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Infection of tomato leaf curl New Delhi virus (ToLCNDV), a bipartite begomovirus with betasatellites, results in enhanced level of helper virus components and antagonistic interaction between DNA B and betasatellites

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Abstract Tomato leaf curl New Delhi virus (ToLCNDV) (*Geminiviridae*) is an important pathogen that severely affects tomato production. An extensive survey was carried out during 2003–2010 to study the diversity of begomoviruses found in tomato, potato, and cucurbits that showed symptoms of leaf puckering, distortion, curling, vein clearing, and yellow mosaic in various fields in different regions of India. Ten begomovirus isolates were cloned from infected samples and identified as belonging to the species ToLCNDV. A total of 44 % of the samples showed association of betasatellites, with CLCuMuB and LuLDB being the most frequent. The ToLCNDV cloned component DNA A and DNA B were agroinoculated on *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) plants with or without betasatellites, CLCuMuB or LuLDB. The viral genome levels were then monitored by real-time polymerase chain reaction at different time points of disease development. Plants co-inoculated with betasatellites showed enhanced symptom severity in both *N. benthamiana* and tomato, as well as increases in helper viral DNA A and DNA B levels. The DNA B and betasatellites acted antagonistically to each other, so that the level of DNA B was 16-fold greater in the presence of betasatellites, while accumulation of betasatellites, CLCuMuB and LuLDB, were reduced by 60 % in the presence of DNA B. DNA B-mediated symptoms predominated in CLCuMuB-inoculated plants, whereas betasatellite-mediated leaf abnormalities were prominent in LuLDB-co-inoculated plants. Inoculation with the cloned components will be a good biotechnological tool in resistance breeding program.

Keywords Begomovirus · Tomato leaf curl New Delhi virus · Antagonism · DNA B · Betasatellites

Introduction

Tomato leaf curl disease is caused by whitefly-transmitted geminiviruses belonging to the genus *Begomovirus* of the family *Geminiviridae*. This disease is the major constraint in improving tomato production in India. Plants affected with this disease show vein clearing, yellow mottling, crinkling, puckering, and upward or downward curling of leaves. They have a characteristic bushy appearance and accompanying poor fruit setting and sterility.

Viruses of the family *Geminiviridae* are characterized by twinned particles (18×30 nm) that encapsidate a circular, single-stranded (ss) genome DNA (~2.7 kb length) (Stanley 1985). The members are differentiated into four genera, *Mastrevirus*, *Topocuvirus*, *Curtovirus*, and *Begomovirus*,

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based on vector transmission, host range, and genome organization (Fauquet et al. 2008). The genus *Begomovirus* includes viruses transmitted by the whitefly, *Bemisia tabaci* Genn.; these cause devastating diseases in several crops. Begomoviruses with a bipartite genome have their essential viral functions divided into two DNA components referred to as DNA A and DNA B. The DNA A component encodes the coat protein (CP), replication initiation protein (Rep), replication enhancer (REn), and transcriptional activator protein (TrAP), whereas the genes encoding the movement functions are located on DNA B. The open reading frames (ORFs) of DNA A and DNA B are arranged in two divergent clusters and are separated by an intergenic region, referred to as the common region (CR), which contains sequences that are conserved between both components. The CR contains the origin of replication, which consists of a conserved hairpin structure with a nonanucleotide sequence in the loop and Rep binding iteron sequences located upstream of the hairpin (Hanley-Bowdoin et al. 1999; Rojas et al. 2005). The presence of iteron sequences in DNA B similar to those in DNA A ensures binding between the DNA A-encoded Rep and the cognate DNA B.

Tomato leaf curl disease in India is caused by both monopartite and bipartite begomoviruses. To date, 14 species have been reported (http://www.ncbi.nlm.nih.gov) from the Indian subcontinent. Of all the begomoviruses affecting tomato crops, tomato leaf curl New Delhi virus (ToLCNDV) (Padidam et al. 1995; Srivastava et al. 1995), a bipartite begomovirus, is the most predominant virus in northern India, infecting elite cultivars (Sahu et al. 2010). The virus causes severe leaf curl and, so far, no resistance source is available. The majority of Old World (OW) monopartite begomoviruses that infect tomato are associated with ssDNA molecules of 1.3 kb length, referred to as betasatellites (Briddon et al. 2001; Briddon and Stanley 2006). Betasatellites are dependent on their helper virus (DNA A) for replication, encapsidation, and movement within plants and are required, in many cases, for symptom induction in the primary hosts from which they have been isolated (Briddon et al. 2001; Jose and Usha 2003; Saunders et al. 2004; Li et al. 2005).

Betasatellites encode an ~13.5-kDa protein known as βC1, an "A"-rich region and a 150-nt length sequence, known as a satellite conserved region, which is conserved between all betasatellites (Briddon et al. 2002). The positionally conserved βC1 is a pathogenicity determinant (Cui et al. 2004; Saunders et al. 2004; Saeed et al. 2005; Briddon and Stanley 2006; Guo et al. 2008; Yang et al. 2008) that suppresses RNA silencing (Cui et al. 2005; Gopal et al. 2007; Sharma et al. 2010) and enhances viral DNA levels in plants (Mansoor et al. 2003; Saunders et al. 2004). Although the majority of betasatellites are associated with OW monopartite begomoviruses, in rare instances, betasatellites have been detected along with bipartite begomoviruses like ToLCNDV (Sivalingam et al. 2010), mung bean yellow mosaic India virus (MYMIV) (Rouhibakhsh and Malathi 2005; Qazi et al. 2007; Ilyas et al. 2010) and tomato yellow leaf curl Thailand virus (TYLCTHV) (Guo et al. 2009).

In the last 8 years (2003–2010), during our investigation into tomato begomoviruses, we frequently found ToLCNDV associated with betasatellites. In the present communication, we report the occurrence of ToLCNDV along with betasatellites in seven host species. Infectivity of one of the isolates from tomato was analyzed in detail in tobacco (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*). We investigated the possibilities that DNA B and betasatellites act in a synergistic manner when co-inoculated and that DNA A of a bipartite virus can maintain itself if association occurs with betasatellites in the absence of DNA B. The replication level of each genomic component was monitored by real-time polymerase chain reaction (PCR) at different time points during disease development. We found that both plant species showed enhanced symptom severity when co-inoculated with betasatellites. In the presence of the DNA B component, the accumulation of betasatellites, CLCuMuB and *Luffa* leaf distortion betasatellite (LuLDB), were reduced.

Materials and methods

Virus source

A survey was carried out between 2003 and 2010 to determine the distribution and genetic diversity of begomoviruses that infect solanaceous and cucurbitaceous crops (Table 1). Tomato, potato, and cucurbit plant samples were collected if they showed symptoms such as leaf puckering, leaf distortion, yellow mosaic, vein clearing, and leaf curl. A total of 137 samples were collected and analyzed for the presence of begomoviruses.

