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BRIEF REPORT

Tomato leaf curl Bangalore virus (ToLCBV): infectivity and enhanced pathogenicity with diverse betasatellites

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Abstract Genomic components of a begomovirus isolated from tomato plants showing leaf curl and stunting symptoms in farmer's fields at Hessarghatta village near Bangalore, India, were cloned by rolling-circle amplification. The virus was identified as a variant of strain C of the species *Tomato leaf curl Bangalore virus* and designated as *Tomato leaf curl Bangalore virus*-C[India:Hessarghatta:2008], ToLCBV-C[IN:Hess:08]. The betasatellite isolated from these samples belongs to the betasatellite species *Tomato leaf curl Bangalore betasatellite*. ToLCBV-C[IN:Hess:08] induced severe symptoms in *Nicotiana benthamiana* and *Solanum lycopersicum* plants when co-inoculated with the cognate betasatellite, *Tomato* leaf curl Bangalore betasatellite–[India:Hessarghatta:2008], ToLCBB-[IN:Hess:08] and with two other non-cognate betasatellites, *Cotton* leaf curl Multan betasatellite–[India:SriGanganagar:2002] and *Luffa* leaf distortion betasatellite–[India:Luffa:2004].

Tomato leaf curl disease, the most destructive disease of tomato crops all over the world, is caused by viruses belonging to the genus *Begomovirus* in the family *Geminiviridae* [3]. The members of the genus *Begomovirus* have typical geminate particles encapsidating a single-stranded circular DNA genome, infect dicotyledonous plants and are transmitted by the whitefly *Bemisia tabaci*. Begomoviruses of New World and some of Old World viruses have bipartite genome with [5] two DNA components: DNA A has the coat protein gene on the viral strand and replication initiation protein and transcription activation protein genes on the complementary strand, and DNA B has the gene for the nuclear shuttle protein on the viral sense strand and for the movement protein on the complementary strand. However, the majority of Old World begomoviruses are monopartite and have a DNA A component that is associated with a single-stranded DNA molecule half the length of DNA A. These molecules, which are required for production of typical symptoms in the primary hosts from which they were isolated, are designated as betasatellites [2] and are dependent on DNA A for replication, encapsidation and transmission. There is no sequence identity between DNA A and betasatellites other than a potential stem-loop structure containing the nonanucleotide sequence TAATATTAC, which represents the origin of viral-strand replication for all geminiviruses. The betasatellites have three conserved features: (1) a 100-nt stretch of sequence upstream of the stem-loop region that is conserved in all betasatellites, referred to as the satellite conserved region, (2) an 'A'-rich region and (3) only one open reading frame in the complementary strand, encoding a product known as βCl. The product of ORF βCl is a pathogenicity determinant and a PTGS suppressor that helps in viral DNA accumulation [2].

Tomato leaf curl disease was first reported in northern India by Vasudeva and Sam Raj in 1948 [13]. At present, the disease is widespread and is a major problem throughout the country, causing 27–100% yield loss, depending on the stage of infection [8]. The symptoms of the disease are leaf curling, vein clearing, reduction in leaf lamina, vein enation and, overall stunting of plants. There are seven monopartite and three bipartite begomoviruses that cause leaf curl disease in India [3], of which tomato

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leaf curl Bangalore virus is widely prevalent in the southern and southwestern parts of the country [7, 8]. Although ToLCBV was characterized earlier, until now, the infectivity of cloned components has not been established. The present communication summarizes work in that direction; it describes the infectivity of an isolate of ToLCBV on *Nicotiana benthamiana* and *Solanum lycopersicum* with cognate and non-cognate betasatellites. Co-inoculation with the cognate and two non-cognate betasatellite enhanced the severity of symptoms in both the hosts.

