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Full Length Article

In Planta Interaction and Transreplication of Distinct Begomoviruses and their Associated Components

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Abstract

The studies described here were intended to examine the transreplication and interactions abilities of a widespread ToLCNDV, and an emerging begomovirus PeLCV associated with its cognate betasatellite TbLCuB. PeLCV, a monopartite begomovirus, has been characterized from many important crops, vegetables and weeds along with its associated TbLCuB. The DNA-B of bipartite ToLCNDV genome has been successfully transreplicated by the DNA-A of different bipartite begomoviruses, albeit with low frequency. Whether PeLCV can transreplicate DNA-B of ToLCNDV is unknown. To unravel this notion, both these viruses were inoculated to the model *Nicotiana benthamiana* plants in all possible combinations and the *in planta* existence of viral components were verified by PCR and Southern blot hybridization. The results demonstrated that PeLCV transreplicated and maintained ToLCNDV DNA-B. Whereas, ToLCNDV DNA-A could not transreplicate TbLCuB. Analyses of Rep proteins structure of ToLCNDV and PeLCV revealed a structural resemblance, whereas putative iteron-binding sequences of PeLCV were compatible with the Rep-binding iterons of ToLCNDV-B. The results suggested that PeLCV and ToLCNDV DNA-B can interact synergistically and can be disastrous under field conditions.

Keywords: Begomovirus; Interaction; PeLCV; ToLCNDV; TbLCuB; Transreplication

Introduction

The viruses in the genus *Begomovirus* and family *Geminiviridae* are small geminate shaped particles containing a circular, single-stranded (css) DNA genome and depends upon a distinct whitefly species *Bemisia tabaci* for long-distance transmission between plants or across the fields. The majority of the New World (NW) begomoviruses genome are bipartite and cssDNA (DNA-A and DNA-B) of almost similar in size with each other (Hanley-Bowdoin *et al*. 2013). However, the majority of Old World (OW) begomoviruses are monopartite with a distinct DNA-A component only. The DNA-A is ~2800 nucleotides (nt) in length and it characteristically encode 4–5 open reading frames (ORFs) in the two orientations, respectively. Whereas in DNA-B BC1 and BV1 are present in opposite orientation and have roles in virus movement across the cells and into the phloem, respectively (Noueiry *et al*. 1994). All the begomovirus-encoded genes are situated either on virion or on complementary sense and have non-coding region to separate them (called intergenic region [IR]) that includes the promoter elements. This IR is commonly conserved between both genomic components and therefore it is called common region (CR). It covers origin of replication (*ori*), which also have hairpin nona-nucleotide (TAATATT/AC) sequences (hallmark of whole family *Geminiviridae*), additionally it also have iteron-related domain where Rep binds during replication (Hanley-Bowdoin *et al*. 2000). The transreplication of associated (or occasionally non-associated) DNA-Bs or the DNA-satellites is mainly dependent on the iterons sequences in the N-terminus of replication associated (Rep) protein of DNA-A (Hanley-Bowdoin *et al*. 2000). Iterons are direct repeat sequences for Rep binding, introduces a nick at the nona-nucleotide sequences to start rolling circle replication (Gladfelter *et al*. 1997).

Some OW monopartite begomoviruses occurs in association with DNA-satellites alphasatellite and/or betasatellite. According to the current classification, alphasatellites belong to a sub-family *Geminialphasatellitinae* in the family *Alphasatellitidae* (Briddon *et al*. 2018). Alphasatellites carry a cssDNA genome with a size equal to half of the helper virus genome and solely encodes their own Rep protein. Sometimes, the presence of an alphasatellite necessitates the association of a betasatellite during a monopartite begomovirus infection in

the OW. However, the occurrence of monopartite begomoviruses is very limited in the NW and few studies on bipartite begomoviruses have reported the alphasatellite association (Paprotka *et al*. 2010; Romay *et al*. 2010). On the other hand, betasatellites are members of genus *Betasatellite* in the family *Tolecusatellitidae*. In the OW, betasatellites carry a smaller genome (ca 1350 nt) and often establish a disease complex with monopartite begomoviruses. Such associations are indispensable for their encapsidation, transmission and replication. In return, betasatellites assist their helper begomoviruses with their single ORF (βC1) to establish the infection, pathogenicity and to tackle plant defense responses effectively. Since their first discovery in 1999 (Saunders *et al*. 2000), ~500 complete betasatellites sequences have been submitted in the GenBank databases, which is indicative of their importance in disease etiology. Betasatellites encode single gene product (βC1) to commence their role to suppress both TGS and PTGS (Zhou 2013), pathogenesis (Saeed *et al*. 2005; Qazi *et al*. 2007), expanding host plants range (Amin *et al*. 2010), suppress host defense by down regulating certain plant hormones (Zhang *et al*. 2012), *in planta* virus movement (Saeed *et al*. 2007), bind to RNA/DNA (Cui *et al*. 2005), modulate the developmental microRNAs levels (Amin *et al*. 2011), form multimers and interact with host-encoded factors (Cheng *et al*. 2011).