Cloning of viral genome

The viral double-stranded replicative form (RF) DNA was isolated from potato samples affected by leaf curl by cesium chloride ($CsCl_2$) density gradient centrifugation (Stanley and Townsend 1985). Alternatively, viral RFs were enriched by rolling circle amplification (RCA) using φ29 polymerase (Haible et al. 2006) and used for cloning. Both products produced by $CsCl_2$ density gradient and RCA were digested with different endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Xba*I). The ~2.7-kb fragments representing full-length DNA A and DNA B components and ~1.3-kb fragments representing alphasatellites or betasatellites generated were purified and cloned into pUC18. The virus isolates from *Luffa*, bitter gourd, and some tomato samples were cloned

Table 1 Detection of ToLCNDV infection in leaf curl-affected plants and associated betasatellites during survey 2003–2010

Host	Location/state	No. of samples analyzed	No. of samples PCR positive for		Betasatellite identified as
			ToLCNDV full-length primers	β01/β02 primers	
Tomato (*Solanum esculentum*)	New Delhi/Delhi	18	17	14	CLCuMuB
	Sonepat/Haryana	9	9	8	TYLCTHB
	Ludhiana/Punjab	7	5	Multiple bands	CLCuMuB
	Meerut/Uttar Pradesh	3	3	3	–
	Pune/Maharashtra	8	4	1	PaLCuB
	Nasik/Maharashtra	6	2	2	TYLCTHB
	Naogaon/Maharashtra	4	1	–	TYLCTHB
					–
Potato (*Solanum tuberosum*)	Anand/Gujarat	5	5	–	PaLCuB
	Meerut/Uttar Pradesh	3	3	1 (0.7 kb)	BYVB, PaLCuB
	Kanpur/Uttar Pradesh	7	7	–	–
	Hapur/Uttar Pradesh	5	3	1	PaLCuB
	Chandigarh/Punjab	4	1	–	–
Sponge gourd (*Luffa cylindrica*)	Sonepat/Haryana	11	11	7	LuLDB
	New Delhi/Delhi	9	9	9	LuLDB
	Meerut/Uttar Pradesh	3	3	3	LuLDB
Bitter gourd (*Momordica charantia*)	Sonepat/Haryana	5	3	3	LuLDB
	New Delhi/Delhi	5	4	3	LuLDB
	Meerut/Uttar Pradesh	3	2	2	LuLDB
Cucumber (*Cucumis sativus*)	Dabawali/Haryana	12	12	2	LuLDB
Watermelon (*Citrullus lanatus*)	Paniwala/Haryana	3	1	–	–
Long melon (*Lagenaria siceraria*)	Paniwala/Haryana	7	7	1	LuLDB

– not detected

by PCR amplification using abutting full-length DNA A (NDVAF/NDVAR) and DNA B (NDVBF/NDVBR) (Table 2) and universal betasatellite primers (Briddon et al. 2002). The presence of any other monopartite tomato begomoviruses was detected using a set of primers (TMPF/TMPR) (Table 2) designed on the basis of the multiple alignment of the complete nucleotide sequence of DNA A from 41 begomovirus isolates. Recombinant clones were screened for the presence of viral inserts and the selected clones (nine tomato, seven potato, six *Luffa*, and two bitter gourd clones) containing the desired fragments were sequenced.

Sequence analysis

Sequences of the recombinant plasmids were determined with an ABI automated sequencer (Applied Biosystems, Perkin Elmer, Foster City, CA, USA) at the University of Delhi, South Campus, India. Complete nucleotide sequences of the full-length genomes were aligned using MUSCLE (Edgar 2004) and percentage pairwise nucleotide identity plot was made using the SDTv1.0 program (http://web.cbio.uct.ac.za/SDT). Phylogenetic analysis was performed using BioEdit v7.0.9.0 (Hall 1999) with Mega 5.05 (Tamura et al. 2011) (neighbor-joining trees with 1,000 bootstrap replicates).

Construction of infectious clones

Infectivity studies were conducted for one tomato isolate, JID, from Pune. Both the DNA A (pJID27) and DNA B (pJID17) genomic components were subcloned as partial tandem repeats (PTRs) in pBin19 (Bevan 1984). A full-length fragment of ~2.7 kb, produced from PT3 (Pune-Tomato-3) by PCR amplification with primer pair NDVAF/NDVAR, was cloned into the pGEMT-Easy vector (Promega, Madison, WI, USA) to yield pJID27 and then sequenced. The 0.2-mer (600 bp) fragment was digested with *Xba*I (2,251 nt)–*Bam*HI (126 nt) of ToLCNDV DNA A (pJID27) and was cloned into pBin19 to generate a 0.2-mer, p0.2JID27. A full-length copy of the pJID27 genome obtained by digestion with *Xba*I was inserted into p0.2JID17 to produce a 1.2-mer tandem repeat, p1.2JID27.

Table 2 Primers used in the present study

Primer ID	Forward sequence (5′–3′) Reverse sequence (5′–3′)	Amplicon length	Region in the genome amplified
NDVAF NDVAR	ATTCTAGAACGTCTCCATCTTT GTTCTAGAATGGGGTGTTTTCCA	2.7 kb	Full-length genome
NDVBF NDVBR	TCTCTAGAACTGATCTGGTGTCTCT GTTCTAGAGAGAGAAACTGCTGCTTCT	2.7 kb	Full-length genome
CPF CPR	AAGATATGGATGGATGAGAAC ACATAATTATTAACCCTAACAA	274 bp	Conserved region of coat protein gene
BC1F BC1R	TCTCTCAAGGATAAGAATCC ATCTGCTTGCCCATGATTC	319 bp	Conserved region of movement protein
CBC1F CBC1R	CAGGATCCATGACAACGAGCGG TAGAGCTCTTAAACGGTGAAC	356 bp	ORF βC1 of CLCuMuB
LBC1F LBC1R	TAGGATCCATGGATCACTCACAACC AAGAGCTCTTAAACGGTGAACTTC	449 bp	ORF βC1 of LuLDB
TMPF TMPR	TGGCATGCAACTGTCACCGGT TTGCATGCCACTTCCTCAA	2.7 kb	Full-length genome

Sequence analysis of the recombinant clones of pUC18 (MBI Fermentas, Vilnius, Lithuania) revealed that clone pJID17 from the same sample of PT3 was the DNA B component of ToLCNDV, which was taken for further construction of PTR for infectivity studies. The 0.6-mer (1.6 kb) fragment was digested with *Hin*dIII (2,017 nt)–*Bam*HI (935 nt) and was cloned into pBin19, producing a 0.6-mer, p0.6JID17. The full-length *Bam*HI fragment was inserted into calf intestinal alkaline phosphatase-treated p0.6JID17 to produce the 1.6-mer tandem repeat, p1.6JID17. The orientation was checked by restriction with *Bam*HI and *Xba*I (for DNA A) and *Bam*HI and *Hin*dIII (for DNA B), which released 2.7 kb fragments.

The betasatellites used in the present study were cotton leaf curl Multan betasatellite–[India:SriGanganagar:2002] (CLCuMuB–[IN:Sri:02]) GenBank accession no. AY083590 and *Luffa* leaf distortion betasatellite–[India:Luffa:2004] (LuLDB–[IN:Lu:04]) GenBank accession no. AY728262. Construction of PTRs of both the betasatellites was as described in Tiwari et al. (2010).

Agroinoculation

The PTR constructs of ToLCNDV DNA A (TA) and ToLCNDV DNA B (TB), CLCuMuB (CB), and LuLDB (LB) were mobilized into *Agrobacterium tumefaciens* strain EHA105 by triparental mating (Hood et al. 1993). Infectivity was checked by agroinoculation of PTRs into 3-week-old seedlings of *N. benthamiana* and 2-week-old seedlings of tomato cv. Pusa Ruby by a stem pricking method. The plants were maintained under 16/8 h light/dark periods, 18,000 lx, 28–30 °C, and 85 % relative humidity for 3–4 weeks at the National Phytotron Facility, IARI, New Delhi until they were scored for symptoms and analyzed for viral DNA.

