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CHARACTERIZATION OF CIRCULAR DNA MOLECULES FROM COTTON PLANTS WITH LEAF CURL DISEASE

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Abstract

Leaf curl disease of cotton is the most devastating disease of cotton in Pakistan. *Phi29* DNA polymerase that amplifies circular DNA molecules was used to characterize circular DNA molecules found in infected plants. *Phi29* DNA polymerase amplified product was digested with different restriction enzymes and was randomly cloned. Clones were sequenced and their homology to DNA sequences available in NCBI database was estimated. The usual components consisting of begomoviruses and their associated satellites reported previously from cotton were found associated with the disease. The comparison also identified clones of non-viral origin. The most prominent among them were plasmid-like molecules of chloroplast origin. These molecules were only amplified in infected plants and were not at detectable levels in healthy plants. Other circular molecules included transposon sequences of cotton origin, their recombinants and derivatives of varying sizes. Other circular molecules showed homology to sequences related to host defense such as resistance genes, Argonaute etc. There were other circular molecules that showed no homology to any DNA sequence in the database. Some molecules were recombinant of known and unknown DNA sequences. We propose that these circular molecules are amplified due to suppression of RNA silencing based host defense by begomovirus infection.

Key words: Begomoviruses, Cotton, Chloroplast, Transposons, Circular molecules.

Introduction

Cotton (*Gossypium hirsutum*) has a soft, fluffy natural fiber that grows in a boll around the seeds and used primarily as a raw material in textile industry. The plant is actually a shrub native to tropical and subtropical regions around the world. As more than 60% of the total foreign exchange earnings comprise textile exports, thus the success or failure in cotton crop production has direct bearings on foreign exchange. A number of factors including poor farming practices, low quality seed, excessive rains at sowing time, high temperature at flowering stage, late harvesting of wheat, pest attack and the most annoying cotton leaf curl disease (CLCuD) are the major limiting factors for cotton production in Pakistan.

Many economically important crops are prone to geminiviruses infection. Geminiviruses have circular, single stranded DNA genomes and are comprised of nine genera. Genus begomovirus includes the viruses of most economic significance. These are either mono or bipartite viruses: monopartite ones making complexes with alpha and betasatellites. Betasatellites are required for pathogenicity and symptom determination while there is no clear role of alphasatellite known till date (Briddon *et al.*, 2004, Cui *et al.*, 2004, Saunders *et al.*, 2004). These are transmitted by whitefly, and their widespread distribution and diversity is attributed mainly to their vector coupled with the distribution of plant material worldwide due to international trade.

Different circular molecules are known to occur in plants. These include plant viruses, plasmid like molecules of chloroplast and mitochondrial origin, helitrons, repetitive sequences etc. It is reported that extra chromosomal circular DNA formation is enhanced upon any type of stress causing DNA damage (Cohen & Lavi, 1996). Upon prolonged heat stress, attenuation in the epigenetic regulation of repetitive elements has been observed in *Arabidopsis* very recently (Pecinka *et al.*, 2010). The stress might be of any type; abiotic or biotic including pathogen attack like virus infection. Several epigenetic changes are thought to be associated with such type of infections. These changes are considered to be responsible for the altered transcriptional activity of genes, transposons, non-coding RNAs and looping out of circular molecules.Although the origin and the diversity of plant DNA viruses have been extensively studied but exact mechanism for induction of circular molecules have not been investigated thoroughly.

Cytosine methylation has been found associated with transcriptionally silent genomic sequences including transposons, heterochromatin, repeat sequences etc. (Cokus *et al.*, 2008; Lister *et al.*, 2008). While working on different eukaryotic organisms, several evidences provide information that short interfering RNAs (siRNAs) involved in RNA interference pathway guide DNA methylation in a homology dependent manner that suppresses transposons activity, thus shielding the integrity of genome (Martienssen *et al.*, 2005; Henderson & Jacobsen, 2007). In addition, several examples have been documented showing gene regulation controlled by DNA methylation in the past few years. One of such studies is rice endosperm biogenesis regulated by DNA methylation (Zemach *et al.*, 2010). Plants also use post-transcriptional gene silencing (PTGS) i.e. homology dependent mechanism of RNAi as a defense measure against viruses (Lucioli *et al.*, 2003; Mubin *et al.*, 2007). So after getting entry into the plant system, viruses face two major threats from host side; transcriptional gene

