# Cloning and Partial Characterization of Cotton Leaf Curl Burewala Virus From Khanewal

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## ABSTRACT

Begomoviruses are a serious threat to cotton production throughout the world. In Pakistan, enormous crop losses occur as a result of cotton leaf curl disease (CLCuD) caused by begomoviruses. Molecular characterization of begomoviruses has made possible the identification and analysis of begomoviruses prevalent in a host plant. Infected cotton leaf sample (C-59) was obtained from area around Khanewal during 2011. The total DNA was isolated from the infected sample by Cetyl trimethyl ammonium bromide (CTAB) method. An expected size band of approximately 1100bp, covering coat protein region of the virus, was amplified using universal primers. The amplified product was T/A cloned and sequenced to its entirety. DNA sequence showed 99% nucleotide sequence identity to each of Cotton leaf curl Burewala virus ((CLCuBuV; Accession No HF549184)) and Cotton leaf curl Kokhran virus (CLCuKV; Accession No AJ002449)). Since CLCuBuV is a recombinant of CLCuKV and Cotton leaf curl Multan virus and the coat protein region of CLCuBuV was derived from CLCuKV that is most probable reason that the available sequence showed identity with CLCuBuV as well as CLCuKV. A complete characterization of full length virus will determine whether isolate C-59 is CLCuBuV or CLCuKV. Literature indicates that there is no existence of CLCuKV within the region and CLCuBuV is dominating within Indo-Pak.

***Key Words:*** Cotton leaf curl Burewala virus, Cotton leaf curl Multan virus, Cotton leaf curl Kokhran virus, Cotton leaf curl disease, C5, Coat protein, Replication enhancer protein.

## 1. INTRODUCTION

### 1.1. Begomoviruses

Begomoviruses have been divided into two groups based on genome organization. The genome is either monopartite or bipartite. Monopartite genome constitutes only DNA A whereas bipartite genome is composed of DNA A and DNA B components. The genome replication takes place through double stranded DNA intermediates (Hanley-Bowdoin et al., 1999).

### 1.2. Bipartite Begomoviruses

Bipartite Begomoviruses have two genomic components designated as DNA A and DNA B (Lazarowitz, 1992). Six genes are coded by DNA A whereas DNA B codes two genes. Coat protein gene (CP or V1) and the pre-coat protein (V2) gene are encoded on the virion sense strand whereas replication-associated protein gene (Rep or C1), transcriptional activator protein gene (TrAP or C3), replication enhancer protein gene (REn or C3) and the C4 gene are found on the complementary sense strand of DNA A. Movement protein (MP) and the nuclear shuttle protein (NSP) are coded on the DNA B component (Kallender et al., 1998).

#### 1.2.1. Function of the Virion Sense Proteins

The coat protein plays a role in encapsidation of ssDNA, movement of the begomovirus and insect transmission (Sharma and Ikegami 2009). Silencing suppression and pathogenicity determination is carried out of by the pre-coat protein (Yadava et al., 2010).

#### 1.2.2. Function of the Complementary Sense Proteins

Located in the IR is the origin of replication containing a nonanucleotide sequence (TAATATT/AC) that forms a stem loop structure. Binding of Rep to the viral iterons positioned upstream of the origin of replication present in the IR initiates viral DNA synthesis (Nagar et al., 1995). Transcriptional activator protein (TrAP) blocks hypersensitive response to the begomovirus infecting a plant (Mubin et al., 2010). It also acts as a silencing suppressor and functions in the up-regulation of virion sense genes (Yang et al., 2007). Replication enhancer protein (REn) is involved in viral DNA replication (Pasumarthy et al., 2011). The C4 protein is involved in the determination of symptoms and virus movement (Jupin et al., 1994). All these six proteins are encoded on the DNA A component (Figure 1.1).

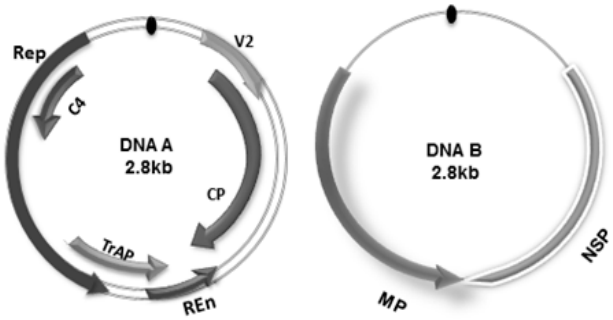


**Figure 1.1.** Genomic maps of DNA A and DNA B components of a begomovirus.

### 1.2.3. Function of the Proteins on DNA B

Movement protein (MP) aids in the movement of the bipartite begomovirus across plasmodesmata. The virus is transported from the nucleus to the cytoplasm with the help of nuclear shuttle protein. Both proteins are encoded on the DNA B component (Hanley-Bowdoin et al., 1999) (Figure 1.1).