Quantification of viral DNA

Southern hybridization

Plants were observed for symptom development and newly emerging leaves of 28 days postinoculation (dpi) plants were harvested for Southern blot analysis. DNA was extracted from 100 mg of leaf tissue from systemically infected and uninoculated plants following the GEM-CTAB method (Rouhibakhsh et al. 2008). DNA from three plants was pooled to make one sample and two such samples were analyzed by Southern blotting.

Total DNA (5 μg) was electrophoresed on 1.2 % agarose gels, transferred to nitrocellulose membranes, and hybridized with a [α^{32}P]-dCTP-labeled probes using a standard protocol (Sambrook et al. 1989). Viral DNA was detected using a radiolabeled probe specific for DNA A (a 274-bp fragment containing the conserved region from the ORF of the *AV1* gene) or a probe specific for the DNA B component (a 318-bp fragment conserved for the *BC1* gene). Fragments of the respective *βC1* genes were amplified and used as probes to analyze the replication levels for betasatellites. Radiolabeled probe was prepared and hybridized by using a random primer labeling kit (Bangalore Genei Ltd., Bangalore, India). Hybridization was detected using a Storage Phosphor System Cyclone® Plus (Perkin Elmer, Shelton, CT, USA). Images were analyzed using OptiQuant Version 5.0 (Perkin Elmer, Shelton, CT, USA), assigning a value of 1 to the signal intensity of samples from plants inoculated with DNA A alone and expressing the increase or decrease in the signal intensity relative to this in the other lanes.

Relative quantitative analysis

Total plant genomic DNA was extracted from 100 mg of leaf tissue collected at regular intervals of 7, 14, 21, and 28 dpi and was subjected to quantitative real-time PCR (qRT-PCR) using LightCycler® 480 Real-Time PCR System (Roche Diagnostics GmbH, Penzberg, Germany) coupled with the DNA binding fluorescent dye SYBR Green I. Specific primers were used for the quantification of all four components: DNA A (CPF/CPR), DNA B (BC1F/BC1R), CLCuMuB (CBC1F/CBC1R), and LuLDB (LBC1F/LBC1R) (Table 2). Levels of all four components were measured for the inoculations done in six combinations, as depicted in Table 5. DNA from three plants was pooled to make one sample for analysis.

The reaction mixture (20 μl) consisted of 2X SYBR Green Master Mix (Roche Diagnostics GmbH, Penzberg, Germany), 0.2 μM specific primers, and 500 ng of the template. The PCR conditions were kept as 95 °C for 10 min, followed by 40 cycles of 10 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C for amplification. All amplified products were analyzed by recording fluorescence and by gel electrophoresis. The threshold cycle (Ct) at which the significant increase in fluorescence occurs was calculated using software version LCS480 1.5.0.39 provided with the LightCycler® 480.

Data were expressed in terms of the concentration of DNA by preparing standard curves using recombinant clones of respective components. Tenfold dilution series were made by taking 100 ng of the corresponding clone as starting material to construct standard curves for each target. The plasmid DNA was serially diluted to final concentrations of 10^9–10^{15} pg/μl (1 μl per reaction). The concentrations of different components in the inoculated samples of all six different combinations were determined using a relative quantitative method that combines two absolute quantification reactions: one for the target-specific genes and the other for the endogenous reference gene. The quantified results of the target genes were compared (AV1—DNA A; BC1—DNA B; βC1—CLCuMuB/LuLDB) with β-actin gene from the respective samples. Standard curves were prepared for the target genes and the endogenous β-actin gene: these were compared to the experimentally derived levels in each sample and were expressed as fold changes relative to the endogenous gene.

Standard error bar graphs were prepared based on three independent experiments. Melting curve analyses were performed to verify the specific product formation. The amount of each gene under study was normalized to the internal control β-actin and analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Leaf samples were collected from diseased tomato, potato, and cucurbit plants for the screening of the diversity of begomoviruses. Out of 137 samples tested, 82 % showed ToLCNDV (~2.7 kb) and 44 % showed the presence of the betasatellites, indicating their frequent association. The results for ToLCNDV detection in the collected symptomatic samples and its association with betasatellites are summarized in Table 1. No amplification was seen with primers specific for monopartite begomoviruses, indicating the absence of other monopartite tomato begomoviruses in the samples analyzed in the present study.

Cloning, sequencing, and identification of the full-length viral genome and betasatellites

Comparison of the complete nucleotide sequence of the DNA A component of 10 clones from 21 isolates with the sequences of tomato begomoviruses revealed a 94–98 % identity to tomato leaf curl New Delhi virus–[India:New Delhi:Severe:1992] ToLCNDV–IN[IN:ND:Svr:92] and ToLCNDV–IN[IN:ND:Mild:92]. Hence, the isolates were identified as belonging to the species ToLCNDV, as recommended by the International Committee on Taxonomy of Viruses study group on *Geminiviridae* (Kings et al. 2011), where a threshold value of 89 % sequence identity was used as the basis for demarcation of species. GenBank accession numbers and the proposed isolate descriptors for the clones obtained as part of this study are listed in Table 3.

The heat map (Fig. 1) generated clearly showed that, though 55 isolates have been identified as belonging to the species ToLCNDV, only 14 isolates share sequence identity between 96 and 99 %. The majority of isolates (36) share 90–95 % and 5 isolates share sequence identity between 86 and 90 %. On the basis of nucleotide sequence identity, we propose that these isolates can be demarcated as strains A, B, and C. From the phylogenetic tree constructed on the basis of DNA A nucleotide sequence alignment, three clusters can be identified: one major cluster comprising virus isolates from solanaceous hosts with the exception of two isolates from ash gourd and pumpkin; a second cluster including mainly isolates from cucurbitaceous hosts originating from both Southeast Asia and Indian subcontinent; and a third cluster comprising isolates from okra, tomato, eggplant, and chili pepper (in the Supplementary Material Fig. S1).

Four betasatellite species had associations with the 21 isolates of ToLCNDV being studied here. A betasatellite species demarcation criterion of 78 % sequence identity (Briddon et al. 2008) identified these as CLCuMuB and tomato yellow leaf curl Thailand betasatellite (TYLCTHB) in tomato, papaya leaf curl betasatellite (PaLCuB) in potato,

Table 3 Database accession numbers and proposed isolate descriptors for the clones obtained in this study

Host	Isolate descriptor	DNA A	DNA B	Betasatellite
Tomato	ToLCNDV–IN[IN:Pune:JID:2010]	HQ141673	HQ141674	HM989847
Tomato	ToLCNDV–IN[IN:Pune:X:2010]	HM345979, FJ468356	HM803117	EF095958
Tomato	ToLCNDV–IN[IN:ND:del:2010]	EF068246	EF408038	EF068245, AY438562
Potato	ToLCNDV–IN[IN:Meerut:PM:2010]	EF043231	EF043232	EF043234
Potato	ToLCNDV–IN[IN:Meerut:PM:2010]	AY286316	AY158080	–
Potato	ToLCNDV–IN[IN:Hapur:PH:2010]	EF043230	EF043233	–
Luffa	ToLCNDV–IN[IN:ND:JLD:2010]	HM989845	HM989846	–
Luffa	ToLCNDV–IN[IN:ND:LD:2010]	AY939926	AY939924	AY728262, DQ020491
Bitter gourd	ToLCNDV–IN[IN:ND:BD:2010]	–	DQ020490	AY817151

Species—tomato leaf curl New Delhi virus (ToLCNDV)