silencing (TGS) that leads to viral DNA methylation, and post transcriptional gene silencing (PTGS). Both mechanisms of RNAi are triggered by siRNAs in their pathway. In response, viruses, both RNA and DNA viruses encode suppressors of gene silencing. Pns10 protein of *Rice dwarf virus*, an RNA virus, suppresses PTGS in *Nicotiana benthamiana* (Zhou *et al.*, 2010). P19 of *Tomato bushy stunt virus* (TBSV), P20 of *Cucumber necrosis virus* are suppressors of RNA silencing in RNA viruses (Silhavy *et al.*, 2002; Hao *et al.*, 2011). Geminiviruses also encode such silencing suppressors which inhibit PTGS (Vanitharani *et al.*, 2004) and TGS causing a genome wide hypomethylation (Buchmann *et al.*, 2009), so the epigenetic status of plant genome may be compromised upon geminiviruses infection.

Mostly PCR based detection methods have been used for the study of plant viruses. Rolling circle amplification (RCA) through *Phi29* DNA Polymerase offers a unique opportunity to study the diversity of circular molecules of viral origin that are not discovered earlier. *Phi29* DNA polymerase is a replicative polymerase from *Bacillus subtilis* phage *Phi*29 (ϕ29) having exceptional strand displacement and processive synthesis properties. This polymerase may amplify any circular molecule to which random hexamer primers bind irrespective of the nature of molecule. So, the technology also offers opportunity to study the diversity of circular molecules by cloning the molecules of non-viral origin randomly. Circular molecules of mitochondrial origin have already been reported during an experiment for the screening of curtoviruses (ssDNA viruses). For the diagnosis of BCTV in sugar beet, initially these molecules were considered as false positives in uninfected control plants. Later on, these molecules were sequenced and in BLAST search, these were found to be nearly identical with sugar beet mitochondrial plasmids (Homs *et al.*, 2008). Here we found that diverse circular molecules were also associated with CLCuD, and these molecules were amplified upon virus infection only as we could not detect them in the samples taken from healthy plants. Further, we can say host defense related gene sequences become activated upon virus infection and are more copious. The role and importance of these molecules is still to be investigated.

Materials and Methods

Sample collection and DNA extraction: Samples were collected from infected cotton plants showing leaf curl disease symptoms from different areas of Sindh and Punjab provinces of Pakistan. Some samples were collected from glasshouse of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad. The genomic DNA was extracted from leaf samples by standard method as described by Doyle & Doyle, 1990.

Quantification of DNA using spectrophotometer: To quantify the extracted genomic DNA, samples were diluted 6/54 with distilled water and absorbance was measured at 260nm using spectrophotometer standard protocol. At this wavelength optical density (O.D.) of 1 was equivalent to 50µg/mL of DNA.

Amplification of circular DNA molecules using *Phi29* DNA polymerase: *Phi29* DNA polymerase was used for the amplification of circular molecules, following rolling circle amplification (RCA) method (Fire & Xu, 1995). 1-2µl i.e.100-200ng of genomic DNA was added in a mixture containing 50 µM random hexamer primers, 2 µl (10X) *Phi29* DNA polymerase reaction buffer and 1mM dNTPs. To denature the double stranded DNA, the mixture was heated at 94°C for 3 minutes cooled to room temperature and then 5-7 units of *Phi29* DNA polymerase were added. To eliminate the accumulated pyrophosphate, 0.02 units of pyrophosphatase were also added. The whole mixture was kept at 30°C for 20-24hrs. Phi product was heated at 65^0C for 10 minutes to inactivate the enzyme.

Digestion of amplified product using restriction enzymes and cloning: Different restriction endonucleases were used for the digestion of concatameric product to yield monomers. Enzymes used were *Eco*RI, *Hind*III, *Sal*I, *Xho*I and *Bam*HI. A cloning vector (pTZ57R) was also digested with the same enzymes. Restricted RCA product and vector were purified using phenol-chloroform and then ligated in 3:1 ratio, respectively. Ligation mixture was incubated at 16°C overnight and transformed into competent *E. coli* cells the next day. Through blue-white screening, white colonies were picked and cultured in liquid LB media.

Miniprep plasmid isolation through kit: Plasmids were isolated with GeneJET Plasmid Miniprep kit (Fermentas) using the standard protocol supplied with the kit and then sent for sequencing.

Sequence Analysis: Sequences obtained were assembled using SeqMan and EditSeq (DNA Star: sequence analysis software). These sequences were compared with the database sequences by doing NCBI nucleotide BLAST (Basic Local Alignment Search Tool).

