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ORIGINAL ARTICLE

Molecular characterization and phylogenetic analysis of tomato leaf curl Palampur virus, a bipartite begomovirus, associated with *Cucumis sativus* L. in Pakistan

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Abstract

Leaf samples of *Cucumis Sativus* L. (*C. sativus*) (Family; *Cucurbitaceae*) showing vein thickening, mild leaf curling and leaf enations were collected from the farmer's field. Amplification of the full-length viral molecules was performed through rolling circle amplification (RCA). Cloning of the full-length viral molecules was done through standard cloning procedure followed by sequencing. Sequence similarity analysis and phylogenetic studies showed that the virus associated with leaf curling and enations in *C. sativus* was a bipartite begomovirus, where DNA-A and DNA-B showed highest nucleotide sequence homology of 98% and 97% to tomato leaf curl Palampur virus (ToLCPMV) from India. Attempts to isolate betasatellites and alphasatellites through PCR using RCA product as template, did not result in any amplification. A maximum likelihood phylogenetic tree grouped DNA-A and B components with other isolates from India. SDT was used to find the pairwise identity scores of different sequences of ToLCPMV present in the database. Phylogenetic analysis showed that sequences of ToLCPMV DNA-A and B components in this study share high degree of homology with existing viruses and are isolates of ToLCPMV-India. Infectious molecules of both components (Accessions, MG252783 and MG252784, respectively) were constructed for infectivity analysis to fulfill the Koch's postulate. Infectivity analysis revealed that ToLCPMV DNA-A is infectious to model host plant *Nicotiana benthamiana* and viral accumulation was confirmed through Southern blot analysis. Accumulation of DNA-B was confirmed through PCR. Infectivity analysis was also conducted using the original host, *C. sativus,* but plants were unable to survive the agroinoculation. To our knowledge this is the first report of ToLCPMV associated with *C. sativus* L. in Pakistan.

Keywords ToLCPMV · Begomovirus infection · *Nicotiana benthamiana* · *Cucumis sativus* · Infectivity analysis · Phylogenetic analysis

Abbreviations

RCA	Rolling circle amplification
SDT	Species demarcation tool
ToLCPMV	Tomato leaf curl Palampur virus
MEGA	Molecular evolutionary genetics analysis
NW	New world
CMV	Cucumber mosaic virus
SqMV	Squash mosaic virus
WMV-1	Watermelon mosaic I
WMV-2	Watermelon mosaic 2
ZYMV	Zucchini yellow mosaic
DNA	Deoxyribose nucleic acid
CTAB	Cetyltrimethylammonium bromide
ICTV	International committee on taxonomy of viruses
GUS	β-Glucuronidase
DIG	Digoxygenin
CP	Coat protein

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BLAST Basic local alignment search tool
PCR Polymerase chain reaction

Introduction

Geminiviridae encompasses nine genera; *Begomovirus*, *Mastrevirus*, *Topocuvirus*, *Curtovirus*, *Turncurtovirus*, *Eragrovirus*, *Becurtovirus*, *Grablovirus* and *Capulavirus* (Zerbini et al. 2017). Of these the genus *Begomovirus* encompasses agronomically the most important viruses. Begomoviruses are exclusively transmitted by the whitefly *Bemisia tabaci* and infect only dicotyledonous plants in the warmer parts of the world. In the New World (NW) the vast majority of begomoviruses are bipartite, with a genome consisting of two ssDNA components known as DNA-A and DNA-B. Only two monopartite begomoviruses, with genomes consisting of a homolog of the DNA-A component of bipartite begomoviruses, have been identified in the NW (Sánchez-Campos et al. 2013; Melgarejo et al. 2013; Macedo et al. 2018). By contrast the majority of begomoviruses in the Old World have monopartite genomes and are associated with small ssDNA satellites known as betasatellites and alphasatellites (Zhou 2013).