The association between pedilanthus leaf curl virus (PeLCV) and tobacco leaf curl betasatellite (TbLCuB) is an emerging threat (Munir *et al*. 2018). PeLCV was initially identified in *Pedilanthus tithymaloides*, in Southeast Asia (Tahir *et al*. 2009). Since its first discovery, PeLCV has proliferated much and has been characterized from many important crops, vegetables and weeds (Srivastava *et al*. 2014; Saritha *et al*. 2016; Zaidi *et al*. 2016a; Ismail *et al*. 2017; Munir *et al*. 2018; Yasmin *et al*. 2017). PeLCV induces growth stunting, thick leaf veins and upward leaf curling, which become more severe in the presence of TbLCuB. On the other hands, tomato leaf curl New Delhi virus (ToLCNDV) is a common bipartite begomovirus in the territory of monopartite begomoviruses in Southeast Asia. ToLCNDV infects elite cultivars of tomato crop in Pakistan and India (Sahu *et al*. 2010). ToLCNDV was initially characterized about 20 years ago (Padidam *et al*. 1995) from solanaceous crops in India. Until now, it has been wide-spread in areas of Indonesia, Iran, Spain, Tunisia, Italy, and Morocco (reviewed by (Zaidi *et al*. 2016b), as a result of its cross-continent spreading. ToLCNDV and its DNA-A have the ability to transreplicate CLCuMB (Saeed *et al*. 2007; Iqbal *et al*. 2017) and TbLCuB (Shahid *et al*. 2021). Moreover, different virus-encoded suppressors enhanced *in planta* movement and titre of ToLCNDV (Iqbal *et al*. 2020). Moreover, betasatellite molecules are found associated with ToLCNDV (Singh *et al*. 2012; Jyothsna *et al*. 2013; Akhter *et al*. 2014; Hameed *et al*. 2017). ToLCNDV-B is required by pepper leaf curl Lahore virus to cause a symptomatic infection (Shafiq *et al*. 2010). Apart from these, ToLCNDV-B has been found associated with BYVMV in okra (Venkataravanappa *et al*. 2015).

In natural infection, multiple viruses can be present in the same host and can interact with each other antagonistically or synergistically. This research is an investigation to empirically predict the interaction and trans-replication abilities of a monopartite begomovirus, PeLCV with bipartite begomovirus, ToLCNDV and vice versa.

Materials and Methods

Origins of viruses and satellites

Infectious constructs of ToLCNDV DNA-A ([hereafter referred to as TV]; accession# U15015), ToLCNDV DNA-B ([hereafter referred to as TB]; accession# U15016), PeLCV ([hereafter referred to as PV]; accession# AM712436) and TbLCuB ([hereafter referred to as TbB]; accession# AM955608) (Padidam *et al*. 1995; Ilyas *et al*. 2010). All the constructs were transformed into GV3101 Agrobacterium strain using electroporation (Just *et al*. 2017).

Agrobacterium-mediated inoculation

Agrobacterium tumefaciens cultures bearing recombinant plasmids for each component were inoculated in *N. benthamiana* plants during 4–5 weeks along with mock inoculated controls. Five plants for each combination were inoculated on the underside of 2–3 leaves with a sterile 1 mL syringe using 0.5–1 mL inoculums as described earlier (Iqbal *et al*. 2017). The inoculated plants were kept in growth chambers in completely controlled environment. The plants were regularly visited to observe symptom development on a daily basis. The experiment was repeated twice.