Southern hybridization: To know the titer and concentration of amplified chloroplast molecules, Southern blot analysis was performed (Southern, 2006). For this, the probe used was clone NT9, the blast result of which has 100% homology with "*Gossypium hirsutum* cultivar coker310 FR chloroplast". Total genomic DNA of infected cotton samples, DNA of the root of infected cotton plant and of whitefly was loaded and run in the gel. It was transferred to Hybond nylon membrane and hybridized to the probe mentioned above.

Results

The dilutions of total genomic DNA extracted from infected cotton samples were used in RCA reaction as template DNA. 1 µl of Phi product was loaded in 1% agarose gel to estimate the quantity of amplification. A single high molecular weight band was obtained when the gel was exposed to UV light (Fig. 1). 1 µl of Phi product was digested with selective hexacutter endonucleases which were supposed to be unique sites for geminivirus components. The restricted product was then cloned and sequenced. The fragments cloned were of different sizes ranging from 0.25kb to >3kb (Fig. 2).

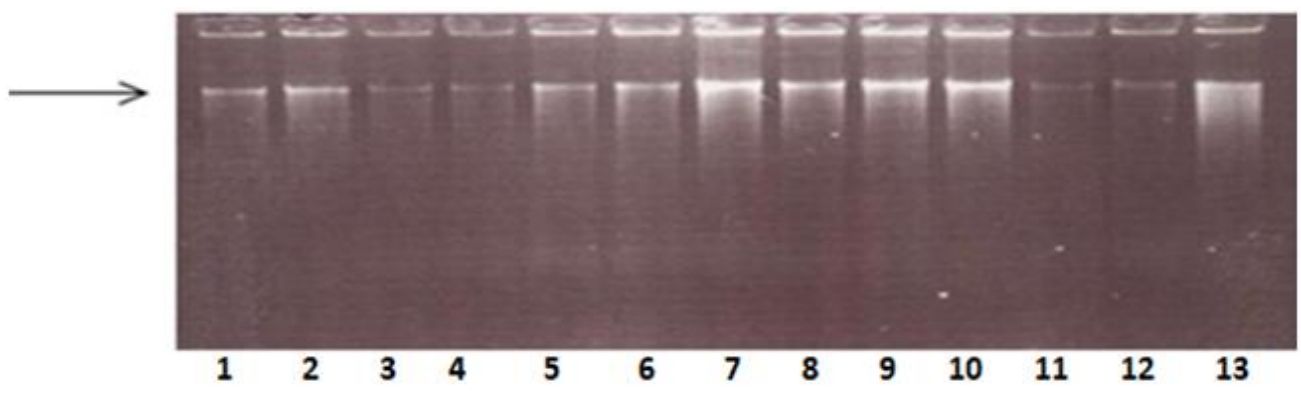

Fig. 1. Rolling circle amplification (RCA) of the DNA samples using Phi29 DNA Polymerase. Product of RCA loaded in 1% agarose gel. Arrow shows the high molecular weight RCA product.

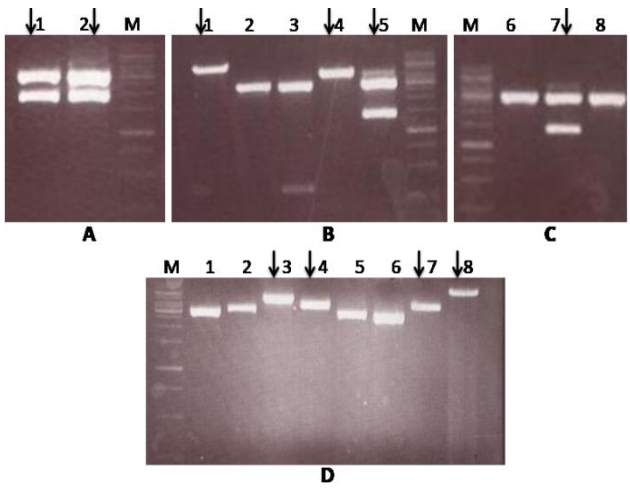

Fig. 2. Random cloning showing different sizes of fragments. pTZ57R is the vector used for these clones. M represents 1 kb DNA marker. A) A 2kb fragment was released after restriction with *Eco*R1, B & C) Using *Xho*I some of the clones were linearized as in 1st and 4th well while in 5th and 7th, 1.4kb fragments were released. D) Phi product was digested with *Eco*RI, the clones were linearized using *Sac*I.

