merciful, who is the Lord of this universe, Creator of mankind and king of wisdom. He gifted us with the ability to observe, think and research by providing us dynamic sense. All respect and honors to the **Holy Prophet Hazrat Muhammad (Peace be upon Him)**. The eternal educator, the everlasting source of leadership, the torchbearer of truth and messenger to the whole world.

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Table of Contents

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Tables

List of Figures

ABBREVIATIONS

RoLCuV	*Rose leaf curl virus*
DLCV	*Duranta leaf curl virus*
BCTV	*Beet curly top virus*
BLAST	Basic local alignment tool
bp	Base pair
CaCl$_2$	Calcium chloride
CP	Coat protein
CR	Common region
CTAB	Cetyl trimethyl ammonium bromide
V2	Precoat protein
NSP	Nuclear shuttle protein
MP	Movement protein
OW	Old world
NW	New world
Rep	Replication associated protein
REn	Replication enhancer
Rpm	Revolution per minute
TE	Tris-EDTA
TAE	Tris acetate EDTA
RCA	Rolling circle amplification
SCR	Satellite conserved region

PaLCrV   Papaya leaf curl virus

HoYVV   Hollyhock yellow vein virus

PeLCuV   Pedilanthus leaf curl virus

MalYVV   Malvastrum yellow vein virus

ABSTRACT

Duranta repens is an evergreen ornamental plant and has many medicinal uses. It has been shown to possess antimicrobial, antioxidant, and insecticide properties. Its phytoconstituents are reported as the basis of its efficacious therapeutic properties. The leaf curl disease of Duranta repens is prevalent in Pakistan and exhibits symptoms typical of begomoviral infection. In present study,some of the infected plants having symptoms of severe leaf curling were observed in the vicinity of NUST, Pakistan during 2018.The investigation of begomovirus and betasatellite association having symptoms of duranta disease was done by sequence analysis through PCR. Universal primers WTGF/WTGR and Beta01/Beta02 were used for amplification of ~1.5 kb and ~1kb DNA fragment for begomovirus (DNA-A) and β-DNA respectively. The highest nucleotide sequence identity (90%) and phylogenetic interpretations revealed close relation of begomovirus (DNA-A) and β-satellite spotted in *D. repens* with *Rose Leaf Curl Virus* (RoLCuV). This result indicated that *D. repens* plants infested with RoLCuV and related satellite DNA act as reservoir that could lead to increased incidence of rose leaf curl disease in Pakistan and could also lead to the evolution of new virus and satellite species by recombination.

Chapter 1

1. Introduction

Geminiviruses are the plant viruses that belong to the family *Geminiviridae* (Murphy, 1995)**.** Almost 1100 years ago, geminivirus disease symptoms were described in the poem written by Empress Koken (Saunders, Bedford, Yahara & Stanley, 2003)**.** The importance of geminiviruses was largely recognized after MSV and BCTV were identified in 1974 (Bock, Guthrie & Woods, 1974).Geminivirus consists of plant infecting viruses that are transmitted through insects having a circular ssDNA genome ~2.8 kb in size which is non-enveloped. The viruses are responsible for worldwide crop losses mainly food and vegetable crops, fibre crops and ornamental plants (Navas-Castillo et al. 2011).Harrisson et al. in 1977 given the term geminivirus because of their nature twinned icosahedral particles. In last few decades,geminivirus like genomic components were detected by high performance sequencing and rolling circle amplification (Roossinck et al. 2015).The classification of geminivirus at genus level has been developed by International Committee on Taxonomy of Viruses (ICTV). *Geminiviridae* consists of nine genera i.e. *Begomovirus, Turncurtovirus , Curtovirus, , Grablovirus, Becurtovirus ,Eragrovirus ,Mastrevirus, Topocuvirus* and *Capulavirus*. They are classified on the basis of gene organisation, pairwise sequence identities based on allele, host rang, and insect vector (Zerbini et al. 2017).

"Bean golden mosaic virus gives rise to the term Begomovirus" (van Regenmortel et al. 1997). *Bemisia tabaci* is the insect vector and is responsible for transmission of begomoviruses and these are widespread all over the world. On the basis of their phylogenetic distribution, they are designed as the New World (NW) and Old World (OW) viruses (Rybicki, 1994).The difference between OW and NW is that the genome of OW are monopartite and contained either DNA-A linked with alpha or betasatellite molecules or simple DNA-A whereas the genome of NW begomoviruses are bipartite containing DNA-A and DNA-B (Briddon & Stanley, 2006). DNA-A and DNA-B,both have size of 2.7-2.8 kb and both bidirectionally encodes their own ORFs. Six ORFs are encoded by monopartite viruses, where four are in complementary sense orientation and two in virion sense orientation. The ORFs of the complementary sense are referred to as AC1 – AC4 and virion sense ORFs are referred to as AV1 and AV2 (Fontenelle et al., 2007).

Begomovirus DNA-A and DNA-B have no ORF and sequence in common, but share a small region which is known as CR of ~200bp.In the case of CLCrV and ToLCGuV respectively,there are rare exemptions where DNA-A and DNA-B CR vary between 60-63% similar(Chakraborty, Pandey, Banerjee, Kalloo & Fauquet, 2003).The most significant feature of CR is TAATATT/AC, which is a nine nucleotide sequence also known as nonanucleotide, and is also retained among all viruses. Another important aspect of CR is the existence of an iterated order called iteron sequence. DNA-A encodes its own Rep protein while DNA-B does not encode Rep protein and depends on helper DNA-A for its replication (Chatterji, Beachy & Fauquet, 2000).

"The Satellites molecules are also associated with begomoviruses.In Northern Australia the first DNA satellite molecule (ToLCV-sat) related with Tomato leaf curl virus was identified. Its length was 682 nucleotides, and also has a noncoding DNA satellite,and it shared no important order with the helper DNA virus in homology. A virus is dependent for encapsidation and replication process on helper begomovirus and does not require ToLCV-sat for infection" (Dry, Krake, Rigden and Rezaian, 1997) (Roshan, Kulshreshtha and Hallan, 2017). Satellites,especially monopartite begomoviruses, have often been found with begomoviruses (Jyothsna et al., 2013).Begomovirus-related satellite molecules are three: alphasatellite, betasatellite and recently characterized deltasatellite.

Betasatellites molecules have an A-rich region, an ORF (betaC1) and a strongly preserved ~100 bp section called SCR. The multifunctional protein betaC1 in betasatellite molecule,that plays a vital role in the defense mechanism by helping to increase overall pathogenicity. They have no sequence homology with their helper virus except in stem loop of IR which is a nonanucleotide sequence (TAATATTAC) (Briddon et al., 2003). While alphasatellites are not true satellites because they are molecules that are self-duplicating molecules, and relied for their movement on helper virus. They encode a single replication associated Rep protein (Mansoor et al., 1999) and TAGTATTAC, which resembles nanoviruses,is the nonanucleotide inside the hairpin.The precise function of alphasatellites has not yet identified but it has been observed that alphasatellites replication overpowers hosts' PTGS (Nawaz-ul-Rehman, Nahid, Mansoor, Briddon & Fauquet, 2010).