The genomes of monopartite begomoviruses (DNA-A components of bipartite begomoviruses) encode genes in both virion- and complementary-sense orientations. The genes in the virion-sense encode the coat protein (which forms the characteristic geminate capsid, is involved in insect transmission and movement in plants) and the (A)V2 protein (which is involved in virus pathogenicity, movement and may overcome small RNA-based host defenses (Wang et al. 2014; Mubin et al. 2007, 2010, 2018) whereas those in the complementary-sense, encode the replication-associated protein (Rep; a rolling-circle replication initiator protein), the transcriptional-activator protein [TrAP, encoded by the (A)C2 (also known as the (A)L2 for NW geminiviruses) gene; which is involved in the up-regulation of late viral gene expression, modulates host gene expression and may overcome small RNA-based host defenses], the replication enhancer protein (which is involved in creating a cellular environment that is conducive for virus replication) and the (A)C4 protein (which may be a pathogenicity determinant and may overcome small RNA-based host defenses) (Sharma and Ikegami 2008; Fondong 2013). Cucurbits are infected by the both DNA and RNA viruses throughout the world. The most important viruses are cucumber mosaic (CMV), squash mosaic (SqMV), watermelon mosaic I (WMV-1), watermelon mosaic 2 (WMV-2), and zucchini yellow mosaic (ZYMV). SqMV is an exception, which is seed-borne virus in melon and is transmitted by beetles, all other major viruses are transmitted by several aphid species (Ibaba et al. 2016; Sabanadzovic et al. 2016; Nagendran et al. 2016).

Pakistan, as an agricultural country, has a favorable climate for the cultivation of vegetables in all seasons around the year. Cucumber (*Cucumis sativus* L.) belonging to family *Cucurbitaceae* is an important summer vegetable cultivated during the spring season. During the last two decades, production of vegetable crops has drastically been reduced due to viral diseases. The common viruses involved in the yellow mosaic infection of cucurbits are Cucumber green mottle mosaic virus (CGMMV), Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus (WMV), Papaya ring spot virus (PRSV) and Squash leaf curl China virus (SLCCNV) (Ali et al. 2004; Tahir et al. 2010). Tomato leaf curl Palampur virus (ToLCPMV) has been investigated as an infectious agent of leaf curl disease in cucurbits, tomato, cucumber and muskmelon (Namrata et al. 2010; Heydarnejad et al. 2009; Esmaeili et al. 2015; Malik et al. 2011). This virus is relatively new introduction in agro ecological zone of Pakistan. Leaf samples from *C. sativus* plants showing virus/virus-like symptoms such as curling of leaves, veins swelling and enations were collected from the farmer's fields in Lahore, Pakistan and new strain of ToLCPMV was isolated from cucumber in Pakistan. To our knowledge this is the first report of tomato leaf curl Palampur virus that causes infection of *C. sativus* in Pakistan.

Materials and methods

Virus sources and DNA extraction

Cucumis sativus showing typical symptoms of begomovirus infection, i.e., leaf curling, vein thickening and leaf enations were collected from farmer's fields in 2011 from Lahore, Pakistan. Non-symptomatic plants were taken as negative control. Young leaves were collected, labeled and transported on ice to lab and stored at − 80 °C. Total DNA was extracted from leaf samples by CTAB method described by Doyle and Doyle (1990). The concentration of DNA was determined by Bio spectrophotometer (Eppendorf AG Model: 22331 Hamburg, Germany).

Viral DNA detection and cloning using rolling circle amplification

Total DNA extracted from infected leaves of *C. sativus* was subjected to rolling circle amplification (RCA) (Blanco et al. 1989). The RCA product was restricted using different restriction enzymes, i.e., *Sal*I, *Bgl*II, *Hin*dIII, *Kpn*I and *Eco*RI. Fragments of size 2.8 Kb, which could be begomovirus, were generated by restriction/digestion with *Hin*dIII

enzyme. The restricted product was gel eluted and cloned into the pBluescript II KS/SK [+] that was also digested with *Hin*dIII. Total of 20 clones were partially sequenced and then subsequently two full-length clones were completely sequenced through dideoxy method of sequencing to generate clones with lab names MU6 and MU7. The dilution of RCA product was used to amplify satellites associated with helper virus using universal primers of betasatellite, i.e., BETA01 F5′CCGGTACCACTACCTACGCAGCAG CC′3 BETA02 5CCGGTACCTACCCCCCAGGGGTAC AC′3 and and alphastellite, i.e., Alpha01 FCTGCAGATA ATGTAGCTTACCAG, Alpha02 CTGCAGATCCTCCAC GTGTATAG).

Sequence and phylogenetic analysis

Sequences were assembled and analyzed by the Lasergene DNA analysis package (v8; DNAStar Inc., Madison, WI, USA). Pairwise comparisons for sequence similarities were produced using the SDT program as recommended for geminiviruses species demarcation (Zerbini et al. 2017). Phylogenetic trees were generated, first by aligning the sequences using CLUSTAL-W, followed by Maximum likelihood method of phylogenetic tree construction in MEGA7 program (Kumar et al. 2016). The accession numbers for MU6, i.e., DNA-A and MU7 i.e., DNA-B are GenBank: (MG252783) and (MG252784), respectively. The other begomovirus sequences were downloaded from GenBank and virus abbreviations are used as described by ICTV (Zerbini et al. 2017). Species demarcation tool (SDT analysis) (Martin et al. 2010) was used to identify the pairwise sequence identities among sequences.

