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Molecular Identification and Characterisation of a Begomovirus Associated with Tomato Vein Clearing Disease in Karnataka

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Abstract

Tomato leaf curl disease is an important challenge to tomato cultivation around the world. Along with a sharp decline in plant growth and productivity, the disease is characterized by curling, puckering, crinkling and stunting of the leaves. An unusual symptom was noted in tomato originating from Karnataka state (India) consisting of leaf curl associated with vein clearing and interveinal chlorosis. PCR- mediated detection showed the presence of ToLCNDV but it was not associated with any of the satellite molecule. The partial genome sequencing of AV1 gene showed that more than 97 per cent identity with ToLCNDV isolates. The isolate under investigation was recognized as a ToLCNDV isolate and given the designation ToLCNDV_GKVK based on high sequence identities and phylogenetic linkages of partial DNA-A genome with ToLCNDV isolates. Although the isolate was neither internally nor externally seed-borne, the transmission experiments indicated that it was effectively spread by vector and sap transmission.

Keywords : Tomato leaf curl, Begomovirus, Transmission, Vector, Mechanical, Seed

Tomato (*Solanum lycopersicum* L.) is a key vegetable crop within the Solanaceae family, cultivated for its edible fruit across tropical and subtropical regions worldwide (Achari *et al.,* 2019). Tomatoes are valued not only for their taste but also for their nutrition, containing 42 µg of vitamin 'A', 0.037 mg of vitamin 'B1', 0.594 mg of vitamin 'B3', 0.4 mg of nicotinic acid, 14 mg of vitamin 'C', 7.9 µg of vitamin 'K', 2573 µg of lycopene, 24 mg of phosphorus and 237 mg of potassium per 100 g of raw fruit. Tomatoes have grown in popularity because of their processing potential as well as their medical benefit, particularly the antioxidant properties of ascorbic acid and lycopene (Rick, 1969).

India ranks second to China in tomato production on a worldwide scale. In 2021-22, the country produced around 20,300.19 tons of tomatoes, accounting for nearly 9.63 per cent of total vegetable production. The area under tomato cultivation has increased from 546.93 million hectares in 2005-06 to 865.29 million hectares in 2021-22, resulting in a total production of 20,300.19 tons, up from 99.67 thousand tons in 2005-06 (Ghalawat *et al.,* 2024). Tomato cultivation occupies 65,545 hectares in Karnataka with an annual output of 2.06 million tons and a yield of 31.37 tonnes per hectare (Anonymous, 2017). Bengaluru, Belagavi, Tumakuru, Chikkaballapura, Kolar, Hassan, Haveri, and Davanagere are the primary tomato-growing districts in Karnataka (Achari *et al.,* 2019).

Tomato plants are highly susceptible to various plant diseases, many of which have spread globally with significant economic impact. Among these, tomato is particularly affected by leaf curl virus disease, primarily caused by begomoviruses from the family

Geminiviridae. These begomoviruses possess circular, single-stranded DNA genomes enclosed within twin geminate particles (Harrison and Robinson, 1999). Tomato leaf curl virus (ToLCV), a prominent pathogen in numerous regions of India (Vasudeva & Samraj, 1948 and Sastry & Singh, 1973) is transmitted by the whitefly *Bemisia tabaci*. Infected tomato plants display a range of symptoms, including leaf curling, yellowing, stunted growth and partial or complete sterility if infection occurs early in plant development (Saikia and Muniyappa, 1989).

ToLCV is present in approximately 50 per cent of tomatoes cultivated from July to November and 100 per cent of tomatoes planted from February to May (Padidam *et al.,* 1995 and Ramappa *et al.,* 1998). Several ToLCV isolates have been cloned and sequenced around the country. Northern Indian isolates contain a bipartite genome (DNA A and DNA B) with two circular ssDNA of about 2.5-2.7 kb [Tomato leaf curl New Delhi virus (ToLCNDV)], whereas Southern Indian isolates have a monopartite genome (DNA A) with a single circular ssDNA (Hong and Harrison, 1995). Given this background, a study was conducted to determine the begomovirus status in connection to tomato vein clearing disease in Karnataka, India.

Material and Methods

Virus Source

During summer of 2022, the tomato plants exhibiting the typical symptoms of vein clearing were collected from tomato plot, 'F' block, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka state of India. Infected leaf and ripened fruit samples were collected from infected tomato plants. Collected isolate was designated as GKVK1 isolate.

