



Full Length Article

Construction of an Infectious Chimeric Geminivirus by Molecular Cloning Based on Coinfection and Recombination

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Abstract

In the evolution of geminiviruses, genetic recombination plays a key role in ecological, biochemical and evolutionary processes. They evolve as results of prehistoric inter-genus recombination whereas intra-genus recombination of geminiviruses leads to the emergence of agriculturally important plant pathogens. Geminiviruses are transmitted by leafhoppers, treehoppers and whiteflies. An isolate of *Chickpea chlorotic dwarf virus* (CpCDV), a monopartite dicot infecting mastrevirus is transmitted by leafhopper. In contrast, *Cotton leaf curl burewala virus* (CLCuBuV), a monopartite begomovirus, was acquired and transmissible by whitefly to various host plants. In this study, we remove the *coat protein* (CP) gene of CpCDV by PCR and replaced with that of PCR amplified CLCuBuV-CP. The resultant infectious clone of chimeric CpCDV is produced later named mastrebegomo chimera (pGII0000MBC). By using Agroinfiltration technique (*Agrobacterium*-mediated inoculation) the chimeric clone along with wild type Clone CpCDV and CLCuBuV are injected on tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana benthamiana* Domin.) seedlings and get symptoms after 21dpi (days post inoculation). Later, the symptoms appeared on plants were compared with healthy plants. This study elaborates our awareness about the genetic recombination and coexistence of mastreviruses with begomovirus globally including Pakistan and will provide the basic information regarding their management. © 2017 Friends Science Publishers

Keywords: Coat protein gene replacement; Mastrebegomo chimeric infectious clone; Symptomatic infection on plants

Introduction

Host range of Geminiviruses is becoming broader than expected in terms of their occurrence and geographical distribution (Lefeuvre *et al.*, 2010). Major factors including the whitefly biotypes dominance, emergence and recombination of viruses by expanding their host range and the betasatellite ability to interact with manifold begomoviruses contribute significantly to the emergence and resurgence of geminiviruses (Seal *et al.*, 2006). In monocot and dicot infecting mastreviruses, very similar ecological, evolutionary and biochemical factors trigger recombination patterns irrespective of the differences in host range (Martin *et al.*, 2011). Acquisition of recombination in some mastreviruses leading to the establishment of some novel traits that prompt the emergence of pathogens (Varsani *et al.*, 2008).

Geminiviruses are classified into seven genera by insect vector i.e., whitefly (Genus: begomoviruses), leafhopper (Genera: mastreviruses, becurtovirus) beet leafhopper (Genus: curtovirus) and treehopper (Genus:

topocovirus) along with their genome organization and range of host (Varsani *et al.*, 2014). However, the insect vector of eragrovirus and turncurtovirus are still unknown. Rresearch reports illustrate that all Geminiviruses possess single stranded DNA (ssDNA) molecules, which is ~2500 to ~3100 nucleotides long, encapsidated in twinned icosahedral coat/capsid. Except begomovirus which are both monopartite as well as bipartite, remaining six genera of geminiviruses have circular monopartite genome encode only few genes and they depend on host plant replication machinery for their own replication (Hanley-Bowdoin *et al.*, 1999; 2004).

Chickpea chlorotic dwarf virus (CpCDV) is a circular, ssDNA, monopartite, leafhopper transmitted mastrevirus with genome ranges from ~2.5 – ~2.7 kb was first reported in India by Horn *et al.* (1993) in *Cicer arietinum* L. Their genomes consist of two proteins on virion strand i.e., one is movement protein (MP) for cell-to-cell movement and another is structural protein i.e., coat protein (CP) for insect (vector) transmission, encapsidation, systemic infection and also act like nuclear shuttle protein (NSP) of bipartite

begomoviruses (Kotlizky *et al.*, 2000; Boulton, 2002). Whereas complementary strand encode two replication associated proteins (other geminiviruses have only one replication protein) namely Rep A and Rep B, both share ~70% amino acid sequence and required for replication of virus by rolling circle amplification by using host DNA polymerase (Liu *et al.*, 1998, 1999; Boulton, 2002). The complete genome organization of CpCDV is clearly depicted in Fig. 1a.

