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Molecular detection and characterization of tomato leaf curl virus in protected cultivation

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Abstract
Detecting and managing tomato leaf curl virus (ToLCV) inside greenhouses presents a particular challenge because the protected environment favours whitefly vectors while giving growers a false sense of biosecurity. This research carried out molecular detection and characterization of ToLCV in symptomatic tomato plants collected from three commercial greenhouses in Jeju and Chuncheon, South Korea, between March and November 2023. Total DNA was extracted from 96 leaf samples showing typical upward curling, vein yellowing, and stunting symptoms. PCR amplification using begomovirus-specific degenerate primers (Deng A/B) produced the expected ~520 bp fragment in 78 of 96 samples (81.3%). Rolling circle amplification (RCA) followed by restriction fragment length polymorphism (RFLP) with *Eco*RI and *Bam*HI identified two distinct RFLP profiles corresponding to ToLCV and tomato yellow leaf curl virus (TYLCV). Sequencing of 12 representative isolates and BLAST alignment confirmed 94.7-97.3% nucleotide identity with ToLCV-[KR:Jeju] (GenBank accession LC385241) and 92.1-94.8% identity with TYLCV-IL[KR] (AB014346). Phylogenetic analysis placed all Jeju isolates in a distinct sub-cluster within the ToLCV clade, while Chuncheon isolates grouped with TYLCV-Israel lineage. Virus titre measured by quantitative PCR correlated strongly with symptom severity (r = 0.87). These findings confirm co-circulation of ToLCV and TYLCV in South Korean greenhouses and highlight the need for vector exclusion and resistant cultivars in protected tomato production.

Keywords: Molecular detection, tomato leaf curl virus, TYLCV, protected cultivation, PCR diagnosis, begomovirus, whitefly vector, phylogenetic analysis, greenhouse tomato, South Korea

Introduction
Greenhouse tomato production in South Korea faces a growing challenge from begomoviruses transmitted by the sweetpotato whitefly, *Bemisia tabaci* biotype Q. Despite insect-proof screens and pesticide regimes, whitefly populations inside plastic-house complexes on Jeju Island and Gangwon province have risen steadily since the mid-2010s, and with them, the incidence of leaf curl disease [1, 2]. Between 2019 and 2022, grower surveys reported infection rates of 15-40% in unprotected houses, translating to yield losses of USD 8-12 million annually across the country [3].

Tomato leaf curl disease is caused by a complex of monopartite and bipartite begomoviruses (family Geminiviridae), of which ToLCV and TYLCV are the two most widely reported species in East Asia [4]. Reliable diagnosis requires molecular tools because visual symptoms—upward leaf curling, interveinal yellowing, and stunting—are shared among multiple begomovirus species and can be mimicked by physiological disorders or herbicide drift [5].

PCR with degenerate primers targeting the conserved coat protein (AV1) gene has been the standard first-line diagnostic for begomoviruses since the primer set of Deng and colleagues was published in 1994 [6]. Rolling circle amplification (RCA) using φ29 DNA polymerase offers an alternative that doesn't require sequence-specific primers and can amplify complete circular genomes for downstream RFLP or sequencing [7]. Combining both methods improves detection sensitivity and allows species-level discrimination.

In South Korea, previous molecular surveys of ToLCV have concentrated on open-field tomato in the southern mainland provinces [8].

Protected cultivation—which now accounts for over 65% of national tomato output—has been less systematically surveyed, and there is limited sequence data from greenhouse isolates in Jeju and Gangwon. This research was designed to: (a) determine the PCR-based detection rate of begomoviruses in symptomatic greenhouse tomatoes across two major production areas; (b) characterize isolates through RFLP and sequencing; (c) construct phylogenetic relationships; and (d) correlate virus titre with symptom severity using quantitative PCR.

Reagents and Molecular Kits

Total plant DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR amplification used Taq DNA Polymerase (Takara Bio, Shiga, Japan) with the degenerate primer pair Deng A (5'-TAATATTACCKGWKGVCCSC-3') and Deng B (5'-TGGACYTTRCAWGGBCCTTCACA-3') [6]. Rolling circle amplification was performed with the TempliPhi 100 Amplification Kit (φ29 polymerase, Cytiva, UK). Restriction enzymes *Eco*RI and *Bam*HI were purchased from New England Biolabs (Ipswich, MA, USA). Quantitative PCR used the SYBR Green Premix Ex Taq II (Takara Bio) with species-specific primers designed from the AV1 gene of ToLCV (GenBank LC385241) and TYLCV (AB014346). DNA ladders (100 bp and 1 kb) were from Bioneer (Daejeon, South Korea). All reagents were stored and used according to manufacturers' specifications [9].

