Characterization of Tomato leaf curl New Delhi virus associated with leaf curl and yellowing disease of bitter gourd from Karnataka

Abstract

Aims: The objective of this study was to identify the ToLCNDV isolate responsible for leaf curl and yellowing disease in bitter gourd crops in Karnataka, India.
Study design: Molecular characterisation and transmission study
Place and Duration of Study: The laboratory studies were conducted in the Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, GKVK, Bangalore-560065
Methodology: The sample was collected and subjected to molecular characterization using specific primer for ToLCNDV and sent for sequencing. Vector and sap transmission studies were also carried out.
Results: PCR-mediated detection revealed the presence of ToLCNDV, although it was not coupled with any satellite molecules. The partial genome sequencing of the AV1 gene revealed more than 97% similarity with ToLCNDV isolates. The isolate under investigation was identified as a ToLCNDV isolate and given the name ToLCNDV_GKVK_BT due to high sequence identities and phylogenetic links between the incomplete DNA-A genome and ToLCNDV isolates. The transmission experiments demonstrated that it was effectively transmitted via vector and sap transfer.
Conclusion: This study identified the ToLCNDV isolate responsible for leaf curl and yellowing disease in bitter gourd crops in Karnataka, India. Identifying, characterizing at the molecular level, and understanding the transmission of begomoviruses are essential steps in developing effective management approaches to minimize agricultural losses caused by these viruses. Therefore, these findings are important for identifying the leaf curl disease of bitter gourd.

Keywords: Bitter gourd, Begomovirus, ToLCNDV, Transmission, Vector, Mechanical

1. INTRODUCTION

Bitter gourd (*Momordica charantia* L.), a member of the Cucurbitaceae family, is a popular vegetable in home gardening. Its origins are believed to be in tropical Asia, with widespread cultivation in Pakistan, China, India, Bangladesh, Malaysia and parts of tropical Africa [1]. In India, bitter gourd is grown over 109 hectares with a production yield of 1330 metric tons, and Madhya Pradesh ranks highest in production with 229.91 tons. Traditionally, bitter melon has been valued in various medicinal systems for its health benefits, offering essential nutrients and aiding in disease prevention. It contains diverse bioactive compounds, including alkaloids, polypeptides, vitamins, and minerals. Among these, two types of saponins type oleanane and cucurbitane type triterpenoids are notable [2].

Bitter gourd productivity is significantly affected by diseases such as downy and powdery mildew, fusarium wilt, white rot, damping-off, root rot, bacterial wilt, leaf spot, mosaic, watermelon bud necrosis and viral infections that cause leaf curl and distortion. A particularly impactful viral disease is caused by the tomato leaf curl New Delhi virus (ToLCNDV), which leads to symptoms like yellow mosaic patterns, upward leaf curling, and smaller fruits. ToLCNDV, initially identified in tomatoes in India in 1994, has become a critical agricultural issue for crops in India and neighboring regions. This virus has been reported across India [3] and in neighboring countries, including Pakistan [4,5], Bangladesh [6] and Thailand [7]. ToLCNDV is a bipartite begomovirus from the *Geminiviridae* family and is transmitted by the whitefly *Bemisiatabaci*. This study aimed to investigate the causal agent behind the leaf curl

and yellowing disease in bitter gourd through molecular characterization and transmission methods.

2. MATERIAL AND METHODS

2.1 Virus source

In the summer of 2023, bitter gourd plants showing characteristic yellowing symptoms were gathered from the cucurbit plot in 'F' block at the University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India. Samples of infected leaves and ripe fruits were obtained from the affected bitter gourd plants. The isolated sample was designated as GKVK3.

2.2 DNA isolation and PCR based detection of begomovirus

To verify the presence of ToLCNDV in bitter gourd samples displaying yellowing symptoms, total genomic DNA was extracted from bitter gourd leaves using the CTAB method [8]. PCR was conducted with primers specific to the ToLCNDV, targeting an amplicon size of 730 bp. Additionally, PCR was performed with primers specific to alphasatellite and betasatellite to detect satellite molecules in the samples (Table 1). The resulting amplicon, representing a partial genome of the ToLCNDV, was submitted for Sanger sequencing at Eurofin Genomics India Pvt. Ltd., Bangalore, India.