The replication process of DNA is mainly responsible for creating variety in the viral genome through recombination-dependent replication which is also known as rolling circle mechanism (Preiss & Jeske, 2003).The evolutionary process which considerably affects geminivirus genomic evolution is called Recombination (Saeed and Samad, 2017). There is also a pseudo-recombination in the begomovirus genus that is responsible for genetic diversity. The diversification of the begomovirus between different species, different genera and variants of the same virus is responsibility of both phenomenon,contributing to the capability to adjust in diverse environments. The new viruse emerged as a result of mutations in the viral genome of new elements (Briddon, Patil, Bagewadi, Nawaz-ul-Rehman & Fauquet, 2010; Saeed and Samad, 2017). Variation in satellite molecules i.e. betasatellites paying more role in virulence (García-Arenal, Fraile and Malpica, 2001). So now these types of viruses have become more effective and have surpassed the resistance of the host and contributed to an outbreak (Tsai et al., 2011). In the recombination of begomoviruses the breakpoints are maintained with hot spots in the 5′-end of the intergenic region and in the Rep N-terminal portion (Lefeuvre, Lett, Reynaud and Martin, 2007; Martin and Rybicki, 2000; Prasanna and Rai, 2007). The recombination events are responsible for their diversity. Different viral strains have appeared in *Tomato leaf curl virus* due to recombination events (Kirthi, Maiya, Murthy and Savithri, 2002). Likewise, a recombinant CLCuMuV and CLCuKoV, *Cotton leaf curl Burewala virus,* was developed to break the resistance of the host plant and improve the infectious process (Amrao et al., 2010). Begomoviruses was suggested to be important in their evolution due to high recombination ability and is emerging as a devastating phytopathological problem. Recombination events play a crucial role in driving host switches (Lefeuvre & Moriones, 2015).Begomovirus infections have been known for centuries. With a massive economic losses, infects a wide variety of crops, ornamental plants, weeds etc. Due to their rapidly mutating behavior, these ssDNA viruses became the topic of interest by researchers globally. RDP (Martin and Rybicki, 2000), GENECONVE (Padidam et al., 1999), CHIMAERA, MAXIMUM CHI^2, recscan (Martin et al., 2005), GENECONVE (Padidam et al., 1999) and SISTER SCAN (Gibbs et al., 2000) methods are implemented in rdp2 (Martin et al., 2004) can be used to classify recombination parts that are exchanged in begomoviruses (Saeed & Samad, 2017).

The annual loss due to cotton leaf curl disease (CLCuD) was estimated to be more than one million dollors in Pakistan. In Latin America, ToLCuV caused a yield loss of 100% of tomato crops. The

reduction in cassava yield in Africa was caused by cassava mosaic disease which also resulted in shortage of food (Khatri, Nahid, Fauquet, Mubin & Nawaz-ul-Rehman, 2014).

Duranta repens is an evergreen ornamental plant and has many medicinal uses. It has been shown to possess antimicrobial, antioxidant, and insecticide properties. Its phytoconstituents are reported as the basis of its efficacious therapeutic properties. In Pakistan duranta leaf curl disease is prevalent and shows typical symptoms as of Begomovirus infection. In duranta repens isolates of tomato leaf curl New Delhi virus (ToLCNDV) and Papaya leaf curl virus (PaLCuV) were related to each other, whereas isolates of catharanthus yellow mosaic virus (CaYMV),with no satellite molecules was identified in D. repens (Anwar & Tahir, 2017).In Pakistan D. erecta was found to be infected by PeLCV, CaYMV and ToLCB. This may result in reduction of crop production in Pakistan and may also result in production of new viruses and satellite molecules by recombination.

Objectives

1. To characterize monopartite Begomovirus (es) from *Duranta repens* in the vicinity of NUST, Islamabad Capital territory of Pakistan.
2. To better understand the functions of Begomovirus and betasatellite in the infected plant.

Chapter 2

2. Literature review

2.1. Family: Geminiviridae

Geminiviridae consists of plant-infecting viruses that are transmitted by insect vector and have circular non-enveloped ssDNA genome of ~2.8 kb. These viruses are accountable for worldwide losses of food and vegetable crops, fibre crops and ornamental plants (Navas-Castillo et al., 2011).

Harrisson et al. in 1977 given the term geminivirus because of their nature twinned icosahedral particles. The genomic components of geminiviruses are filled in two incomplete icosahedra that contains 22 pentameric capsomeres (Hesketh et al., 2018).DNA polymerase is not encoded by virus particle but replication depends on rolling circle and recombination mechanisms (Jeskey, 2009). For the synthesis of complementary strand they wholly depend on the host and also utilize replication factors of their respective host. There is a bidirectional transcription that leads to the production of many overlapping transcripts (Brown et al., 2012).

2.2. Classification of Geminiviruses

In the last decades, Geminivirologists succeeded in the identification of many novel geminivirus like genomic components by using advanced molecular tools i.e high throughput sequencing and rolling circle amplification (Roossinck et al., 2015). The classification of geminivirus at genus level has been developed by International Committee on Taxonomy of Viruses (ICTV). *Geminiviridae* consists of nine genera i.e. *Begomovirus, Turncurtoviru, Grablovirus, Curtovirus, Becurtovirus ,Eragrovirus ,Mastrevirus, Topocuvirus* and *Capulavirus*. They are classified on the basis of gene organisation, pairwise sequence identities based on allele, host rang, and insect vector (Zerbini et al. 2017).

Figure 1: Genome organization of the representatives' geminiviruses belonging to different genera in the geminiviridae family

2.3. Evolution in Begomoviruses

As a result of mutation or genetic exchange by recombination results in the evolution of Plant viruses (García-Arenal, Fraile & Malpica, 2001). Begomoviruses frequently exploit gene flow mechanism for variation that results their ability to challenge environmental changes such as host resistance (Botstein, 2019 & Martin, van der Walt, Posada & Rybicki, 2005).Thus, begomoviruses

was suggested to be crucial in their evolution due to high recombination ability and emergence as a devastating phytopathological problem; recombination plays important role in driving host switches and the onset of these viruses (Lefeuvre & Moriones, 2015).

Three distinct geminiviruses namely East African cassava mosaic virus (EACMV), MSV and TYLCCNV have been studied for point mutations and have been reported for mutation frequency of~10-4 point substitutions/site/year. Intragenic recombination among geminiviruses in the Indian subcontinent has facilitated epidemics of cotton leaf curl disease (CLCuD) (Hameed, Shakir & Zaidi, 2019).

2.4. Begomoviruses

Bean golden mosaic virus gives rise to the term begomovirus (van Regenmortel et al., 1997).Whitefly *Bemisia tabaci* is its transmission vector and transmits these viruses in a persistent and circulative manner. The agricultural output of many agricultural countries including Pakistan is suffering at the expanse of begomoviruses (Brown et al., 1995).It causes devastating losses to dicotyledonous plants with estimated losses of several billion dollars per year (Harrison and Robinson, 1997; Malathi and Varma, 2003).

2.4.1. Classification of Begomoviruses

Begomoviruses on the basis of their phylogenetic distribution are designed as New World (NW) [Americas] and Old World viruses (OW) [Asia, Africa, Europe and Australasia]. OW viruses are monopartite and contained either DNA-A linked with alpha or betasatellite molecules or simple DNA-Awhereas the genome of NW begomoviruses are bipartite containing DNA-A and DNA-B (Briddon & Stanley, 2006). Reports have shown that among OW viruses about 15% were bipartite (Ha, et al., 2008; Mondal et al., 2019).Among begomovirus genome DNA-A is conserved component and has genes vital for viral movement, infection and replication process (Mondal et al., 2019). Moreover, OW begomoviruses comprise V2 gene in the DNAA component, absent from NW viruses (Rybicki & Stanley et al. 2005).There is a conserved amino acid sequence at the N-Terminal of NW viruses coat protein (CP),while the OW lack this motif(Harrison et al., 2002).