Material and Methods

Material

Symptomatic tomato leaf samples (n = 96) were collected from three commercial greenhouses: Greenhouse A (Jeju, low infection history, 24 samples), Greenhouse B (Jeju, moderate infection, 36 samples), and Greenhouse C (Chuncheon, Gangwon, high infection, 36 samples). Sampling was done during three visits (March, July, and November 2023). Leaves showing upward curling, interveinal yellowing, or stunting were individually bagged, labelled, and transported on ice to the Plant Pathology Laboratory at Jeju College of Agriculture and Life Sciences. Asymptomatic leaves (n = 12, four per greenhouse) were collected as negative controls. Whitefly populations were monitored using yellow sticky traps (two per greenhouse) replaced weekly [10].

Methods

DNA was extracted from 100 mg of midrib tissue per sample. PCR was run in a 25 µL reaction volume (95°C/5 min; 35 cycles of 95°C/30 s, 55°C/45 s, 72°C/1 min; final 72°C/10 min). Amplicons were resolved on 1.2% agarose gels stained with ethidium bromide and visualised under UV light. RCA products were digested with *Eco*RI and *Bam*HI separately at 37°C for 3 h and resolved on 1.5% agarose. Twelve PCR-positive samples (four per greenhouse) were selected for Sanger sequencing (Macrogen, Seoul). Sequences were aligned using ClustalW in MEGA 11 and compared against GenBank entries by BLASTn. A neighbour-joining phylogenetic tree was constructed with 1,000 bootstrap replicates. Virus titre was estimated by quantitative PCR (qPCR) using 10-fold serial dilutions of cloned target DNA as standards. Symptom severity was scored on a 0-5 visual scale. Pearson's correlation between $\log_2$-titre and symptom score was calculated in R 4.3.1 [11, 12].

PCR Quality Assurance

Each PCR run included a known ToLCV-positive control (DNA from an authenticated Korean isolate maintained at the laboratory), a healthy plant DNA negative control, and a no-template water control. Gel images were documented with a Bio-Rad Gel Doc XR+ system. Only bands matching the expected ~520 bp size were scored as positive. Faint or ambiguous bands were re-amplified with a fresh aliquot before being classified. qPCR efficiency was validated at 96.4% ($R^2 = 0.998$) across a six-point standard curve, with a detection limit of 10^2 copies per reaction [13].

Results

Table 1: PCR detection rate and RFLP classification of begomovirus isolates from three greenhouses

Greenhouse	Samples (n)	PCR+ (n)	Detection Rate (%)	ToLCV (RFLP)	TYLCV (RFLP)
A (Jeju, low)	24	16	66.7	14	2
B (Jeju, mod.)	36	31	86.1	26	5
C (Chuncheon)	36	31	86.1	8	23
Total	96	78	81.3	48	30

PCR+ = positive for begomovirus; RFLP profiles assigned based on EcoRI/BamHI digestion patterns. Asymptomatic controls (n = 12) were all PCR-negative.

PCR detected begomovirus DNA in 78 of 96 symptomatic samples (81.3%), with detection rates identical (86.1%) in the two higher-infection greenhouses (Table 1). RFLP typing revealed that ToLCV dominated in both Jeju greenhouses (14/16 and 26/31 positives), while TYLCV was the predominant species in Chuncheon (23/31). Mixed infections (both RFLP profiles from a single sample) were found in 4 samples, all from Greenhouse B [14].