2.3 Sequence analysis

The AV1 gene sequences from the GKVK3 virus isolate were analyzed against sequences available in the NCBI database using Blastn [9]. The sequences showing the highest blast scores, indicating over 90% similarity with the GKVK3 isolate's AV1 gene, were retrieved from the NCBI GenBank database and aligned with Clustal X2 software. To calculate pairwise identity for the AV1 gene between the GKVK3 isolate and viral sequences from GenBank, the Species Demarcation Tool (SDT) [10] was employed (Table 2). Phylogenetic trees were then constructed using the neighbor-joining method with default settings in the MEGA X software package [11].

2.4 Insect transmission

The GKVK3 isolate was transferred to healthy bitter gourd plants within an insect-proof glasshouse using whiteflies (*B. tabaci*Genn.), with a 12-hour acquisition access period (AAP) and a 24-hour inoculation access period (IAP). Symptoms in infected plants were monitored, and the inoculated plants were tested using PCR with specific primers.

2.5 Sap transmission

For sap inoculation, sap from the GKVK3 isolate was applied to healthy tomato plants at the two true-leaf stage, with leaves pre-dusted with 600-mesh carborundum [12]. After inoculation, plants were rinsed with water to remove excess inoculum and kept in insect-proof micro-cages to observe symptom development. PCR testing was conducted on the inoculated plants to confirm infection by the ToLCV isolate.

Table 1: Primer sequence and PCR condition of ToLCNDV, betasatellite and alphasatellite specific primers

Sl. No.	Primer	Sequence data	PCR condition/sec	Amplicon size (bp)	Reference
1.	ToLCNDV-Forward	5'TACGGATCCATATGATGATGTCGAAGCGACCAG CA3'	94 ◦C–50 50 ◦C–45 72 ◦C–90	□730	Naganur, 2018
	ToLCNDV-Reverse	5'TAGAAGCTTTTAATTTGTGACCGAAC3'			
2.	Betasatellite-Forward	5' GGTACCACTACGCATCGCAGCAGCC 3'	94 ◦C–50 sec 52 ◦C–60 sec 72 ◦C–90 sec	□1350	Briddon *et al.,* 2002
	Betasallite-Reverse	5' GGTACCTACCCTCCCAGGGGTACAC 3'			
3.	Alphasatellite-Forward	5' CTGCAGATAATGATGTAGCTTACCAG 3'	94 ◦C–50 sec 50 ◦C–45 sec 72 ◦C–90 sec	□1400	Bull *et al.,* 2003
	Alphasatellite-Reverse	5' CTGCAGATCCTCCACGTGTATAG 3'			

Table 2: Reference sequences of begomovirus AV1 gene used for sequence comparision and phylogenetic analysis of begomovirus associated with yellowing disease in bitter gourd

