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Whiteflies are Not Responsible for Transmission of Chickpea Chlorotic Dwarf Virus and Mastrebegomo Chimeric Virus

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SK conducted the experiment and analysed the samples and data. MZR and UH designed the experiments. SS, KJ and FK reviewed the article. MSH critically reviewed the experimental design and research data and also provided diagnostic support.

Key words
Whiteflies, Geminivirus coat protein, Mastrevirus, Begomovirus, Mastrebegomo chimeric virus

ABSTRACT

Gemini virus coat protein (CP) is an important element of vector specificity and mandatory for insect transmission as well regardless of the type of gemini virus. This study was planned to conduct virus acquisition and transmission experiments using screen cages in an insectary for the period of one month. For this purpose non-viruliferous whiteflies (B cryptic species) were reared and used to acquire the viruses for 48-72 h from the agroinfiltered symptomatic tobacco (*Nicotiana benthamiana* Domin.) and tomato (*Solanum lycopersicum* L.) plants and these were liberated to healthy plants for transmission of viruses. It was evident from the results that the whiteflies were incapable to acquire and transmit chickpea chlorotic dwarf virus (CpCDV)-a mastrevirus and mastrebegomo chimeric virus (MCV) from the symptomatic tobacco and tomato plants to healthy plants. Whiteflies also failed to acquire and transmit the cotton leaf curl Kokhran virus-Burewala strain (CLCuKoV-Bu)- a begomovirus from infected to healthy tobacco plants due to the inability of whiteflies to feed on *Nicotiana benthamiana* Domin. leaves. Although whitefly successfully acquired and transmitted the CLCuKoV-Bu from symptomatic tomato leaves to healthy one whose presence later confirmed by DNA extraction from whiteflies and symptomatic plants followed by PCR amplification of CP gene and full length genome of viruses for authentication of results. So, it has been confirmed that whiteflies were not responsible for transmission of CpCDV and MCV from diseased to healthy plants.

INTRODUCTION

Transmission by insect vector is a definite event in the life cycle of a virus. This is achieved by the interaction of receptors (vector) with the specific virus encoded elements. Viruses lack the intrinsic motility so they are spread by means of their specific vectors. Plant viruses mainly transmitted by arthropod vectors including aphids, whiteflies, leafhoppers, treehoppers, plant hoppers, thrips and beetles (Gallet *et al*., 2018). A large number of plant-infecting mastreviruses cause compelling reduction in economically important monocotyledonous and dicotyledonous plants (Stanley *et al*., 2005). The processes of recombination and co-infection of different geminiviruses have turned these groups of viruses into a potent threat to the crops worldwide (Hamza *et al*., 2018).

Geminiviruses are single stranded plant infecting DNA viruses (~2500 to ~3100 nucleotides) encapsidated in twin icosahedral coat and are transmitted in a persistent, circulative and non-propagative way (Hanley-Bowdoin *et al*., 1999, 2004). They do not replicate in their insect vector (Akhtar *et al*., 2014). It is reported that the coat protein (CP) of geminiviruses assumes a prime part in infection transmission by vector specificity (Briddon *et al*., 1990). Geminiviruses are divided into nine genera (Varsani *et al*., 2017). Among them Begomovirus is the largest genus transmitted by whiteflies (*Bemisis tabaci* Gennadius.) and has the ability to co-infect with Mastrevirus (second largest genus) transmitted by leafhopper (*Orosius albicinctus* Distant.; Khalid *et al*., 2017a). To the best of our knowledge three reports have been published of co-infection of dicot infecting mastrevirus together with begomovirus infecting the same host plants i.e., weed (*Xanthium strumarium* L.), cotton (*Gossypium hirsutum* L., *Gossypium arboretum* L.) and squash (*Cucurbita pepo* L.) (Mubin *et al*., 2012; Manzoor *et al*., 2014; Hameed *et*

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al., 2018; Fahmy *et al*., 2015).