DNA Isolation and PCR Based Detection of Begomovirus

To confirm the presence of begomovirus in tomato samples showing vein-clearing disease symptoms, total genomic DNA was extracted from tomato leaves using the CTAB method (Lodhi *et al.,* 1994). PCR was carried out with primers specific to the tomato leaf curl New Delhi virus, aiming for an expected amplicon size of 730 bp. In addition, PCR was performed with alpha satellite and beta satellite-specific primers to check for the presence of satellite molecules in the samples (Table 1). The resulting

Table 1

Primer sequence and PCR condition of ToLCNDV, beta satellite and alpha satellite specific primers

Primer	Sequence data	PCR condition/sec	Amplicon size (bp)	Reference
ToLCNDV-Forward	5'TACGGATCCATATGATGATG TCGAAGCGACCAGCA3'	94 °C–50 50 °C–45 72 °C–90	□ 730	Naganur, 2018
ToLCNDV-Reverse	5'TAGAAGCTTTTAATTTGTG ACCGAAC3'			
Betasatellite-Forward Briddon *et al.,* 2002	5' GGTACCACTACGCATCGCA	94 °C–50 sec GCAGCC 3'52 72 °C–90 sec	□ 1350 °C–60 sec	
Betasallite-Reverse	5' GGTACCTACCCTCCCAGG GGTACAC 3'			
Alphasatellite-Forward	5' CTGCAGATAATGATGTAG	94 °C–50 sec CTTACCAG 3'50 72 °C–90 sec	□ 1400 °C–45 sec	Bull *et al.,* 2003
Alphasatellite-Reverse	5' CTGCAGATCCTCCACGT GTATAG 3'			

amplicon, representing a partial begomovirus genome, was submitted for Sanger sequencing (Eurofin Genomics India Pvt. Ltd., Bengaluru, India).

Sequence Analysis

The AV1 gene sequences of the virus from the GKVK1 isolate were compared with sequences that were available in the NCBI database using Blastn (Altschul *et al.,* 1990). Sequences with the highest blast scores, indicating over 90 per cent similarity with the GKVK1 isolate (AV1 gene), were obtained from the NCBI GenBank database and aligned using Clustal X2 software. The Species Demarcation Tool (SDT) (Muhire *et al.,* 2014) was used to calculate pairwise identity for the AV1 gene of the GKVK1 isolate and viral sequences from GenBank (Table 2). Phylogenetic trees were then constructed using the neighbor-joining method with the default parameters in the MEGA X software package (Kumar *et al.,* 2018).

Insect Transmission

The obtained GKVK1 isolate was transferred to healthy tomato plants (Arka vikas) in an insect-proof glasshouse *via* whitefly (*B. tabaci* Genn.) using a 12-hour acquisition access period (AAP) and a 24-hour inoculation access period (IAP). Symptoms of infected plants were observed. The resulting inoculated plants were evaluated using a specific primer in PCR.

TABLE 2

Reference sequences of begomovirus AV1 gene used for sequence comparision and phylogenetic analysis of begomovirus associated with vein clearing disease in tomato

Details	Accession No.	Abbreviation
Tomato leaf curl virus (India: Kolar: Tomato)	AF321929.1	ToLCV
Tomato leaf curl virus (India:Kannur:Tomato)	AJ810353.1	ToLCV
Tomato leaf curl Bangalore virus (India: Karnataka:Tomato)	MN095551.1	ToLCBV
Tomato leaf curl Bangalore virus (India: Madurai: Tomato)	KP164859.1	ToLCBV
Tomato leaf curl Bangalore virus (Sri Lanka: Batticaloa: Tomato)	PP935253.1	ToLCBV
Tomato leaf curl Karnataka virus (India: Sagoni Kalan, Bhopal:Tomato)	PQ106819.1	ToLCKV
Tomato leaf curl Gujarat virus (India: Varanasi, UP: Tomato	NC004558.1	ToLCGV
Tomato leaf curl New Delhi virus (India:Tomato)	U15015.2	ToLCNDV
Tomato leaf curl New Delhi virus (India: Cucumber)	MH883329.1	ToLCNDV
Tomato leaf curl New Delhi virus (India: Uttarakhand:Tomato)	MZ781422.1	ToLCNDV
Tomato leaf curl New Delhi virus(India: Bolarum, Hyderabad: Bittergourd)	MT976080.1	ToLCNDV
Tomato leaf curl Palampur virus (India:Himachal Pradesh:Tomato)	AM884015.2	ToLCPalV
Tomato leaf curl Kerala virus (India: Tomato)	EU910141.1	ToLCKerV
Tomato leaf curl Sri Lanka virus (Sri Lanka:Bandarawela:Tomato)	NC004647.1	ToLCSKV
Tomato leaf curl Pune virus (India: Pune: Tomato)	KP178732.1	ToLCPuV
Tomato leaf curl Rajasthan virus (India: Rajasthan: Tomato)	DQ339117.1	ToLCRJV
Tomato leaf curl Joydebpur virus (India:Mung bean)	JQ654460.1	ToLCJDV
Chilli leaf curl Ahmedabad virus (India: Dharwad:Chilli)	MW760321.1	ChiLCV
Squash leaf curl China virus (India: Tindivanam,TN:Ashgourd)	KJ584150.1	SLCCNV
Cotton leaf curl Bangalore virus (India:Cotton)	AY705380.1	CLCuBaV
Bhendi yellow vein mosaic virus (India:Madurai:Bhendi)	AF241479.1	BYVMV
Mungbean yellow mosaic India virus (India:Cowpea)	AF481865.2	MYMIV
Ageratum enation virus (India: Pantnagar:Tomato)	JX436472.1	AEV

