Identification and Sequence Analysis of Tomato Leaf Curl New Delhi Virus

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Abstract [**Objectives**] This study was conducted to detect and analyze tomato leaf curl New Delhi virus (ToLCNDV). [**Methods**] Through PCR detection, sequence analysis, and pathogenicity verification, tomato leaf curl New Delhi virus (ToLCNDV) was identified in zucchini exhibiting systemic disease symptoms during a 2024 outbreak in Qingzhou City, Shandong Province, and was designated as ToLCNDV-SD. [**Results**] Specific primer amplification showed that all eight diseased samples produced bands of 504 bp (DNA-A) and 892 bp (DNA-B). Sequencing analysis revealed that ToLCNDV-SD DNA-A shared 96.10% homology with an Indonesian melon isolate (LC421834.1), while DNA-B showed 88.31% homology with a Malaysian bitter gourd isolate (MW248678.1). Phylogenetic analysis indicated its closest relationship with Southeast Asian cucurbit-infecting isolates. Friction transmission tests confirmed that the virus could spread mechanically, inducing typical symptoms 14 d after inoculation with positive PCR detection. [**Conclusions**] This study provides important insights for understanding the epidemic mechanisms and control strategies of ToLCNDV in China.

Key words Zucchini; Tomato leaf curl New Delhi virus (ToLCNDV); Identification; Sequence analysis

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Tomato leaf curl New Delhi virus (ToLCNDV) belongs to the genus *Begomovirus* in the family Geminiviridae and is a bipartite begomovirus. Upon infecting host plants, it induces severe symptoms such as leaf curling, swelling of veins in young leaves, shortened internodes, reduced fruit size, and rough fruit peel[1-3]. The virions are bipartite, measuring approximately 18 nm × 30 nm in size. The genome consists of two single-stranded circular DNA components: DNA-A (about 2 739 nt) encoding six functional proteins, and DNA-B (about 2 724 nt) encoding two functional proteins. Isolates from different regions or hosts may exhibit slight variations in genome size. The virus has two strains: ToLCNDV and ToLCNDV-Spain, sharing 91% - 94% nucleotide sequence similarity in their genomes[4]. It can be transmitted by whiteflies (*Bemisia tabaci*) in a recurrent and persistent way[5-6], as well as through mechanical means during agricultural activities. Additionally, it spreads via vegetative propagation materials (*e.g.*, seed tubers) and naturally through seeds and pollen. Long-distance transmission occurs through seed trade[7]. ToLCNDV causes devastating diseases in various crops, including Solanaceae and Cucurbitaceae. When infecting Cucurbitaceae plants, the virus leads to severe symptoms such as stunted growth, leaf crinkling, uneven leaf surface, reduced leaf size, yellowing, downward curling, and fruit malformation[6].

The virus was first identified in India in 1948 and has since spread to regions including the Middle East, Far East, North Africa, and parts of Europe, such as Pakistan, India, Taiwan region (China), Thailand, Indonesia, and Tunisia[8-9]. In 2019, ToLCNDV was listed as a quarantine pest by EPPO. ToLCNDV was first detected in Taiwan region, China, in 2010[10] and subsequently spread to mainland China in 2021[11]. The virus has since affected over 600 hm^2 of melon crops in regions including Ningbo (Zhejiang Province), Nantong (Jiangsu Province), and Shanghai, causing economic losses exceeding 100 million yuan[12].

In 2024, a large-scale viral disease outbreak occurred in greenhouse-cultivated zucchini in Qingzhou, Shandong Province. Infected plants exhibited typical systemic infection symptoms, including leaf curling, darkened leaf margins, reduced leaf area, swollen and thickened veins, pronounced wrinkling, yellowing, mosaic mottling, shortened internodes, and stunted growth, leading to significant yield reduction. PCR-based molecular testing ruled out common zucchini viruses such as cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), and cucumber green mottle mosaic virus (CGMMV) as potential pathogens. Given the high similarity between the observed symptoms and those caused by tomato leaf curl New Delhi virus (ToLCNDV), ten field-collected diseased samples were subjected to PCR amplification using specific primers. The products were analyzed by 1.0% agarose gel electrophoresis, followed by Sanger sequencing and BLAST alignment of positive samples to identify the causative pathogen.