In the co-infection of mastrevirus with begomovirus, mastrevirus not only expanded its host range but also has amplified individually from host plant (Khalid *et al*., 2017b). However, at that time it was still unclear whether the leafhopper transmits the CpCDV to new host plant or the whitefly did this job due to transencapsidation of begomovirus CP to mastrevirus genome. Hence to explore this aforementioned aspect Khalid *et al*. (2017b) was designed a study to replace CP gene of CpCDV (Genus *Mastrevirus*, Family: Geminiviridae, Vector: leafhopper) with the CP gene of Cotton leaf curl Kokhran virus-Burewala strain (CLCuKoV-Bu; Genus: *Begomovirus*, Family: Geminiviridae, Vector: whiteflies) in order to successfully construct mastrebegomo chimeric virus.

In present study, transmission experiment of CpCDV, CLCuKoV-Bu and mastrebegomo chimeric virus (MCV) was performed by releasing non-viruliferous whiteflies into screen cages containing triplicates of experimental and control tobacco and tomato plants. Results indicated different efficiencies of acquiring and transmitting the viruses by *B. tabaci.*

MATERIALS AND METHODS

Collection and rearing of non-viruliferous whiteflies

Initially, the population of whiteflies *B. tabaci* (B-biptype) was collected from cotton, *Gossypium hirsutum* L. Whiteflies were reared in whitefly-proof screen cages at 26±2 ºC on healthy cotton plants in an insectary (31.29° N, 74.17° E) at Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The cryptic species of whiteflies population was confirmed by doing PCR targeting mitochondrial cytochrome oxidase 1 genes mtCO-F and mtCO-R specific primer pair. The virus free populations of whiteflies were monitored by sampling 15 adults/generation doing PCR using universal primers for mastrevirus and begomovirus as mentioned in Table I.

Preparation of host plants

Healthy tomato plants (*Solanum lycopersicum* L.) and tobacco plants (*Nicotiana benthamiana* Domin.) were used. Three replicates of plants at the three-true-leaf stage were injected with infectious clones of CpCDV (GenBank accession no. KP881605), CLCuKoV-Bu (GenBank accession no. HF567942) along with CLCuMB and MCV separately (Santi *et al*., 2008) whose infectivity was previously checked on tobacco and tomato plants by using agroinfiltration method (Khalid *et al*., 2017b). Typical leaf curl symptoms were observed on inoculated plants as shown in Figure 1. Each cage contained triplicates of tobacco and tomato plants agroinfiltered with respective infectious clone.

Table I. Primers used in this study for amplifying the desired fragments.

No	Primers/ Primer sequence 5` → 3`	PCR product size
1.	CpCDV coat protein	
	ChicCP-F GAGCTCAGGAATCAGAATCAGC *	~738 bp
	ChicCP-R GGTACCTACTCACACAATGAAACA **	
2.	CLCuKoV-Bu coat protein	
	BurCP-F GAGCTCCTGGTAATAAGGGCTAG *	~771 bp
	BurCP-R GGTACCGACCAATCATCTTC **	
3.	CpCDV	
	ChicFL-F ATATTTTGATTGGAATCTGAAGTTCTTG	~2.6 kb
	ChicFL-R AATATGTTTTCCTTACCTACCCTAAATG	
4.	CLCuKoV-Bu	
	BurFL-F ACGCGTATGGGCTGCGAAGTTSAGAC	~2.8 kb
	BurFL-R ACGCGTGCCGTGCTGCTGCCCCCATTGTCC	
5.	CLCuMB	
	Beta01 GGTACCACTACGCATCGCAGCAGCC	~1.4 kb
	Beta02 GGTACCTACCCTCCCAGGGGTACAC	
6.	MastreBegomo chimeric virus	
	MasBego-F GAGCTCTGATTCCTAGCCG	~2.6 kb
	MasBego-R CCATCCTTGATGAAGCCTCCA	
7.	*Mitochondrial cytochrome oxidase* 1 gene	
	mtCO-F GAAGTTTATGTTCTTATCTTACCAG	~ 780 bp
	mtCO-R AGCTGGTTTATTAATCTTTCATTCTA	

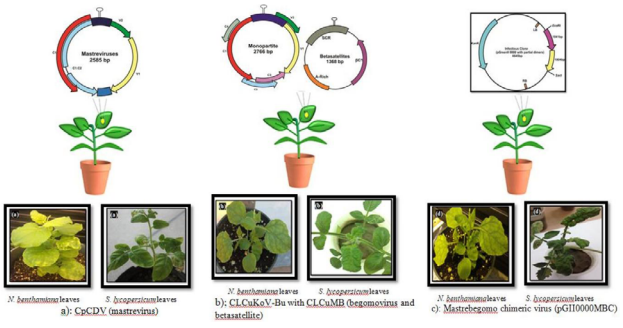

Fig. 1. Agroinfiltration of different infectious clones in tobacco (*Nicotiana benthamiana* Domin.) and tomato (*Solanum lycopersicum* L.) plants (a) CpCDV (mastrevirus) (b) CLCuKoV-Bu with CLCuMB (begomovirus and betasatellite) (c) mastrebegomo chimeric virus.