Construction of infectious clones of virus

Infectious clones for both components of the begomovirus were generated in pBIN19 binary vector (Dry et al. 1997). For infectious molecule of DNA A, MU6 was restricted with *Hin*dIII and *Eco*RI producing two fragments of 1215 bp with origin of replication (confirmed through sequencing) and 1541 bp. Fragment of 1215 bp was cloned in pBIN19 at *Hin*dIII and *Eco*RI to generate pBIN19-ToLCPMVA.1.2. Then full-length molecule was also cloned in same construct at *Hin*dIII site to generate pBIN19-ToLCPMVA.4.0. For infectious molecule of DNA B, MU7 was restricted with *Hin*dIII and *Bam*HI producing two fragments of 1100 bp with origin of replication (confirmed through sequencing) and 1600 bp. Fragment of 1100 bp was cloned in pBIN19 at *Hin*dIII and *Bam*HI to generate pBIN19-ToLCPMVB.1.1. Then full-length molecule was also cloned in same construct at *Hin*dIII site to generate pBIN19-ToLCPVMB.3.9.

Agrobacterium-mediated inoculation of plants

The binary vectors pBIN19-ToLCPMVA.4.0 and pBIN19-ToLCPMVB.3.9 were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze–thaw method (Wise et al. 2006). Later, agro-infiltration was performed by injecting 0.2 ml of culture into *Nicotiana benthamiana* and *C. sativus* leaves as described previously (Ma et al. 2012). Seeds of *N. benthamiana* and *C. sativus* were sown on mixed soil containing sandy loam and peat moss. Pots were kept under controlled conditions with 16 h/day light and the temperature between 22 and 28 °C in growth room for the germination of seeds. After 15–20 days of seed germinations, small seedling was transferred in pots and maintained in growth room. Hoagland's nutrient solution was prepared and used as source of nutrient. The inoculation details are presented in Table 1.

Detection of viral replication in inoculated plants

Total genomic DNA was isolated from the systemically infected plant tissue upon appearing symptoms, using CTAB

Table 1 Layout of the infectivity experiment: representing the inoculations of *Nicotiana benthamiana* and *Cucumis sativus* plants with ToLCPMV

Crop/vegetables used for agro-inoculation	Virus/infectious clones	Plants used for inoculation + control plants	Days post inoculation (dpi)	Symptoms	Southern blotting and PCR analysis
Nicotiana benthamiana	ToLCPMV/(pBIN19-ToLCPMVA.4.0 and pBIN19-ToLCPVMB.3.9)	15 + 15	10–12	15 plants showed severe symptoms of leaf curling and vein thickening, control plants were symptomless	All Plants were positive for ToLCPMV through PCR and two selected plants were positive through Southern blot analysis
Cucumis sativus (Cucumber)	ToLCPMV/(pBIN19-ToLCPMVA.4.0 and pBIN19-ToLCPVMB.3.9)	15 + 15	8–10	Inoculated patches were dead leading towards plant wilting and then death	

method (Doyle and Doyle 1990). Viral DNA was detected by PCR with the universal primer pairs (Briddon and Markham 1994). For Southern blot hybridization analyses, total genomic DNA (500 ng) was equally loaded in each lane and separated on 1.5% agarose gel. The DNA was later transferred onto nylon membrane, as recommended by manufacturer (Hybond N+; Amersham). The Digoxygenin (DIG) labeled PCR products were amplified for coat protein (CP) gene of DNA-A component by the primers (CP-F 5′-ATG TGGAAGCGTACCGCCGATA-3′, CP-R 5′-TAATTCACG ATCCTGTCATAAAAGTA-3′) to be used as probe, as recommended by the manufacturer (Roche, PCR DIG Probe Synthesis Kit).

Results

Cloning and sequencing of viral components from *Cucumis sativus*

Leaf samples of *Cucumis sativus* showing symptoms of begomovirus infection, i.e., leaf curling, vein swelling, leaf enations were collected from farmer's field in Lahore, Pakistan (Fig. 1a). There were fields of cultivated crops like cotton and other vegetable crops around the *C. sativus* field.