### 1.3. Monopartite Begomoviruses

A sole genomic element of approximately 2.8 kb termed as DNA A constitutes the genome of monopartite begomoviruses. This single genomic constituent holds genes that are responsible for all viral functions in its entirety (Tan et al., 1995). Some monopartite begomoviruses require a betasatellite for their symptom modulation. The size of the betasatellite is half the size of the helper virus and depends on it for the encapsidation, replication, insect transmission and movement. Betasatellite shares no sequence similarity with the helper component although the nonanucleotide sequence is conserved (Zhou et al., 2003). (Figure 1.2).

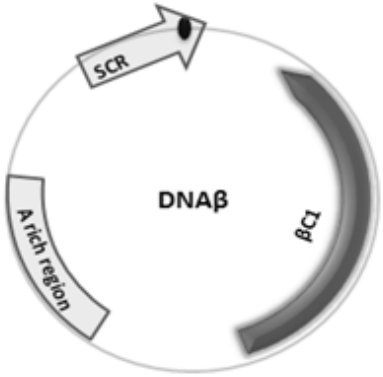


**Figure 1.2**. Betasatellite molecule identified in begomoviruses.

### 1.3.1. Function of Betasatellites

The only gene encoded by the begomovirus betasatellite is the βC1 gene, encoded on the complementary sense strand, required for the virulence of the helper begomovirus. A region rich in adenine and a satellite conserved region (SCR) are located on the virion sense strand (Rojas et al., 2001) (Figure 1.2). Betasatellites can be trans-replicated by any helper component in a non-specific way. The best example is the trans-replication of Cotton leaf curl Multan betasatellite (CLCuMB) by Ageratum yellow vein virus (AYVV) (Saunders et al., 2008).

### 1.4. Importance of Begomoviruses and Cotton Leaf Curl Disease

The devastating losses of crops such as tomato, bean, cotton and cucurbits in USA at the end of 20th century due to the dissemination of begomoviruses by Bemisia tabaci have been reported (Henneberry et al., 2000) Pakistan economy has suffered due to losses in cotton production as a result of Begomovirus infection with additional losses in cucurbits, cassava and pepper production (Briddon and Markham, 2000). The major disease affecting cotton in Pakistan since 1967 is the cotton leaf curl disease (CLCuD) first reported from Multan. It is a disease complex that involves leaf curling and formation of enations on the underside of leaves. (Hussain et al., 1991). The disease in Pakistan occurred in 1967 in the Punjab province with a decline in the disease incidence at the end of the 20th century due to the establishment of resistant cotton varieties against it. The disease has emerged as a serious threat to cotton production since 2001 due the development of overpowering breakdown of resistance in the disease causing begomovirus. Until 2003 occurrence of CLCuD in Sindh was irregular with no serious consequences to the cotton production in Sindh than in other localities of Pakistan but in the year 2003 the disease has appeared to be severe and devastating (Mansoor et al., 2003).

## 2. MATERIALS AND METHODS

Infected cotton leaf samples were collected from area around Khanewal during 2011. The leaves were stored at -80°C until further processing.

### 2.1. Diagnosis of the Viral DNA

The method employed for the isolation of total nucleic acid was similar to that set forth by Doyle and Doyle in 1990. The extracted DNA was subjected to polymerase chain reaction using universal primers (Table 2.1).

**Table 2.1.** Primer pairs for partial amplification of begomoviruses.

| Primer | Sequence |
|---|---|
| CLCV1 | CCGTGCTGCTGCCCCCATTGTCCGCGTCAC |
| CLCV2 | CTGCCACAACCATGGATTCACGCACAGGG |

### 2.2. Transformation and Cloning

The PCR product run on 1% agarose gel was gel eluted using Silica Bead DNA Gel Extraction Kit # K0153. Ligation of the purified DNA with the pTZ57R/T vector was performed (InsTAcloneTM PCR Cloning Kit). Competent E. coli DH5α cells were prepared using calcium chloride method as mentioned by Cohen et al. (1972). Transformation of competent E. coli DH5α cells was followed using standard protocol as mentioned by Sambrook and Russell, 2001.