2.4.2. Genome Organization of Begomoviruses

"Begomoviruses have been categorized as monopartite and bipartite by determining the organization of their genome. DNA-A (~2.7 kb) and DNA-B (~2.6 kb) are constituents of bipartite begomoviruses while the component DNA-A (~2.7 kb) alone is characterized by monopartite begomovirus. The genomic components of monopartite and bipartite viruses have partly overlapping bidirectional ORFs. DNA-A has six ORFs i.e AV1/V1 and AV2/V2 genes are present in sense strand and AC1/C1, AC2/C2, AC3/C3, and AC4/C4 genes are present in the antisense strand. However, some bipartite begomoviruses have also been identified with the presence of AC5/C5 ORF" (Fontenelle et al. 2007; Kheyr-Pour et al. 2000). DNA-B has two ORFs i.e BV1 gene is present in the sense strand and BC1 gene in the antisense strand" (Hanley Bowdoin et al. 2000).

2.4.3. Satellites Associated with Begomoviruses

"The Satellites molecules are also associated with begomoviruses.In Northern Australia the first DNA satellite molecule (ToLCV-sat) related with Tomato leaf curl virus was identified. Its length was 682 nucleotides, and also has a noncoding DNA satellite, the homology of which share no important sequence with the helper DNA virus. A virus relies on helper begomovirus for encapsidation by the viral CP and replication and does not require ToLCV-sat for infection" (Dry, Krake, Rigden and Rezaian, 1997) (Roshan, Kulshreshtha and Hallan, 2017). Satellites,especially monopartite begomoviruses, have often been found with begomoviruses (Jyothsna et al., 2013).Begomovirus-related satellite molecules are three: alphasatellite, betasatellite and recently characterized deltasatellite.

2.4.4. Betasatellites

Betasatellites contain single ORF, coding for βc1 protein, which plays a significant and well established role in pathogenicity determination, accelerates helper DNA replication and suppresses plant defenses (Saunders et al. 2004).Unlike alphasatellites, replication of betasatellites depends

on the Rep gene of helper virus. An A-rich region is found on both the satellites while betasatellite also has a satellite conserved region (SCR).

The βc1 protein determines movement , satellite and viral titer and host range (Saundres et al. 2000).The also modulate symptom appearance in hosts by invading replication of helper virus(Briddon et al., 2001).

2.4.5. Alphasatellites

Alphasatellites are molecules encoding their own Rep protein, thus, capable of self-replication. An A-rich region of 153-169 nucleotides, downstream of the rep gene is common to all alphasatellites. Alphasatellites along with monopartite begomovirus contributes in modifying the symptoms and causes less DNA to accumulate, however the process is still unknown (Briddon et al., 2004).

Chapter 3

3. Materials and Methods

3.1. Sample Collection

Duranta repens leaf samples having usual begomovirus symptoms of vein yellowing were collected from NUST, Islamabad in 2019. Young leaves were collected from symptomatic plant and were kept at low temperature (-80°C).

3.2. Total Genomic DNA Extraction

For total DNA extraction protocol of Doyle & Doyle (1990), with some modifications was followed.Leaf sample of 1g was crushed in mortar and pestle using liquid nitrogen until it turned into a fine powder. It was then transferred into falcon tubes containing pre-heated 25ml CTAB isolation buffer solution (2% Cetyltrimethyl ammonium bromide, 0.2% mercaptoethanol, 100mM Tris-Hcl, 20mM EDTA and 1.4 M NaCl) at 60°C for 15 minutes. Falcon tube was placed in water bath with constant shaking at 60°C for 30 minutes.

After half an hour of heating and shaking, the mixture was cooled down at 25°C for 3 minutes. It was then transferred into 2 falcon tubes equally (12.5ml each).Isoamyl alcohol solution and Chloroform were added in the ratio of 1:24 in both tubes. It was then mixed to form an emulsion. For separation of phases centrifugation was performed at 4000rpm maximum for 15 minutes at 25°C in Eppendorf centrifuge 5804R.Upper clear phase containing DNA from both the falcons was carefully transferred into a new falcon tube with pipette, leaving behind a few ml of clear phase to avoid contamination of DNA with proteins and RNA. Equal amount (almost 20 ml of chilled isopropanol) was added into the supernatant. Tube was mixed gently. White threads of DNA became visible. Centrifugation of precipitated DNA at 4000 rpm was carried out for 10 minutes. Upper phase having supernatant was discarded and DNA was pellet down. DNA washing buffer (10mM ammonium acetate and 76% ethanol) was used to wash the pellet and pellet after washing was then put into a new Eppendorf tube along with the buffer. Eppendorf tube was centrifuged and supernatant was discarded. Pellet was incubated at 37°C for 30-60 minutes and then mixed until

dissolved completely into 1ml of TE buffer (1 mM EDTA and 10mM Tris –HCl) and stored at -20ºC.

3.3. DNA Analysis

3.3.1. Agarose Gel Electrophoresis

Using agarose gel electrophoresis, DNA concentration was determined. Agarose gel of 1% was made by using 100ml of 1XTAE buffer (1mM EDTA, 25mM Tris, 5 mM Glacial Acetic acid, pH 8.0) and dissolving 1g of agarose in it. Mixture was heated until it became clear and upon cooling Ethidium Bromide was added into it. Gel was poured into a gel caster having an appropriate comb and allowed to solidify. Solidified gel was placed into a tank containing 1Xtae buffer so that it is completely immersed into the buffer. Comb was removed carefully.6X loading dye mixed with 5μl of DNA were then loaded into the gel. DNA ladder of 1 kb was also loaded into the well. Agarose Gel was run for 50 minutes at 80 volts and was then observed on Dolphin-Doc plus Image System.

3.4. PCR Amplification of DNA

For all the samples 25 μl reactions were made. The reactions consist of 10X Taq buffer [25mM $Mgcl_2$ and $(NH_4)_2SO_4$], 2mM dNTP's, 100 mM primers(WTG), Taq DNA Polymerase and DNA depending upon its concentration.

For betasatellite amplification, primers β01 and β02 (Briddon et al. 2002). The conditions applied for amplification of required region are as follow.
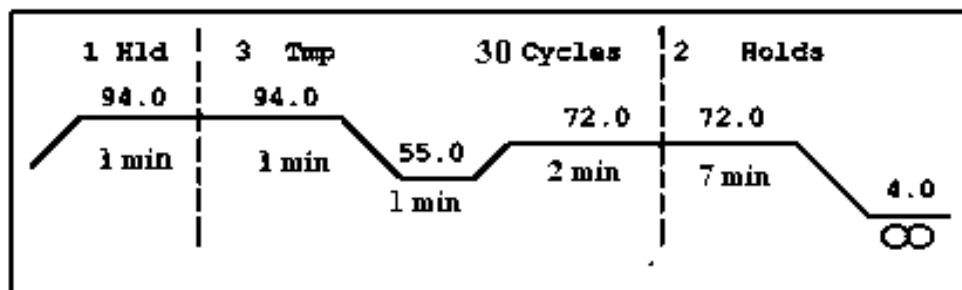
Figure 3.1: PCR conditions used for amplification of DNA A and Betasatellite of Duranta virus.