Total of 20 molecules of size 2.8 kb were cloned from 7 different infected plant samples. Partial sequencing showed that every plant is infected with a bipartite begomovirus, i.e., tomato leaf curl Palampur virus (ToLCPMV). Two clones (MU6, i.e., DNA-A and MU7, i.e., DNA-B) were completely sequenced and their analysis showed that both clones have typical genome organization of bipartite begomoviruses (Zaidi et al. 2017). The sequences obtained were compared with reported sequences in NCBI database using BLAST analysis and results showed that MU6 and MU7 are isolates of ToLCPMV, i.e., ToLCPMV-[PK:LHR:11]-MG252783 and ToLCPMVB-[PK:LHR:11]-MG252784. There was no amplification of 1.4 Kb fragments, i.e., satellite molecules, when RCA was used as template in PCR.

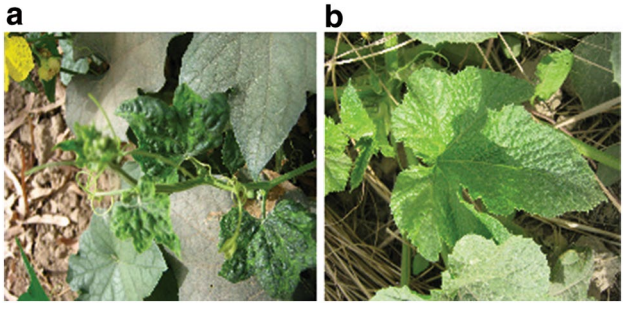

Fig. 1 *Cucumis sativus* plant, **a** symptomatic plant showing vein thickening and leaf curling symptoms; **b** asymptomatic plant

SDT analysis

Sequences were aligned with begomovirus sequences available in databases using MUSCLE and pairwise identity scores were calculated with SDT (species demarcation tool) (Martin et al. 2010). ToLCPMV-[PK:LHR:11]-MG252783 shared 98% and 97% nucleotide identity, with Indian isolates ToLCPMV-[IN:Punjab:10]-KC456161 and ToLCPMV-[IN:Him Pra:06]-AM884015 while 93–94% with Pakistan and Iranian isolates (Fig. 2a). ToLCPMVB-[PK:LHR:11]-MG252784 shared 97% nucleotide identity with Indian isolates ToLCPMVB-[IN:Pb:Tom:10]-KC456162 and ToLCPMVB-[IN:Him:Sol:06]-AM992534 and 96% homology with Pakistan isolate ToLCPMVB-[PK:Vehari:CucMelo:05]-FR856888. It showed 88–90% sequence homology with Iranian isolates ToLCPMVB-[IR:Jir3:T4P:Cuc:07]-FJ660430 and ToLCPMVB-[IR:Jir7:T11P:Cuc:07]-FJ660426 (Fig. 2b). Nucleotide sequence homology of both components with the most closely related begomoviruses was above the 97% threshold for species demarcation, thus confirming that the begomoviruses found infecting *C. sativus* in Pakistan are isolates of ToLCPMV.

Sequence and phylogenetic analysis

A phylogenetic dendrogram based upon an alignment of complete nucleotide sequences of the genomes of selected begomoviruses and the sequences of isolates from this study are shown in (Fig. 3). In a BLAST search, the complete sequence (2756nt) of ToLCPMV-[PK:LHR:11]-MG252783 showed 98% nucleotide sequence identity with tomato leaf curl Palampur virus from India, i.e., ToLCPMV-[IN:Punjab:10]-KC456161. The phylogenetic tree further confirmed that ToLCPMV-[PK:LHR:11]-MG252783 forms a cluster with other isolates from India i.e., ToLCPMV-[IN:Rauke:09]-KF663700 and ToLCPMV-[IN:Punjab:10]-KC456161 (Fig. 3a). Phylogenetic tree showed that the ToLCPMVB-[PK:LHR:11]-MG252784 forms a cluster with other isolates in the region ToLCPMVB-[IN:Hima:Sol:06]-AM992534, ToLCPMVB-[IN:Hima:Rumex:13]-KT895906, ToLCPMVB-[IN:Pb:Tom:10]-KC456162, ToLCPMVB-[PK:Vehari:Cuc:05]-FR856888 and ToLCPMVB-[PK:Chicha:Cuc:05]-FR851930 (Fig. 3b). ToLCNDV DNA-B, (ToLCNDVB-[IN:ND:Luffa:10]-HM989846), a bipartite virus from same genus was included in the phylogenetic analysis as an outgroup. In phylogenetic tree (neighbor joining), Iranian isolates are grouped in cluster I, whereas cluster II includes both Indian and Pakistani isolates (Fig. 3).