The begomovirus genome components, DNA A and its cognate betasatellite were cloned from total DNA extracted by the GEM-CTAB method [10] from young leaves of tomato plants with leaf curl disease collected from fields at Hessarghatta village, near Bangalore, India. The circular DNA was enriched by rolling-circle amplification (RCA) [6], and RCA products were restricted with endonucleases *Bam*HI and *Kpn*I. The 2.7 and 1.3-kb fragments, predicted to represent the full-length genome, were cloned in the vector pUC18 in *Escherichia coli* DH5α cells following standard protocols [11]. The clones were confirmed by Southern hybridization with radiolabelled MYMIV-[IN:ND:Bg3:91] probe for DNA A and CLCuMB-[IN:Sri:02] probe for betasatellite and further authenticated by sequencing (Perkin-Elmer-7000) at Delhi University, South Campus. The virus species and strain of the begomovirus and betasatellite of the present isolate were distinguished into species based on the recommendations made by Fauquet et al. [5] and Briddon et al. [2]. Based on the nucleotide sequence identity of 95.4%, the begomovirus was identified as variant of the strain C of the species *Tomato leaf curl Bangalore virus* and the isolate descriptor is *Tomato leaf curl Bangalore virus*–C[India:Hessarghatta:2008], ToLCBV-C[IN:Hess:08], GenBank accession no: GU474418. The betasatellite belongs to the species *Tomato leaf curl Bangalore betasatellite* and designated as *Tomato leaf curl Bangalore betasatellite* [India:Hessarghatta:2008], ToLCBB-[IN:Hess:08], GenBank accession no:GU984046.

To confirm their infectivity, the DNA A and DNA betasatellites were cloned as a complete or partial tandem repeat in the binary vector pBin19. The *Bam*HI-to-*Sal*I (1.1 kb) fragment of the *Bam*HI clone pBA6, representing the DNA A component of ToLCBV-C[IN:Hess:08], was separated by gel electrophoresis, purified and cloned in pBin19 vector to give pBin A0.4 mer. After confirming the presence of the insert, the full-length DNA A component, released as a *Bam*HI fragment, was ligated with *Bam*HI-linearized pBin A 0.4 mer to give pBinA1.4mer. The tandem orientation of the constructs was verified by restriction with *Hind*III, which was expected to release a 2.7-kb fragment in tandem orientation. In the case of *Tomato leaf curl Bangalore betasatellite* [India:Hess:08], a clone having a complete tandem repeat was obtained while cloning concatamers of the RCA product. This complete tandem repeat in the vector pUC18 was released by *Pvu*II restriction, and the 2.6-kb fragment was gel-purified and cloned at the *Sma*I site of pBin19 to give pBin B 2.0mer.

The non-cognate betasatellites used in the present study are *Cotton leaf curl Multan betasatellite* –[India:SriGanganagar:2002] CLCuMB–[IN:Sri:02] GenBank accession no: AY083590; *Luffa leaf distortion betasatellite*–[India:-Luffa:2004] (LuLDB-[IN:Lu:04]) AY728262; *Papaya leaf curl betasatellite* -[India: Meerut:2006] PaLCuB-[IN:Mee:06] EF053234; and *Tomato leaf curl Varanasi betasatellite* [India:Varanasi1: 2003] ToLCVB-[IN:Var1:03] AY438559. In all of these cases, DNA β clones in pGEM-T Easy Vector were used for making constructs. Full-length DNA β was amplified from the clone by using the β01/β02 primer pair [1]. The PCR fragment was restricted with *Kpn*I and gel-purified. The plasmid DNA of β clones in pGEM-T Easy Vector was linearized (4.3 kb) by partial restriction with *Kpn*I. To this fragment, *Kpn*I-restricted 1.3-kb PCR product was ligated to give a 2.6-kb tandem dimer in pGEM-T Easy Vector. The 2.6-kb fragment in pGEM-T Easy Vector was released by *Pvu*II restriction and cloned at the *Sma*I site of pBin19 to give complete tandem repeats.

ToLCBV-C[IN:Hess:08] (pBA6) was agroinoculated, either alone or with ToLCBB-[IN:Hess:08], CLCuMB-[IN:Sri:02], LuLDB-[IN:Lu:04], PaLCuB-[IN:Mee:06], or ToLCVB-[IN:Var1:03] on *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) cv. Pusa Ruby seedlings. The bacteria containing the DNA A and betasatellite constructs were mixed thoroughly and adjusted to an OD of 1.0, and 40-µl suspensions were introduced through pinpricks of the stem of 21- and 15- day-old *N. benthamiana* and tomato seedlings, respectively. Plants were maintained for 40 days at the National Phytotron Facility at 25 ± 2°C, relative humidity 85, daylight 18,000 Lux.