Acquisition of CpCDV, CLCuKoV-Bu and MCV by whiteflies

About 30-40 adult whiteflies were collected into a collection vial from repeated aspirations to lessen physical damage to the insects by using aspiraion device (Polston and Capobianco, 2013). These non-viruliferous whiteflies

were released into each screen cage containing triplicates of each infectious clone infected tobacco plant and tomato plants (symptomatic). Whiteflies fed on a virus infected plant for 48-72 h. After three days of feeding, plant pots were removed from the screen cages (Dennehy *et al*., 2005).

Transmission of CpCDV, CLCuKoV-Bu and MCV by whiteflies

Triplicates of healthy tobacco and tomato plants were placed in the screen cages separately having viruliferous whiteflies for feeding trial/transmission for the period of 72 h. Three days were required for the whiteflies to transmit the virus to healthy plants. After three days all the plants were taken out from the cage and placed in another virus and whitefly free cage for growth and expression of symptoms (Ambuja *et al*., 2018).

Amplification, cloning and sequencing of the CpCDV, CLCuKoV-Bu and MCV from infected leaf tissues

Total genomic DNA was isolated from the infected leaf samples using the CTAB method (Doyle, 1990). The initial CpCDV, CLCuKoV-Bu and MCV detection was carried out using degenerate PCR primers pair (ChicCP-F/ ChicCP-R and BurCP-F/BurCP-R; Table I), to amplify ~ 738 bp and ~ 771bp fragments of the CP gene of CpCDBV and CLCuKoV-Bu. The relative sizes of virus CP were visualized by using 0.8 % agarose gel electrophoresis. On the basis of the resultant CP sequences, the universal primers for mastrevirus and begomovirus and an abutting primer pair for chimeric CpCDV genome (ChicF-F/ ChicF-R, BurF-F/ BurF-R, MasBego-F/ MasBego-R; Table I) was designed to amplify the full-length genomes. Moreover, universal primers pairs Beta01/Beta02 (Briddon *et al*., 2002) were employed to amplify betasatellites from the same samples infected with CLCuKoV-Bu. All the amplicons were subsequently cloned into TA cloning vector pTZ57R/T (InsTAclone PCR cloning kit, ThermoScientific) and sequenced in their entirety from First BASE Laboratories Sdn Bhd, Malaysia.

Detection of virus DNA in whiteflies

For detection of infectious clones of CpCDV, CLCuKoV-Bu and MCV acquisition and retention, the DNA of whiteflies was extracted using the method by Barro and Driver (1997) with minor modifications and presence/absence of respective virus genome was detected by PCR by using primers depicted in Table I.

Sequence comparisons

All the obtained sequences were initially analyzed using BLASTn with already submitted sequences available in the NCBI GenBank database (http:www.ncbi.nlm.nih.gov).

RESULTS

In present study, transmission experiment was performed by releasing non-viruliferous whiteflies (B cryptic species) as insect vector. This study comprised screen cages containing triplicates of experimental and control tobacco and tomato plants. Results indicated that the efficiency of acquiring and transmitting CpCDV, CLCuKoV-Bu and MCV by *B. tabaci* was different. It was found that the whiteflies transmitted the begomovirus successfully and the presence of CLCuKoV-Bu along with CLCuMB in whiteflies and symptomatic tomato plants was confirmed by amplifying CP gene using PCR followed by amplification of full length genome (sequencing and blast with NCBI further confirmed the presence of respective begomovirus isolates) and the plants were showed typical symptoms of cotton leaf curl disease (CLCuD). However, whiteflies were incapable to transmit CLCuKoV-Bu from agroinfiltered symptomatic tobacco plants to healthy one.

Furthermore, whiteflies failed to transmit mastrevirus and mastrebegomo chimeric virus as well and the experimental tobacco and tomato plants remained healthy after one-month trial of viruliferous whiteflies (previously exposed to the CpCDV and MCV infected plants for three days) in screen cages.