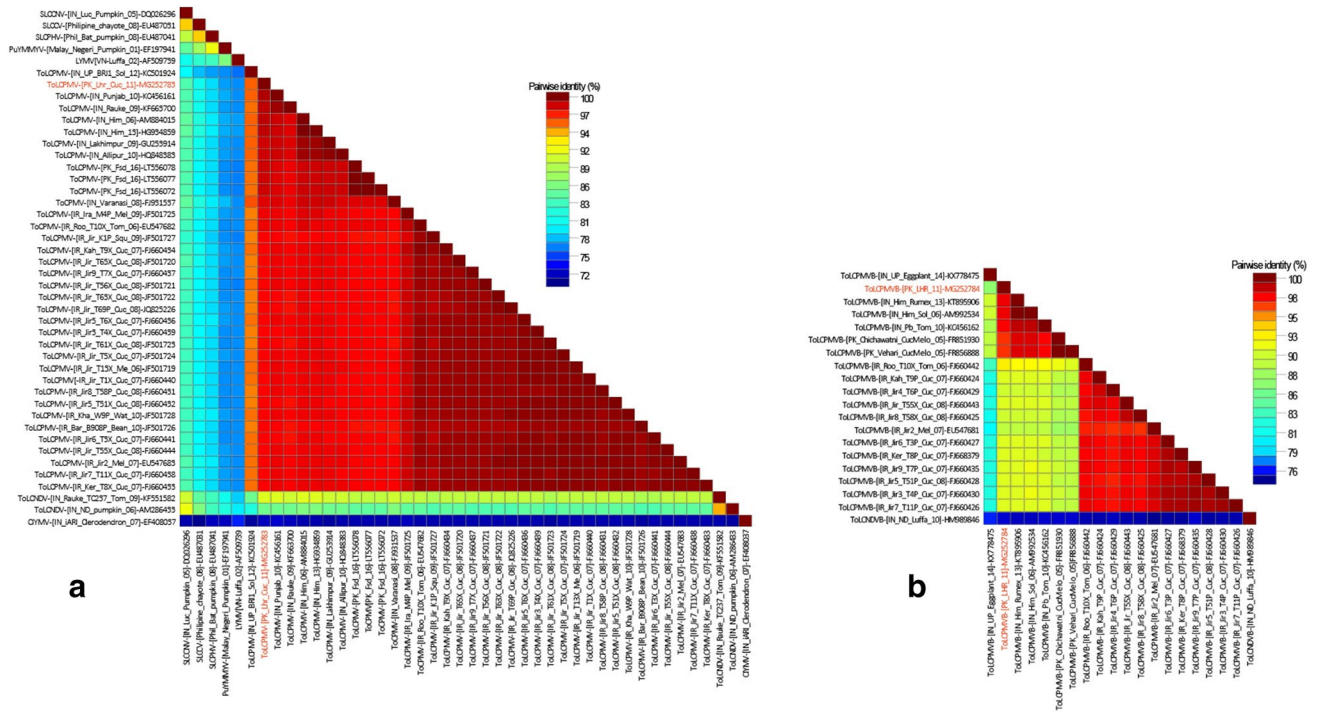

Fig. 2 Color-coded matrix of pairwise nucleotide identity inferred from alignments of complete, **a** DNA-A and **b** DNA-B sequences of ToLCPMV and selected bipartite begomoviruses. The matrix uses a discontinuous range of three shades of color (red, green and blue) differentiating two cut-off values representing the strain (93–94%, green–red) and the species (90–91%, blue–green) demarcation thresholds of begomoviruses. Identities were calculated with SDT v. 1.2 (Martin et al. 2010). Isolates from this study are indicated with red color

Infectivity analysis of tomato leaf curl Palampur virus (ToLCPMV)