Total nucleic acid (10 µg) extracted from leaf tissue of *N. benthamiana* and tomato plants was fractionated by electrophoresis in a 1.2% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham). The DNA fragments, representing the coat protein gene or full-length betasatellite were labeled with [α-^{32}P]-dCTP and used in hybridization. The Southern blots were washed three times with low-stringency wash buffer, 2×SSC and 0.1% SDS, and signals were detected by phosphorimaging, using a Cyclone Plus Storage Phosphor System (Perkin Elmer).

The infectivity of the present isolate was studied by inoculating partial tandem repeats of ToLCBV-C[IN:Hess:08] alone or with its cognate betasatellite ToLCBB-[IN:Hess:08]. *N. benthamiana* and tomato plants inoculated with plain *Agrobacterium tumefaciens* cells without any construct served as controls. Inoculation with ToLCBV-C[IN:Hess:08] alone (Fig. 1a) produced mild

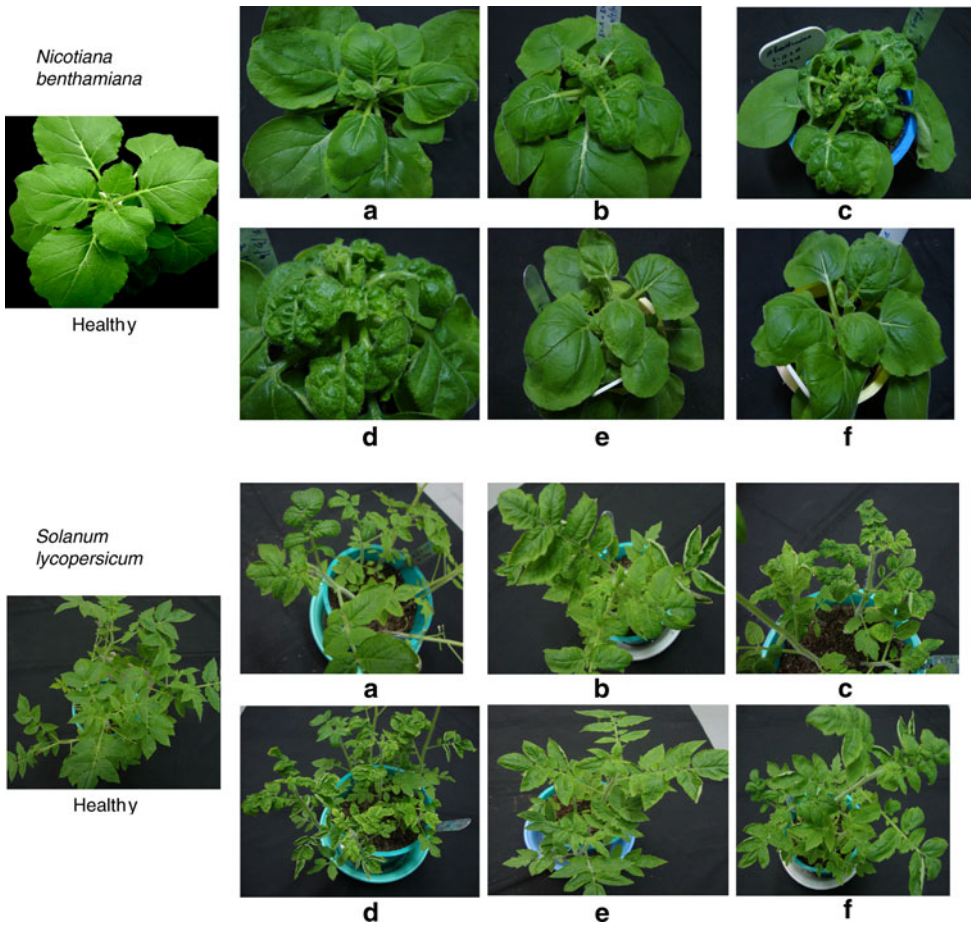

Fig. 1 Infectivity of ToLCBV-C[IN:Hess:08] and ToLCBB-[IN:Hess:08] in *N. benthamiana* and tomato plants. Plants inoculated with *Agrobacterium tumefaciens* cells without the construct served as controls. Plants were photographed 21 days after agroinoculation with ToLCBV-C[IN:Hess:08] alone (**a**), with cognate ToLCBB-[In:Hess:08] (**b**), and with non-cognate betasatellites CLCuMB-[IN:Sri:02] (**c**), LuLDB-[IN:Lu:04] (**d**), PaLCuB-[IN:Mee:06] (**e**), and ToLCVB-[IN:Var1:03] (**f**)

leaf curl symptoms, very mild backward rolling and stunting in *N. benthamiana*. In tomato, (*Solanum lycopersicum*) ToLCBV-C[IN:Hess:08] produced mild downward curling and mosaic appearance of leaf lamina. In both *N. benthamiana* and tomato, symptoms did not progress further to express typical leaf curl. However, it is evident from Southern blot analysis (Fig. 2a) that DNA A alone was infectious and systemically spread in both *N. benthamiana* and tomato, though severe symptoms were not seen.