Materials and Methods

Experimental materials

Plant materials: Ten young leaf samples were randomly collected from zucchini plants showing chlorosis, yellowing, wrinkling, and curling symptoms in greenhouse trials in Qingzhou, Shandong Province. Healthy young zucchini leaves were used as negative control.

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Reagents: DNA extraction kits were purchased from Tiangen Biotech (Beijing) Co., Ltd.

Primer synthesis and gene sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd.

Experimental mathods

Virus detection in field zucchini samples Total DNA was extracted from leaves using the CTAB method following the manufacturer's instructions of the Tiangen kit (TIANGEN DP350-02). Virus-specific primers ToLCNDV-A-504F/R[13] and ToLCNDV-B-892F/R[14] (Table 1), designed based on published literature, were used for PCR amplification. The PCR amplification system was 20 μl in volume, containing 10 × Easy Taq DNA Buffer (Mg^{2+}): 2 μl, High Pure dNTPs: 1.6 μl, Easy Taq DNA polymerase (5 U/μl): 0.2 μl, DNA: 1 μl, 10 μmol/L forward and reverse primers: 0.4 μl each, supplemented with double-distilled water to 20 μl. The PCR conditions were as follows: 95 ℃ for 3 min, 32 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 1 min, and 72 ℃ for 5 min. The PCR amplification products were detected by 1.0% agarose gel electrophoresis, followed by gel extraction and sequencing at Sangon Biotech (Shanghai) Co., Ltd.

Table 1 Primers used for amplification of ToLCNDV genes

The purpose	Name	Sequence	Fragmennt size//bp
Detection of ToLCNDV DNA-A	ToLCNDV-A-504F	GGGTTGTGAAGGCCCTTGTAAGGTGC	504
	ToLCNDV-A-504R	TGTACAGGCCATATACAACATTAATGC	
Detection of ToLCNDV DNA-B	ToLCNDV-B-892F	AATACACGCGTAAGGAAATATGT	892
	ToLCNDV-B-892R	AGTCATGGGCTAGCAGATCG	

Phylogenetic tree analysis The obtained ToLCNDV gene sequences were aligned and analyzed using BLAST and ClustalX software on the NCBI website. Cluster analysis and phylogenetic tree construction were performed using MEGA 11.0 software, and the Neighbor-Joining (NJ) method was adopted for phylogenetic tree construction. The bootstrap value was set to 1 000.

Pathogenicity assay Zucchini seeds were sown in sterilized growth medium (a volume ratio of nutrient soil to vermiculite at 1∶1) and cultivated in a controlled-environment growth chamber set at (26 ± 1) ℃, with a 16 h light/8 h dark photoperiod and a relative humidity within 60% – 70%. Virus inoculation was performed by the friction inoculation method when the seedlings reached the full-expanded cotyledon stage.

(1) Preparation of inoculum: Typical infected leaf tissues were mixed with 0.01 mol/L PBS buffer at a 1∶2 (*w/v*) ratio and thoroughly ground in a sterile mortar to obtain a crude viral extract.

(2) Inoculation treatment: Carborundum powder was evenly spread on the true leaves of zucchini as an abrasive. Wearing sterile gloves, the viral extract was gently rubbed onto the pretreated leaf surface using a finger. After 1 min of inoculation, the leaf surface was rinsed with distilled water, followed by 12 h of shaded moisture treatment to promote viral infection. The control group used an equal volume of PBS buffer instead of viral extract. Each treatment included three biological replicates, and a total of 30 seedlings were inoculated.