Preliminary PCR-mediated diagnostics

After two weeks of inoculation (WI), the inoculated *N. benthamiana* plants for each combination were subjected to total genomic DNA extraction using extraction buffer (EB) available in Extract-n-Amp Plant PCR Kit (Sigma-Aldrich). Systemic leaves were partially isolated by punching the leaf between the tube and cap following the addition of 50 μL EB. The tubes were incubated for 10 min at 95^{o}C/and 1 μL template for PCR was directly taken from the supernatant in the individual PCR reactions with primers specific for each genomic component (Table 1).

Genomic DNA isolation, PCR and Southern blot hybridization

Total genomic DNA was isolated at 4WI by harvesting newly emerging leaves and a slightly changed miniprep method was used for extractions (Dellaporta *et al*. 1983). PCR-mediated diagnostics were performed for detection of

each inoculated virus and/or betasatellite components using respective set of primers (Table 1). Total genomic DNA (10 μg) of one plant for each combination were loaded into agarose gels (1.5%) and electrophoresed in 1.5 X TAE buffer, respectively. Equal loading of genomic DNA was ensured for Southern blot hybridization. After electrophoresis, the DNA were transferred onto Hybond nylon membranes (Amersham, the Netherlands), probed with radioactively [P-32P] dCTP labelled PCR-amplified CP gene fragment for TV and PV, MP fragment for TB, and βC1 gene fragment for TbB, respectively. The protocol from Just *et al*. (2017) was followed for Southern blot hybridization and phosphorimager (Bio-Rad) was used to detect hybridization signals.

Results

Healthy *N. benthamiana* inoculated with PV showed severe symptoms with leaf curling and stunted leaves with severity index 4 (Fig. 1A and Table 2), following (Sattar *et al*. 2019). The presence of PV was detected with PCR and later with Southern blot hybridization (Fig. 2A). The plants co-inoculated with PV along with its associated TbB exhibited severe downward curling and crumpling of the leaves, growth stunting and vein thickening phenotype (Fig. 1B and Table 2). These symptoms progressed further and the plants showed severe stunting in growth at 4 WI with severity index 5. The infection and presence of both PV and TbB was confirmed by PCR at 2 and 4 WI in all the inoculated plants. The presence of PV and TbB was also detected in Southern blot (Fig. 2A and C).

Co-inoculation of PV with TB in Nb plants led to curled leaves with thick veins comparable to TV and TB infection (Fig. 1E, Table 2). Later, at 4 WI the leaves become shorter and vein thickening and upward leaf curling phenotype was more prominent with severity index 3. No downward leaf curling (which, in most cases, are characteristics of betasatellite presence) were observed. The presence of PV and TB was confirmed at 2 and 4 WI using PCR, respectively. Similarly, the Southern blot hybridization results also successfully detected the presence of PV and TB (Fig. 2A and D), respectively.

The plants inoculated with TVonly failed to exhibit symptoms (Fig. 1C and Table 2), however, the occurrence of TV was only confirmed with PCR but no signals were found in Southern blot hybridization (Fig. 2B). The plants inoculated with TV and TB showed leaves with thick veins and upward curling at 2 WI (Fig. 1D and Table 2). The symptoms became more prominent at 4 WI with severity index 4. The presence of both genomic components of ToLCNDV was confirmed using PCR at 2 WI and 4 WI in all plant inoculations. The Southern blot hybridization readily confirmed the accumulation of both TV and TB (Fig. 2B and D).

Plant inoculations with TV and TbB, failed to induce any symptoms at 2 WI. Interestingly the plants started showing mild leaf curling symptoms on the margins of leaves during late infection at 4 WI (Fig. 1F and Table 2). However, TV was detected successfully using PCR at 2 and 4 WI. Furthermore, the presence of TV was also detected using Southern blot hybridization (Fig. 2B); whereas PCR

Table 1: Oligonucleotide primers used in the study, their target and amplicon size

Primers	Primer sequences (5'-3')	PCR product	size
AC1048	GGRTTDGARGCATGHGTACATG	Coat	~579 kb
AV494	GCCYATRTAYAGRAAGCCMAG	Protein	
Beta01	GGTACCACTACGCATCGCAGCAGCC	Betasatellite	~1.4 kb
Beta02	GGTACCTACCCTCCCAGGGGTACAC		
PCRc1	CTAGCTGCAGCATATTTACRARWATGCCA	T-B	~0.6 kb
PBL1v2040	GCCTCTGCAGCARTGRTCKATCTTCATACA		