3.5. Gene Elution from PCR Product

Required fragment was cut from the gel using razor blade and was kept in Eppendorf tube. Monarch Gel Extraction Kit (NEB#T1020) for DNA was used for gene clean according to manufacturer's instruction. Gel Dissolving Buffer in a 4:1 amount was added in Eppendorf containing the excised band. It was then heated in a heat block for 10 minutes at 48^OC. The tubes were vortexed after short intervals to mix the content evenly. The sample was then loaded onto the column and centrifugation was performed at 13,000 rpm for one minute. The flow through was discarded and centrifugated again. 200 μl of DNA Wash Buffer was applied and the same parameters were performed for centrifugation. Centrifugation was performed to elute the DNA of desired fragment. This gene clean product was used for ligation.

3.6. Cloning of PCR Product into PTZ57R/T Vector

Gene product was ligated with pTZ57R/T vector. The kit used was InsTAclone™ PCR Cloning Kit, Fermentas, as mentioned by the manufacturer. Vector contains unique restriction sites (Figure 3.2). A total of 20 μl reaction was prepared in an Eppendorf tube containing 1x Ligation buffer,

~98 ng of purified DNA, 165 ng restricted vector and 5 units of T4 DNA ligase were also added. The incubation of reaction was done overnight at 4ºC .Next day it was processed for transformation into DH5α competent cells.
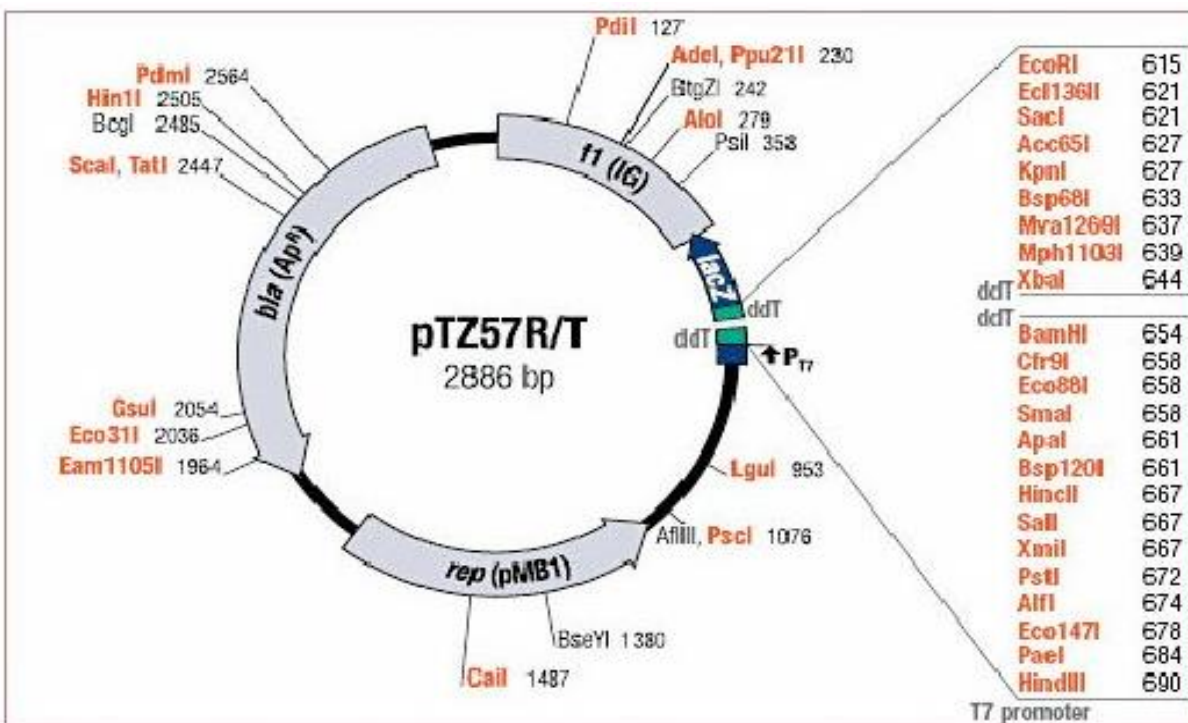
Figure 3.2: shows pTZ57R/T vector used for cloning. Map represents distinctive restriction sites.

3.7. Preparation of DH5α Cells

As defined by Cohen et al (1972), competent cells were basically prepared.The strain used for vector transformation was *E. coli* DH5α. At 37ºC, a single colony of cells were inoculated into 5ml of LB Broth and overnight incubation (14-16 hours) was performed with shaking incubator. 1 ml of overnight culture was inoculated in a 50ml LB medium and incubation was done at 37ºC for 2 hours. The culture was then centrifuged at 4000 rpm for 5 minutes and the cell pellet was observed. The supernatant was pour out and the cell pellet was resuspended in 10ml of cold 50mM $CaCl_2$ and left for 40 mins on ice. As before, the cells were pelleted again and the supernatant was removed. In 1ml of cold 50 mM $CaCl_2$, the cell pellet was resuspended and stored at 4^0C.

3.8. Transformation of Competent Cells

Following the method described by Chang *et al* (2017) *E. coli* cells were transformed. Ligated product was put into Eppendorf tube having DH5α cells and was mixed gently. Then the mixture was put on ice for 30 minutes before giving heat shock at 42°C via water bath. After that, cell mixture was put on ice for 2 minutes. 1m of LB broth was added to Eppendorf tubes containing transformation mixture, cells were allowed to grow for 2 hours at 37°C. Transformation mixture was spread on solid agar plates containing Ampicillin antibiotic and plates were incubated overnight (12-16 hours) at 37°C.

3.9. Selection of Clones and Plasmid Isolation

Next day white colonies were picked one at a time and were transferred to different falcon tubes containing 10 ml LB and 100ug/ml Ampicillin. In a water bath heated at 37°C falcon tubes were incubated for 16 hours on continuous shaking. After incubation and growth 5ml of culture was added to Eppendorf tubes. Centrifugation of tubes was done at 14000 rpm for 1 minute. Miniprep was performed by PureLink® Quick Plasmid Miniprep Kit and the manufacturer instructions was followed. 1 mL of overnight culture was centrifuged and supernatant was removed. 250 μl of Resuspension Buffer (R3) with RNase A was applied to the cell pellet and the pellet was resuspended until it becomes homogeneous. 250 ml of Lysis Buffer (L7) was then applied and gently mixed until the mixture becomes homogeneous by inverting the capped tube. The tubes were incubated for 5 minutes at room temperature. Then 350 μl of Precipitation Buffer (N4) was added and was mixed by inverting the tube immediately. Lysate centrifugation was performed at >12,000 × g for 10 minutes. The supernatant was loaded onto a spin column in a 2-mL wash tube. The column centrifugation was done at 12,000 × g for 1 minute. The flow through was discarded and the column was inserted back into the wash tube. After that, 700 μl of Wash Buffer (W9) with ethanol was applied to the column. The column centrifugation was performed for 1 minute at 12,000 × g. Flow through was discarded and column was placed into the wash tube. The column was centrifuged at 12,000 × g for 1 minute. A clean 1.5-mL elution tube was put in the spin column and 75 μl of preheated TE Buffer (TE) was applied to the center of the column. At room temperature, the column was incubated for 1 minute and was centrifuged at 12,000 × g for 2

minutes. The elution tube contains the purified plasmid DNA was stored at 4°C. Extracted plasmid was confirmed by running 2 µl of plasmid DNA mixed with loading dye in 1% agarose gel.