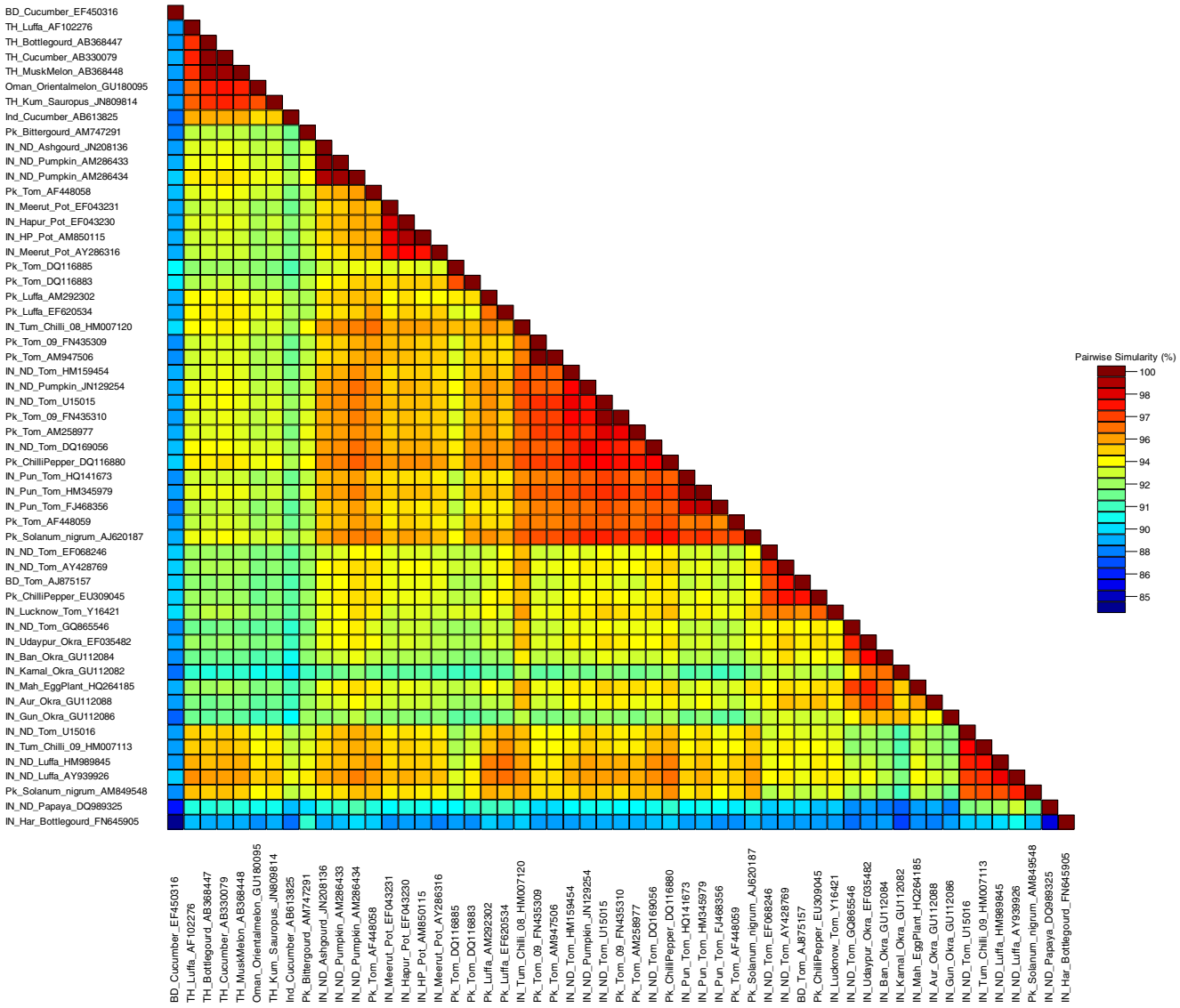

Fig. 1 Graphical representation of percentage pairwise genome scores and nucleotide identity plot of full genomes (55 isolates of ToLCNDV)—prepared using SDTv1.0 (Species Demarcation Tool) (http://web.cbio.uct.ac.za/SDT)

and LuLDB in cucurbits. In addition, both tomato and potato showed subgenomic (deleted versions of betasatellite instead of the expected 1.3 kb when amplified with universal betasatellites primers; Briddon et al. 2002) betasatellites of 0.65 kb in 13 % of the samples; one of these was identified as the Bhendi yellow vein betasatellite (BYVB). Betasatellites isolated from *Luffa* plants were distinct and showed 77 % identity with CLCuMuB and only 48 % identity with all other betasatellites. LuLDB is unique as it has an additional 30 amino acids in the N-terminal region of the ORF of βC1 and shares only 67.7 % identity with that of the CLCuMuB βC1.

Analysis of the infectivity of ToLCNDV

DNA A alone from the bipartite ToLCNDV can maintain itself and cause infection

Agroinoculation of TA alone resulted in downward leaf curling in *N. benthamiana* plants and mild mosaic in tomato plants (Table 4); however, symptoms were milder when compared to those of plants co-inoculated with the TB component. Symptoms also took a somewhat longer time to develop: 8 days for *N. benthamiana* and 10 days for tomato.

Symptom phenotype when TATB are co-inoculated

The PTR constructs of ToLCNDV were highly infectious (100 % infectivity) in both *N. benthamiana* and tomato when TA and TB were inoculated together (TATB). In *N. benthamiana*, systemic symptoms, such as downward leaf curling, appeared within 6 dpi (Table 4). Infected plants exhibited typical downward leaf curling, yellowing of the leaf lamina, and stunted growth at 14 dpi (Fig. 2). Tomato plants inoculated with TATB developed upward leaf curling by 7 dpi; by 12 dpi, the infected plants showed yellow specks over the entire leaf lamina, and by 21 dpi, the yellow specks had coalesced on the leaf lamina and the severity of upward leaf curling and mottling had increased.

Interaction of ToLCNDV DNA A with betasatellites

Co-inoculation of TA with CB (TACB) in tomato resulted in leaf curling, puckering, and reduction of the leaf lamina. Symptoms appeared at 7 dpi and the plants had a stunted appearance. The characteristic yellow mottling symptoms seen following co-inoculation with TB was not seen in this case; otherwise, the symptoms were as severe. In *N. benthamiana*, downward leaf curling, vein clearing, curly shoot, and vein swelling were seen

Table 4 Infectivity and symptoms expressed by ToLCNDV–IN[IN:Pune:JID:2010] inoculated alone and with component B and cognate betasatellites

Inoculum[a]	No. of plants infected/no. of plants inoculated			Symptom appearance–severe symptom expression (days postinoculation)	Symptoms
	Experiment I	Experiment II	Experiment III		
N. benthamiana					
TA	6/6	11/11	15/15	8–14	Downward leaf curl
TATB	6/6	11/11	15/15	6–14	Leaf curl, vein enlargement, yellow mottling, stunting
TACB	12/12	11/11	15/15	4–7	Leaf curl, vein clearing, curly shoot, vein swelling, stunting
TATBCB	6/6	11/11	15/15	4–7	Yellow mottling, leaf curl, vein curling, curly shoot, stunting, bleaching of leaves
TALB	12/12	11/11	15/15	4–7	Severe leaf curl, vein clearing, vein swelling, stunting, bleaching of leaves
TATBLB	6/6	11/11	15/15	4–7	Severe leaf curl, vein clearing, vein swelling, stunting, bleaching of leaves
S. lycopersicum					
TA	17/17	11/11	15/15	10–12	Mild mosaic
TATB	18/18	11/11	15/15	7–12	Leaf curl, yellow mottling
TACB	13/17	11/11	15/15	7–10	Leaf curl, vein clearing, puckering, stunting
TATBCB	12/18	11/11	15/15	5–8	Yellow mottling, leaf curl
TALB	13/17	11/11	15/15	5–8	Severe leaf curl, vein clearing, stunting
TATBLB	12/18	11/11	15/15	5–8	Vein clearing/banding, yellow mottling, stunting