(3) Virus verification: Leaf samples were collected 15 – 20 d after inoculation for DNA extraction and PCR amplification detection.

Results and Analysis

Field symptom observation

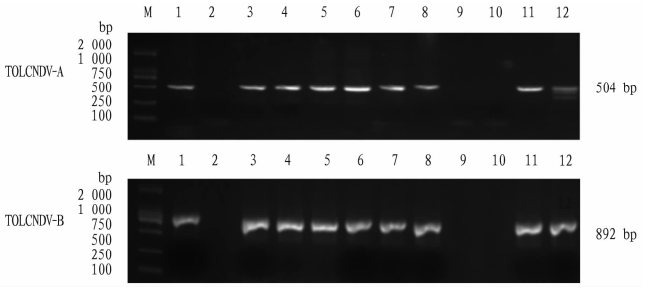
The infected zucchini plants in the field exhibited systemic viral infection symptoms. The leaves showed systemic chlorotic mottling, pronounced yellowing, and were wrinkled with obvious concave and convex deformity and downward curling phenotypes. Plant growth was inhibited, and manifested as shortened internodes and dwarfing. The fruit exhibited abnormal development, showing deformity, lignified hardening of the flesh, and flavor deterioration (Fig. 1). Disease surveys revealed a high population density of whiteflies in affected greenhouses, and infected plants displayed an aggregated distribution pattern, indicating typical insect-mediated virus spread characteristics.

Fig. 1 Suspected zucchini infected with ToLCNDV

PCR detection of zucchini suspected to be infected with ToLCNDV

To determine whether ToLCNDV was present in the zucchini leaves, PCR detection was performed. Based on relevant literature, the primer pairs TOLCNDV-A-504F/R and ToLCNDV-B-892F/R were synthesized and used to conduct PCR amplification on 10 zucchini leaf samples collected from fields in Qingzhou, Shandong Province, which exhibited symptoms suspected to be caused by ToLCNDV infection. The experimental results showed that the expected specific bands of 504 and 829 bp were successfully amplified from eight diseased leaf samples, but the corresponding amplification products were not detected in healthy

control plants (Fig. 2). Sequencing analysis obtained DNA fragment sequences of 485 bp (GenBank accession No.: PV102578.1) and 891 bp (GenBank accession No.: PV394099.1). Since the virus samples were collected from Shandong Province, the virus was designated as ToLCNDV-SD.



M: 2 000 bp marker; 1. Positive control: TOLCNDV-infected leaf 2; Negative control: water; 3 – 12. Zucchini disease samples.

Fig. 2 Zucchini sample PCR detection TOLCNDV electrophoresis chart

Phylogenetic analysis

ToLCNDV has been reported in multiple countries and regions, including India, Indonesia, Pakistan, and China. It exhibits an extremely broad host range, primarily infecting Cucurbitaceae and Solanaceae crops. Additionally, certain ornamental plants and weeds can also be infected.

To elucidate the evolutionary relationship between the ToLCNDV-SD isolate and other reported ToLCNDV isolates, representative sequences from the NCBI database were selected for multiple sequence alignment. The results demonstrated that the nucleotide sequence identity between the ToLCNDV-SD DNA-A isolate and previously reported ToLCNDV isolates ranged from 89.46% to 96.10%. Specifically, ToLCNDV-SD DNA-A showed the closest relationship with the Indonesian melon isolate ToLCNDV-A-ID-Mel-16 (GenBank accession No.: LC421834.1), sharing 96.10% sequence identity. The nucleotide sequence identity between ToLCNDV-SD DNA-B and reported ToLCNDV isolates ranged from 76.08% to 88.31%. In specific, the closest genetic relationship was observed with the Malaysian bitter gourd isolate ToLCNDV-17MY120 DNA-B (GenBank accession number: MW248678.1), and the sequence identity reached 88.31%. From the perspective of hosts, the ToLCNDV-SD isolate showed closer phylogenetic relationships with Cucurbitaceae hosts (*e. g.*, melon and bitter gourd) than with Solanaceae hosts (*e. g.*, tomato) and Acanthaceae hosts (*e. g.*, *Crossandra infundibuliformis*) (Fig. 3). These results suggest that the ToLCNDV-SD viral isolate may originate from cucurbit plants in Southeast Asia.