Blast results: The sequencing results were quite surprising. When these sequences were compared with the sequences in the database through NCBI nucleotide BLAST, they showed homology to a large number of diverse circular molecules in addition to the virus components. It means that the amplified Phi products were not only comprised of viral components but all types of circular molecules were gathered in it. Table 1 shows sequence blast analysis of different clones. Some of the clones have not shown complete homology to any particular sequence in the database, so these are divided into bits and shown with different colors. Some clones are not fully sequenced and need sub sequencing so this region is highlighted with a light red color.

Circular molecules of viral origin i.e. Geminivirus components: DNA A & α-satellite: As per expectations several circular molecules of viral origin were amplified in Phi reaction. For DNA A, we have obtained partial clones. These clones had a sequence homology (>90%) with cotton leaf curl Multan virus DNA A. The samples to which they belonged were collected from Shahdadpur and Tandojam cities of Sindh, Pakistan. A sub-genomic molecule of *Cotton leaf curl Multan virus* (1.6 kb) was cloned because the Phi product was digested with *Bam*HI (not a unique site for DNA A). 1.169 kb sized fragment between two *Bam*HI sites was missing, so the clone contained two complete (AC1 and AC4) and two truncated genes (AC2 and AC3) (Fig. 3A).

Digestions with *Eco*RI and *Xho*I (unique sites for alpha satellites) cut the Phi product into 1.4kb DNA fragments (Fig. 3B). We have four such clones, three of which are fully sequenced while fourth is partially sequenced and needs subsequencing. Comparison of these sequences with the ones in database showed that these were nanovirus like particles or alphasatellites of begomoviruses. Sequence alignment of the full length clones proved that two of them i.e. SO7 and SO9 were nearly the same having 99.9% identity, while SO6 was a different one with only 21% sequence identity. Leaf samples of these clones were collected from the infected plants grown in NIBGE glass house.

Interestingly we found virus sequence integrated in cotton sequences and sequences with no significant homology in a clone (Fig. 4). It was a fragment of V2 of *Cotton leaf curl Gezira virus* (CLCuGV) DNA A with a length of 188bps ranging from position 168 to 355. Leaf samples were collected from Hala (Sindh).

Circular molecules of chloroplast origin: There are three types of circular genome containing organelles in a eukaryotic cell i.e. nucleus, mitochondria and chloroplast. Here we found some chloroplast molecules amplified in the infected cotton samples. Different sized chloroplast molecules ranging from 0.27 kb to >3 kb were amplified during *Phi*29 DNA Polymerase activity. Comparison of these sequences with full length circular genome of chloroplast revealed that these were not derived from a particular region of the chloroplast genome; rather these were amplified from diverse locations within the genome. Most of these molecules were actually inverted repeats found at different locations (Fig. 5). In addition to the intact molecules of chloroplast we also found some chloroplast sequences in conjunction with the sequences of no significant homology and some other sequences (Fig. 6). The titer of such molecules in infected cotton plants is up to detectable level by Southern hybridization (Fig. 7).

Table1. Nucleotide sequence blast analysis of different clones against NCBI nucleotide database. The complete sequence of clones has been divided into bits of 500 bases to analyze each bit.

	1-500	501-1000	1001-1500	1501-2000	2001-2500	2501-3000
SO37 (2.8kb)	NH				*Cotton*	
NT6 (2.1kb)	*Cotton*			NH		
SO5 (2kb)	*Cotton*			NH		
SO14 (2.7)	NH					*Cotton*
SO21 (2kb)	*Cotton*		NH			
SO27 (2.8kb)	*Pt*				NH	
SO48 (2.8kb)	NH				*Cotton*	
SO38 (2.9kb)	NH	*Cotton* NH			*Cotton*	
SO2 (1.734kb)	*Cotton* NH	*Vv* NH *Lj*		*Gr Gh Lj* NH		
NT4 (0.365kb)	*Cotton*	NH				
SO40 (0.23kb)	NH Zf					
SO44 (3kb)	NH	*Cotton* NH	*Cotton*		*Cotton*	NH
SO19 (1kb)	NH					
SO22 (0.54kb)	NH					
SO28 (1.2kb)	NH					
SO29 (0.45kb)	NH					
SO30 (3kb)	NH					NH
SO46 (1.7kb)	NH					
NT29 (2.1kb)	*Cotton*					
SO1 (2.65kb)	*Cotton*					
SO13 (3kb)	*Cotton*					
SO17 (2kb)	*Cotton*					
SO32 (0.69kb)	*Cotton*					
SO39 (2.68kb)	*Cotton*					