Inoculation of ToLCBV-C[IN:Hess:08] with ToLCBB[IN:Hess:08] resulted in typical leaf curl symptoms (Fig. 1b) (Table 1) within 8–12 days postinoculation (dpi) in *N. benthamiana*. The symptoms intensified further into leaf distortion and stunting by 12–15 dpi. When *N. benthamiana* plants were maintained at three different temperatures, 23 ± 2°C, 25 ± 2°C and 28 ± 2°C, distinct expression of symptoms were seen within 8–12 dpi in plants at 23 ± 2°C. Plants maintained at 25 ± 2°C and 28 ± 2°C took nearly 15 days to express symptoms. In tomato, backward rolling of newly emerging leaves was the first symptom, which took 15–21 days to appear. Further symptoms intensified into vein thickening, lamina crinkling and stunting. Diseased plants could be clearly distinguished from healthy control plants only at 30 dpi. In tomato, symptoms appeared only in plants maintained at 25 ± 2°C.

Co-inoculation with non-cognate betasatellites CLCuMB-[IN:Sri:02] and LuLDB-[IN:Lu:04] induced more severe symptoms (Fig. 1c, d) than were induced by ToLCBV-C [IN:Hess:08] with ToLCBB-[IN:Hess:08] (Fig. 1d). In both *N. benthamiana* and tomato inoculated with DNA A and LuLDB-[IN:Lu:04], veinal yellowing in marginal veins (Fig. 1b) appeared as the first symptom, which merged to give a network of yellow veins (Fig. 1d). Symptoms progressed further, and plants showed vein thickening, leaf deformation, leaf crinkling and stunting. Inoculation of ToLCBV-C[IN:Hess:08] with CLCuMB-[IN:Sri:02] resulted in severe downward leaf curling (Fig. 1c) and stunting both in *N. benthamiana* and tomato without yellow vein symptoms. Co-inoculation with PaLCuB-[IN:Mee:06] and ToLCVB-[IN:Var1:03] produced symptoms (Fig. 1e, f) like ToLCBB-[IN:Hess:08].

The presence of helper virus DNA A and betasatellite components in plants was confirmed by Southern blot analysis. In plants co-inoculated with ToLCBB-[IN:Hess:08], there was an increase in helper viral DNA A accumulation compared to plants inoculated with DNA A alone. Especially, a marked increase (Fig. 2b, lanes 4, 5) in