3.10. Confirmation of Clones via Restriction Enzyme Digestion

Clones of *Duranta repens* betasatellite amplified with Beta01/Beta02 primers were digested with *EcoR1* and *HindIII.* Total of 30 µl reaction was performed having 4 ul plasmid DNA, 2ul buffer 1 µl l of enzyme. Reaction tubes were incubated for 2.5 hours at 37°C. 1 % Agarose was used to run the digestion mixture and confirmation of clones was done by looking at the size of fragments obtained.

3.11. Sequence and Phylogenetic Analysis

The extracted plasmid via kit (PureLink® Quick Plasmid Miniprep Kit) was sent to Eurofins, USA for sequencing. While the PCR amplified DNA with WTGs primers was sent to Alpha genomics for sequencing.

After sequencing of begomovirus DNA was done, resulting sequence was analyzed in BLASTN for local alignment based similarity search. Aligned sequences from BLAST was selected and retrieved to build a phylogenetic tree using MEGAX software.

3.12. Structural Analysis

Structural analysis of Duranta virus C4 was done using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) tool.

Chapter 4

4. Results

4.1. Sample Collection

Duranta repens leaves – symptomatic was collected from NUST, Islamabad during 2019 and stored at -80ºC until processed for DNA isolation.

Figure 4.1: showing infected plant with leaf curl and vein yellowing symptoms

4.2. DNA Isolation

Total DNA was collected from infected sample and was stored at -20 ºC until further processing. The good quality DNA was obtained while analyzed on to 1% agarose gel and compared with 1kb DNA ladder (Figure 4.2).
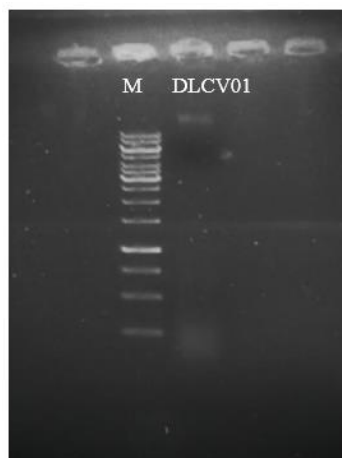
Figure 4.2: Agarose gel electrophoresis of total DNA of Duranta repens. Lane of isolate DLCV01 showing the DNA extracted from symptomatic sample and Lane M showing 1kb Standard DNA marker. Total DNA is above the ladder as its size is greater than the ladder.

4.3. PCR Amplification of DNA

WTG primers WTG-F/WTG-R were used for the amplification of Begomovirus DNA A (section 3.4) and produced a band at approx. 1.5kb (Figure 4.3 B).Betasatellite was amplified using a primer pair Beta01/Beta02 (Briddon et al. 2002; section 3.4).An unexpected band of 980 base pairs in size was obtained using Beta01/Beta02 primers(Figure 4.3 C).
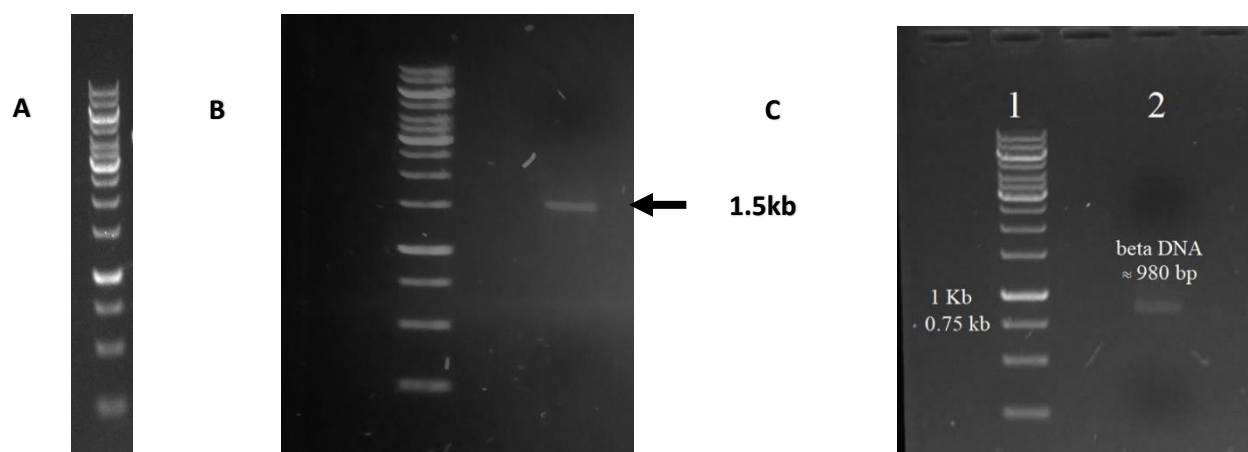

Figure 4.3: A, showing standard DNA marker of 1kb. B, Agarose gel electrophoresis showing amplification product of WTG-F/WTG-R of 1.5kb. C, Agarose gel electrophoresis showing amplified product at 0.98kb of beta01/beta02 primers.

4.4. T/A Cloning of Begomovirus

Band size of 0.9kb for betasatellite was cut and was purified using Monarch DNA Gel Extraction kit after running on gel. Under UV trans-illuminator the purified product was visualized. The resulted product was subjected to ligation in vector pTZ57R/T .DH5α cells of E.coli were used to transform the ligated product. Transformed cells were spread on plates having agar and Ampicillin.

4.5. Selection of clones

Many white colonies were appeared on the plates next day. The colonies were selected and cultured for their plasmid isolation. Isolated plasmid DNA was subjected to restriction digestion analysis.
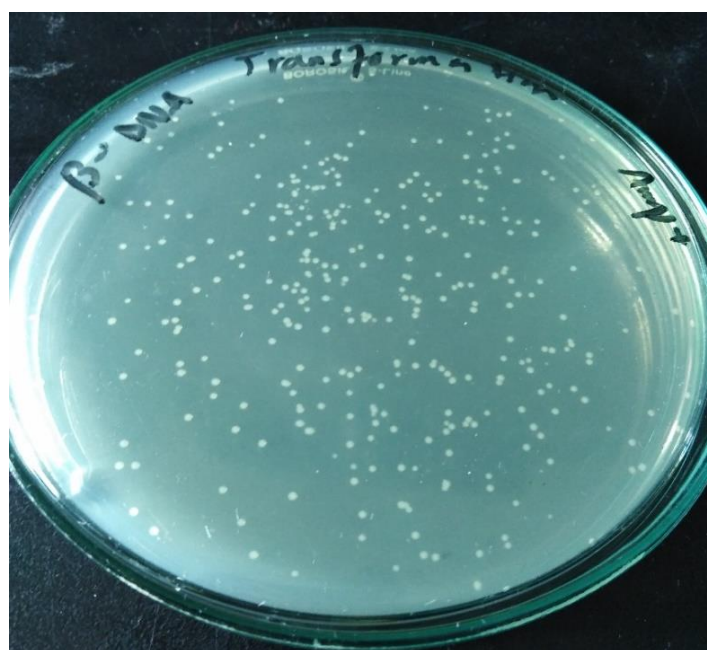