Sap Transmission

For sap inoculation, sap from the GKVK1 isolate was applied to healthy tomato plants at the stage of two fully expanded true leaves with leaves pre-dusted with 600-mesh carborundum (Sohrab *et al.,* 2013). Following inoculation, the plants were rinsed with water to remove any excess inoculum and placed in insect-proof micro-cages to monitor symptom development. The inoculated plants were then tested using PCR to confirm infection by ToLCV isolates.

Seed Transmission

For externally seed borne virus test, 25 seeds from GKVK1 isolates infected plants of tomato were collected. Seeds were placed for external washing for extraction of virus present on the seed surface (Vargas-Mejia *et al.,* 2022). In addition, the aqueous phase under went the CTAB technique of DNA extraction and PCR confirmation was performed to demonstrate the existence of the virus on the seed surface. Following an external seed-borne virus test, then seeds were dissected into their component parts, such as the seed coat, endosperm and embryo to ascertain whether the virus internally seed-borne and if so, to pinpoint its position within the seed. The seed dissects of tomato seeds were utilized to extract genomic DNA and were then subjected to PCR examination.

Results and Discussion

Symptomatology

The infected tomato plants produced interveinal chlorosis along the length of the leaves and size of the newly emerged leaves were reduced. The internodal length of the affected plant was severely reduced, resulting in stunting. As the symptoms worsened over time, the leaf area decreased (Fig. 1). Similar indications of leaf roll and interveinal chlorosis were commonly seen in tomato fields (Macedo *et al.,* 2014 and Fiallo-Olive & Navas-Castillo, 2019).

PCR Based Detection of Begomovirus and Associated Satellite Molecule

Using ToLCNDV specific primer, the PCR analysis of GKVK1 isolate produced an anticipated amplicon size of 750 bp (Fig. 2). No amplification was found for alpha satellite and beta satellite specific primer.

Fig. 1 : Tomato plant showing vein clearing symptom under field conditions

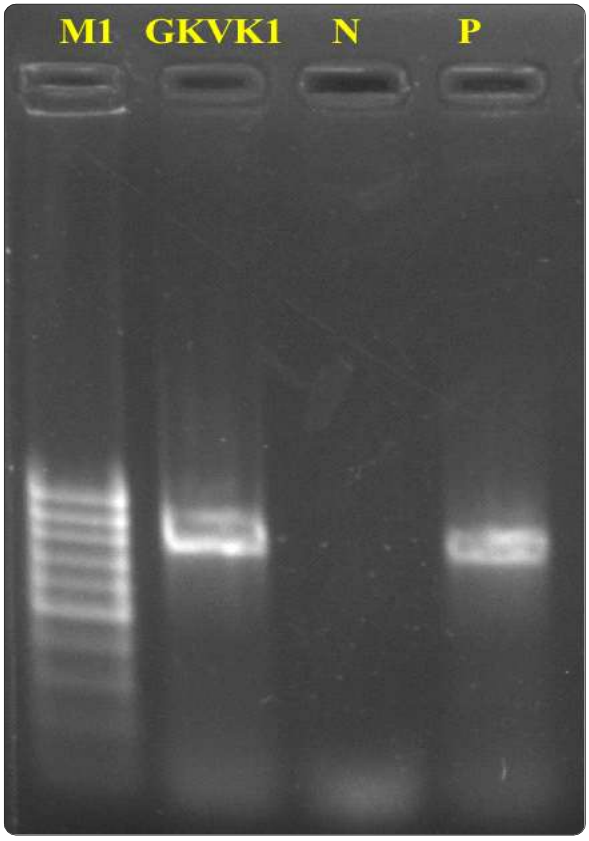

Fig. 2 : Agarose gel electrophoresis of PCR products of begomovirus isolate showing vein clearing symptoms with ToLCNDV specific primer

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

This suggests the presence of begomovirus infection in tomato plants but not associated with any satellite molecule. Later, PCR amplified product was sequenced and sequence analysis showed more than 97 per cent 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 uder accession number PQ435988. Other studies detected begomovirus infection in tomatoes in a similar manner (Macedo *et al.,* 2014 and Rakhonde *et al.,* 2024).