Coat protein (CP) play multifunctional roles including virion formation, systemic infection and transmission by insect vector but it is not required for viral DNA replication. The tripartite interaction between virus-vector-host and coat protein functions is responsible for systemic infection in geminiviruses except in bipartite begomovirus because of the presence of DNA-B, CP is not necessary for progression of symptoms as well as for systemic infection. Nevertheless, its job in spreading viral infection in plants cannot be ignored in monopartite viruses therefore any alteration in CP gene e.g., restriction fragment inversion or deletion of some base pairs result in whitefly non-transmissible capsid mutants (Azzam *et al.*, 1994).

Briddon *et al.* (1989) reported that *Beet curly top virus* (BCTV; beet leafhopper transmitted curtovirus) coat protein (CP) is essential for virus spread and infectivity. However, by replacing the *coat protein* gene of *African cassava mosaic virus* (ACMV; whitefly transmissible begomovirus) with BCTV-CP using appropriate restriction enzymes switched its transmission from leafhopper to whitefly (Briddon *et al.*, 1990). Hofer *et al.* (1997) conducted the same experiment on whitefly transmissible and non-transmissible begomoviruses i.e., *Sida golden mosaic virus* (SiGMV-Co) and *Abutilon mosaic virus* (AbMV), respectively, by applying polymerase chain reaction with specific primers (instead of using restriction enzymes). The resultant AbMV changed its transmission behavior from non-transmissibility to successful transmission.

Co-occurrence of begomovirus and dicot infecting mastrevirus in the same host is now become common and three reports have been published so far. But in these reports the host is different every time belonging to different plant families. Mubin *et al.* (2012) reported this mastrebegomo coinfection in weed (Family: Asteraceae) whereas Manzoor *et al.* (2014) and Fahmy *et al.* (2015) affirmed in cotton (Family: Malvaceae) and squash (Family: Cucurbitaceae), respectively. So, in order to investigate this aforesaid feature, we planned an experiment to remove *coat protein* (CP) gene of CpCDV (Genus: *Mastrevirus*) and in place of it insert the CP gene of CLCuBuV (Genus: *Begomovirus*) in order to make 'MastreBegomo chimera (Both viruses belonging to the same family *Geminiviridae*). We showed that this chimera producing symptomatic infection along with wild type CpCDV and CLCuBuV+CLCuM β on tomato and tobacco plants. Hence, the proposed research work help scientific community to better understands the coinfection with two different Geminiviruses especially due

to vector inspecificity thus lead to the occurrence of revolutionary recombination events occurring with the potential to yield new viruses that could adversely affect agriculture.

Materials and Methods

Selection/Construction of Infectious clones

Infectious clone of CpCDV (GenBank accession no. KP881605.1) and CLCuBuV (GenBank accession no. HF567942) were constructed and checked their infectivity in tomato and tobacco plants along with CLCuM β by using agroinfiltration method (Santi *et al.*, 2008). Final sequences have already been submitted to the NCBI database.

Construction of Recombinant Constructs of CpCDV and CLCuBuV-Polymerase Chain Reaction, Cloning and Sequencing

Coat protein gene of CpCDV was replaced by CLCuBuV by removal of the coat protein coding region of CpCDV using polymerase chain reaction (PCR) amplification (Hofer *et al.*, 1997). The machine was programmed for a preheat treatment of 94°C for 5 min pursued by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. chased by a final incubation of 20 min at 72°C. In case of CpCDV, primers were used in opposite orientation as shown in Table 1 in order to amplify the whole genome of CpCDV deprived of its coat protein gene (~1800bp). PCR-amplified coat protein gene of CLCuBuV (~771bp) was subsequently inserted into coat protein region of CpCDV genome. Specific primers were used for DNA amplification as shown in Table 1. Bands were visualized under UV on gel documentation system.