Figure 4.4: Colonies of DH5α containing β-DNA on LB agar Amp plate

4.6. Confirmation of Clones through Restriction Digestion

Clone of betasatellite of isolate DLCV01 was digested with EcoR1 and HindIII Restriction enzymes. The bands obtained at approx. 1kb for EcoR1 + HindIII and at 2.8kb (Figure 4.5 B).
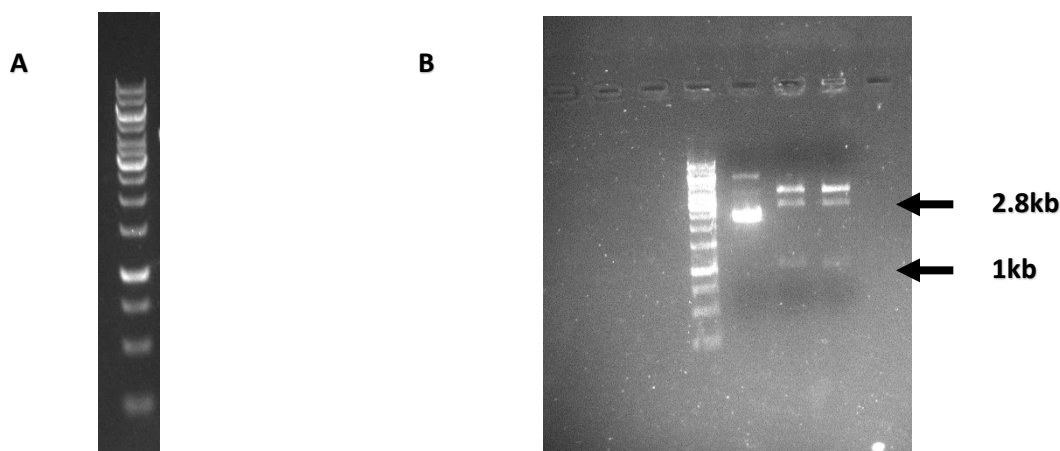
Figure 4.5: A, showing standard 1kb DNA marker. B, Agarose gel electrophoresis of Duranta betasatellite DNA with EcoR1 + HindIII. Gel showing bands at 1kb for both enzymes and 2.8kb kb for plasmid DNA.

4.7. Comparison of islolate DLCV01 with other Begomoviruses

Total size of isolate DLCV01 was found to be 1500 nucleotides. Genomic component of isolate DLCV01 was compared to begomoviruses present in the databases. The analysis revealed its maximum nucleotide sequence identity of 96% with RoLCV.

4.8. DNA Sequence Analysis

The selected sequence (DLCV01) showed the highest identity (90%) and the maximum query coverage with RoLCuV-[PK, Fai, 06] (GenBank Acc. No. GQ478342). This result ensured that the query sequence belongs to the DNA-A of a monopartite Begomovirus and that the suspected virus is an isolate of RoLCuV.

The isolate DLCV01 contains six overlapping ORFS with an arrangement typical of begomovirus (Table 4.1). Pre coat protein (V2) genes and Coat protein (CP) genes were present on virion sense strand while rest of the genes: Replication associated protein (rep) gene, AC5 (pathogenicity

determinant) gene and AC1 gene were present at complementary strand. Hairpin loop was present comprising the conserved nona nucleotide sequence (TAATATTAC).The full length gene C4 were considered for further analysis.

Table 4.1: Residue position, length and protein size of different genes of virus.

Isolate	Percentage Identity	Gene	Start site	End site	Total residues	Protein size	Sequence Identity (%)	Virus
DLCV01 DNA A	90% Rose leaf curl virus	V2	1151	1412	420	139	99%	RoLCV
		AC4	839	360	480	159	75%	PaLCrV
		AC5	1292	1412	126	41	95%	PaLCrV
		AV1	1310	1431	123	40	100%	ChiLCV
		AC1	206	292	87	28	74%	TbCSV
		Rep	109	333	228	75	93%	RoLCV

Sequence analysis revealed that the C4 had the iso-electric point of 10.60, which indicates it to be highly alkaline in nature (Table 4.2). The aliphatic index of C2 was found to be 76.04. The relative volume which a protein occupies by aliphatic side chains is indicated by Aliphatic index (alanine, valine, isoleucine, and leucine) (Benoit et al., 2014); aliphatic index is directly proportional to thermostability, the higher the aliphatic index the higher is the thermostability and vice versa (Ikai, 1980). The instability index showed that C4 is less stable. An instability index above 40 indicates less stability of a protein in vitro. The grand average of hydropathy (GRAVY) of the C4 was -0.457 (sum of hydropathy for all the amino acids divided by the total number of residues gives the GRAVY value of a protein). The polarity of a protein can be determined by a positive GRAVY value (http://bif.uohyd.ac.in/cpc/help_pcp.php). Thus, it was clear that the C4 is a non-polar protein. There has been no study on the polarity of geminiviral proteins so far. However, the polar nature of the viral proteins indicates their water-solubility.

**Table 4.2: Results of Protparam tool from ExPaSy proteomic server**

S.No	Description	C4
1	Isoelectric point	10.60
2	Molecular weight	18229.02
3	Aliphatic index	76.04
4	Instability index	78.87
5	Grand average hydropathicity (GRAVY) index	-0.457

4.9. Multiple Sequence Alignment of C4

To reveal the similarity of the DLCV01-C4 with other C4/AC4 proteins, BLASTp was performed where 37 entries (including our isolate) sharing more than 70% identity were retrieved and MSA was performed.

