Evidence of the Association of *Radish leaf curl virus* with Tobacco Yellow Leaf Curl Disease in Bihar, India

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Abstract A new disease of tobacco with characteristic mild leaf curl and yellowing symptoms was observed in 2007 in commercial plantings in Pusa, Bihar, India. A begomovirus and a betasatellite were found associated with the disease. The associated begomovirus was identified as a strain of *Radish leaf curl virus* (RaLCV) based on nucleotide sequence of the viral genome (2,761 nucleotides; EU194914). The betasatellite (HQ180397) associated with TbYLCD was identified as a variant of Chilli leaf curl betasatellite (ChLCB). Recombination events were detected both in the RaLCV and ChLCB sequences. This is the first report of yellow leaf curl disease of tobacco, and the association of RaLCV with a disease of tobacco.

Keywords Begomovirus · Tobacco · Tobacco yellow leaf curl disease · Betasatellite · *Radish leaf curl virus*

Begomoviruses cause devastating diseases of crops in the tropical and subtropical regions of the world [1, 20, 21]. The genome of these viruses consists of one or two similar-sized DNA components, designated as DNA-A and DNA-B [8]. The DNA-A component encodes proteins required for replication, control of gene expression, and encapsidation. The DNA-B component encodes two proteins involved in intra- and inter-cellular movement of the virus

in plants [17]. The bipartite begomoviruses require both the genomic components, i.e. DNA-A and DNA-B, for systemic infection and symptom induction [16]. Whereas, DNA-A alone of the monopartite begomoviruses can infect and cause disease in the experimental hosts [6], but requires the association of betasatellite molecules to develop typical disease symptoms in the original plant hosts [3]. The betasatellites are shown to have a single ORF β CI [2, 12].

The leaf curl disease of tobacco (TbLCD) has been known for nearly a century. So far, 12 distinct monopartite begomoviruses have been identified to be associated with the leaf curl or leaf curl-like diseases of tobacco in various parts of the world [21]. In India, TbLCD is known to cause serious losses in tobacco production for over 75 years [10, 19]. Based on symptomatology, five different types of leaf curls, designated A, B, C, D and X were considered to be caused by four different viruses in northern India [10]. Later, four different types of leaf curl symptoms have also been observed in southern India [19]. The sequences of the CP gene and IR of the two isolates from Southern India suggested the possibility of association of two distinct viruses with TbLCD in the region [11]. Recently, Tobacco leaf curl Pusa virus (TbLCPuV) and a betasatellite have been reported to be associated with TbLCD in Bihar, India [14]. During a survey of tobacco fields in Bihar, India, in 2007 a distinct yellow leaf curl disease of tobacco (TbYLCD) was observed. Here we report the association of a new strain of *Radish leaf curl virus* (RaLCV) (genus *Begomovirus*) and a betasatellite with TbYLCD.

In 2007, TbYLCD affected plants were collected from the tobacco plantations in Pusa, Bihar, India was subjected to the viral genome was amplified by rolling circle amplification (RCA), from the total nucleic acid extracted from the samples by CTAB method [5], using Φ 29 DNA

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polymerase (Fermentas), as per the protocol described previously [14]. The high molecular weight amplicons were digested with 5 U *Bam*HI, for 15 min at 37°C. The products of ~2.7 kb, separated by gel electrophoresis, were cloned in pUC18 vector. The associated betasatellite was amplified by PCR using betasatellite specific primers beta01/beta02 [4]. The full length ~1.4 kb betasatellite obtained by PCR amplification was cloned in the pGM-T Easy Vector [Promega]. The clones of DNA-A, and betasatellite were sequenced and the sequences were submitted to the GenBank (DNA-A: EU194914; Betasatellite: HQ407397). The sequences were assembled and analyzed using BioEdit [7] and Mega 4 [18], and confirmed by similarity search using BLAST and FASTA from the EBI webpage (<http://www.ebi.ac.uk/>). In order to identify the potential genetic recombination events leading to the evolution of the present isolate of RaLCV, the Recombination Detection Program RDP3 [9] was used.