Sequence Analysis

The blast analysis of the conserved coat protein gene (PQ435988) indicated a sequence identity of more than 97 per cent with ToLCNDV (U15015). The Sequence Demarcation Tool (SDT v1.2) was used to determine pairwise sequence identity with other ToLCNDV and begomovirus isolates. The pairwise sequence identity of the AV1 gene of the GKVK1 isolate revealed the highest nucleotide identity (>91%) with ToLCNDV isolates (U15015, MT976080, MZ781422 and MH883329). The GKVK1 isolate also had 94.70 per cent nucleotide identity with the squash leaf curl China virus (KJ584150). Furthermore, the GKVK1 isolate and other begomoviruses that infect other crops have about 88 per cent of the same genetic makeup (Table 3 and Fig. 3). The phylogenetic analysis of the viruses' partial coat proteins revealed the closest link with ToLCNDV (Fig. 4). The data unambiguously revealed that GKVK1 isolates shared more than 97 per cent identity, which is much larger than the 91 per cent similarity recommended as a threshold for predicting viral strain and less than 90 per cent for identifying Begomovirus species (Brown *et al.,* 2015). As a result, the GKVK1 isolate under examination was identified as a ToLCNDV isolate and given the name ToLCNDV_GKVK due to significant sequence similarities and phylogenetic links between the incomplete DNA-A genome and ToLCNDV isolates. Based on nucleotide sequence analysis, the Pantnagar begomovirus isolate has a maximum identity of 98 per cent with the ToLCNDV (Rakhonde *et al.,* 2024). Similarly, the begomovirus isolate from Hyderabad shared a maximum similarity of 93 per cent with the ToLCNDV, according to nucleotide sequence analysis (Rajasri *et al.,* 2010) and 91-99 per cent with ToLCNDV from Chitrakoot, India (Agnihotri *et al.,* 2018).

Transmission Studies

The vector transmitted tomato plants expressed mild curling symptoms after 14 days of incubation period [Fig. 5(a)]. Out of 15 plants inoculated *via* vector, 12 plant samples amplified for ToLCNDV specific primer [Fig. 6(a)]. The vector transmission rates of GKVK1 isolate on tomato plant was generally high with 80.00 per cent rate of transmission (Table 4). This suggests that GKVK1 isolate in tomato efficiently transmitted by whiteflies. The sap transmitted tomato plants exhibited yellowing symptoms after 14 days of incubation period [Fig. 5(b)]. Out of 15 plants inoculated *via* sap, 7 plant samples amplified for

TABLE 3

Per cent nucleotide sequence identities of AV1 region of Tomato leaf curl New Delhi virus with other selected begomoviruses species sequences retrieved from NCBI GenBank

Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
NC004558	100																							
AF241479	74.20	100																						
MN095551	73.10	78.20	100																					
AF321929	60.50	62.40	99.50	100																				
AJ810353	74.20	80.20	92.10	98.90	100																			
KP164859	74.00	79.60	91.60	97.90	97.30	100																		
PP935253	73.10	79.90	90.00	98.40	95.80	95.80	100																	
KP178732	74.20	79.80	89.40	99.50	94.60	95.20	95.20	100																
MW760321	75.20	81.20	86.00	93.10	89.20	89.30	89.10	88.70	100															
NC004647	75.10	78.50	85.50	97.30	86.50	86.30	86.30	85.60	86.50	100														
DQ339117	76.80	80.80	81.70	62.00	85.60	85.70	85.90	84.60	83.50	82.70	100													
PQ106819	76.70	81.20	82.70	61.40	86.30	86.60	86.40	85.60	83.80	83.00	95.30	100												
EU910141	75.50	79.90	82.10	59.50	86.30	86.60	86.50	84.80	82.60	81.50	89.20	90.50	100											
AY705380	74.40	82.40	82.40	61.60	86.30	86.10	86.40	85.70	83.90	81.70	87.80	89.00	86.60	100										
JX436472	78.30	80.20	79.10	77.70	81.20	80.90	81.10	81.10	81.50	81.20	87.80	87.80	86.40	82.60	100									
JQ654460	76.90	81.60	79.00	78.70	81.70	82.20	81.70	81.80	82.50	82.40	87.70	87.70	86.00	82.90	90.10	100								
*PQ435988	75.00	78.00	80.10	59.50	77.60	77.00	77.30	76.60	77.50	79.60	85.00	82.40	79.80	80.30	80.70	80.50	100							
U15015	74.70	76.90	79.10	60.60	77.30	76.90	76.90	76.80	77.00	79.20	84.30	81.70	79.50	80.30	80.70	80.70	97.10	100						
MT976080	74.70	78.30	79.80	69.40	77.20	76.80	77.00	77.20	77.10	79.10	83.50	81.80	79.00	81.40	80.80	80.40	96.00	95.10	100					
MZ781422	72.40	75.50	83.40	58.30	77.90	77.90	78.40	78.10	78.90	77.70	82.40	80.00	77.40	80.50	79.80	77.70	94.50	95.00	96.70	100				
MH883329	75.00	77.80	79.60	61.60	77.50	77.20	77.40	77.10	77.60	79.30	84.10	81.80	79.80	80.90	81.00	81.00	96.40	96.60	97.50	97.60	100			
KJ584150	74.20	78.00	78.50	61.40	77.30	76.70	76.80	76.90	77.70	79.40	82.70	81.70	80.40	80.20	81.80	80.90	94.70	94.80	93.60	93.80	94.70	100		
AM884015	73.00	75.70	76.40	62.10	76.80	76.40	76.50	75.90	77.70	77.60	81.30	79.20	78.30	78.70	78.60	78.90	84.30	84.20	83.60	82.70	84.10	84.20	100	
AF481865	69.90	69.60	68.60	58.70	69.90	69.80	70.30	69.40	69.00	68.30	70.00	69.40	70.90	69.70	70.20	71.10	70.00	71.40	70.60	71.30	71.00	70.10	69.70	100