[a] Plants were inoculated with *Agrobacterium* cultures harboring constructs for the infectivity of ToLCNDV DNA A (TA), ToLCNDV DNA B (TB), CLCuMuB (CB), and LuLDB (LB)

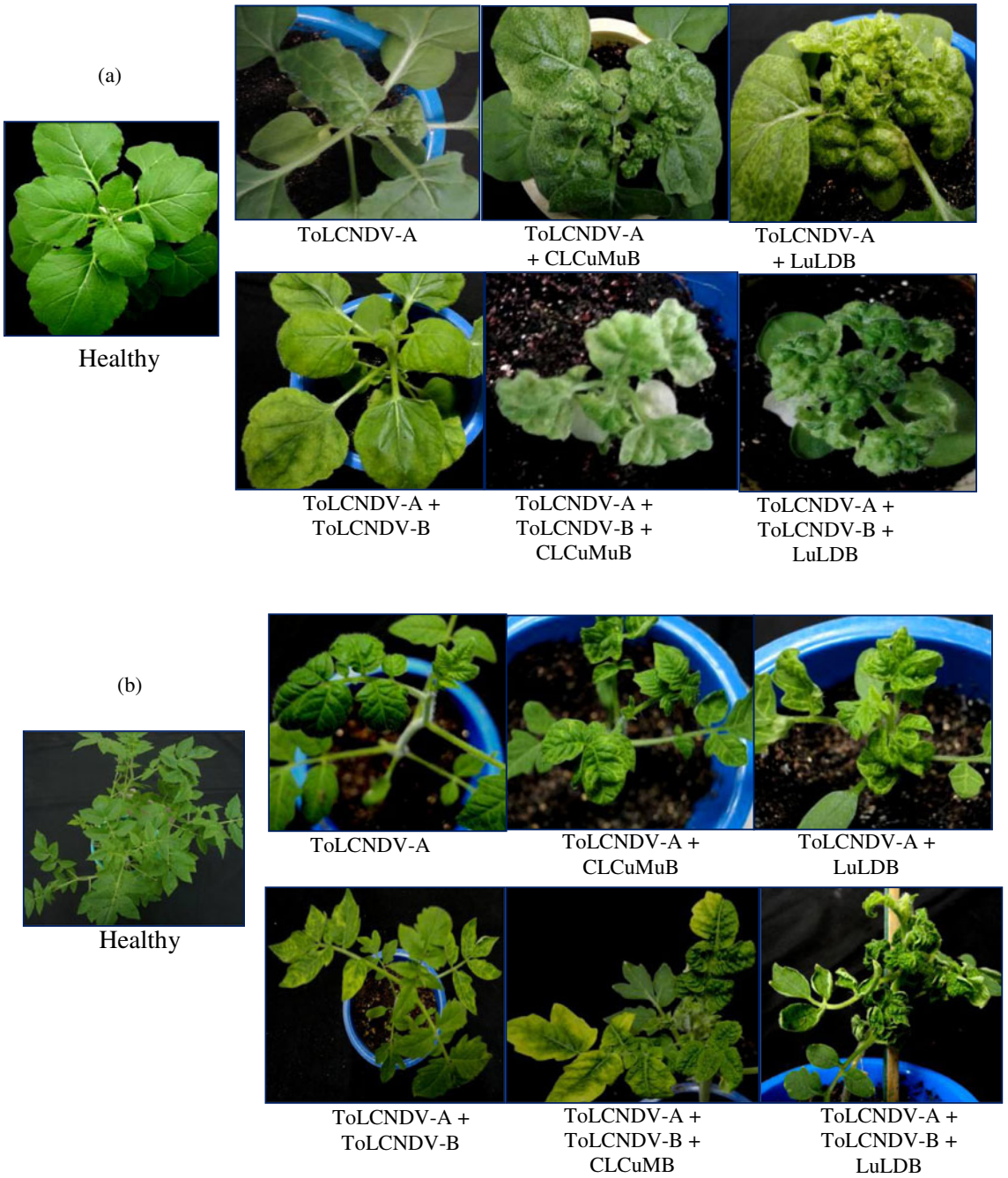

Fig. 2 Symptoms induced by plants following agroinoculation with infectious constructs in different combinations as labeled on each photograph at 15 dpi. **a** *N. benthamiana*, **b** tomato

within 4 dpi. Co-inoculation of TA and LB (TALB) in tomato resulted in symptoms more severe than in TACB-inoculated tomato plants and symptoms appeared in fewer days (5–7 dpi) compared to TACB. The symptoms include severe leaf curling, veinal yellowing and puckering. In *N. benthamiana*, thickening of veins and interveinal chlorosis appeared within 4 dpi; the latter symptom intensified to give a totally discolored/bleached appearance to the leaves.

Understanding the synergistic/antagonistic relationship between DNA B and betasatellites

Interesting results were seen when TA and TB were co-inoculated with CB or LB (TATBCB or TATBLB). Differences were very evident in tomato plants. The tomato plants inoculated with TATBCB showed yellow mottling, upward curling, and leaf lamina reduction. The addition of CB enhanced the severity, reduced the number of days

for symptoms to appear, and produced increased puckering. In contrast, the plants inoculated with TATBLB showed the same veinal yellowing that had been seen in the plants inoculated with TALB. However, severity of symptom was more in TATBLB-inoculated plants.

Nearly 100 % infection was seen in all six combinations in both *N. benthamiana* and tomato. The general trend observed with co-inoculation with betasatellites was an increase in severity and a reduced time for the appearance of symptoms.

Semiquantitative analysis of replication levels of DNA A, DNA B, and betasatellites

Although ToLCNDV is a bipartite virus, plants inoculated with TA alone showed good replicative levels of DNA A at 28 dpi in the young leaves (Fig. 3a, lanes 3 and 4), indicating good replication and systemic movement of DNA A. However, the level was only 50 % of what was visualized in plants inoculated with TATB (Fig. 3a, lanes 5 and 6). The DNA A level was ~1.17-fold higher in plants inoculated with TATB than in plants inoculated with TACB or TALB. The DNA A level was further enhanced by 1.99-fold in plants inoculated with TATBLB (Fig. 3a, lane 13), when compared to plants inoculated with TATBCB (Fig. 3a, lane 9).

In contrast, the level of DNA B was ~4.17-fold higher in plants inoculated with TATB in combination with either CB or LB (Fig. 3b, lanes 11, 12, 13, and 14) than in plants inoculated with only TATB (Fig. 3b, lanes 5 and 6). In the plants co-inoculated with the two betasatellites, the DNA B level was 1.14-fold higher in plants inoculated with TATBCB than in plants inoculated with TATBLB.

The betasatellite levels were reduced at least by 60 % in plants inoculated with TATBCB or with TATBLB (Fig. 3c, lanes 9, 10, 13, and 14) when compared to plants inoculated with TACB or TALB.