In order to create the chimeric/recombinant clone of two different viruses, PCR amplified DNA amplicons ~1800bp and ~771bp of CpCDV and CLCuBuV respectively were elutriated from agarose gel by means of *Gel Purification kit by Fermentas* as described by the manufacturer, quantified and cloned into the vector pTZ57R/T (InsT/Aclone™ PCR Cloning Kit, Fermentas) separately in order to create restriction sites i.e., KpnI and SacI (Fig. 1). Both fragments were ligated with each other called MastreBegomo chimera. RCA (rolling circle amplification) of this chimeric DNA was done and cloned this RCA restricted product with KpnI into the pGEM3Zf⁺ vector (Promega, Madison, WI). This chimeric clone (~2600bp+~3200bp) was now named as pG3Z⁺MBC. While the partial dimers were sub cloned into the binary vector, pGreenII0000 as shown in Fig. 2. For the production of recombinant plasmids, two fragments of the sizes ~1841bp and ~1804bp kb were cloned into the sticky site of pGreenII0000 (Promega, Madison, WI). Molecular vector and inserts were ligated in a 20 μ L reaction mixture containing ~170 ng molecular vector and

Table 1: List of oligonucleotide primers used for amplifying the desired fragments

Sr. No.	Geminivirus	Primer name	Primer sequence	Restriction sites
i.	CpCDV			
	Forward primer	CpMutF	<u>GAGCTCTGATTCTAGCCG</u>	SacI
	Reverse Primer	CpMutR	<u>GGTACCTGTTTCATTGTGTGAGTAG</u>	KpnI
ii.	CLCuBuV			
	Forward Primer	BurCPF	<u>GAGCTCCTGGTAATAAGGGCTAG</u>	SacI
	Reverse Primer	BurCPR	<u>GGTACCGACCAATCATCTTC</u>	KpnI

The underlined sequences are the restriction enzyme sites engineered for cloning of both fragments

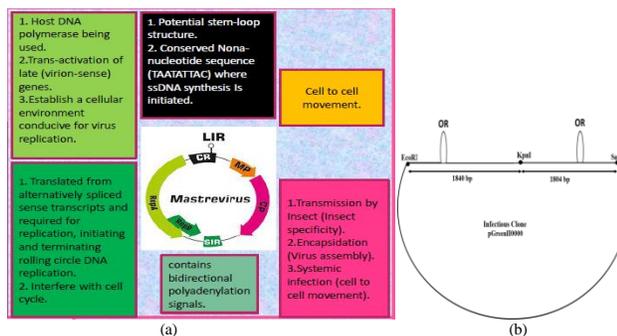


Fig. 1a: Genome organization of CpCDV (Mastrevirus) (b). Physical map of partial dimers of CpCDV into binary vector pGreenII0000 (infectious clones)

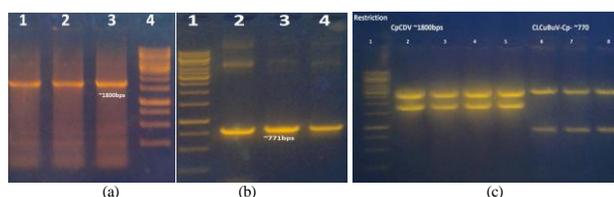


Fig. 2: Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of CpCDV and CLCuBuV. (a) Lane. 1-3 depicted ~1800bp of CpCDV genome amplified by using specific primers i.e., CpMutF and CpMutR (devoid of coat protein gene, which was later confirmed by sequencing) and Lane 4: 1Kb DNA ladder (b) Lane 1: 1Kb DNA ladder and Lane 2-4: amplified ~771bp fragment of CLCuBuV coat protein gene (c) Restriction of cloned pTZ57R/T. Lane 1: 1Kb DNA ladder Lane 2-5 & 6-8 restriction with KpnI and SacI in order to create restriction sites of PCR amplified inserts of CpCDV ~1800bp and CLCuBuV-CP gene ~771bp respectively

~560 ng inserts (1:3 ratio approx.), 2 μ L 10X ligation buffer and 1 μ L T4 DNA ligase by using the formula mentioned at http://www.insilico.uni-duesseldorf.de/Lig_Input.html. The ligation mixture was put up overnight at 16°C and the subsequently transformed into *E. coli* (DH5 α) competent cells. This construct was named as pGII0000MBC.