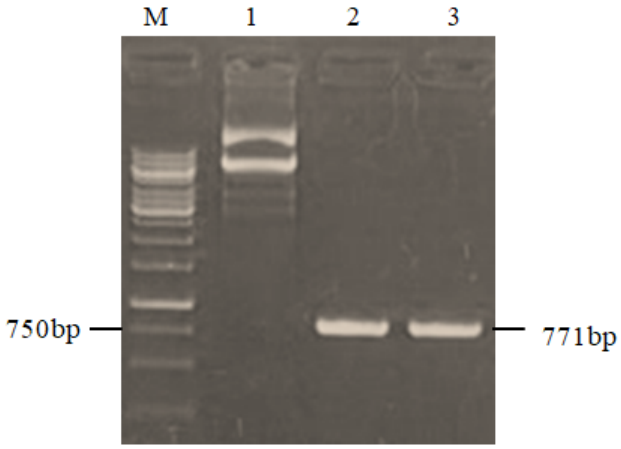

Fig. 2. PCR amplification of CLCuKoV-Bu -CP gene. Lane 1 shows no band visualized from infected tobacco plants. Lanes 2-3 shows ~ 771 bp CP gene of CLCuKoV-Bu in whiteflies and tomato plants, respectively. Lane M, 1kbp DNA ladder.

Although all the agroinfiltered plants i.e., CpCDV,

CLCuKoV-Bu + CLCuMB and MCV showed typical symptoms of the infectious clones due to the DNA replication of viruses in plants which showed that chimeric virus was constructed successfully and has the ability to replicate into host plant cells. It is evident that the PCR of full length begomovirus was positive and confirmed by sequencing. So, circular DNA of virus was definitely found in plant cell. PCR of whiteflies was negative indicating that may be virus particles were not present in plant cell. It is believed that somehow the virion formation of chimeric virus was failed mainly due to the absence of specified encapsidation signals for mastrevirus genome in begomovirus CP region. So that whiteflies did not recognize the chimeric virus and did not transmit virus to the healthy plants.

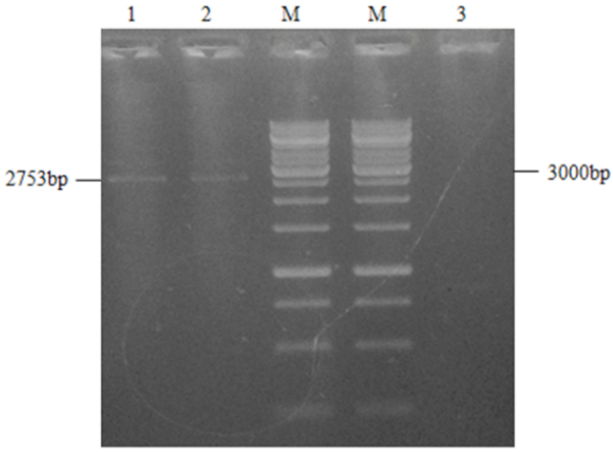

Fig. 3. PCR amplification of full length genome of CLCuKoV-Bu. Lane M, 1kbp DNA ladder. Lanes 1-2, 2753 bp fragment of CLCuKoV-Bu full length fragment amplified by using BurFL-F and BurFL-R specific primers in whiteflies and infected tomato plants respectively. Lane 3, No CLCuKoV-Bu full length fragment is amplified from symptomatic tobacco plants.

DISCUSSION

Whitefly has been a serious insect pest of horticultural and agricultural crops in many other tropical and sub-tropical countries of the world including Pakistan for more than 20 years (Qiu *et al*., 2009; De Barro *et al*., 2005; Perring, 2001; Brown *et al*., 1995). *B. tabaci* is a composite of cryptic species that cover 37 species, differentiated mainly by DNA markers (Firdaus *et al*., 2013; De Barro *et al*., 2011) and differ in host range, virus transmission, insecticidal resistance and their ability to cause plant ataxia. Among them, Middle East Asia Minor 1 (MEAM1) (earlier termed B cryptic species) and the Mediterranean (MED) (formerly termed Q cryptic species) species are the most ruinous and predominant globally (Brown, 2007; Brown *et al*., 1995). Displacement of one species with another under agricultural practices and international trade are the main causes of worldwide circulation of MEAM1 and MED (Hu *et al*., 2011). Understanding the transmission mechanisms of begomoviruses by *B. tabaci*, and the identification of virus and insect proteins involved in the translocation of the virus in the vector may elevate the efforts made to design new ways for virus control. In addition to the affliction potential of begomoviruses for cotton crop, the host range of begomoviruses is very diverse, which includes a great majority of dicotyledonous plants (Mugiira *et al*., 2008).