*GKVK1 isolate AV1gene sequence of present study

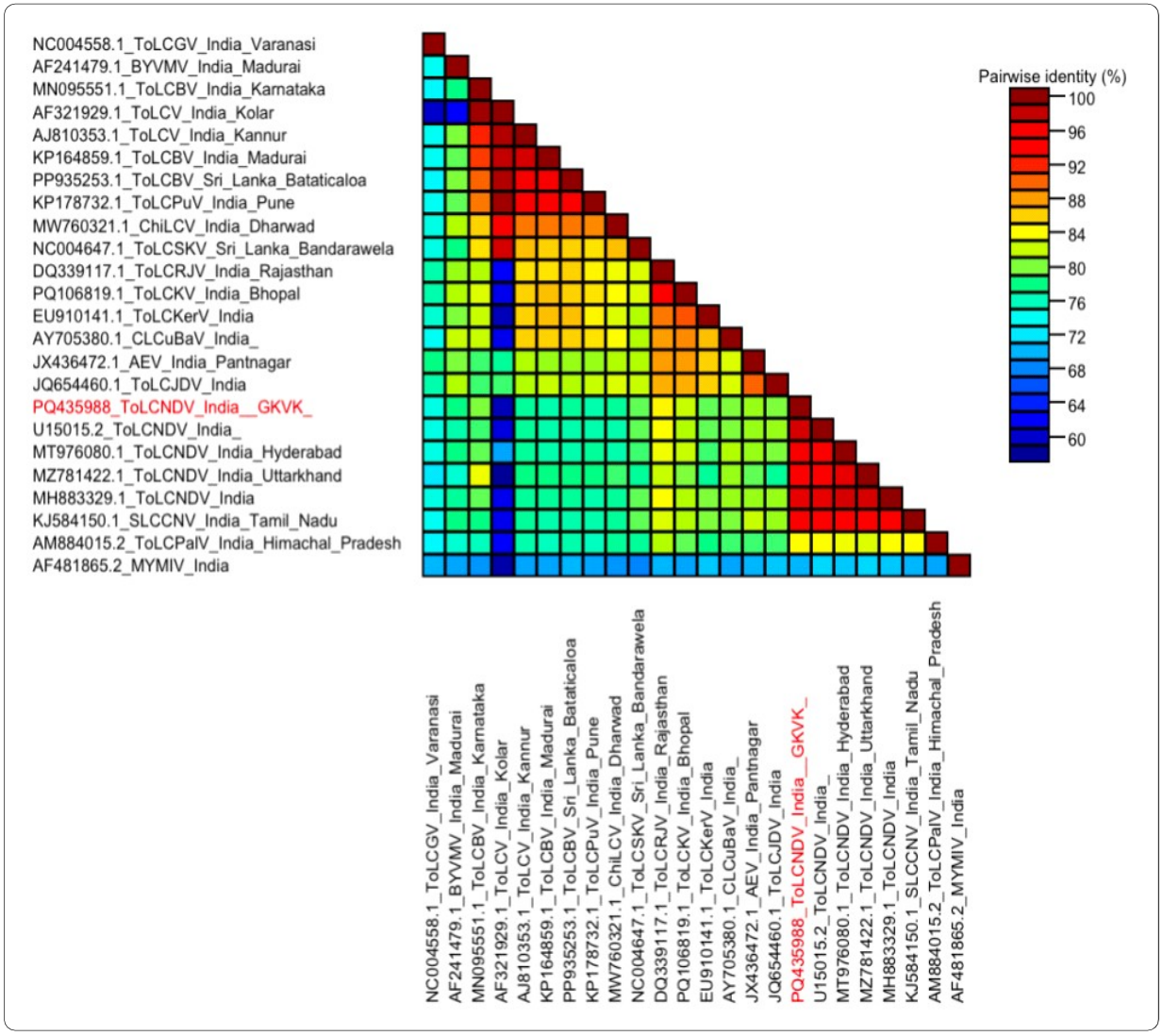

Fig. 3 : Two dimensional color-coded matrix of pairwise identity scores of the AV1 gene of Tomato leaf curl New Delhi virus in the present study with other selected begomovirus sequences using sequence demarcation tool (SDTv1.0)

ToLCNDV specific primer [Fig. 6(b)]. The sap transmission rate was relatively low with a transmission rate of 46.47 per cent (Table 4), suggesting that direct sap transmission is generally less effective in tomato plants compared to vector transmission. PCR analysis of tomato seeds from plants infected with the GKVK1 isolate showed no viral presence on the seed surface or within components such as the seed coat, endosperm and embryo. This finding indicates that the GKVK1 isolate of ToLCNDV is not seed-borne, either externally or internally.

Janssen *et al.* (2022) reported that feeding tomato plants with groups of 5, 20 and 50 viruliferous *B. tabaci* resulted in 15, 30 and 100 per cent of plants showing symptoms and testing positive for the virus, respectively. Similarly, Sakthivel *et al.* (2023) used twenty whiteflies to transmit ToLCNDV in bitter gourd, achieving a transmission efficiency of 93.33 per cent. In this study, twenty whiteflies successfully transmitted ToLCNDV in tomato with an 80.00 per cent transmission rate. Although the GKVK1 isolate's sap transmission rate was relatively low, it produced yellowing and vein clearing 15 days post-