Sl. No.	Details	Accession No.	Abbreviation
1.	Tomato leaf curl virus (India: Kolar: Tomato)	AF321929.1	ToLCV
2.	Tomato leaf curl virus (India:Kannur:Tomato)	AJ810353.1	ToLCV
3.	Tomato leaf curl Bangalore virus (India: Karnataka:Tomato)	MN095551.1	ToLCBV
4.	Tomato leaf curl Bangalore virus (India: Madurai: Tomato)	KP164859.1	ToLCBV
5.	Tomato leaf curl Bangalore virus (Sri Lanka: Batticaloa: Tomato)	PP935253.1	ToLCBV
6.	Tomato leaf curl Karnataka virus (India: Sagoni Kalan, Bhopal:Tomato)	PQ106819.1	ToLCKV
7.	Tomato leaf curl Gujarat virus (India: Varanasi, UP: Tomato	NC004558.1	ToLCGV
8.	Tomato leaf curl New Delhi virus (India:Pollachi,TamilNadu:Bittergourd)	KM275601.1	ToLCNDV
9.	Tomato leaf curl New Delhi virus (India: Bagalkot:Ridge gourd)	MW505931.1	ToLCNDV
10.	Tomato leaf curl New Delhi virus (India: Vettaikaranpudur,TN:Ridgegourd)	OR678733.1	ToLCNDV
11.	Tomato leaf curl New Delhi virus(India: Bolarum, Hyderabad: Bittergourd)	MT976080.1	ToLCNDV
12.	Tomato leaf curl Palampur virus (India:HimachalPradesh:Tomato)	AM884015.2	ToLCPalV
13.	Tomato leaf curl Kerala virus (India: Tomato)	EU910141.1	ToLCKerV
14.	Tomato leaf curl Sri Lanka virus (Sri Lanka:Bandarawela:Tomato)	NC004647.1	ToLCSKV
15.	Tomato leaf curl Pune virus (India: Pune: Tomato)	KP178732.1	ToLCPuV

16.	Tomato leaf curl Rajasthan virus (India: Rajasthan: Tomato)	DQ339117.1	ToLCRJV
17.	Tomato leaf curl Joydebpur virus (India:Mung bean)	JQ654460.1	ToLCJDV
18.	Chilli leaf curl Ahmedabad virus (India: Dharwad:Chilli)	MW760321.1	ChiLCV
19.	Squash leaf curl China virus (India: Tindivanam,TN:Ashgourd)	KJ584150.1	SLCCNV
20.	Cotton leaf curl Bangalore virus (India:Cotton)	AY705380.1	CLCuBaV
21.	Bhendi yellow vein mosaic virus (India:Madurai:Bhendi)	AF241479.1	BYVMV
22.	Mungbean yellow mosaic India virus (India:Cowpea)	AF481865.2	MYMIV
23.	Ageratum enation virus (India: Pantnagar:Tomato)	JX436472.1	AEV

Table 3: Assessment of transmission efficiency of begomovirus isolate on bitter gourd through vector and sap transmission

Sl. No.	Transmission mode	No of plants expressed symptoms after 14 days/ Total no. of plants inoculated	Types of symptoms expressed after 14 days of inoculation	Number of samples positive in PCR	Transmission efficiency (%)
1.	Vector transmission	12/15	Mosaic, chlorosis	14/15	93.33
2.	Sap transmission	3/15	Mild mosaic	7/15	46.67

3. RESULTS AND DISCUSSION

3.1 Symptomatology

The infected bitter gourd plants displayed leaf yellowing along with slight curling of newly developed leaves. The internodal length in these affected plants was significantly shortened, leading to stunted growth. As the symptoms progressed, the leaf area gradually diminished (Figure 1). Similar symptoms, including yellow mosaic and mild leaf curling, were observed in bitter gourd plants in eastern Uttar Pradesh [13]. Ash gourd infected with ToLCNDV also produced similar yellowing and mosaic symptoms [14].

Figure 1: Bitter gourd plant showing yellowing along with leaf curl symptom under field conditions

3.2 PCR based detection of ToLCNDV and associated satellite molecule

Using ToLCNDV specific primer, the PCR analysis of GKVK3 isolate produced an anticipated amplicon size of 750 bp (Figure 2). No amplification was found for alphasatellite and betasatellite specific primer.This suggests the presence of begomovirus infection in bitter gourd plants but not associated with any satellite molecule. Later, PCR amplified product was sequenced and sequence analysis showed more than 96% identity with ToLCNDV isolates collected from other parts of India in the GenBank of NCBI. This partial sequence of AV1 gene was deposited in NCBI Gene Bank data base under accession number PQ435992. Other studies detected ToLCNDV infection in bitter gourd in a similar manner [15, 16].