Fresh cultures of *Agrobacterium tumefaciens* cells transformed with the empty vector pBIN19 and binary vectors harboring infectious molecules, i.e., pBIN19-ToLCPMVA.4.0 and pBIN19-ToLCPVMB.3.9 were cultured under standard conditions. Activated cultures were used for inoculation of *N. benthamiana* and *C. sativus* plants. *Agrobacterium* mediated inoculation of infectious molecules, i.e., pBIN19-ToLCPMVA.4.0 and pBIN19-ToLCPVMB.3.9. in *N. benthamiana* resulted in upward leaf curling, leaf darkening and stunted growth at 10–12 dpi (Fig. 4a, b). Control plants inoculated with empty pBIN19 vector did not show any symptoms (Fig. 4c). Details of inoculations are enlisted in Table 1. The inoculations experiments were repeated three times. Inoculated patches in *C. sativus* leaves became dead, leading to wilting and death of whole plant. DNA from systemic leaves of infected *N. benthamiana* plants was subjected to RCA and PCR to confirm the presence of replicating MU6 and MU7 (Data not shown). The replication and movement of virus in systemic leaves was also checked with Southern blot hybridization (Fig. 4d). For this purpose CP of ToLCPMV was used as a probe. Purified PCR product of the CP was used as +ve control while DNA isolated from a *N. benthamiana* plant inoculated with simple vector pBIN19 was used as –ve control. Viral symptoms on *N. benthamiana* were severe and signals in Southern blot analysis were almost of same intensity (Fig. 4d).

Discussion

Viral diseases involving DNA as well as RNA viruses are a major threat to agriculture and food security in Pakistan. Begomovirus, i.e., ssDNA viruses, transmitted by whiteflies (*Bemisia tabaci*), associated diseases specifically have become a greater threat to economically significant food and fiber crops (Hanley-Bowdoin et al. 2013; Sanz et al. 2000). This is mainly due to global proliferation in the population and spreading of insect vectors, trans-continental shipment of plant resources and also due to the appearance of new and more aggressive whitefly biotypes (Varma 2003). These viruses cause a variety of symptoms including vein yellowing, yellow mosaic, and leaf curl and are spreading at an alarming speed due to a high rate of recombination (Seal 2006; Pita et al. 2001). Several viruses are invading new ecological zones, where they were not reported before. There are number of reports showing potyviruses and RNA viruses such as Cucumber green mottle mosaic virus (CGMMV),