Genetic Analysis

Recombinant DNA techniques were applied as depicted by Sambrook and Russell (2001). DNA modifying enzymes and restriction endonucleases were used as

manufacturers recommended. The sequence and adjacent sequences of the replaced coat protein of CpCDV determined using dideoxy chain termination method with BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA according to the manufacturer's instructions.

Inoculation of Plants by Agroinfiltration Procedure

Binary vector pGII0000MBC was introduced into *Agrobacterium tumefaciens* LBA4404 by heat shock transformation method (Grimsley *et al.*, 1987). The resulting transformed bacterial strains were grown overnight at 30°C on Rifampicin Kanamycin selection plates and used to infiltrate 6 to 8 week-old greenhouse grown *Lycopersicon esculentum* leaves (maintained s at 25–30°C with a 16 h photoperiod) (Santi *et al.*, 2008). Momentarily by centrifugation at 5,000 \times g/5 min., the bacteria were pelleted and then resuspended in infiltration buffer (10 mM of 2-(Nmorpholino) ethanesulfonic acid (MES), pH5.5 and 10 mM MgSO₄) to OD600 = 0.2. By using syringe without needle, the consequential bacterial suspensions were infiltrated, either alone or as a mixture of several strains (Table 2) into abaxial side by puncturing the entirely expanded leaves of tomato and tobacco plants (Huang and Mason, 2004). Symptoms appeared after 18–21 dpi (days post-inoculation) and were recorded. After symptom induction total plant leaves nucleic acid were extracted using CTAB method (Doyle and Doyle, 1990). Samples were analyzed by Gel electrophoresis and PCR (Mullis, 1990).

Results

Confirmation of Recombinant Constructs of CpCDV and CLCuBuV

Whole genome of CpCDV (~1800bp) was amplified except coat protein (CP) region by using primer pair (i) but only coat protein region of CLCuBuV (~770bp) was amplified by using primer pair (ii) by means of polymerase chain reaction (primers sequence depicted in Table 1). (Fig. 2a and b) clearly showed the amplification of both fragments. These two fragments were cloned in pTZ57R/T separately and restrict with SacI and KpnI in order to create the restriction sites in both fragments as depicted in Fig. 2c.

Table 2: Combination of infectious clones agroinfiltrated to plants

Constructs (Infectious clones)	Figure
CLCuBuV+ CLCuMβ- Begomovirus	4 (a) and 5 (a)
CpCDV- Mastrevirus	4 (b) and 5 (b)
MastreBegomo chimera- pGII0000MBC + CLCuMβ	4 (c) and 5 (c)
MastreBegomo chimera- pGII0000MBC + CLCuMβ	4 (d) and 5 (d)
Healthy Plants	4 (e) and 5 (e)

Afterwards these fragments were purified and ligated with each other (recombinant virus/MastreBegomo chimeric virus). At this stage a new CpCDV was constructed having coat protein of CLCuBuV. This MastreBegomo chimera was cloned into pGEM3Zf⁺ with KpnI site and construct was named as pG3Z⁺MBC (as shown in Fig. 3a, b). Afterwards infectious clone was constructed in binary vector pGreenII0000 and named as pGII0000MBC and it was confirmed by restriction with KpnI (as shown in Fig. 3c) and sequencing.

Agrobacterium-mediated Transformation

Three constructs (infectious clones) were mechanically injected into plants viz' tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana benthamiana* Domin.) by agroinfiltration method either alone or in combination as shown in Table 2. Characteristic vein clearing, more greener leaves than usual and curling of juvenile leaves were appeared in plants inoculated with CLCuBuV along with CLCuMβ as shown in Fig. 4a and 5a. However, the symptoms of mastrevirus appeared as chlorotic lesions and curling and crumpling in the upper leaves of stunted plants as illustrated in 4b and 5b. Further, it was noticed that the symptoms were more severe in plants infected with mixed infection of mastrebegomo chimera (pGII0000MBC + CLCuBuV+ CLCuMβ: Fig. 4c and 5c than those associated with (pGII0000MBC + CLCuMβ: Fig. 4d and 5d. This data was recorded by comparing the symptoms of experimental plants with control (healthy) plants as depicted in Fig. 4e and 5e.