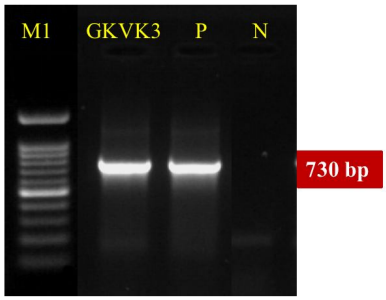

Figure 2: Agarose gel electrophoresis of PCR products of ToLCNDV isolate showing yellowing symptoms with ToLCNDV specific primer

M1-100 bp ladder; N - Negative control; P - Positive control

3.3 Sequence analysis

The BLAST analysis of the conserved coat protein gene (PQ435992) showed over 96% sequence identity with ToLCNDV (MT976080). Pairwise sequence identity with other ToLCNDV and begomovirus isolates was determined using the Sequence Demarcation Tool (SDT v1.2). The pairwise analysis of the AV1 gene from the GKVK3 isolate revealed the highest nucleotide identity (>91%) with ToLCNDV isolates (MT976080, OR678733, KM275601, and MW505931), and over 94% identity with the squash leaf curl China virus (KJ584150). Additionally, the GKVK3 isolate shared approximately 88% genetic similarity with other begomoviruses infecting various crops (Figure 3). Phylogenetic analysis of the partial coat proteins indicated a close relationship with ToLCNDV (Figure 4). The data clearly showed that GKVK3 isolates had over 97% sequence identity, surpassing the 91% threshold for strain classification and the 90% threshold for Begomovirus species identification [17]. Consequently, the GKVK3 isolate was classified as a ToLCNDV isolate and designated as ToLCNDV_GKVK_BT due to the high sequence and phylogenetic similarities between its incomplete DNA-A genome and ToLCNDV isolates. In nucleotide sequence comparisons, the Pantnagarbegomovirus isolate exhibited a maximum identity of 98% with ToLCNDV [18]. Similarly, the Hyderabad begomovirus isolate shared up to 93% similarity with ToLCNDV [19] and between 91%-99% with ToLCNDV from Chitrakoot, India [20].

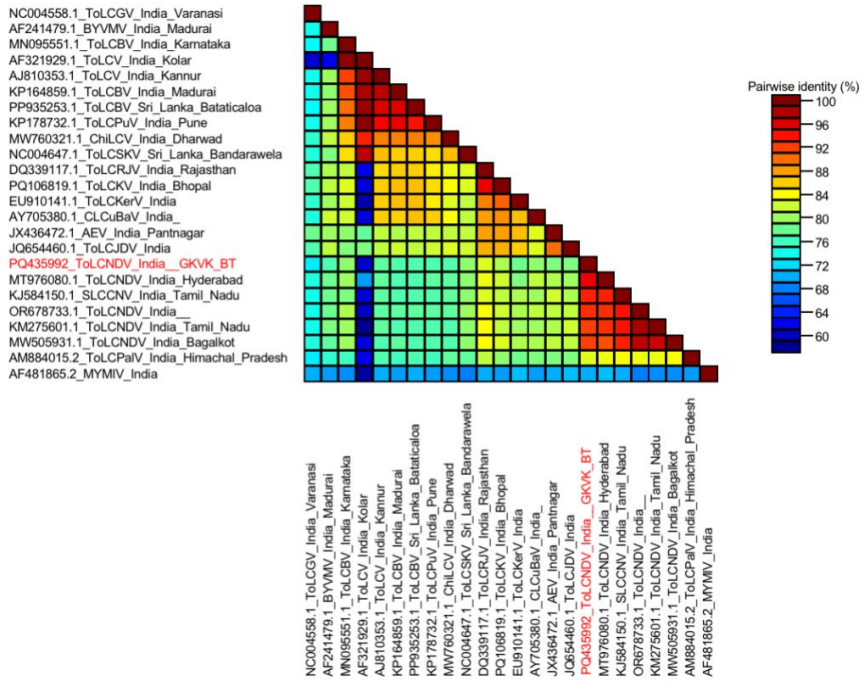

Figure 3: Two dimensional color-coded matrix of pairwise identity scores of the AV1 gene of Tomatoleaf curl New Delhi virusin the present study with other selected begomovirus sequences using sequence demarcation tool (SDTv1.0)