Assessment of the aggressiveness of the pathogen at different stages of pathogenesis

Genomic DNA components were quantified by measuring the target quantity in the DNA extracted from agroinoculated plants at four time points: 7, 14, 21, and 28 dpi (Table 5) by qRT-PCR (Fig. 4). The standard curves showed a linear relationship between the amount of input DNA and the Ct values for all the four templates (TA, TB, CB, and LB) over a range of 6 log units (in the Supplementary Material Fig. S2). Melt curves show single products produced in amplification of both viral and β-actin genes (in the Supplementary Material Fig. S3).

The level of DNA A in plants inoculated with TA alone was very low at 7 dpi, even though symptoms had started to appear and the accumulation increased by sixfold by 21 dpi, after which a significant fall in the level of DNA A was seen by 28 dpi, which was almost same to that of the level of TA at 7 dpi (Table 5). A similar trend of accumulation in DNA A level was seen in all the combinations of inoculation, except TACB, while a consistent decrease in the level of DNA A from 7 dpi (17.5 pg) to 28 dpi (3.5 pg) was observed in plants inoculated with TACB. Plants inoculated with TATB had high DNA A levels during the first week, but they showed a slight dip by 14 and 21 dpi, followed by a drastic increase, up to 4 ng (a fourfold increase), by 28 dpi. Comparison of the samples inoculated with the two betasatellites indicated that the viral titer of the DNA A genome

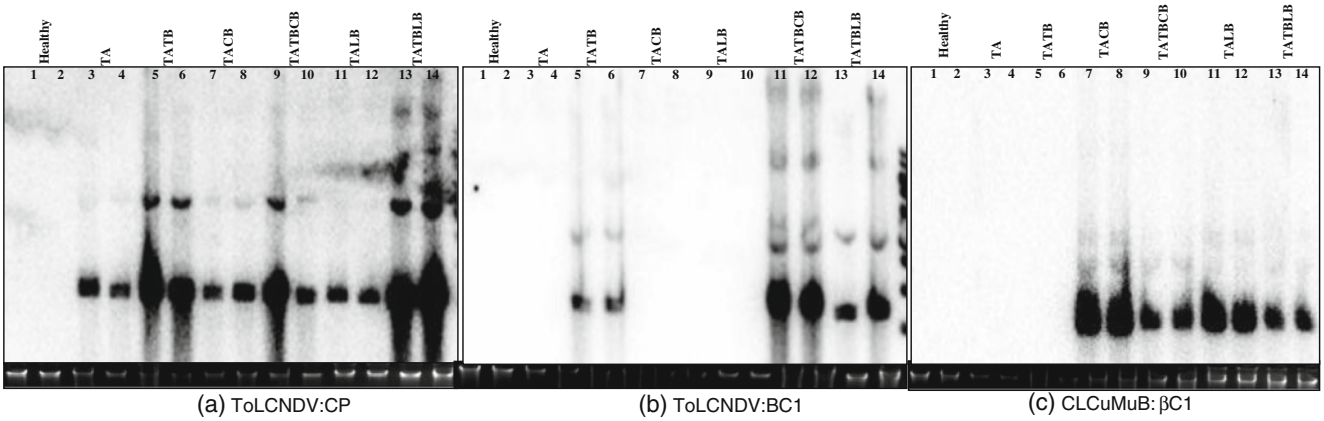

Fig. 3 Southern blot analysis showing relative accumulation of viral DNA from tomato plants agroinoculated with infectious constructs of ToLCNDV in six different combinations at 28 dpi. The blots were hybridized with probes specific to DNA A (CP gene) (**a**), DNA B (BC1 gene) (**b**), CLCuMuB (βC1 gene) (**c**). Each lane was loaded with 5 μg ethidium bromide stained DNA. **a**, **c** *Lanes 1* and *2* healthy, *lanes 3* and *4* TA, *lanes 5* and *6* TATB, *lanes 7* and *8* TACB, *lanes 9* and *10* TATBCB, *lanes 11* and *12* TALB, *lanes 13* and *14* TATBLB; **b** *lanes 1* and *2* healthy, *lanes 3* and *4* TA, *lanes 5* and *6* TATB, *lanes 7* and *8* TACB, *lanes 9* and *10* TALB, *lanes 11* and *12* TATBCB, *lanes 13* and *14* TATBLB where *TA* DNA A, *TB* DNA B, *CB* CLCuMuB, and *LB* LuLDB. The higher bands represent concatemeric forms of viral replicative DNA

Table 5 Absolute concentration (in nanograms) of the viral genomic components at different stages of pathogenesis

Viral component	Days postinoculation	TA	TATB	TACB	TATBCB	TALB	TATBLB
		Concentration in ng					
DNA A	7 dpi	0.0039	0.8000	0.0175	0.1100	0.0037	0.6500
	14 dpi	0.0100	0.4000	0.0090	0.6500	0.0063	0.6000
	21 dpi	0.0250	0.0480	0.0052	0.1100	0.0180	2.7500
	28 dpi	0.0035	4.0000	0.0035	2.7500	0.0041	5.500
DNA B	7 dpi	–	0.5500	–	0.0850	–	1.5000
	14 dpi	–	0.7500	–	2.5000	–	0.1150
	21 dpi	–	0.8500	–	2.0000	–	1.7500
	28 dpi	–	0.9000	–	2.2500	–	1.0000
CLCuMuB	7 dpi	–	–	0.0500	0.0010	–	–
	14 dpi	–	–	0.0550	0.0250	–	–
	21 dpi	–	–	0.0850	0.0450	–	–
	28 dpi	–	–	0.0475	0.0100	–	–
LuLDB	7 dpi	–	–	–	–	0.0300	0.7500
	14 dpi	–	–	–	–	0.0370	0.0360
	21 dpi	–	–	–	–	2.0000	0.0500
	28 dpi	–	–	–	–	0.7600	0.0310

Absolute concentrations of all the four components TA, TB, CB, and LB were estimated by extrapolating logarithmic values of Ct on the standard curves of the respective genes taken for analysis