4.10. Phylogenetic Analysis of Isolate DLCV01

MEGAX was utilized for phylogenetic analysis. The construction of tree was based on the alignments of most similar begomoviruses sequence available in the database using neighbor joining (NJ) algorithm. The Sequences considered here are shown in Figure 4.5.From the tree it is evident that isolate DLCV01 is closely related and cluster together with RoLCV. They have high sequence identity and a high bootstrap value. Isolate DLCV01 is highlighted and outgroup used in tree is BCTV that can be seen segregated from other genera of geminivirus.
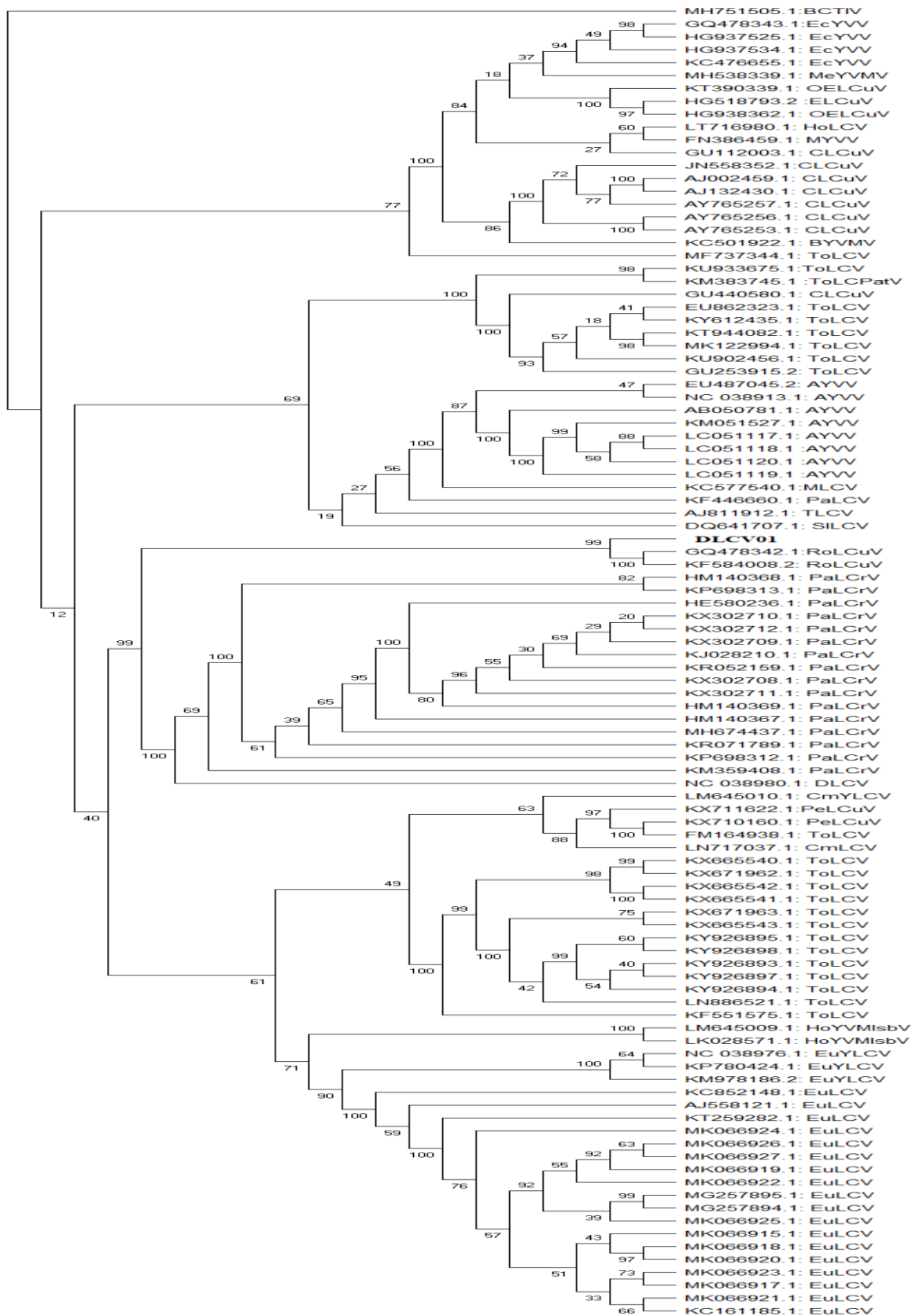
4.11. Structural Analysis

The protein (C4) sequence was then subjected to I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) server for homology modelling. It is a highly rated server used for the production and prediction of automated protein structures. When an amino acid sequence is submitted, I-TASSER works by using various threading alignments and iterative structural assembly simulations to construct a 3D atomic model. By employing 10 threading templates I-TASSER server predicted five tertiary structures of C4 protein. According to the Z-score values, the 10 chosen templates have good alignment, accordin. The five models supported by the server have C-score values ranging from −3.71 to −4.93. For the future refinement protocol (Fig.4.6-a), the highest C-score model derived from homology modelling was selected in this analysis. An expected TM score of 0.31 and RMSD value of 12.6±4.3Å are seen in the results.The TM score, which dissolves all the fluctuations associated with the RMSD values,is evaluated to examine the similarities between two protein structures.

4.11.1. Validation of the model stability

The stability of the model was confirmed through ProSA-web; which verifies 3D models of protein structures for potential errors. For a particular input structure, it calculates an overall quality score. The model under study, was considered to be suitable as it exhibits Z-score of −3.93, which is seen in a graph containing the z-scores of all experimentally determined proteins in PDB (Fig.4.6-b).
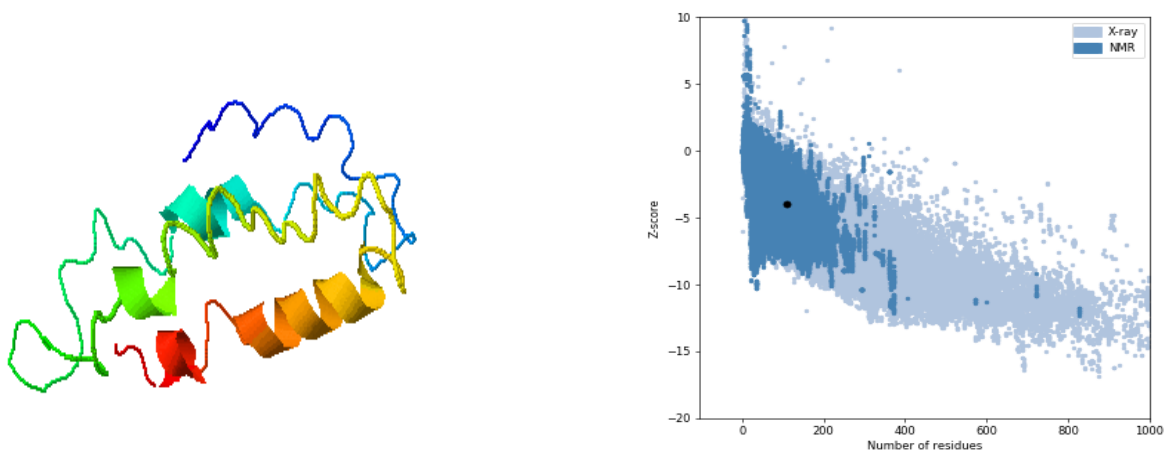
Figure 4.6: Protein modelling and validation**. (a)** The final 3D model of the C4 protein obtained after homology modelling on I-TASSER**. (b)** ProSA-web, giving a Z-score of −3.93.

4.11.2. Molecular docking of C4 protein with BIN2 (ASK etha)

In order to check the strength of C4 protein, Molecular docking of protein was performed with BIN2 (ASK etha), which belongs to multigene family of *Arabidopsis thaliana* and plays an important role in brassinosteroid signaling pathway. For initiating an interaction of the C4 protein with BIN2, the binding pockets of protein was determined by the CASTp 3.0 server. The surface area of the pocket was 443.381 Å and a molecular surface volume was 1080.654 Å. CPORT predicted the provided active interface amino acid residues: S10, E12, F20, L35, S36, H38, R39, M40, S41, R42, P43, I44, W45, R47 from the C4 protein; and M1, A2, N31, E33, P34, K35, Q36, T37, I38, S39, E41, A42, M43 from the BIN2 protein. To drive the docking procedure, these active residues were used. From the HADDOCK server, docked complex with the highest position was selected which have minimum intermolecular energies and have the lowest average pairwise backbone RMSD at the interface. The relative binding   affinity (ΔG) of C4-BIN2 complex was -9.3 Kcal/mol. While, a total of 14 hydrogen bonds were formed between C4 and BIN2 protein active residues. (Fig 4.7).