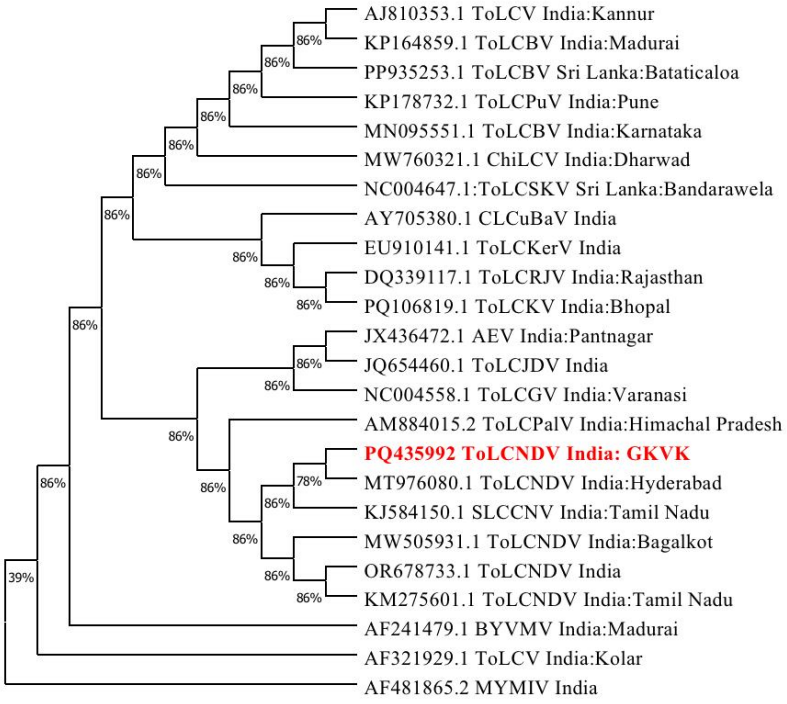

Figure 4: Phylogenetic tree of ToLCNDV isolate collected from bitter gourd constructed by a neighbor-joining method using species specific primer

3.4 Transmission studies

The vector transmitted bitter gourd plants expressed mild curling symptoms after 14 days of incubation period [Figure 5(a)]. Out of 15 plants inoculated *via* vector, 14 plants samples amplified for ToLCNDV specific primer [Figure 6(a)]. The vector transmission rates of GKVK3 isolate on bitter gourd plants was generally high with 93.33per cent rate of transmission (Table 3). This suggests that GKVK3 isolate in bitter gourd efficiently transmitted by whiteflies. The sap transmitted bitter gourd plants exhibited yellowing symptoms after 14 days of incubation period [Figure 5(b)]. Out of 15 plants inoculated *via* sap, 7 plants samples amplified for ToLCNDV specific primer [Figure 6(b)]. The sap transmission rate was relatively low, with a transmission rate of 46.47% (Table 3), suggesting that direct sap transmission is generally less effective in bitter gourd plants compared to vector transmission. It is reported that feeding tomato plants with groups of 5, 20, and 50 viruliferous *B. tabaci* resulted in 15%, 30%, and 100% of plants showing symptoms and testing positive for the virus, respectively [21]. Similarly, twenty whiteflies were used to transmit ToLCNDV in bitter gourd, achieving a transmission efficiency of 93.33% [22]. In this study, twenty whiteflies successfully transmitted ToLCNDV in tomato with an 80.00% transmission rate. Although the GKVK1 isolate's sap transmission rate was relatively low, it produced yellowing and vein clearing 15 days post-inoculation. Sap transmission of ToLCNDV in ridge and sponge gourds also resulted in symptoms and severe infection three weeks post-inoculation [12, 23]. Other ToLCV strains have been shown to transmit *via* sap in tomato [24, 25].