### 2.3. Screening of Potential Clones

Plasmid DNA was isolated using Alkaline lysis method. Double digestion of the isolated plasmid with 5 units each of EcoR1 and HindIII was performed to confirm the presence of insert. Plasmid isolated using kit protocol (FavorPrepTM Nucleic acid extraction) was sent for sequencing.

## 3. RESULTS

### 3.1. Diagnosis of the Viral DNA

PCR amplification of the extracted DNA using universal primer designed for the partial characterization of begomovirus revealed a fragment of approximately 1.1kb on 1% agarose gel (Figure 3.1).

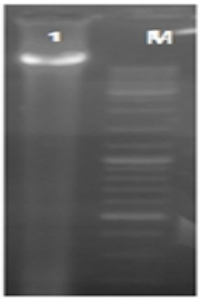


**Figure 3.1.** Total DNA (T. DNA) extracted using CTAB method. Lane 1: Contains T. DNA of C-59. Lane M: Standard 1kb ladder.

### 3.2. Transformation and Cloning

The purified DNA was ligated into T/A cloning vector (pTZ57R/T) vector for transformation. The ligated product was used to transform DH5@ cells. Blue white colonies appeared on the agar plate containing ampicillin, IPTG and XGAL after 16 hours incubation. White colonies were selected for the screening of clones containing the required DNA fragment.

### 3.3. Screening of Potential Clones

Double digestion of the isolated plasmid with 5 units each of EcoR1 and HindIII generated two fragments: one fragment of approximately 3000bp or 3kb and the other of nearly 1200bp or 1.2kb. The larger band indicated the plasmid DNA while the small band indicated that the partial amplified DNA had been cloned (Figure 3.2).

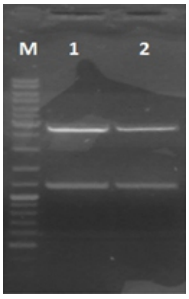


**Figure 3.2.** Gel picture showing double digestion of Plasmid DNA with EcoR1 and Hind111. Lane 1 shows double digestion of plasmid DNA obtained for clone 1. Lane 2 shows the double digestion of plasmid DNA obtained for cone 2.

### 3.4. Sequence Analysis

#### 3.4.1. Sequence Analysis Using NCBI BLAST and ORF Finder

Nucleotide BLAST results showed 99% sequence identity with the isolate CLCuBuV-[PK-Okara: 11] (Accession number: HF549184) and CLCuKV-Fai [PK: Fai1] (Accession number: AJ496286).

Sequence analysis was done using open reading frame finder (ORF finder). Three partial ORFs were found in C-59. In frame amino acid number of each protein translated form the partial ORFs were predicted and compared with that in the full length isolates (Table 3.1) and amino acid sequence identity is shown in Table 3.2.

**Table 3.1.** Predicted ORFs based on nucleotides.

| ORFs in C-59 | Predicted number of nucleotides in C-59 | Position of ORFs in a typical begomovirus |
|---|---|---|
| **CP** | 378 – 1062 | 292 - 1062 |
| **Ren** | 1059 -1211 | 1059 - 1463 |
| **C5** | 378 – 807 | 283 - 807 |

**Table 3.2.** Amino acid sequence identity.

| ORFs | Predicted number of amino acids in C-59 | Predicted Amino acids | Predicted number of amino acids in matched isolates | Percent identity |
|---|---|---|---|---|
| CP | 208 | 256 | Cotton leaf curl Kokhran virus (Accession number: NC_004583) | 99% |
| REn | 50 | 134 | Cotton leaf curl Burewala virus (Accession number: AM774301) | 98% |
| C5 | 161 | 175 | Cotton leaf curl Burewala virus (Accession number: FN645929) | 99% |

#### 3.4.2. Construction of Sequence Map

The sequence map shows that the C5 gene and the CP gene overlap with each other but in different orientation. Some part of REn overlaps with the CP gene. The CP ORF is present in the virion sense strand while REn ORF is located on the complementary sense strand.

### 3.5. Phylogenetic Analysis

The phylogenetic dendrogarm based on multiple sequence alignments between partial sequence obtained in the present study and with sequences available in the database showed its presence between CLCuBuV and CLCuKV (Figure 3.3).