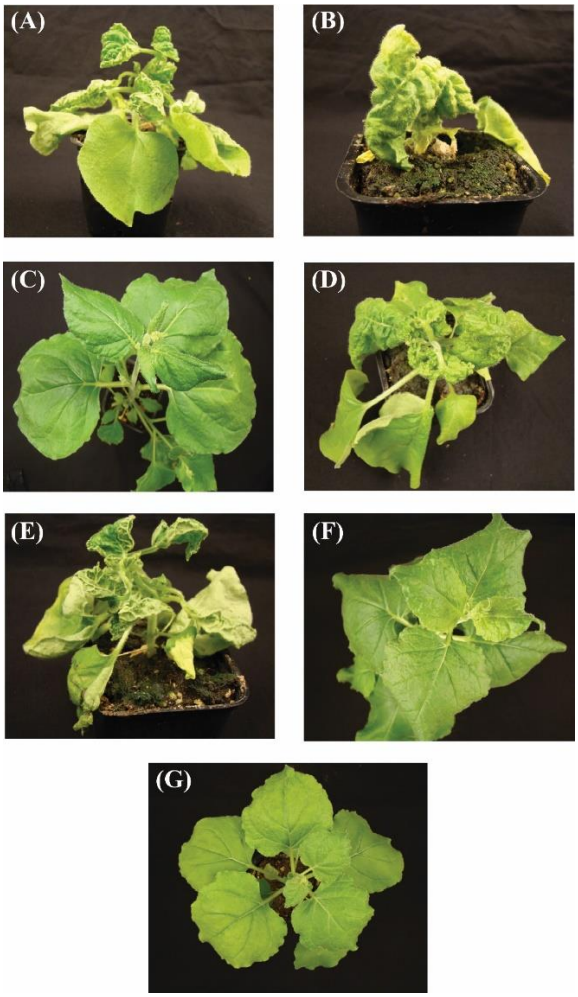

Fig. 1: Inoculation of *Nicotiana benthamiana* plants with PV (**A**), PV and TbB (**B**), TV (**C**), TV and TB (**D**), PV and TB (**E**), TV and TbB (**F**) and negative control (**G**). All the photographs were taken at 6 weeks of post-inoculation using Sony DSC-H300 camera, after two weeks of post-inoculation. Abbreviations used are: pedilanthus leaf curl virus (PV), tobacco leaf curl betasatellite (TbB), tomato leaf curl New Delhi virus DNA-A (TV), and tomato leaf curl New Delhi virus DNA-B (TB)

Table 2: Symptoms and severity induced by PeLCV and ToLCNDV upon inoculation with different combinations in *N. benthamiana* plants and Southern blot hybridization

Inoculation	Severity index*	Symptoms	Southern blot hybridization		
			Begomovirus	TbB	To-B
PV	4	Severe leaf curling, growth stunting	(+)	(NI)	(NI)
PV + TbB	5	Very severe leaf curling, stunted plant growth, leaf crumpling	(+)	(+)	(NI)
PV+TB	3	Severe leaf curling, vein thickening	(+)	(NI)	(+)
TV	1	No symptoms	(-)*	(NI)	(NI)
TV + TB	4	Leaf curling, vein thickening, short leaves	(+)	(NI)	(+)
TV + TbB	1	Mild downward leaf curling	(+)	ND	(NI)
Mock control	0	No symptoms	ND	ND	ND

Pedilanthus leaf curl virus (PV), tomato leaf curl New Delhi virus (ToLCNDV), and its DNA-A (TV), and DNAB (TB), tobacco leaf curl betasatellite (TbB), NI = not included, and ND= not detected. *Disease severity index (0-5) was used following Sattar et al. (2019)
* Detected in PCR but not by Southern blot hybridization