4. CONCLUSION

Identifying, characterizing at the molecular level, and understanding the transmission of begomoviruses are essential steps in developing effective management approaches to minimize agricultural losses caused by these viruses. This study identified the ToLCNDV isolate responsible for leaf curl and yellowing disease in bitter gourd crops in Karnataka, India. Future researchers could use similar techniques to recognize common symptoms caused by

various begomoviruses across different crops. In areas where the disease is prevalent, a combined approach including vector control and the use of resistant cultivars should be developed to mitigate the risk.

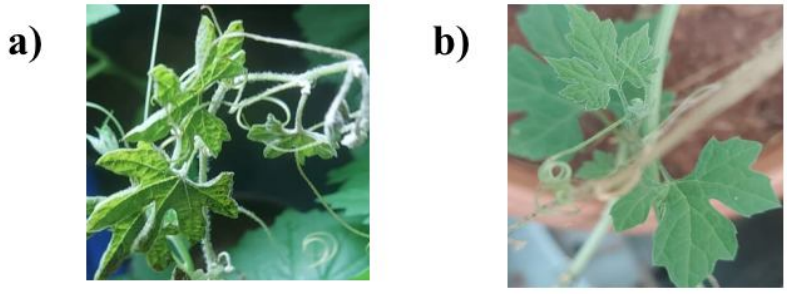

Figure 5: Leaf curl symptom expression in bitter gourd plants inoculated with GKVK3 isolates through a) Whitefly transmission and b) Sap transmission

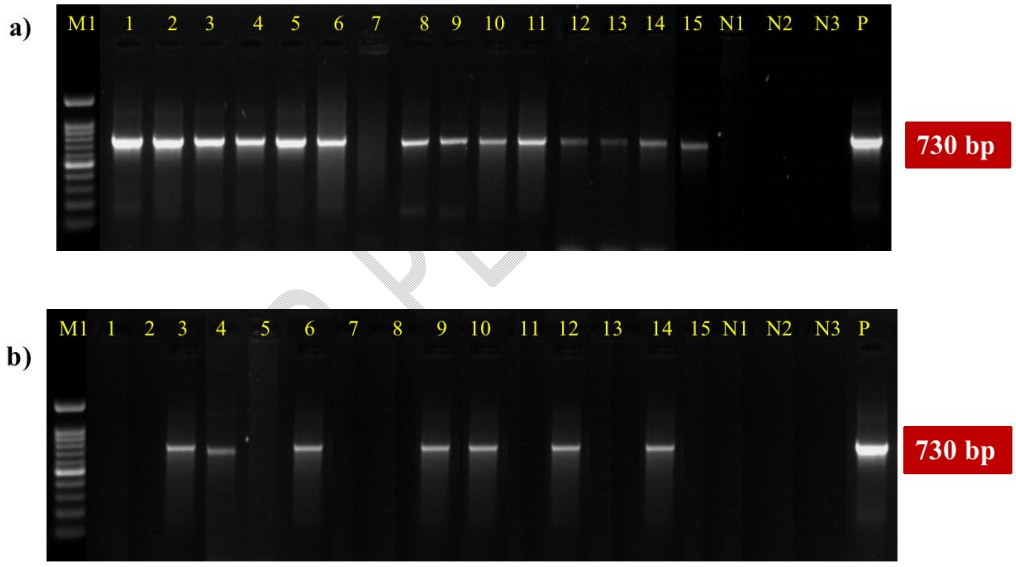

Figure 6: Agarose gel electrophoresis of PCR products from bitter gourd plants inoculated with GKVK3 isolates *via* a) Whitefly transmission and b) Sap transmission

ETHICS APPROVAL

This research is observational. It has been certified by the Navsari Agricultural University Research Ethics Committee that no ethical clearance is needed.

CONSENT TO PARTICIPATE

The study did not involve any human volunteers in its investigation.

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