Vv= *Vitis vinifera*, Lj= *Lotus japonicus*, Zf= Zebrafish DNA sequence, Pt= *Populus trichocarpa*, NH= No significant homology

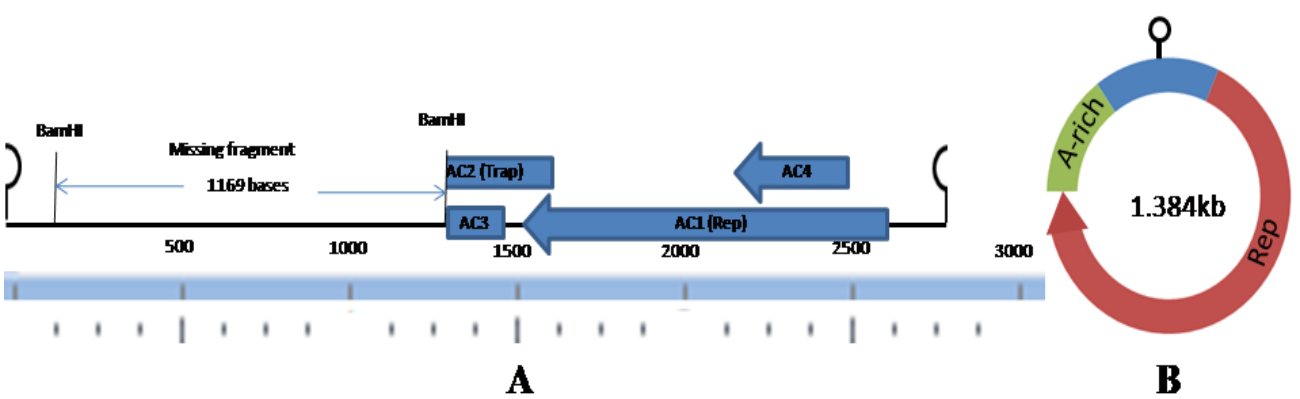

Fig. 3. Geminivirus components cloned through RCA. A) Partial clone of *Cotton leaf curl Multan virus* (1.5 kb). B) α-satellite, a full length molecule.

Circular molecules of transposon sequences: Several molecules were found having homology with transposons and transposon like sequences. Some sequences comprise of transposons plus sequences having no homology with any sequence in nucleotide database. There were other hybrid molecules having transposon sequences fused with the endogenous sequences of cotton, probably because some transposable elements captured the host genes. There is no specificity for the size of such molecules but most of them fall within the size range of geminivirus particles. Their normal size range is 1.4 kb to >3 kb but we found a 64 bases fragment too. Different transposons amplified were mutator like DNA transposons, copia like and gypsy like retrotransposons. Fig. 8 gives a comprehensive look on different types of transposon containing molecules cloned and sequenced.

Circular molecules showing homology to host defense related sequences: A complex of host defense related gene sequences become activated upon virus infection. Some circular molecules having homology to host defense related sequences were also amplified and cloned. These include resistance gene analogs (*Gossypium hirsutum* isolate Gh-RGA2 disease resistance related genomic sequence) and one of the proteins implicated in RNA silencing pathway, Argonaute4 (AGO4). Such molecules are induced only in infected plant and not found in DNA extracted from healthy plant.

Circular molecules of unknown origin: Though the components of unknown origin were found in association with chloroplast like sequences and transposons but there were some intact circular molecules as well, having no homology to sequences in the database. There is no specificity for the size of such molecules.

Circular molecules are amplified upon virus infection: In Southern hybridization experiments, it was observed that the probes used to detect chloroplast molecules and transposons were bound only to DNA from infected

samples and not to the healthy plant DNA, used as a negative control. These results suggested that all types of circular molecules discussed were amplified only from the total DNA extracted from infected cotton plants and not from the healthy plant genome.