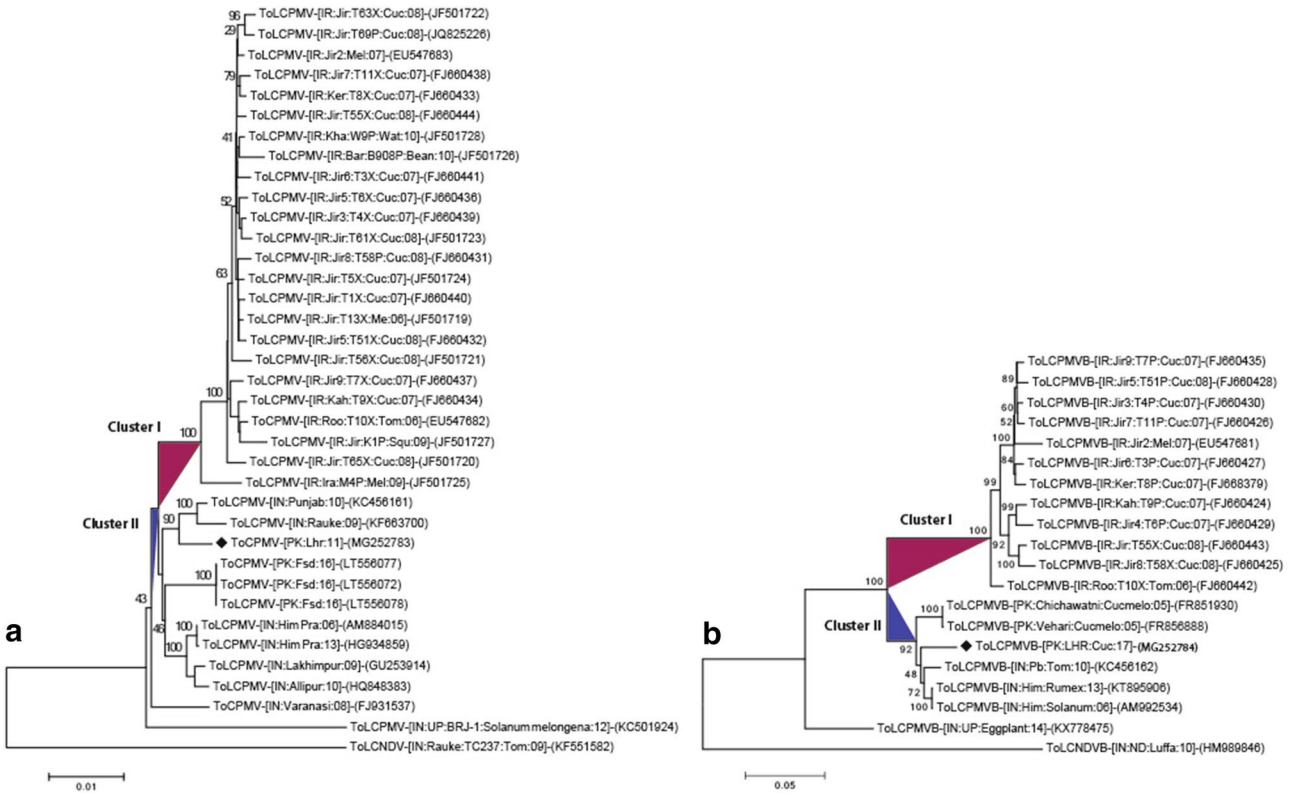

Fig. 3 Phylogenetic dendrogram based upon selected complete sequences of ToLCPMV; **a** DNA-A component, **b** DNA-B component. Begomovirus sequences of both components used for comparison were downloaded from GenBank. The database accession number and complete description in each case is given. The sequences associated with leaf curling and vein thickening disease of *C. sativus* are indicated by a square shape. Two clusters of sequences are shown, Cluster I (maroon color) and Cluster II (blue color). Isolates from this study are indicated with a square shape. The values of 0.01 and 0.05 represent the genetic variation for the length of the scale

Zucchini yellow mosaic virus (ZYMV), Watermelon mosaic virus (WMV), Papaya ring spot virus (PRSV) and Squash leaf curl China virus (SLCCNV) infecting cucurbits in Pakistan (Ali et al. 2004; Tahir et al. 2010). In our study we have found an isolate of ToLCPMV which is frequently infecting cucurbits (Heydarnejad et al. 2009; Malik et al. 2011).

As compared to ToLCNDV, the ToLCPMV is a recently identified begomovirus, reported from bitter cucumber, Rumex sp, tomato, pumpkin, squash, cucumber, bitter gourd, common bean, water melon, chilies, and muskmelon from India and Iran (Namrata et al. 2010; Heydarnejad et al. 2009; Esmaeili et al. 2015). In Pakistan ToLCPMV was first detected in 2007 infecting Bitter gourd (*Momordica charantia* L.), showing typical symptoms of the disease including chlorosis, leaf crumpling, vein thickening, and stunting of plants (Ali et al. 2010). ToLCPMV was also able to develop a synergistic interaction with a potyvirus, Zucchini yellow mosaic virus (ZYMV) while infecting muskmelon to produce a severe leaf curl disease (Malik et al. 2011). The present study deals with molecular and phylogenetic characterization of ToLCPMV from naturally infected cucurbit (*Cucumis. sativus* L.). Symptoms found in cucurbit were diverse, i.e., leaf curling marginal and interveinal leaf chlorosis, stunting and leaf crumpling. Upon sequence analysis, we found an isolate of ToLCPMV along with its cognate DNA-B component. When infectious molecules of ToLCPMV were agro-inoculated on model plant *N. benthamiana,* the virus replication was observed and confirmed through Southern blotting in systemic leaves. Repeated attempts to amplify satellites using RCA product as a template in the PCR, failed to identify their association with the ToLCPMV. These results showed that despite of co-circulation of different satellites in the same region, this virus has not captured any of them. Earlier cucurbit hosts were infected with ToLCNDV (Nagendran et al. 2017; Fortes et al. 2016; Nagendran et al. 2016) but now it is becoming clear that a new bipartite begomovirus ToLCPMV is becoming a major viral threat to cucurbits. The intercropping of cucurbits and other crops such as chilies, cotton and especially tomato (a natural host for ToLCPMV) is a common practice in the farmer's fields, so they may become the infection source for the next tomato-growing season. Therefore, we suggest discouraging the intercropping of tomato and cucurbits to prevent very likely outbreak