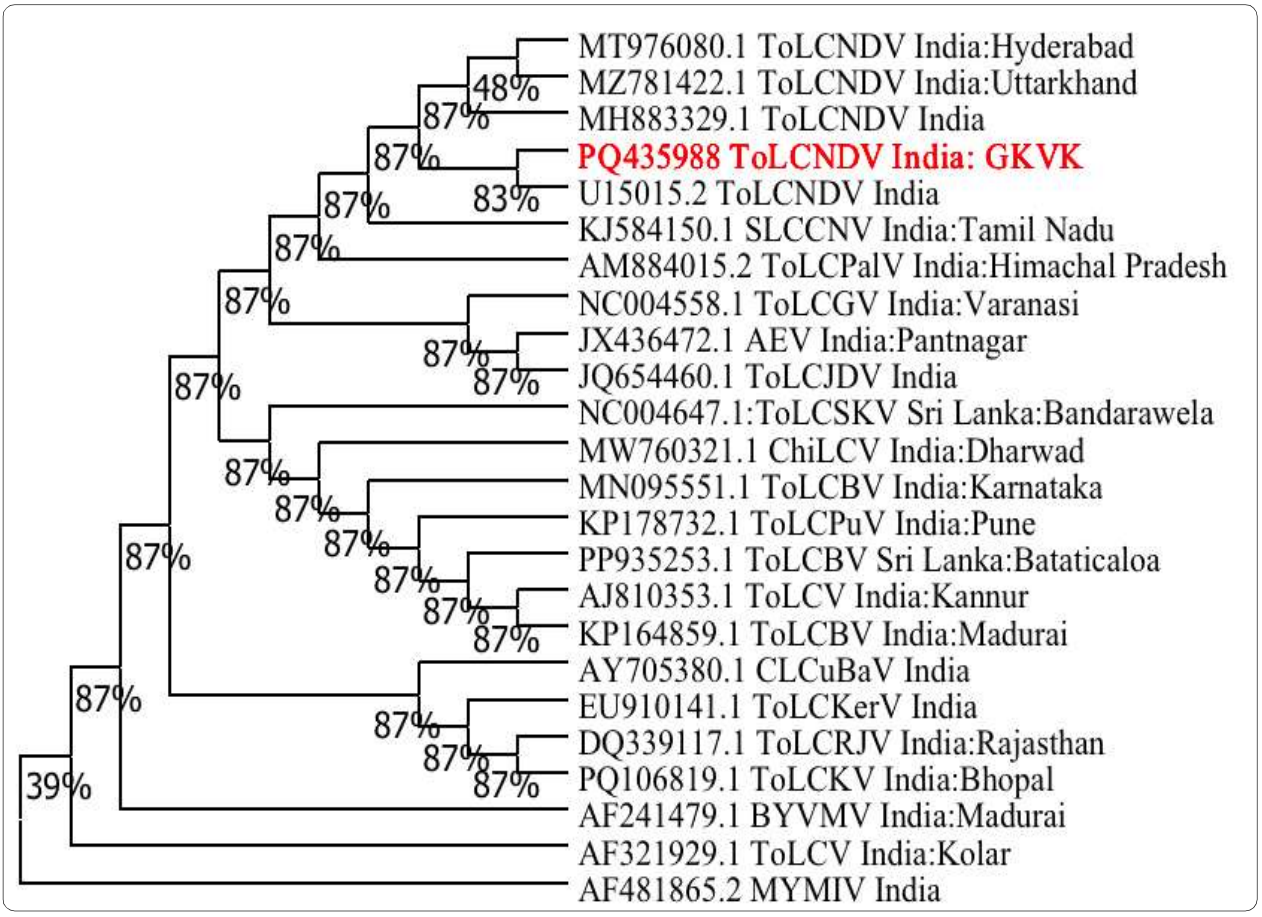

Fig. 4 : Phylogenetic tree of ToLCNDV isolate collected from tomato constructed by a neighbor-joining method using species specific primer

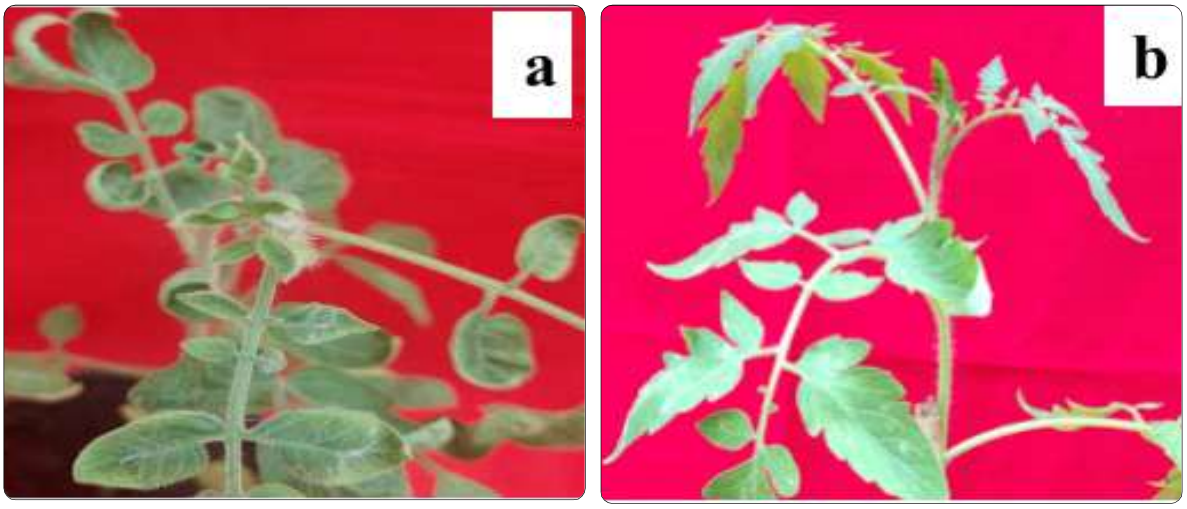

Fig. 5 : Leaf curl symptom expression in tomato plants inoculated with GKVK1 isolates through a) Whitefly transmission and b) Sap transmission

TABLE 4

Assessment of transmission efficiency of begomovirus isolate on tomato through vector, sap and seed transmission

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 (%)
Vector transmission	8/15	Mild curling	12/15	80.00
Sap transmission	3/15	Yellowing	7/15	46.67