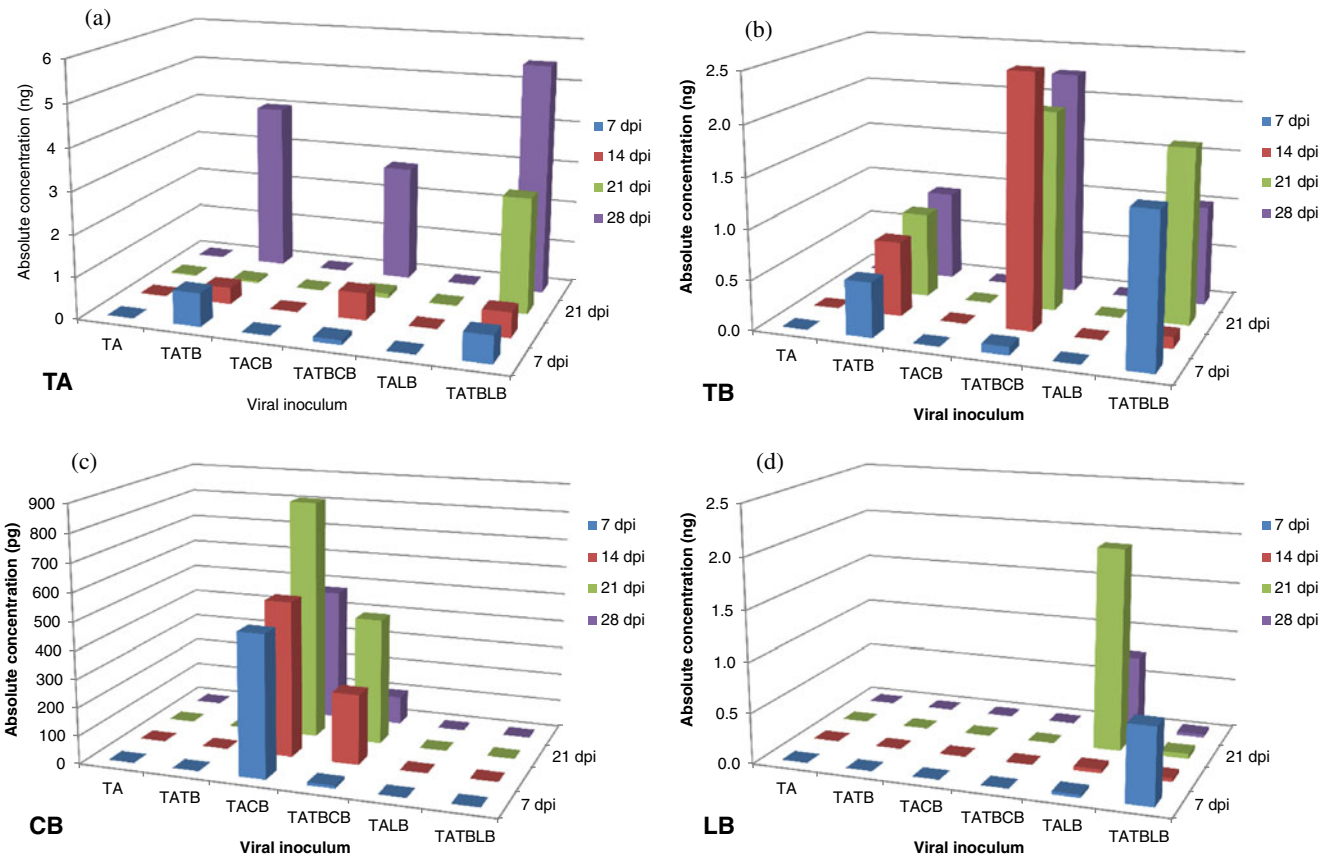

Fig. 4 Graphs showing the accumulation or absolute concentration of the genomic components estimated by qRT-PCR analysis. ToLCNDV DNA A (**a**, *TA*), ToLCNDV DNA B (**b**, *TB*), CLCuMuB (**c**, *CB*), and LuLDB (**d**, *LB*) in different stages (7, 14, 21, and 28 dpi, respectively) of the disease development in six combinations of agroinoculations studied: TA, TATB, TACB, TATBCB, TALB, and TATBLB

was twofold larger when combined with LuLDB, i.e., plants inoculated with TATBLB (Fig. 4a) compared with plants inoculated with TATBCB.

The DNA B level was 550 pg initially (7 dpi) and it was maintained at an 800- to 900-pg level up to 28 dpi in plants inoculated with TATB. Interesting results were seen in plants inoculated with TATBCB or TATBLB, where the DNA B level was enhanced in the presence of the betasatellite when compared to plants inoculated with TATB. This increased accumulation of DNA B was more prominent in plants inoculated with TATBCB than in plants inoculated with TATBLB; a 4.5-fold increase in DNA B level was observed when it was inoculated along with CLCuMuB (Table 5 and Fig. 4b). Plants inoculated with TATBLB had a high initial level but this declined as time progressed.

These results were also well-corroborated by the symptom phenotype. The DNA B-mediated symptom of yellow mottling dominated in plants inoculated with TATBCB, whereas leaf distortion and veinal chlorosis are more striking symptoms in plants inoculated with TATBLB. Between the two betasatellites used for experiment, *trans*-replication mediated by TA was more efficient in the very first week for CB, and this was maintained up to 28 dpi. In contrast, the replication level of LuLDB improved only at 21 dpi. In both cases, in the presence of TB, the betasatellite level was gradually reduced at least by 60 % in plants inoculated with TATBCB and plants inoculated with TATBLB when compared to plants inoculated with TACB or TALB (Fig. 4c, d). Standard error bar graphs were prepared based on the data of three independent experiments (in the Supplementary Material Fig. S4).

Whitefly transmission of tripartite components

Transmission of the viral genomic components from plants inoculated with TATB, with TATBCB, or with TATBLB to healthy tomato seedlings was evaluated using 50 adult whiteflies. After 24 h of acquisition access period and 24 h incubation access period, plants were maintained in insect-proof cages for symptom expression. Typical symptoms to those seen in plants inoculated with TATB were seen in 80 % of the plants. PCR amplification showed that all three components were transmitted efficiently in 8, 11, and 7 plants out of 12 plants inoculated (67 % transmission efficiency) in three different experiments.

Discussion

This study conducted over the past 8 years clearly showed that the association of betasatellites with the bipartite begomovirus, ToLCNDV, is frequent (44 %). How this tripartite association affects viral pathogenicity was examined in the work communicated here. Molecular characterization of nearly 55 isolates of ToLCNDV though has been completed; Koch's postulates have been established only for three isolates (U15015, AY428769, and HQ264185). In this context, this communication examining in detail the infectivity of an isolate of ToLCNDV is important. Results clearly establish that, though the isolate under study is a bipartite begomovirus, it efficiently *trans*-replicates betasatellites and interacts with them, resulting in increased viral pathogenicity.

ToLCNDV association with betasatellites

The majority of betasatellites are found associated with OW monopartite begomoviruses (Briddon and Stanley 2006); two exceptions of bipartite begomoviruses are MYMIV (Rouhibakhsh and Malathi 2005) and ToLCNDV (Sivalingam et al. 2010). More instances of bipartite virus association with betasatellites were reported, for example, by Green and Tsai (as cited in Bull et al. 2004) who isolated two betasatellites, TomLCDβ01-BD and TomLCDβ01-IN, from tomato samples in which DNA B components were also present. Qazi et al. (2007) showed that the leaf crumpling symptom in mung bean plants was caused by MYMIV and a betasatellite. TYLCTHV isolates from China are associated with betasatellites and co-inoculation produced more severe symptoms and also enhanced viral DNA accumulation (Guo et al. 2009). We suggest that ToLCNDV has been postulated to have gained betasatellites over a period of time when it would have been present in mixed infections, along with a monopartite begomovirus in a permissible host. Once it has gained a betasatellite, we infer, from our agroinoculation experiment data presented here, that the betasatellite will be continued to be maintained.

Symptom phenotypes of ToLCNDV with DNA B and betasatellites

Tomato plants inoculated with DNA A alone showed systemic infection and mild downward leaf curl symptoms by 10 dpi. Yellow mottling symptoms, followed by upward leaf curling phenotype, distinguish the plants inoculated with DNA A and DNA B from plants inoculated with DNA A alone and plants inoculated with DNA A and betasatellites. The movement protein of bipartite begomoviruses (MP-ORF BC1) is considered to be the major symptom determinant (von Arnim and Stanley 1992), so that transient or constitutive expression of this protein will induce disease-like symptoms in several hosts (Pascal et al. 1993; Ingham et al. 1995; Duan et al. 1997; Hou et al. 2000). Contrary to this interpretation, Hussain et al. (2005) suggested that the nuclear shuttle protein (NSP-ORF BV1) plays a major role in symptom development in the case of ToLCNDV. The most important role played by MP is to facilitate the exit

of begomoviruses from the phloem (Rojas et al. 2001). We suggest that exit from vascular tissue and interaction with other cellular proteins may result in DNA B-mediated yellow mottling symptoms seen in plants inoculated with DNA A and DNA B. Alternatively, the MP may interfere with the movement of an essential protein/metabolite, resulting in the yellow mottling symptom. The specific role of NSP or MP in typical yellow mottle symptom expression in ToLCNDV is being studied by site-directed mutagenesis.