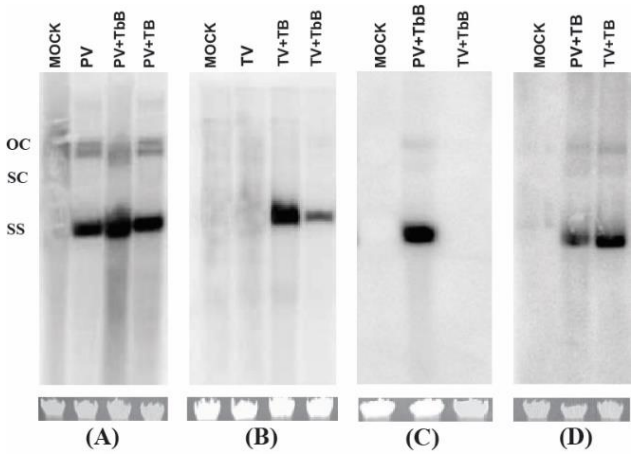

Fig. 2: Southern blot hybridization of the inoculated plants to assess trans-replication. PCR products using primers AC1048/AV494 were used as probes to detect PV (**A**). Lane 1: negative control, Lane 2: PV, Lane 3: PV/TbB and Lane 4: PV/TB, respectively. Similarly, PCR products were used as probe to detect TV (**B**). Lane 1: negative control, Lane 2: To -A, Lane 3: TV/TB and Lane 4: TV/TbB, respectively. The PCR products using primers Beta01/Beta02 were used to detect the presence of TbB (**C**). Lane 1: negative control, Lane 2: PV/TbB and Lane 3: TV/TbB. The PCR products using primer PCRc1/PBL1v2040 were used to detect the presence of TB (**D**). Lane 1: negative control, Lane 2: PV/TB and Lane 3: TV/TB, respectively. All the blots were probed with the α^{32}P radiolabeled PCR products of the respective components. Different viral DNA-forms are abbreviated as open circular (OC), supercoiled (SC) and single stranded (SS) and are indicated on the right side of each panel. Other Abbreviations used are: pedilanthus leaf curl virus (PV), tobacco leaf curl betasatellite (TbB), tomato leaf curl New Delhi virus DNA-A (TV), and tomato leaf curl New Delhi virus DNA-B (TB)

and Southern blot hybridization results could not confirm TbB presence and replication, respectively (Fig. 2C).

Analyses of nucleotide pairwise alignment and structures of CPs encoded by TV and PV demonstrated a high sequence similarity (93.75%) despite their less structure resemblance (Fig. 3). Whereas an opposite was observed for the Rep proteins, both Rep proteins shared less sequence similarity (78.97%) but their structures resemblance was higher, compared to the CP (Fig. 3).

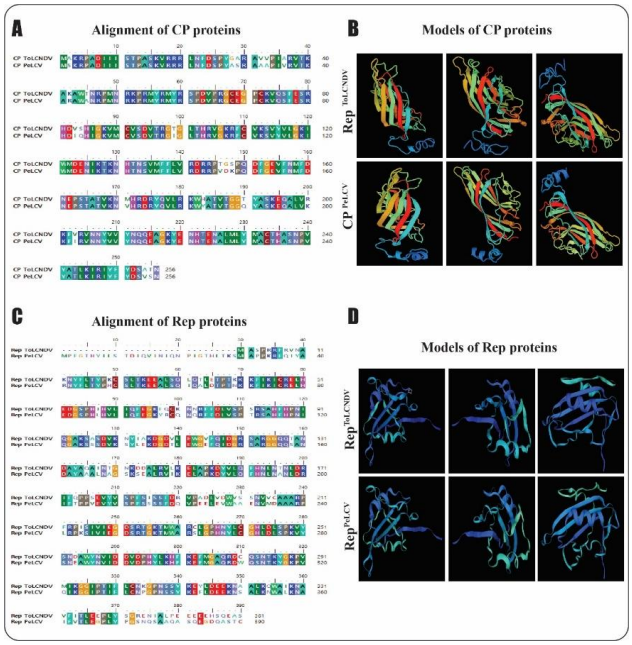

Fig. 3: Nucleotide pairwise alignment of CPs encoded by TV and PV (**A**) and their structure (**B**). Nucleotide pairwise alignment of Rep proteins encoded by TV and PV (**C**) and their structure (**D**), respectively. Abbreviations used are: coat protein (CP), pedilanthus leaf curl virus (PV), replication associated protein (Rep), and tomato leaf curl New Delhi virus DNA-A (TV)

Analysis of the putative iteron-binding sequence (IBS) of TV, TB and PV suggested that these viral components shared highly similar and compatible Rep-binding iterons. Sequence of IBS and structural resemblance could be a contributing factor to transreplication of TB by PV.