In the survey of tobacco growing areas in and around Pusa in 2007, 5–30% incidence of TbYLCD was observed. The affected plants developed distinct yellowing and leaf curl symptoms (Fig. 1), resulting in severe economic losses to the growers. The sequence of the full-length viral DNA-A consisting of 2,761 nucleotide (GenBank accession EU194914) had typical genome organization of the old world begomoviruses, with two ORFs in the virion sense (AV1 and AV2), and five ORFs in the

complementary sense (AC1–AC5). The ORFs AV1 (307–1,074 nt encoding the coat protein) and AV2 (147–455 nt encoding a nonstructural protein) were in the virion sense, and ORFs AC1 (2,611–1,529 nt encoding the replication associated protein), AC2 (1,623–1,222 nt), AC3 (1,478–1,077 nt), AC5 (615–301 nt) and AC4 (2,454–2,155 nt) were in the complementary sense. The coding region was separated by a non coding intergenic region (IR) of 297 nt. The IR sequence exhibited the features characteristic of begomoviruses, consisting of a putative stem-loop structure with the conserved non-nucleotide sequence, TAATATTAC in the loop, a TATA box motif at 2,675–2,678 nt and a repeated iteron sequence GGGTCCT (2,628–2,633 and 2,664–2,670 nt) at the 5' of the TATA motif. The full length nucleotide sequence the betasatellite [Accession No.HQ180397] associated with TbYLCD was 1,376 nt long. It had typical betasatellite-like structural features containing an A-rich region between 780 and 1,060 nt and ORF β C1 located on the complementary sense strand encoding a protein of 152 aa, satellite conserved SCR consisting of 150 nt and containing the nonanucleotide (TAATATT/AC) sequence.

The FASTA analysis showed highest (90.1%) nucleotide sequence identity with *Radish leaf curl virus* (RaLCV-[IN:Var:Ra:03]) described from India, and showed less than (58.3%) nucleotide sequence identity with *Tobacco leaf curl Cuba virus*. The highest amino acid sequence identity of AV2 (85.2%) of the present isolate was with *Tobacco curly shoot virus* (TbCSV), AC1 (90.0%) with *Euphorbia leaf curl virus* (EuLCV), AC4 (91.0%) with *Tomato leaf curl Patna virus* (ToLCPaV-[IN:Luc:Cot:10]), and AV1 (98.4%), AC3 (99.2%) and AC5 (79.8%) with RaLCV. The IR had the highest nucleotide sequence identity (72.9%) with *Tobacco leaf curl Thailand virus* (TbLCThV). The full length nucleotide sequence of the viral DNA of the present isolate shared 58.3–90.1% sequence identity with the other selected begomoviruses compared in this study (Fig. 2). Based on the nucleotide sequence identity and phylogenetic analysis (Fig. 2), the present isolate was found to be closely related to the isolates of RaLCV, reported from India.

The betasatellite isolated in this study had 41.6–91.7% sequence identity with the betasatellites known to occur in India and some other selected betasatellites compared in this study (Fig. 3). The present isolate was most closely (>78% nucleotide sequence identity) related to Chilli leaf curl betasatellite (ChLCB) and Tomato leaf curl betasatellite (ToLCB; 76.7–77.7% sequence identity); all the other betasatellites compared in this study were distantly related with <61.0% sequence identity. Similar affinity was also observed for the amino acid sequence of β C1 (data not shown). Phylogenetically the betasatellite associated with TbYLCD grouped with the other isolates of ChLCB and

Fig. 1 Yellow leaf curl disease of tobacco

Fig. 2 Phylogenetic tree constructed by the alignment of full-length nucleotide sequences of DNA-A of begomoviruses associated with TbLCD and TbYLCD, and some selected begomoviruses listed here. The sequences were aligned in MEGA4 using the ClustalW, and the tree was constructed by Neighbor-joining method. The horizontal distances are proportional to genetic differences amongst different begomoviruses; vertical distances are arbitrary. The numbers at each node represent the percentage boot strap scores (1,000 replicates). The open box highlights the branch formed by different isolates of RaLCV. The following viruses were included in this study: CLCuMV (Cotton leaf curl Multan virus), CYVMV (Croton yellow vein mosaic virus), EuLCV (Euphorbia leaf curl virus), HYVMV (Honeysuckle yellow vein mosaic virus), HYVV (Honeysuckle yellow vein virus), PaLCuV (Papaya leaf curl virus), RaLCV (Radish leaf curl virus),