In the present study, compared to plants inoculated with DNA A alone, plants inoculated with DNA A and CLCuMuB and plants inoculated with DNA A and LuLDB expressed symptoms distinct from those shown by plants inoculated with DNA A alone or by plants inoculated with DNA A and DNA B, indicating the contribution of βC1 protein of the betasatellites to symptom expression. Severe symptoms like leaf crinkling, vein thickening, twisting of petioles (curly shoot), and vein banding are seen in plants inoculated with CLCuMuB and LuLDB. In recent years, viral RNA silencing suppressors were identified as responsible for a significant proportion of morphological and developmental aberrations (Shimura and Pantaleo 2011). In this regard, βC1 that is encoded by different betasatellites acts as a posttranscriptional gene silencing (PTGS) suppressor (Amin et al. 2011; Cui et al. 2005). Severe abnormalities like leaf curling, enations, shoot bending, and vein thickening are seen when βC1 protein is expressed either by transformation or through the PVX vector in *N. benthamiana* (Cui et al. 2004; Kon et al. 2007). These extreme severe symptoms observed by CLCuMuB or LuLDB inoculation are assumed to be due to βC1, a potential PTGS suppressor. Alternatively, developmental defects and the morphological changes may be brought about by βC1 by altering host gene expression through interference with microRNA pathways (Yang et al. 2011).

In the present work, inoculation with DNA A component with or without betasatellites and DNA B allowed the separation of the host responses to DNA A, DNA B, and betasatellite-encoded suppressors. The clear-cut DNA B-mediated yellow mottling symptom and the betasatellite-mediated leaf distortion (CLCuMuB) and yellow vein banding (LuLDB) symptoms in tomato served as excellent markers for understanding the dominance of each component. In plants inoculated with TATBCB, yellow mottling predominated, in combination with leaf curling, while in plants inoculated with TATBLB, leaf abnormalities and yellow banding were dominant even though the LB DNA level was reduced.

Systemic movement of ToLCNDV DNA A, alone and in the presence of betasatellites

The present work indicated that DNA A alone could move systemically; however, the fewer number of days required for symptom expression when co-inoculated with betasatellites suggested that betasatellites facilitated the systemic movement of DNA A. In plants inoculated with DNA A alone, viral DNA could be detected in Southern blots at 28 dpi in young unfurling leaves distal to the site of inoculation. However, Padidam et al. (1995), Sahu et al. (2010), and Pratap et al. (2011) did not detect the replication of viral DNA in distal tissue in the case of DNA A alone inoculated plants. In the absence of DNA B, ToLCNDV DNA A appears to function like a monopartite begomovirus wherein CP/AV2/AC4 mediates viral DNA transport across nuclear and cellular boundaries. Like SLCMV (Saunders et al. 2002), the present isolate of ToLCNDV DNA A has vestigial functional characteristics of a monopartite begomovirus. The systemic infection in plants inoculated with DNA A along with betasatellites clearly showed that movement of ToLCNDV DNA A, a bipartite begomovirus, is facilitated by both the CLCuMuB and LuLDB. These results confirm the observations by Saeed et al. (2007), who showed that the βC1 protein of a betasatellite that was associated with cotton leaf curl disease could transport ToLCNDV DNA A from the nucleus to PD sites in the infected cell.

Competitive replication between DNA B and betasatellites

The qRT-PCR and Southern blot analysis showed that ToLCNDV efficiently *trans*-replicated both betasatellites. *Trans*-replication of full-length and defective betasatellites has been shown for many distinct begomovirus species/betasatellite combinations (Saunders et al. 2002, 2008; Mansoor et al. 2003). The only other bipartite begomoviruses demonstrated to *trans*-replicate betasatellite were SLCMV (Saunders et al. 2002), TYLCTHV (Guo et al. 2009), and the New World bipartite begomovirus CbLCuV (Nawaz-ul-Rehman et al. 2009). These observations clearly indicate that no distinct demarcation exists between monopartite and bipartite begomoviruses for their ability to *trans*-replicate betasatellites. The origin recognition by the helper viral DNA-encoded Rep is suggested to be more relaxed for betasatellites. Briddon and Stanley (2006) suggested that βC1 expression may be involved in reprogramming the infected cells to provide conditions more suitable for begomovirus replication.

In the present investigation, real-time PCR was used to quantify the viral DNA component level in parallel with disease development. Comparison of plants inoculated with DNA A and plants inoculated with DNA A and betasatellites indicated only a marginal increase in DNA A level. This result suggested that co-inoculation of DNA A with CLCuMuB or LuLDB does not lead to the accumulation of helper viral DNA, which contradicts results seen with AYVV/AYVB and CLCuMuV/CLCuMuB combinations (Mansoor et al. 2003; Saunders et al. 2004), where helper

viral DNA accumulation is enhanced. Tiwari et al. (2010) showed that, when ToLCBaV is inoculated with several betasatellites (ToLCBB, TYLCTHB, PaLCuB, LuLDB, and CLCuMuB), enhanced DNA level was seen only with the cognate betasatellite ToLCBB. In contrast, Kumari et al. (2011) did not see viral DNA accumulation in plants inoculated with ToLCRnV and cognate betasatellite ToLCRnB. Interaction of DNA A with betasatellites, therefore, may vary between each virus and betasatellite combination and no predictable pattern may be discernible.

The levels of DNA A and DNA B were enhanced 1.7-fold and 4.2-fold, respectively, in plants inoculated with DNA A, DNA B, and a betasatellite when compared with plants inoculated with DNA A and DNA B. The most unexpected result was seen in the betasatellite levels, which were reduced by 60 % when compared to plants inoculated with DNA A and a betasatellite. In this tripartite interaction, DNA B dominates, as the betasatellite level is considerably reduced. DNA A-encoded Rep is the only protein that initiates replication of both DNA B and betasatellites. Reduction in betasatellite level is attributed to the competition between DNA B and betasatellite for Rep. Initiation of DNA B replication may be favored as it has Rep binding iteron sequences that are the same as in DNA A, but which are absent in betasatellites. Lin et al. (2003) showed that iteron elements are not essential for ToLCV satellite DNA replication, but the presence of these sequences does seem to favor better replication of DNA B compared to betasatellites, as seen in our experiments.

The selective advantages of the presence of the betasatellite appear to be an enhanced viral DNA level and greater interference with host defense responses (either through RNAi-related or defense gene-related pathways). In a tripartite interaction, although DNA B and betasatellites are antagonistic to each other, the cumulative disease severity is high, so that the association is bound to emerge as advantageous. However, whether an increase will occur in the association of betasatellites with bipartite begomoviruses is debatable. Both ToLCNDV and TYLCTHV represent a group of emerging bipartite begomoviruses that show opportunistic association with betasatellites. Available data indeed suggest that a bipartite virus may pick up a betasatellite and that, depending on the host it infects, the association may emerge as a permanent one, leading to a different disease scenario. In this context, the present study is valuable as it indicates how this association will behave in permissible hosts like tomato. The robust protocol of agro-inoculation of cloned components described in this paper is bound to help in understanding the molecular mechanism of viral pathogenesis.

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