(a)
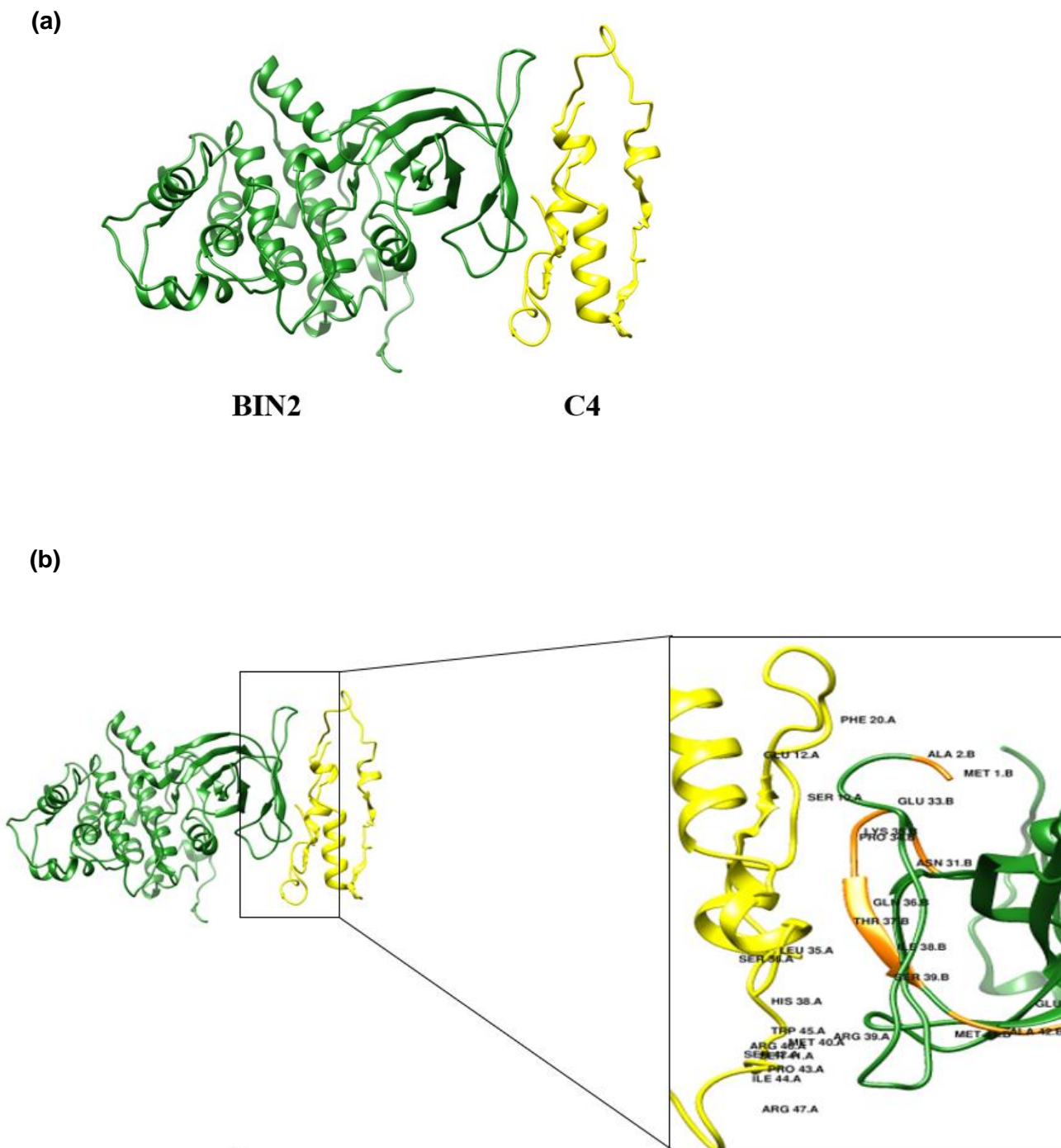

BIN2　　　　　　　　**C4**

(b)

Figure 4.7: Molecular docking of C4 and BIN2 (ASK etha)(a) with protein colored yellow and BIN2 colored sea green(b) while active residues of the complex present at the interface.

Chapter 5

5. Discussion

Begomoviruses have been infecting important crops, vegetables and ornamental plants at serious destructive levels (Tahir et al., 2010). Begomoviruses in Pakistan are a major threat to cotton crops as the total losses because of cotton leaf curl disease (CLCuD) in 2010 were up to 20 percent..It has been observed that bipartite begomoviruses are responsible for emergence of new diseases in plants. Satellite DNA molecules are responsible for varying symptoms in infected plants. Along with this, there are increasing reports of emergence of new species of begomoviruses which suggest that this virus has a high mutation rate and infecting a wide range of host plants.

Infected *Duranta repens* leaf samples showing typical begomovirus symptoms of vein yellowing were collected. Infected *Duranta* vein yellowing sample named as isolate DLCV01exhibited plant infected with a complex of begomovirus and a betasatellite. DNA sequencing of isolate DLCV01 was found to be 1500 nucleotides showed the highest identity (96%) and the maximum query coverage with RoLCuV-[PK,Fai,06] (GenBank Acc. No. GQ478342). This result ensured that the query sequence belongs to the DNA-A of a monopartite Begomovirus and that the suspected virus is an isolate of RoLCuV.

This is the first report of RoLCV infecting *Duranta repens* in Pakistan. Previously DLCV has been reported to infect Duranta. For this reason Duranta can be considered as an alternative host for RoLCV during off season.

The sequence analysis of DNA showed that the virus contains six overlapping ORFs: Pre coat protein (V2) and Coat protein (CP) genes were present on virion sense strand while rest of the genes; Replication associated protein (rep) gene, AC5 (pathogenicity determinant) gene and AC1 gene were present at complementary strand. Hairpin loop was present comprising the conserved nona nucleotide sequence (TAATATTAC).C4 gene has full length so it was considered for further analysis. Sequence analysis revealed that the C4 had the iso-electric point of 10.60, which indicates it to be highly alkaline in nature. The Index of Instability showed that C4 is less stable. The grand average of hydropathicity (GRAVY) of the C4 was -0.457. Thus, it was clear that the C4 is a non-polar protein. There has been no study on the polarity of geminiviral proteins so far. However, the polar nature of the viral proteins indicates their water-solubility.

Phylogenetic analysis, using Neighbor joining algorithm of isolate DLCV01 is closely related and cluster together with RoLCV. They have high sequence identity and a high bootstrap value. Isolate DLCV01 is highlighted and outgroup used in tree of other genera of geminivirus is Becurtovirus (BCTIV), can be seen segregated from the other sequences.

The 3D model of C4 protein was effectively accessed through I-TASSER and the structure stability was confirmed through ProSA-web, which means that most of the residues are present in favourable locations, suggesting that the model is of sufficient quality. While the docking results indicates that C4 protein are having good interaction with BIN2 (ASK etha) protein in favorable pose with which was explained by high Binding affinity.

It was evident from the current results obtained that Duranta is being infected by a new species of begomovirus. In Pakistan a sharp analysis is necessary to keep a record of begomoviruses infecting ornamental plants, their alternative hosts and the emergence of new species as a consequence of recombination in order to overcome the begomoviruses. These results also proved that duranta surely is an alternative host of RoLCV. Hence, all these conditions should be kept in mind while devising an effective strategy against rose leaf curl disease (RoLCD).

Chapter 6

6. References

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