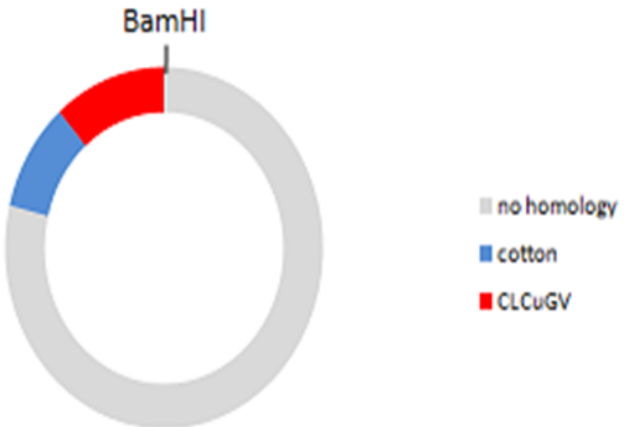

Fig. 4. A circular molecule having a fragment of CLCuGV integrated between sequences of no homology and cotton sequence.

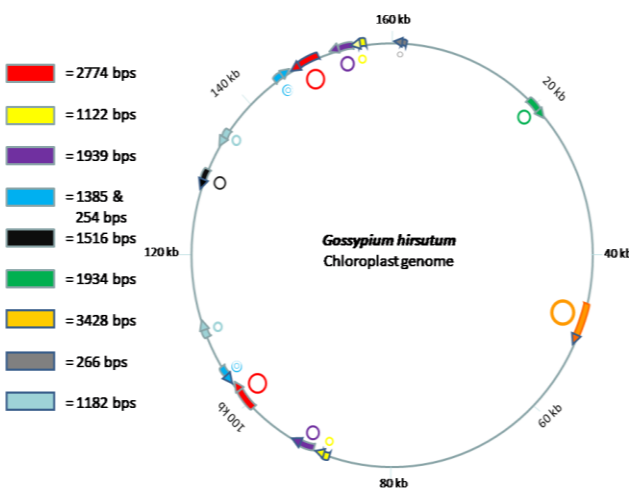

Fig. 5. Representation of looping out of small circular molecules of chloroplast genome. The big circle shows chloroplast genome of *Gossypium hirsutum* (160,301 bps). Smaller circles represent looping out of sequences denoted by differently colored arrows. Mini-circle within circle (blue) shows a small molecule looped out separately (254 bps) lying within bigger molecule sequence.

Discussion

Rolling circle amplification (RCA) by the use of *Phi*29 DNA polymerase is an isothermal technique (at 30°C) for the amplification of circular DNA molecules.The technique possesses a list of advantages which make it a routine practice in molecular cloning lab. Isothermal nature of technique requires no thermocycler. Sequence information of the template DNA is not needed because random hexamer primers are used. The amplification of all types of circular molecules reveals great power of RCA using *Phi*29 DNA polymerase. *Phi*29 has been successfully used to clone the complete begomovirus components (Inoue-Nagata *et al.*, 2004). It is only because of RCA that we have amplified some unknown sequences along with the known ones. It means that still we don't have completeinformation about the understanding of leaf curl disease. So this area is still to be investigated. The research on unknown sequences may suggest their role in relation to leaf curl disease.

RNA silencing has been regarded as the ubiquitous mechanism for defense against the foreign nucleic acids in eukaryotes (Ding, 2000; Baulcombe, 2002). It is an established fact that RNAi has some biological role in transposon silencing (Tabara *et al.*, 1999). Now we know that there are some epigenetic modifications linked to RNAi like DNA methylation and histone modifications or acetylation responsible for contributing towards heterochromatin. So induction of elevated levels of transposons suggests that RNAi has been overcome by the viral suppressors of gene silencing. Suppression of RNAi causes a global demethylation in the genome. Perhaps the amplification of other known and unknown circular molecules is also an expression of the suppression of RNA silencing or demethylation of DNA.