Discussion

Mixed infections of the same or heterologous viruses in a same host plant are quite common phenomena in the fields (Annisaa *et al*. 2021; Ban *et al*. 2021). In such mixed infections, viruses interact synergistically and induce more severe symptoms than individual infection (Pruss *et al*. 1997), or interfere the heterologous virus infection (Crespo *et al*. 2020), or partially complement the infection

of the heterologous viruses (Malyshenko *et al*. 1989). Additionally, during such interactions, viruses possibly recombine with each other (Hou *et al*. 1998; Sanz *et al*. 2000; Pita *et al*. 2001; Saunders *et al*. 2001) and exchange their genomic components or satellites molecules (Saunders *et al*. 2002; John *et al*. 2008). Such interactions between geminiviruses and heterologous components have been empirically proved in various studies (Sattar *et al*. 2019; Iqbal *et al*. 2020; Shafiq *et al*. 2021).

The results in transreplication studies showed that PV have the ability to successfully transreplicate and maintain TbB and TB. However, precise mechanism by which the putative origin of replication of TB was recognized by PV-encoded Rep is unclear. The interaction between the Rep and iterons sequences in DNA-A is a determinant of the ability of helper begomovirus for DNA-B replication (Argüello-Astorga *et al*. 1994; Gladfelter *et al*. 1997). Iterons of begomoviruses vary in a way that the Rep of unrelated or same species is not allowed to perform the replication (Argüello-Astorga *et al*. 1994). Two universal notions have been proposed, the "universal Rep" hypothesis states that Rep proteins may have more origin recognition relaxation properties. The second idea postulates that iteron sequences allow the Rep to recognize them; and referred to as “universal iteron” (Nawaz-ul-Rehman *et al*. 2009). The similarity of the IBS sequence and the structural resemblance of the Rep proteins seem to be consistent with both these hypotheses. We cannot predict the exact phenomena lying beneath, and thus it necessitates further studies. The translation of TB indicates that, regardless of an infection occurred in model plants, both PV and TV can act synergistically and potentially could break host resistance under field conditions.

Although, different betasatellites are increasingly being identified in the presence of ToLCNDV under field conditions (Bull *et al*. 2004; Jyothsna *et al*. 2013; Zaidi *et al*. 2016b), but TV failed to transreplicate and maintain the TbB. Although the symptomatic infection of bipartite begomoviruses require both components, rarely DNA-A is sufficient to cause asymptomatic infections on its own. (Klinkenberg and Stanley 1990; Evans and Jeske 1993; Briddon *et al*. 2001; Iqbal *et al*. 2017). The results obtained here are thus consistent to the previous findings. Possibly, this phenomenon is a peculiarity of ToLCNDV. However, further investigations are required to yield a concrete conclusion.

Nearly in all the studies investigated the interaction of a monopartite begomovirus with a cognate betasatellite resulted in plants with an enhanced titer of virus than those plants that are infected with the virus only (Saunders *et al*. 2000; Briddon *et al*. 2001; Zhou 2013). Similarly, plants co-inoculated with a combination of DNA-A and betasatellite led to a more severe infection than with the plants with DNA-A infection alone (Iqbal *et al*. 2017). Our results confirmed these earlier reports however; our results are slightly different as replication of TV was detected in the presence of betasatellite.

In general, it seems that there is an antagonistic interaction between the betasatellite and the DNA-B. According to some previous studies, carried out using a ToLCNDV isolate and two betasatellites, a weak association between DNA-B and betasatellite was suggested, which may be due to antagonism between them (Jyothsna *et al*. 2013). Further studies are highly recommended to fully determine the possibility and kind of antagonistic interaction between bipartite begomoviruses and betasatellites.

Conclusion

It is likely that with intensive agriculture in southeast Asia the chances of mixed infection of multiple begmoviruses and DNA-satellites will increase. Therefore, the chances of emergence of new begomoviruses will be higher, either due to recombination (true or pseudo- recombination) or due to component/satellite capture. However, our results are corroborating with the previous studies, indicate that predicting the outcome of such encounters is a daunting task, which cannot be mapped on timescale. It will be of interest to co-inoculate different begomoviruses and DNA-satellites in different combinations to different plant species via different inoculation methods including whitefly and allow them to replicate for prolonged period to unravel the possible outcome of such interactions.

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Author Contributions

MNS, MAA and KAA designed and performed the experiment. MNS, SEE and ZI prepared the initial draft of the manuscript. ZI did pairwise sequence alignment and inferred homology models. MNS and ZI substantially improved the final copy of the draft.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

All the data related to this study is included in the article, further inquiries can be directed to the corresponding author.

Ethics Approval

No humans and/or animals were used as research subject during this study.

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