Discussion

In virus evolution, role of genetic recombination is dynamic (Martin *et al.*, 2005). One of the prospective benefits of such recombination is in sexual reproduction in order to access the joint genetic resources of distantly unrelated and related viral species (Rice, 2002; Keightley and Otto, 2006). It is evident, however, that many recombining viruses exhibit scant facts of genetic shuffling even though having the plausible immoral genetic exchanges as reported by Manzoor *et al.* (2014) a noticeable strain of *Chickpea chlorotic dwarf virus* (dicot infecting Mastrevirus)

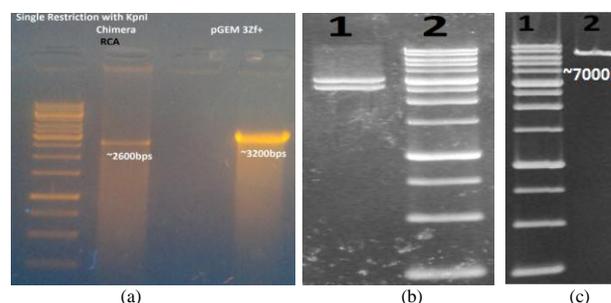


Fig. 3: Agarose gel electrophoresis (a) restriction of RCA product of MastreBegomo chimera (pG3Z⁺MBC) and pGEM3Zf⁺ with KpnI followed by ligation. (b) Lane 1. confirmation of ligation of pG3Z⁺MBC by separation of pGEM3Zf⁺ and pG3Z⁺MBC with KpnI restriction Lane 2. 1Kb DNA ladder (c) Restriction of pGII0000MBC. Lane 1. 1Kb DNA ladder. Lane 2. Restriction of cloned pGII0000MBC with KpnI resulted in linear form of pGII0000MBC

coinfection with *Cotton leaf curl burewala virus* in cotton (non-host of CpCDV) by means of trans-encapsidation in the genome of CpCDV with the begomovirus CP. However, at this time no evidence is available to propose that intergeneric trans-encapsidation is responsible for vector inspecificity, although by replacing/flip-flop the CP genes betwixt two geminiviruses related to distant genera lead to a change in vector species for virus transmission (Briddon *et al.*, 1990). Specifically, it is obvious that in foreign genetic background viral genome regions that have hardly minor genetic connections with other viral genome regions perform better functions than do those with wide-ranging interaction networks (Martin *et al.*, 2005; Lefeuvre *et al.*, 2007; Woo *et al.*, 2014).

In this paper we have constructed a chimeric CpCDV namely pGII0000MBC. From sequence data, CpCDV belongs to the mastrevirus of the family Geminiviridae, which are transmitted by leafhopper (Horn *et al.*, 1993). The coat protein of geminiviruses is indispensable for acquisition and performs a major role in insect/vector specificity (Briddon *et al.*, 1990; Azzam *et al.*, 1994; Liu *et al.*, 1997). We replaced the coat protein gene of the leafhopper-transmissible CpCDV (Genus Mastrevirus) isolate with that of the whitefly-transmissible CLCuBuV (Genus: Begomovirus) without altering contiguous sequences. This swap was adequate to construct a whitefly-transmissible chimeric CpCDV. The CpCDV and CLCuBuV (along with CLCuMβ), used for this experiment are transmitted by leafhopper and whitefly to their respective host plants. However, after agroinfiltration of tomato plants, viral DNA of wild type CpCDV, CLCuBuV and chimeric CpCDV were detected in tomato and tobacco plants by PCR and confirmed by gel electrophoresis.