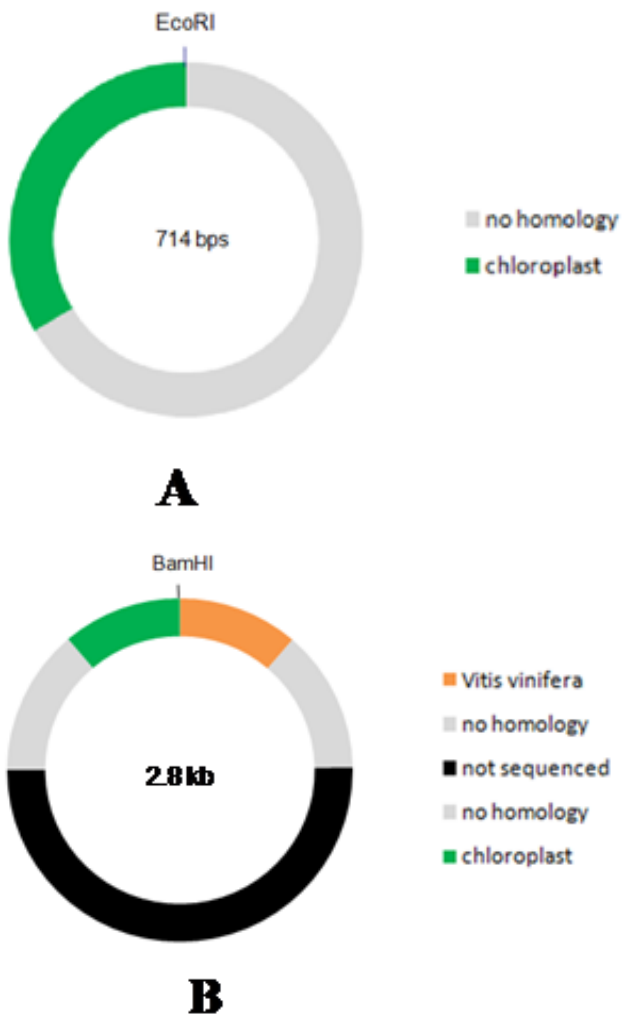

Fig. 6. Circular molecules showing chloroplast molecules in conjunction with other sequences. A) Chloroplast sequence (240 nt) in combination with sequence of no significant homology. B) A 2.8 kb sized circular molecule having a fragment of chloroplast (314 nt) embedded in it.

Leaf curling, vein thickening, vein darkening, yellowing and enations underside the leaves are considered as symptoms of CLCuD (Briddon & Markham, 2000). These symptoms suggested that upon virus infection there were some fluctuations in the copy number of chloroplast molecules. The leaves in which vein darkening occurs, number of chloroplast copy number increases while for yellowing the case was inverse. If the chloroplast copy number increases upon virus infection, there is also another possibility that virus may replicate in chloroplast using the same host resources for its multiplication.

There is enough information available regarding the increased number of chloroplast containing cells in infected plants. Idris *et al.,* (2005) captured a microscopic view of thin cross sections of leaf vein protrusions of cotton leaves infected with CLCuGV sat-DNAs. They observed the proliferation of spongy parenchyma and reduced growth of collenchymatous cells at the site of vein thickening. Qazi *et al.,* (2007) inoculated PVX βC1 in *Nicotiana tabacum* plants through *Agro*-infiltration and reported proliferated growth of spongy parenchymatous cells i.e., cells containing chloroplast, in microscopic observation of transverse sections of leaves at the site of enation. Qazi *et al.,* (2007) also observed a remarkable enlargement of these cells, displacement of vascular bundle and compression of non-affected tissues.

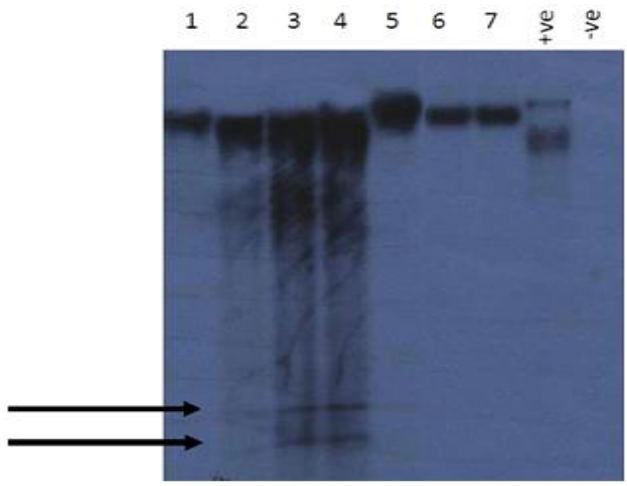

Fig. 7. Detection of small chloroplast molecules in infected cotton. The blot was hybridized with 1.5 kb dig labeled probe amplified from a clone containing a fragment of *Gossypium hirsutum* chloroplast molecule. –ve sample contains genomic DNA of healthy cotton plant grown under aseptic conditions. +ve well has plasmid. Genomic DNA of infected cotton samples from Sindh (1-4), genomic DNA of infected cotton samples from Punjab (5, 6). Well no. 7 contains the genomic DNA of *Nicotian abenthamiana* plant inoculated with CLCuMV along with its β-satellite. Arrows indicate the detection of chloroplast molecules.