TbCSV (Tobacco curly shoot virus), TbLCCoMV (Tobacco leaf curl Comoros virus), TbLCJV (Tobacco leaf curl Japan virus), TbLC-KoV (Tobacco leaf curl Korea Virus), TbLCKoV (Tobacco leaf curl Kochi virus), TbLCPuV (Tobacco leaf curl Pusa virus), TbLCThV (Tobacco leaf curl Thailand virus), TbLCuCuV (Tobacco leaf curl Cuba virus), TbLCYnV (Tobacco leaf curl Yunnan virus), TbLCZV (Tobacco leaf curl Zimbabwe virus), TbLRV (Tobacco leaf Rugose virus), TbMLCV (Tobacco mottle leaf curl virus), ToLCBV (Tomato leaf curl Bangalore virus), ToLCGV (Tomato leaf curl Gujarat virus), ToLCKeV (Tomato leaf curl Kerala virus), ToLCKV (Tomato leaf curl Karnataka virus), ToLCNDV (Tomato leaf curl New Delhi virus), ToLCPmV (Tomato leaf curl Palampur virus), ToLCPaV (Tomato leaf curl Patna virus), ToLCPuV (Tomato leaf curl Pune virus) and ToLCRaV (Tomato leaf curl Rajasthan virus)

synonymous ToLCBDB, and formed a distinct branch with ToLCB (Fig. 3).

The genome of RaLCV showed three potential recombination events. The first event was in the IR (breakpoint at 2,736–35 nt), probability $P = 3.337 \times 10^{-01}$ (RDP), with major parent ToLCBV-A [IN:Ban1] and minor parent ToLCNDV-[IN:ND:05]. The second event was in AV2–AV1 region (breakpoint 341–475 nt), probability $P = 1.406 \times 10^{-01}$, with major parent CLCuMV-His

[IN:ND:99] and minor parent PaLCuV-[IN:Pra:2010]. The third event was in AC1- IR region (breakpoint 2,523–2,713 nt), probability $P = 1.292 \times 10^{-05}$, with major parent ToLCBV-A [IN:Ban1] and minor parent ToLCRaV-[IN:Raj:05]. The betasatellite also showed two potential recombination events. The first event was in the (breakpoint 1,141–255 nt), with the major parent ToLCMaB-[IN: Pun: 04] and minor parent TbCSB-[CN: Yn2:99] with $P = 4.516 \times 10^{-09}$ (RDP). The second event was in the

Fig. 3 Phylogenetic tree constructed (as in Fig. 2) by the alignment of full-length nucleotide sequences of the betasatellites associated with TbLCD, TbYLCD and some other diseases. The *open box* highlights the distinct branch formed by ToLCLB occurring in Laos, and the *shaded boxes* highlight the solanaceous betasatellites which have moved to non-solanaceous crops. The sequences of the following betasatellites were used in this analysis: BYVB (Bhendi yellow vein betasatellite), ChLCB (Chilli leaf curl betasatellite), CLCuMB (Cotton leaf curl Multan betasatellite), KLCuB (Kenaf leaf curl betasatellite), OLCuB (Okra leaf curl betasatellite), PaLCuB (Papaya leaf curl betasatellite), RaLCB (Radish leaf curl

betasatellite), TbCSB (Tobacco curly shoot betasatellite), TbLCB (Tobacco leaf curl betasatellite), TbLCCNB (Tobacco leaf curl China betasatellite), ToLCB (Tomato leaf curl betasatellite), ToLCBB (Tomato leaf curl Bangalore betasatellite), ToLCJoB (Tomato leaf curl Joydepur betasatellite), ToLCLB (Tomato leaf curl Laos betasatellite), ToLCMaB (Tomato leaf curl Maharashtra Betasatellite), ToLCNB (Tomato leaf curl Nepal betasatellite), ToLCPaB (Tomato leaf curl Patna Betasatellite), ToLCVB (Tomato leaf curl Varanasi betasatellite), TYLCCNB (Tomato yellow leaf curl China betasatellite), TYLCVNB (Tomato yellow leaf curl Vietnam betasatellite) and TYLCYnB (Tomato yellow leaf curl Yunnan betasatellite)

(breakpoint at 771–813 nt), probability 1.078×10^{-03} (RDP) with major parent ToLCB-[PK: RYK: 97] and minor parent RaLCB-[IN: Cro: 08].