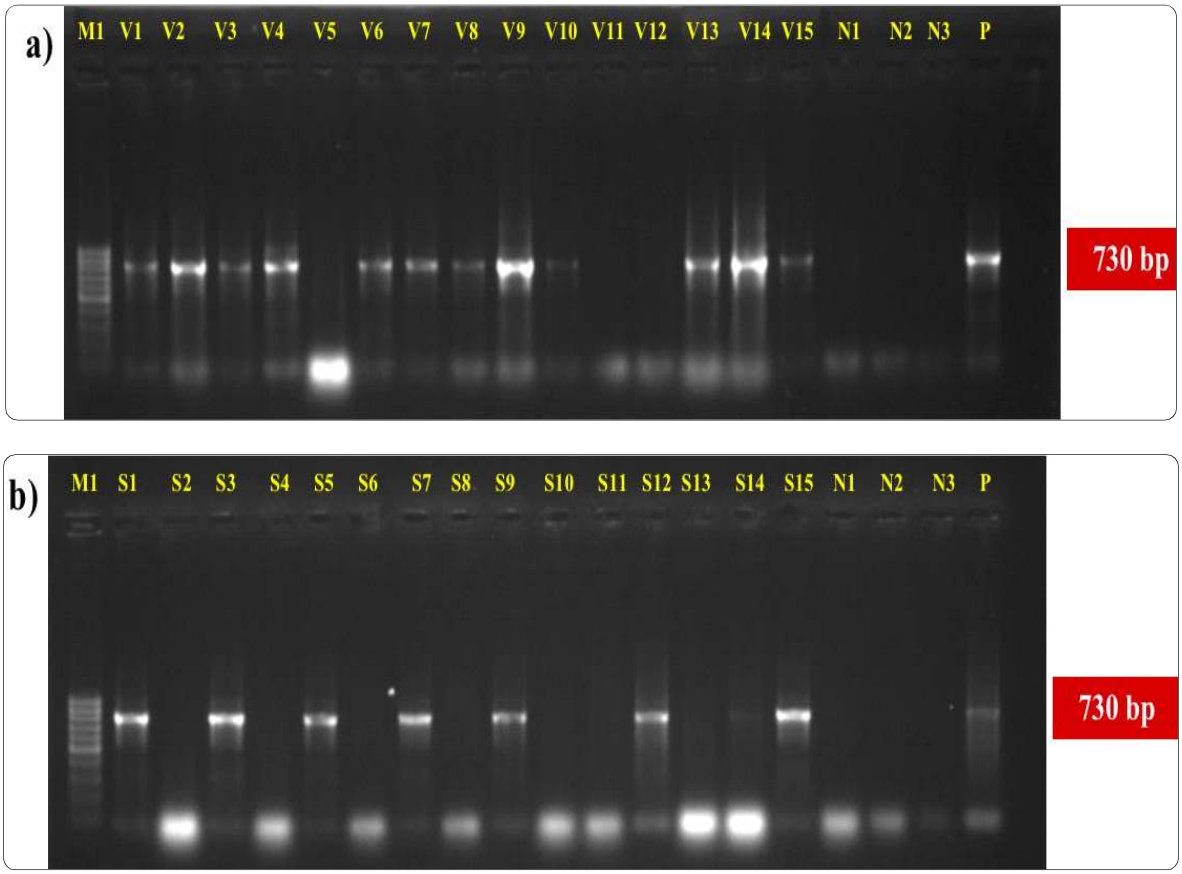

Fig. 6 : Agarose gel electrophoresis of PCR products from tomato plants inoculated with GKVK1 isolates *via* a) Whitefly transmission and b) Sap transmission

inoculation. Sap transmission of ToLCNDV in ridge and sponge gourds also resulted in symptoms and severe infection three weeks post-inoculation (Sohrab *et al.,* 2013 and Kaur *et al.,* 2020). Other ToLCV strains have been shown to transmit via sap in tomato (Chatchawan Kanphanich & Maxwell, 2002 and Chakraborty *et al.,* 2003). However, GKVK1 isolate did not transmit through seeds to the next generation. In a similar study, 180 seedlings from tomato yellow leaf curl Sardinia virus (TYLCSV)-infected plants showed no virus-related symptoms with no viral genomic material detected in their cotyledons or true leaves, confirming that TYLCSV is not seed-transmissible in tomato (Tabein *et al.,* 2021). Conversely, tomato yellow leaf curl virus (TYLCV) has been detected in the reproductive organs of tomato

and *N. benthamiana*, showing an association with seeds during maturation. Yet, after surface disinfecting tomato seeds, TYLCV DNA load decreased significantly, suggesting that the virus is primarily found externally as a contaminant on the seed coat (Perez-Padilla *et al.,* 2020).

Finding viable management strategies and reducing begomovirus-caused agricultural losses is made easier by the identification, molecular characterization and method of transmission of the virus. The ToLCNDV isolate that is producing interveinal chlorosis in tomatoes in Karnataka, India was identified by the current investigation. Future researchers could apply similar methods to identify common symptoms caused by different begomoviruses on different crops. In regions where the disease is more common, an integrated strategy that includes vector control and resistant cultivars must be devised in order to counter the danger.

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