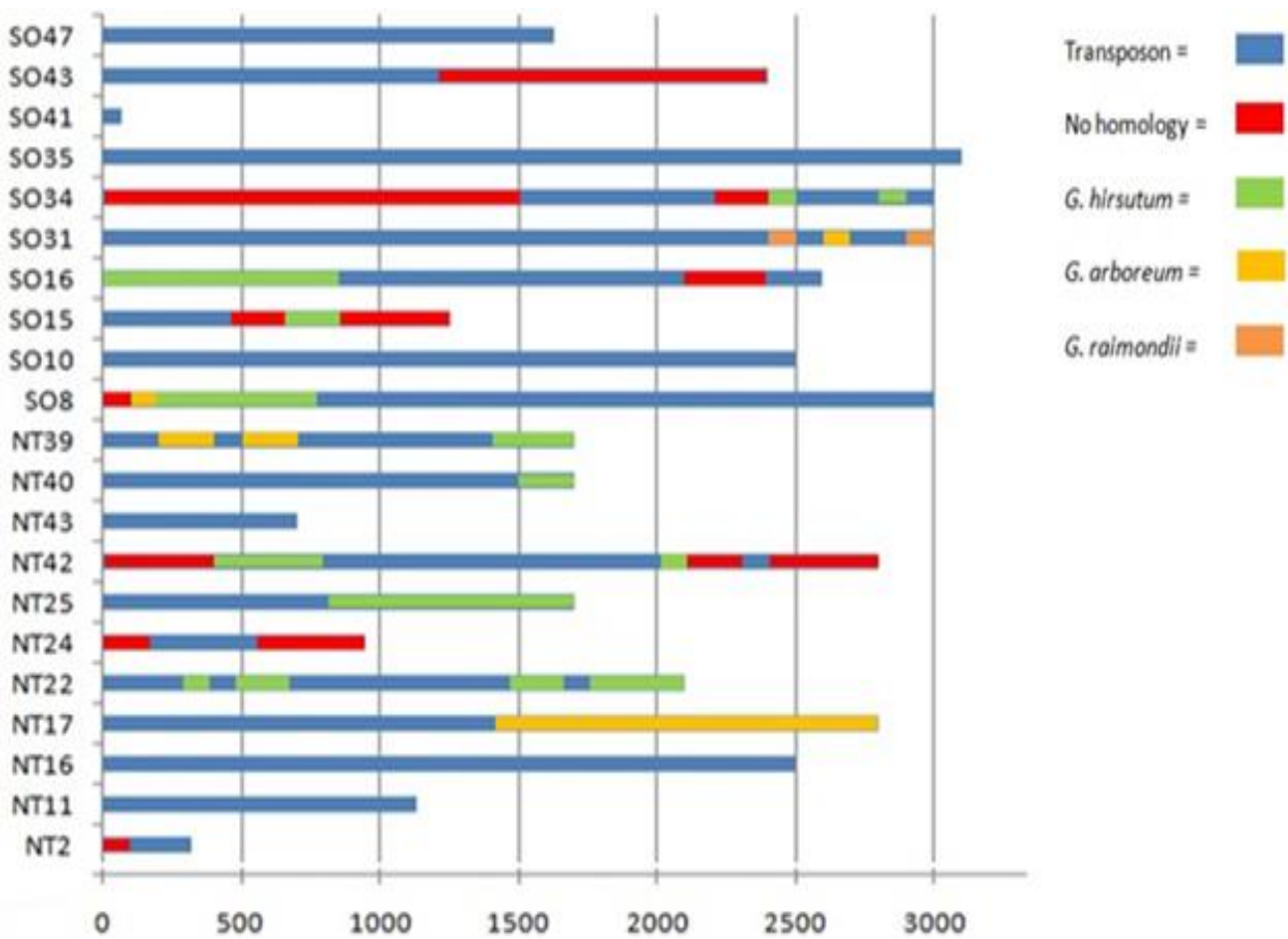

Fig. 8. Graphical representation of clones containing transposons like sequences. Out of 21 clones, eight comprise of only transposon like sequences, while others contain the hybrid molecules consisting of transposons and some other sequences. The graph was generated using Microsoft Office Excel 2007.

Conclusion

CLCuD associated begomovirus components and their interaction with host plants have been studied extensively. Characteristic feature of some virus genes to suppress the RNA silencing based host defense is also well understood. Present study is a sequel of such suppression of host defense which enables the host to make more copies of extra chromosomal circular DNA. These circular molecules include those of chloroplast genome, mitochondrial genome, transposons and other host defense related genome sequences. The role and importance of these molecules in relation to CLCuD need further investigation.

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