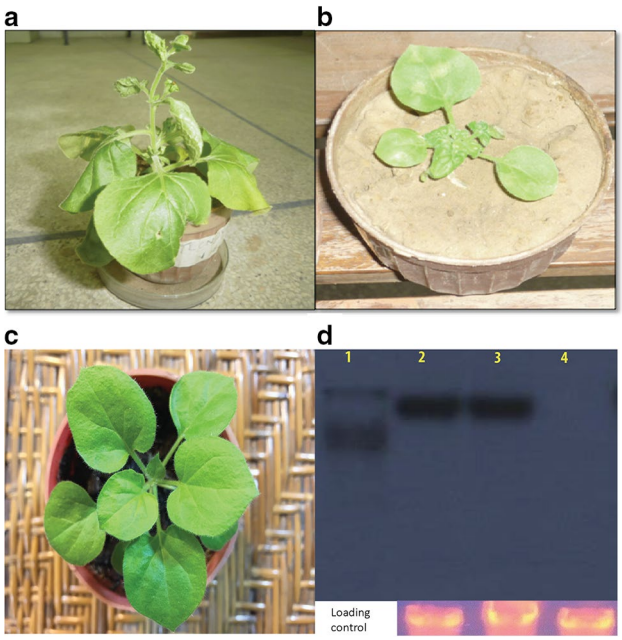

Fig. 4 Infectivity analysis on *Nicotiana benthamiana* plant hosts through the agroinoculation of infectious molecules of ToLCPMV: **a**, **b** *N. benthamiana* plants showing severe leaf curling in systemic leaves 10–12 dpi, **c** *N. benthamiana* plants inoculated with empty pBIN19 vector showing no symptoms, **d** Confirmation of ToLCPMV replication in systemic leaves of *N. benthamiana* through Southern blot analysis after agroinoculation with infectious molecules of ToLCPMV; lane 1 shows positive control, lane 2–3 shows viral replication signal from systemic leaves of *N. benthamiana* plants, lane 4 is negative control, Coat protein gene of ToLCPMV was used as probe. Cloned virus ToLCPMV in pBluescript II KS/SK [+] vector was used as positive control

of ToLCPMV epidemics in tomato fields, which will usually cause a great loss of tomato production. If the cultivation of tomato and cucurbits in the same area is inevitable, the cultivation system, isolation distance, and timing should be carefully considered to make effective management strategies for ToLCPMV control.

The DNA-A component isolated from cucumber in this study is a variant and encodes five ORF (Rep, TrAP, REn, AC4 and AC5) in the complementary-sense and two (pre CP and CP) in the virion-sense are involved in encapsidation and movement (Zaidi et al. 2017).

The sequence analysis of ToLCPMV infecting *C. sativus* showed its 97% sequence homology to ToLCPMV (AM884015) isolated from India and 96% to the ToLCPMV (FJ660444) isolated from Iran. The DNA-B component was 92% sequence similar to the isolate from *Cucumis melo* L. (ToLCPMVB-[PK:Vehari:Cucmelo:05]-FR856888) from Pakistan (Malik et al. 2011). In this association ToLCPMVB was found with a defective NSP protein. It was hypothesized that ZYMV has complemented the DNA-B function. However, in this case, although defective DNA-B was found, but there was no evidence of synergism with RNA viruses. Nucleotide sequence identity of DNA-A component with the most closely related begomoviruses was above 97% threshold for species demarcation, thus confirming that the begomoviruses found infecting *C. sativus* in Pakistan are isolates of ToLCPMV. A maximum likelihood phylogenetic tree grouped DNA-A (ToLCPMV-[PK:LHR:11]-MG252783) into well-supported clades together with ToLCPMV-[IN:Rauke:09]-KF663700 and ToLCPMV-[IN:Punjab:10]-KC456161 isolated from Indian Punjab. Although, at nucleotides level both the Iranian and Indian isolates fall into same species group, but the Indian or Pakistani isolates clustered together instead of Iranian clusters. Same is true for DNA-B, where ToLCPMVB-[PK:LHR:11]-MG252784 closely clustered with Indian isolates instead of Iranian isolates. Iran shares its mountainous border with Pakistan, which is a dry region and agriculture is not practiced. It is possible that ToLCPMV moved to Iran through trade of infected plants fruit or seedlings. However, such speculations need to be confirmed at molecular level.

Conclusion

Phylogenetic analysis showed that the viruses isolated from *C. sativus* in this study is an isolate of ToLCPMV as it shares high degree of homology with existing ToLCPMV. The discovery of ToLCPMV infecting *C. sativus* adds another virus to the list of species that might be part of a complex that causes leaf curl disease in Pakistan, but it is clear that, individually, this species might be a major threat to cucurbit production in Pakistan and surrounding South Eastern countries. Infectivity analysis showed that these viruses are capable of infecting new hosts, i.e., *N. benthamiana* and tomato other than original host i.e., *C. sativus*. So might be in future this virus can cause epidemics to other crops such as tomato, chilies and radish in Pakistan.

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Author contributions MS, SM, AR, WS, MI were involved in performing experiments. MI, MSH, MSNR and MM were involved in data analysis, experimental design. MSH, MSNR, AY and MM wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval Not Applicable.

Informed consent Not Applicable.

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