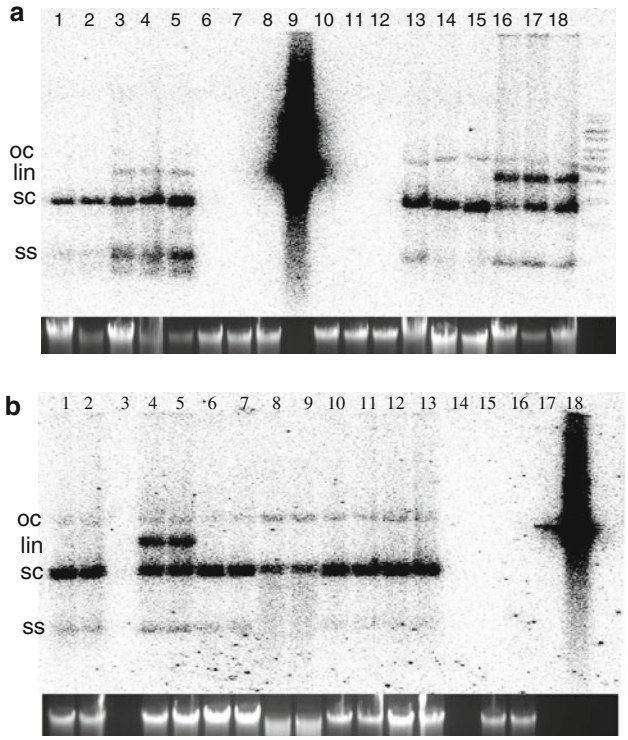

Fig. 2 **a** Southern blot analysis of viral DNA in *N. benthamiana* and tomato plants. DNA extracted from *N. benthamiana* (*lanes 1*, *2*) and tomato plants (*lanes 13*, *14*, *15*) agroinoculated with only ToLCBV-C[IN:Hess:08]; DNA extracted from *N. benthamiana* (*lanes 3*, *4*, *5*) and tomato (*lanes 16*, *17*, *18*) inoculated with ToLCBV-C[IN:Hess:08] and ToLCBB-[IN:Hess:08] DNA extracted from mock-inoculated *N. benthamiana* (*lanes 6*, *7*, *8*) and tomato (*lanes 10*, *11*, *12*) served as negative controls; 2.7-kb linearized DNA of ToLCBV-C[IN:Hess:08] was loaded in *lane 9* as a positive control; blots were probed with [α^{32} P]-dCTP-labeled coat protein gene fragment. **b** Southern blot analysis of viral DNA in tomato plants agroinoculated with cognate and non-cognate betasatellites. DNA extracted from plants inoculated with ToLCBV-C[IN:Hess:08] alone (*lanes 1*, *2*); DNA extracted from plants inoculated with cognate betasatellites ToLCBB-[IN:Hess:08] (*lanes 4*, *5*) with non-cognate betasatellite CLCuMB-[IN:Sri:02] (*lanes 6*, *7*); with LuLDB-[IN:Lu:08] (*lanes 8*, *9*); with PaLuB-[IN:Mee:06] (*lanes 10*, *11*) and with ToLCVB-[IN:Var1:05] (*lanes 12*, *13*). DNA extracted from mock-inoculated plants (*lanes 15*, *16*). Linearized 2.7-kb ToLCBV-[IN:Hess:08] is shown in *lane 18*. *Lanes 3*, *14*, and *17* are empty; blots were probed with [α^{32} P]-dCTP-labeled coat protein gene fragment

the double-stranded linear form was seen in tomato plants inoculated with ToLCBV-C[IN:Hess:08] and ToLCBB-[IN:Hess:08]. This increase was not evident when plants were inoculated with non-cognate betasatellites. A good concentration of betasatellites is seen in plants inoculated with cognate or non-cognate betasatellites (data not shown).

The present work is the first one in which the genome of ToLCBV was cloned along with its betasatellite and in which the fulfillment of Koch's postulates was demonstrated. This was accomplished through rolling-circle amplification to enrich circular DNA in the nucleic acid extract.

This isolate is similar to the majority of Old World begomoviruses, the DNA A of which is infectious by itself and spreads systemically but requires a betasatellite for typical symptom production in primary hosts. Besides the cognate betasatellite, ToLCBV-C[IN:Hess:08] *trans*-replicated four non-cognate betasatellites, although enhanced severity was seen only with two of them, CLCuMB-[IN:Sri:02] and LuLDB-[IN:Lu:04].