Pathogenicity verification of ToLCNDV

To confirm the pathogenicity of ToLCNDV on zucchini, a friction inoculation assay was conducted. The leaves of diseased zucchini plants with ToLCNDV were transferred to healthy zucchini plants. After 15 d of inoculation, all the zucchini plants showed obvious symptoms, the new leaves were obviously yellow and curly, and the plant growth was inhibited and dwarfed (Fig. 4). At 15 d after inoculation, all inoculated plants exhibited clear disease symptoms, including severe yellowing and curling of new leaves, and stunted plant growth (Fig. 4). To further confirm that the symptomatic plants were indeed infected by ToLCNDV, leaves from artificially inoculated zucchini plants showing disease symptoms were collected for DNA extraction. PCR amplification was then performed again using the primer pairs TOLCNDV-A-504F/R and ToLCNDV-B-892F/R. Electrophoresis results confirmed the presence of the virus in leaves of artificially inoculated zucchini plant (Fig. 5). These findings demonstrate that this ToLCNDV isolate can be mechanically transmitted and induce typical symptoms in zucchini, including leaf curling, yellowing, and plant dwarfing.

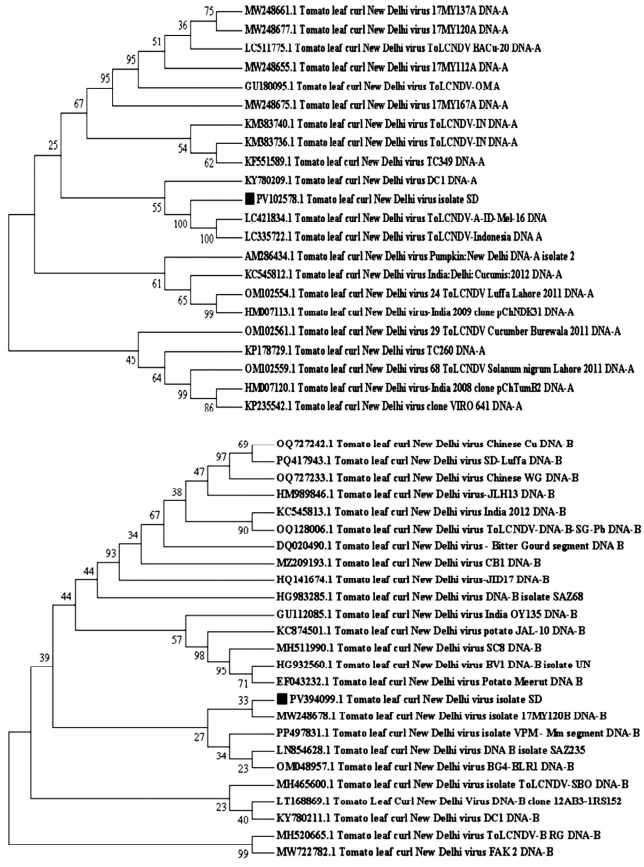
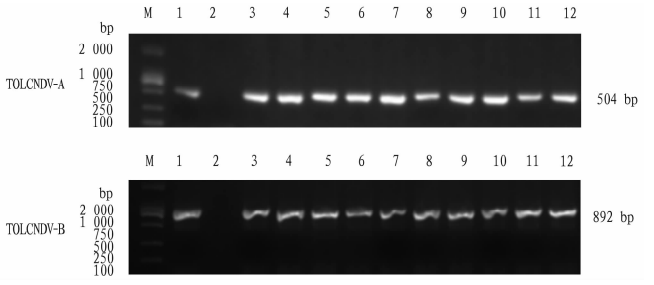


Fig. 3 Construction of the phylogenetic tree for ToLCNDV-SD and other isolates

Fig. 4 Health control and diseasaed zucchini plants at 14 d after artificial inoculation with ToLCNDV



M: 2 000 bp marker; 1. Positive control: TOLCNDV-infected leaf 2; Negative control: water; 3 – 12 Artificially inoculated zucchini leaves showing symptoms.