Co-infection of CpCDV along with a begomovirus hinting towards a changing behavior of the viruses of different genera. There is a very strong likelihood that these two viruses which belong to two different genera, might have evolved themselves to be of a helping nature which made these viruses able to have a synergistic effect to co-infect a host plant regardless to their identity as a member of different genera (Khalid *et al*., 2017a). In this study the reason behind this failure transmission might be that *Nicotiana benthamiana* species of tobacco are not the host of whiteflies (Fig. 3) (Dubey and Ko, 2008; Evans, 2007). *Nicotiana benthamiana* is a widely used experimental host plant for CLCuD symptoms induction by agroinfiltration method but at that time plants were grown enough so whiteflies were unable to suck the sap from plants that resulted in halted transmission. With the maturity of plants, some of the morphological features like waxes, lattices and trichomes are developed that make the feeding more difficult for insects (Fürstenberg-Hägg *et al*., 2013). Nicotine, extracted from mature tobacco, was natural insecticide used in crop protection (Matos, 2000). Moreover, proteinase inhibitor could be expressed in mature tobacco cells and the protein products demonstrated insecticidal activity. Its expression in roots, stems, and leaves could be activated by mechanical injury, jasmonic acid and a-linolenic acid, but not by abscisic acid (Zhang *et al*., 2004). So, in the light of this knowledge whiteflies can acquire and transmit the virus in young tobacco plants.

It is evident from our results that *CP* gene of begomovirus failed to encapsulate the circular ssDNA mastrevirus genome due to the lack of specific encapsidation signals (Fig. 2). So, CP did not encapsidates any circular ssDNA unless of relatively right size. It is assumed that specific encapsidation signals are present in the common region of begomviruses that encapsidates the DNA-A and DNA-B genomic components (Maramorosch *et al*., 1994). In this light it is quite interesting that an engineered African cassava mosaic virus (ACMV) harbouring the

CP of Beet curly top virus (BCTV) was found not only infectious but transmissible also by whiteflies (Briddon *et al*., 1990). In this case there are no specific encapsidation signals in BCTV-CP excepting that it is conserved between begomovirus and curtovirus.

Two mastreviruses MSV and Digitaria streak virus (DSV) could not transencapsidate each other even if they were coinfected to same maize host plant. These were transmissible by two different leafhopper species *viz*., MSV by *Cicadulina mbila* and DSV by *Nesoclutha declivata*. *Cicadulina mbila* can only transmit the MSV to uninfected maize plants. It is apparent that MSV-CP fails to encapsidate the circular ssDNA of DSV (Boulton, 1991). MCV was successfully constructed and replicated its ssDNA. ssDNA moved from cell to cell and produced characteristic symptoms on juvenile leaves of tobacco and tomato plants. It is proposed that due to the absence of encapsidation signals in CP region, MCV failed to produce virions (unable to encapsidate) so whiteflies did not recognize the virus and transmission was failed. In CpCDV and CLCuKoV-Bu CP region, only 18% amino acids were same. This CP amino acids distinction may be a reason for the loss of encapsidation signals in MCV. Furthermore, the packaged genetic information is dictated by the volume of capsid. So, with small sized capsids, strong pressure is exerted on genetic material to lose only that genetic information that is not necessary for virus propagation.

Keeping in view the available information regarding mastreviruses, we have adopted advance molecular techniques, which aimed at finding out the possibility of co-infection of mastreviruses with begomoviruses. It is concluded that whitefly failed to acquire MCV. The reasons might be the absence of some specific encapsidation signals, chimeric virus DNA failed to assemble in quasicosahedral capsid or it may be possible that capsids were not formed as a result of a successful transcription and translation of capsomeres. Moreover, the absence of MCV in the bodies of whiteflies supports the above notion.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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