The mechanism by which the replication initiation protein encoded by viral DNA A recognizes betasatellites is not clear. Our infectivity studies with cognate and non-cognate betasatellites show that all the five betasatellites were *trans*-replicated by ToLCBV-C[IN:Hess:08]. Unlike bipartite begomoviruses, which have well-defined Rep-binding iterative motifs that are identical between DNA A and DNA B, iteron sequences common to DNA A and betasatellite are not seen, which has led to the suggestion that origin recognition is more relaxed for betasatellites. It is possible that Rep protein of helper begomoviruses associated with betasatellites may have relaxed origin-recognition properties, or betasatellites may have sequences that allow recognition by a broader range of Rep proteins [9]. A region between nucleotide co-ordinates 1047 and 1146 (with reference to *Ageratum yellow vein* betasatellite [12]) has been shown to be essential for *trans* replication; comparison of this region in many betasatellite sequences shows that it contains inverted repeats flanking iteron-like sequence. Besides this region, a G-box motif 143 nt upstream of ORF βCl has been shown to be important for efficient gene expression [4] and replication of DNA β. A similar G-box motif was located in cognate and non-cognate betasatellites used in this study, which might have facilitated replication, by helper virus.

Nawaz-ul-Rehman et al. [9] observed that the sequences required for recognition between cognate virus/betasatellite (CLCuMB with CLCuRaV) may differ from those for a non-cognate virus-betasatellite combination (CLCuMB/CabLCV). In the present study, enhanced accumulation of helper viral DNA was seen only in the cognate betasatellite combination and not when co-inoculated with non-cognate betasatellites, despite severe symptom expression. A higher viral DNA level seen only in inoculation with cognate betasatellites is suggestive of a specific interaction between them, which needs to be studied.

Identification of resistance sources and deployment of resistance genes is the only reliable strategy to manage leaf curl disease so that insecticide use can be reduced. Correct identification of resistance genes is possible only when viruses are individually separated and specific virus inoculation and sensitive detection methods are used. Agroinoculation of tomato leaf curl Bangalore virus with the

Table 1 Infectivity and symptoms expressed by ToLCBV-C [IN:Hess:08] inoculated with cognate and non-cognate betasatellites

Host/inoculum	No. of plants showing symptoms/No. of plants inoculated	Type of symptoms	Days until symptom production
Nicotiana benthamiana			
ToLCBV-C [IN:Hess:08]	14/14	Mild leaf curl	10–12
ToLCBV-C [IN:Hess:08] + ToLCBB-[IN:Hess:08]	14/14	Leaf crinkling, stunting	8–12
ToLCBV-C [IN:Hess:08] + CLCuMB-[IN:Sri:02]	14/14	Severe leaf crinkling, leaf curl	8–12
ToLCBV-C [IN:Hess:08] + LuLDB-[IN:Lu:04]	14/14	Veinal yellowing, chlorotic crinkling spot	8–12
ToLCBV-C [IN:Hess:08] + PaLCuB-[IN:Mee:06]	12/14	Leaf crinkling	8–12
ToLCBV-C [IN:Hess:08] + ToLCVB-[IN:Var1:03]	12/14	Leaf crinkling	8–12
Solanum lycopersicum			
ToLCBV-C [IN:Hess:08]	14/14	Downward leaf curling, leaf marginal folding	15–21
ToLCBV-C [IN:Hess:08] + ToLCBB[IN:Hess:08]	14/14	Leaf mottling, asymmetry leaf curl	15–21
ToLCBV-C [IN:Hess:08] + CLCuMB-[IN:Sri:02]	14/14	Severe leaf deformation, lamina reduction, curling	15–21
ToLCBV-C [IN:Hess:08] + LuLDB-[IN:Lu:04]	14/14	Veinal yellowing, leaf curl, Severe leaf deformation	15–21
ToLCBV-C [IN:Hess:08] + PaLCuB [IN:Mee:06]	12/14	Leaf crinkling	15–21
ToLCBV-C [IN:Hess:08] + ToLCVB-[IN:Var1:03]	10/14	Leaf crinkling	15–21

betasatellites described here will help in such resistance breeding programmes.

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