Fig. 5 PCR detection electrophoresis of artificially inoculated zucchini samples for TOLCNDV

Conclusions and Discussion

This study identified ToLCNDV in zucchini plants from Qingzhou City, Shandong Province, and designated it as ToLCNDV-SD. Through molecular detection, sequence analysis, and pathogenicity verification, the genetic characteristics, transmission route and pathogenic mechanism of this virus in zucchini were elucidated. These findings provide important insights for the monitoring and control of ToLCNDV in China.

(1) Molecular identification and phylogenetic analysis of ToLCNDV-SD: PCR detection revealed that specific bands of 504 bp (DNA-A) and 892 bp (DNA-B) were amplified from all eight suspected samples. Sequencing yielded 485 bp (PV102578.1) and 891 bp (PV394099.1) fragments. BLAST analysis showed that ToLCNDV-SD DNA-A shared 96.10% nucleotide identity with an Indonesian melon isolate (LC421834.1), while DNA-B exhibited 88.31% similarity with a Malaysian bitter gourd isolate (MW248678.1). Phylogenetic analysis further confirmed that ToLCNDV-SD was most closely related to cucurbit-infecting isolates (*e.g.*, melon and pumpkin) from Southeast Asia and India, but more distantly related to Solanaceae (tomato) and Acanthaceae (*Crossandra infundibuliformis*) isolates.

(2) Pathogenicity verification and transmission routes: Friction inoculation tests confirmed that ToLCNDV-SD could infect healthy zucchini plants through mechanical transmission, inducing typical symptoms (yellowing and curling of new leaves, plant dwarfing) within 14 d after inoculation. PCR detection confirmed stable viral presence in diseased plants, demonstrating strong pathogenicity and systemic infectivity. ToLCNDV primarily spreads via whitefly-mediated persistent transmission, mechanical transmission through farming operations (*e.g.*, pruning, grafting), and potentially seed transmission. This study confirmed the feasibility of mechanical transmission. Field investigations revealed clustered distribution of infected plants and high whitefly population density, further supporting the critical role of whiteflies in viral spread. Therefore, prevention strategies should simultaneously address vector control and standardized farming operations to reduce transmission risks.

(3) Potential hazards and prevention recommendations: ToLCNDV poses severe risks to Cucurbitaceae crops (*e.g.*, zucchini, melon, bitter gourd) and Solanaceae crops (*e.g.*, tomato, pepper), potentially causing leaf deformity, plant dwarfing, reduced fruit quality, or even complete crop failure. In recent years, the virus has spread rapidly in Asia, Europe, and other regions. In 2019, it was listed as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO). Since its first reported damage in Zhejiang, Jiangsu, and other areas of China in 2021, its distribution range may further expand, necessitating enhanced monitoring and early warning.

This study provides a theoretical basis for the early warning and integrated management of ToLCNDV. Given its mechanical transmission characteristics, standardized disinfection of farming tools and timely removal of infected plants should be prioritized. Additionally, vigilance against whitefly-mediated transmission risks is essential to control the spread of the virus. Furthermore, given the virus's high pathogenicity, rapid transmission and severe impact on fruit quality and yield, developing a host resistance-based integrated management system will be crucial to curb the spread of ToLCNDV. This can be achieved through systematic breeding of resistant germplasm resources or applying gene-editing technologies such as CRISPR/Cas9 to enhance crop resistance traits.

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