Fig 4: Agroinfiltration of different infectious clones in *Nicotiana benthamiana* Domin. (a) CLCuBuV+ CLCuMβ (Begomovirus). (b) CpCDV Symptoms (Mastrevirus). (c) MastreBegomo Chimera (CpCDV+CLCuBuV+CLCuMβ). (d) MastreBegomo Chimera+CLCuMβ. (e) Healthy Plants



Fig. 5: Agroinfiltration of different infectious clones in *Lycopersicon esculentum* Mill. (a) CLCuBuV+ CLCuMβ (Begomovirus). (b) CpCDV Symptoms (Mastrevirus). (c) MastreBegomo Chimera (CpCDV+CLCuBuV+CLCuMβ). (d) MastreBegomo Chimera+CLCuMβ. (e) Healthy Plants

Tremendous genetic changes are responsible for such intergenic recombinations that are neither feasible nor as vigorous as their parents. Coinfection of viruses relating to two different genera e.g. mastrevirus (*Chickpea chlorotic dwarf virus*; CpCDV) and begomovirus (*Cotton leaf curl burewala virus*; CLCuBuV along with its betasatellite) occurs very rarely (Khalid *et al.*, 2017). At present, it is evident that dicot-infecting mastreviruses have tendency to inter-specific recombination (Krabberger *et al.*, 2013). Co-occurrence of a defective mastrevirus with begomovirus was first time reported by Mubin *et al.* (2012) in *Xanthium strumarium* L. (a weed which is non-host of CpCDV). They described that the imperfect CpCDV lack coat protein gene and so depend on begomovirus for its cell to cell movement, encapsidation and transmission).

Saleem *et al.* (2016) stated that two begomoviruses i.e., *Cotton leaf curl multan virus* (CLCuMV) and *Cotton leaf curl kokhran virus* (CLCuKoV) are highly recombinant viruses by means of phylogenetic analyses and

recombination detection programs (RDP). In many recombination events, CLCuMV is a major donor of Rep genes while CLCuKoV donated the CP gene. This intergenic trans-encapsidation may be a reason for the coinfection of begomovirus with dicot infecting mastrevirus (*Cotton leaf curl burewala virus*: CLCuBuV and *Chickpea chlorotic dwarf virus*: CpCDV) in cotton (Manzoor *et al.*, 2014). It is assumed but not evident that by transpassidation of the CpCDV with CLCuBuV-CP, CpCDV could be disseminated by *Bemisia tabaci* Gennadius (begomoviruses specific vector). Although by exchanging/interchanging CP genes tween two divergent genera of geminiviridae leading to a transition in vector species from whitefly to leafhopper (Briddon *et al.*, 1990). Recently, Fahmy *et al.* (2015) reported a coinfection of CpCDV-CLCuBuV in a non-host species of dicot infecting mastrevirus i.e. squash. Full length complete genome of the CpCDV reported first time infecting squash in Egypt with its supposed recombination events beside molecular characterization and phylogenetic analysis.

Such reports of co-occurrence of viruses belonging to different genera of same family situate heavy pressure on recombination events although they have been transmitted by dissimilar vectors. Mastreviruses are spread by leafhopper (*Orosius albicinctus* Distant: Akhtar *et al.*, 2011) whereas begomoviruses are transmissible/spread by whiteflies (*Bemisia tabaci* Gennadius.). It may endorse wide-ranging information to conduct some recombination experiments forcefully with 'Maize streak virus'- a mastrevirus (Schnippenkoetter *et al.*, 2001; Martin and Rybicki, 2002). It is apparent that the *B. tabaci* might be apt to promote and impart our chimeric 'MastreBegomo' named pGII0000MBC confirming the study that coat protein is a fundamental protein for insect specificity in virus transmission (publication under process).

Conclusion

Coinfection of viruses relating to two different genera of geminiviridae is prerequisite of intergenic recombination. Recombination leads not only to host genome diversity but also to increase in already astronomical diversity contained in virus genomes, and it offers multiple new trajectories for virus evolution and co-evolution of mastrevirus and begomovirus that may open new horizons in understanding the intricacy of Cotton leaf curl disease in Pakistan and globally and is the critical area for future research.

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