TbLCD is endemic in India. The affected plants exhibit varying symptoms [10, 19]. A monopartite begomovirus, TbLCPuV, a betasatellite and an alphsatellite were found associated with a TbLCD affected plants, showing characteristic leaf curl and crinkling symptoms and the leaves

remained green [20] in Bihar, India, the same region from where the disease was first described by Pal and Tandon [10]. Two other begomoviruses are also expected to be associated with TbLCD in southern India [11], but they have not been identified so far. In the present study we have characterized a new strain of RaLCV associated with TbYLCD in Bihar, India. The symptoms TbYLCD differed from the TbLCD described earlier from the same

agro-climatic region [14]. In contrast to the TbLCD affected plants, the TbYLCD affected plants showed marked yellowing accompanied by leaf curl and negligible crinkling (Fig. 1). The strains of RaLCV are known to be spreading in the region, causing leaf curl diseases in capsicum and radish crops [13]. Our isolate from tobacco was found to have 89.5–90.1% nucleotide sequence identity with the isolates from capsicum and radish, and also grouped with these isolates in phylogenetic analysis (Fig. 2). A major variation between the present and earlier isolates of RaLCV was found in the IR as it had 52.0–53.7% nucleotide sequence identity, compared to 80.0–99.2% amino acid sequence identity of the products of various ORFs (data not shown). The variation in the IR seems to have mainly resulted due to recombinant events, with the sequences contributed by ToLCBV as a major parent and ToLCNDV and ToLCRaV as minor parents. Interestingly, neither the major nor the minor parents, contributing the recombinant sequences are known to occur in the region, where RaLCV is spreading [21].

However, the recombinant events indicate that RaLCV might have originated in tomato infected with monopartite and bipartite begomoviruses. Phylogenetically, the begomoviruses included in this study formed distinct Asian, African and Cuban clades (Fig. 2). The large Asian clade contained two distinct branches; one branch consisted of the begomoviruses spreading in China, Indian sub-continent and Thailand, and the other branch consisted of the begomoviruses spreading in Japan and Korea (Fig. 2). These findings suggest phylogeographic evolution of the begomoviruses affecting solanaceous crops and some other plant species.

Some of the monopartite begomoviruses require the association of betasatellite molecules to induce typical disease symptoms [2]. In India, considerable variation has been found in the betasatellite molecules associated with the diseases caused by both monopartite and bipartite begomoviruses [15]. However, in tobacco in Bihar, closely related isolates of ChLCB were found associated with both TbLCD [14] and TbYLCD. The isolates of ChLCB are closely related to the isolates of ToLCB, reported to occur in the north-western region of the Indian sub-continent, sharing 76.7–77.7% nucleotide sequence identity. The relationship with all the other betasatellites compared in this study was more distant with nucleotide sequence identity varying from 40.0 to 60.0% (data not shown). This was also reflected in the phylogenetic analysis as the ChLCB isolates grouped together, and formed a distinct cluster with the isolates of ToLCB (Fig. 3). The present isolate of ChLCB was found to be a recombinant betasatellite, with some sequences contributed by ToLCMaB and ToLCB as major parents, although neither of them are known to occur in north-eastern India [15].

Phylogenetic analysis of the nucleotide sequences of betasatellites showed distinct clustering of the betasatellites associated with the diseases of malvaceous and solanaceous crops (Fig. 3). The betasatellites characterized from the solanaceous crops formed five sub-clusters (Fig. 3); sub-clusters I–IV were formed by the betasatellites spreading in the Indian sub-continent, whereas the sub-cluster V consisted of the betasatellites occurring in China and Vietnam. However, the betasatellite (ToLCLB), reported to occur in Laos, formed a separate branch (open box, Fig. 3), suggesting an independent evolution of this betasatellite in South-East Asia. Some betasatellites of sub-clusters II and IV (shaded boxes, Fig. 3) appear to have moved to papaya and radish from solanaceous crops in the Indian sub-continent.

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