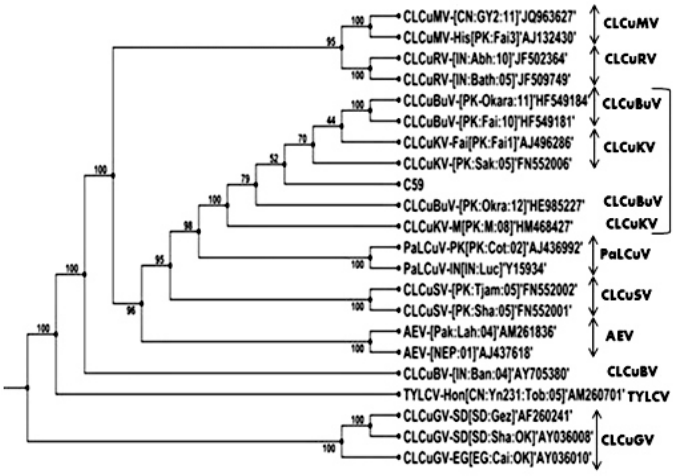


**Figure 3.3.** Phylogenetic tree generated using CLC sequence viewer. Cotton leaf curl Multan virus-Hisar[Pakistan: Faisalabad 3] (Accession number: AJ132430), Cotton leaf curl Mulatn virus -[China:GY2:2011] (Accession number: JQ963627), Cotton leaf curl Rajhasthan virus-

[India:Bathinda:2005] (Accession number: JF509749), Cotton leaf curl-[India:Abohar:2010] (Accession number: JF502364), Cotton leaf curl Burewala virus-[Pakistan:Faisalabad:2010] (Accession number: HF549181), Cotton leaf curl Burewala virus-[Pakistan:Okra:2012] (Accession number: HE985227), Cotton leaf curl Burewala virus-[Pakistan-Okara:2011] (Accession number: HF549184), Cotton leaf curl Gezira virus-Egypt[Egypt: Cairo: Okra] (Accession number: AY036010), Cotton leaf curl Gezira virus-Sudan[Sudan: Shambat: Okra] (Accession number: AY036008), Cotton leaf curl Shadadpur virus-[Pakistan:Shahdadpur:2005] (Accession number: FN552001), Cotton leaf curl Shadadpur virus-[Pakistan:Tandojam:2005] (Accession number: FN552002), Papaya leaf curl virus-India[India: Lucknow] (Accession number: Y15934), Papaya leaf curl virus-Pakistan[Pakistan:Cotton:2002] (Accession number: AJ436992), Ageratum enation virus-[Nepal:2001] (Accession number: AJ437618), Ageratum enation virus-[Pakistan:Lahore:2004] (Accession number: AM261836), Tomato yellow leaf curl virus-Honghe[China: Yunnan231:Tobbaco:2005] (Accession number: AM260701), Cotton leaf curl Kokhran virus-Faisalabad[Pakistan: Faisalabad 1] (Accession number: AJ496286), Cotton leaf curl Kokhran virus-[Pakistan:Sakrand:2005] (Accession number: FN552006) , Cotton leaf curl Bangalore virus-[India:Bangalore:2004] (Accession number: AY705380) and C-59 partial sequence cloned.

## 4. CONCLUSION

Cotton leaf curl disease (CLCuD) has been a main focal point for begomo- virologists. The disease emerged as a local nuisance in Nigeria and was ultimately reported from Sudan with a reduction in cotton production in Gezira (Bailey, 1934). In India, it was reported from Sriganganagar, Rajasthan in 1993 (Ajmera, 1994). Pakistan being the largest exporter of raw cotton depends on its export for foreign exchange. To date, the decline in cotton production in the country is largely due to this disease. Emergence of the disease was reported from Multan in 1967 (Hussain et al., 2001). Since then it has been a major limitation to cotton production in Pakistan. The frequent mutations and recombination in the genomes of begomoviruses rendering them dominant to the disease resistance imparts special emphasis on the molecular characterization of the recombining viruses.

The agent underlying the cotton leaf curl disease (CLCuD) was not clearly characterized at the time of the disease incidence. Mullis et al., 1986 described polymerase chain reaction as an in vitro DNA amplification technique that utilizes various enzymes to amplify a DNA fragment. The technique led to molecular cloning of the amplified DNA. Partial as well as full length sequences of begomoviruses have thus been analyzed in detail. Since 1998, four evident begomovirus species causing CLCuD were prevalent in Pakistan but the begomoviral genome components were not recognized (Zhou et al., 1998). Briddon et al., 1999 reported the presence of DNA A associated with the disease and at the same time Mansoor et al., 1999 reported the presence of betasatellite along with DNA A.

Mixed infections were reported by Zhou et al., 1998 in cotton plants. The Several begomoviruses prevalent at that time and known to cause the disease infected the cotton host plants simultaneously. This had resulted in mixed infections. The resultant of these infections was the emergence of Cotton leaf curl Burewala virus (CLCuBuV) prevalent as a single dominant species infecting cotton in Pakistan. The virus has emerged as a serious threat to cotton production in Pakistan since 2001. It is a recombinant of Cotton leaf curl Kokhran virus (CLCuKV) and Cotton leaf curl Multan virus (CLCuMV). The virus has been previously reported form Vehari, Khanewal, Muzaffargarh, Layyah and Faisalabad. The recombination between the two has resulted in the loss of an intact transcriptional activator protein (TrAP). The coat protein of the CLCuBuV is contributed by the partial sequence of CLCuKV (Hina et al., 2012).

The question arises that why the C-59 partial sequence shares 99% sequence identity to the Cotton leaf curl Burewala virus (CLCuBuV) and Cotton leaf curl Kokhran virus (CLCuKV) partial sequences although both begomoviruses are distinct but closely related? The answer to the question lies in the fact that the virion sense sequences of the CLCuBuV are contributed by CLCuKV ((Amrao et al., 2010a).

The sequence on the virion sense strand of Cotton leaf curl Burewala virus (CLCuBuV) relates to the coat protein (CP) or V1 and the pre-coat protein or V2 (Glick et al., 2008). The universal primer CLCV1/2 used in the present research amplifies some part of CP and V2 along replication enhancer protein (REn) and some part of transcriptional activator protein (TrAP). Sequence analysis using open reading frame finder (ORF finder) gave a significant picture that the partial CP ORF present in the C-59 partial sequence is contributed by Cotton leaf curl Kokhran virus (CLCuKV) whereas partial REn ORF is related to CLCuBuV. Another ORF has been analyzed in Burewala strain. The ORF has been termed as C5. It has been shown to overlap with the CP ORF. The C5 present in the cloned C-59 partial sequence is related to CLCuBuV.

Since, CLCuBuV is a recombinant of CLCuKV and Cotton leaf curl Multan virus and the coat protein region of CLCuBuV was derived from CLCuKV that is most probable reason that the available sequence showed identity with CLCuBuV as well as CLCuKV. A complete characterization of full length virus will determine whether isolate C-59 is CLCuBuV or CLCuKV. Literature indicates that there is no existence of CLCuKV within the region and CLCuBuV is dominating within Indo-Pak region.

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## ABBREVIATIONS

| | |
|---|---|
| µ | Micron |
| $(NH_4)_2SO_4$ | Ammonium sulphate |
| AEV | *Ageratum enation virus* |
| AYVV | *Ageratum yellow vein virus* |
| CLCuAV | *Cotton leaf curl Alabad virus* |
| CLCuBuV | *Cotton leaf curl Burewala virus* |
| CLCuD | Cotton leaf curl disease |
| CLCuGB | *Cotton leaf curl Gezira Betasatellite* |
| CLCuGV | *Cotton leaf curl Gezira virus* |
| CLCuKV | *Cotton leaf curl Kokhran virus* |
| CLCuMB | *Cotton leaf curl Multan Betasatellite* |
| CLCuMV | *Cotton leaf curl Multan virus* |
| CLCuRV | *Cotton leaf curl Rajasthan virus* |
| CLCuSV | *Cotton leaf curl Shadadpur virus* |
| CP/V1 | coat protein |
| CR | common region |
| CTAB | Cetyl trimethyl ammonium bromide |
| EDTA | Ethylene diamine tetra acetate |
| IR | Intergenic region |
| $MgCl_2$ | Magnesium chloride |
| MP | Movement protein |
| NaOH | Sodium Hydroxide |
| NSP | Nuclear shuttle protein |
| OkEV | *Okra enation virus* |
| PaLCuV | *Papaya leaf curl virus* |
| REn/C3 | Replication enhancer protein |
| Rep | Replication associated protein |
| SCR | Satellite conserved region |
| TrAp/C3 | Transcription activator protein |

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