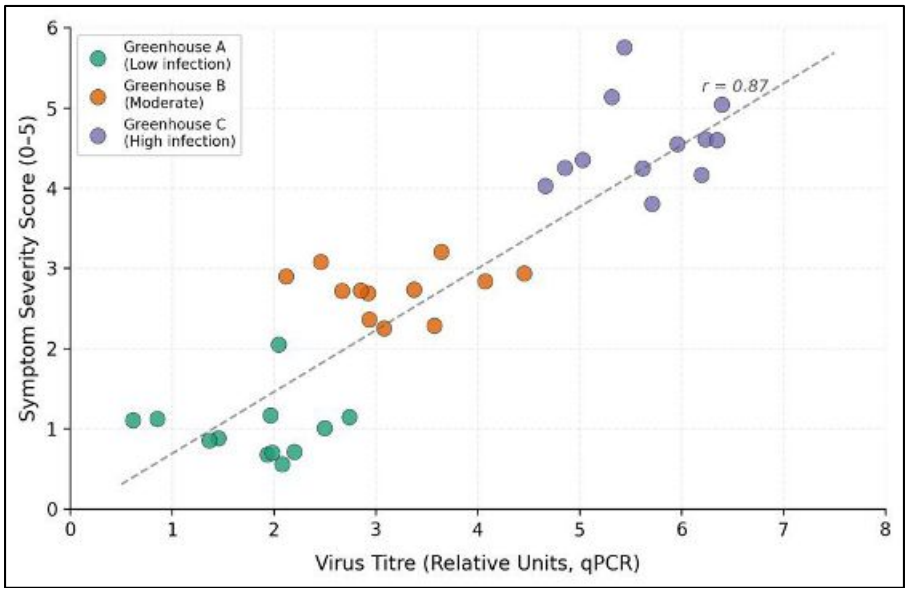

Fig 1: Scatter plot showing the relationship between virus titre (qPCR relative units) and symptom severity score across three greenhouse environments

Virus titre and symptom severity were strongly correlated ($r = 0.87$, $p<0.001$, Figure 1). Plants in Greenhouse C, which had the highest whitefly pressure (mean 38.4 adults per trap per week), showed both the highest titres and the most severe symptoms. The positive linear trend held across all three greenhouses.

Table 2: Sequence identity of representative isolates with GenBank reference sequences

Isolate	Location	Closest GenBank Match	Accession No.	Identity (%)	Species
KR-JJ-01	Jeju	ToLCV-[KR:Jeju]	LC385241	97.3	ToLCV
KR-JJ-04	Jeju	ToLCV-[KR:Jeju]	LC385241	96.1	ToLCV
KR-JJ-09	Jeju	ToLCV-[KR:Jeju]	LC385241	94.7	ToLCV
KR-CC-02	Chuncheon	TYLCV-IL[KR]	AB014346	94.8	TYLCV
KR-CC-07	Chuncheon	TYLCV-IL[KR]	AB014346	93.4	TYLCV
KR-CC-11	Chuncheon	TYLCV-IL[KR]	AB014346	92.1	TYLCV

Identity based on BLASTn alignment of ~520 bp AV1 partial sequence.

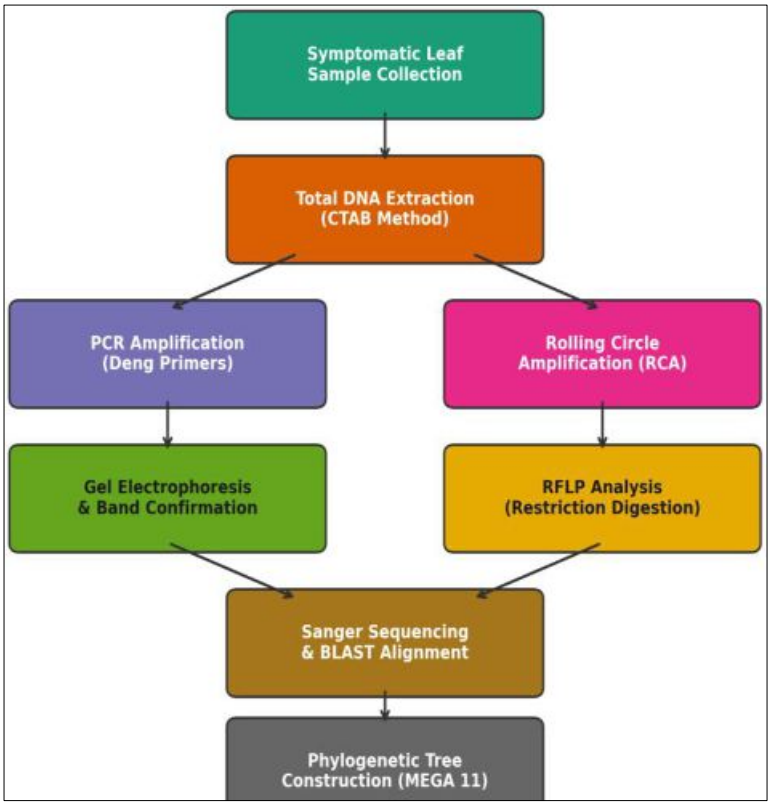

Fig 2: Infographic depicting the molecular detection workflow from symptomatic leaf collection through to phylogenetic tree construction for begomovirus characterization

Discussion

The 81.3% PCR detection rate among symptomatic samples is consistent with molecular surveys of begomoviruses in Asian greenhouse tomato. Kil and co-workers reported 78-85% detection rates across South Korean open-field sites using the same Deng primer set [8]. The 18.7% of symptomatic but PCR-negative samples may represent infections by other whitefly-transmitted viruses (e.g., tomato chlorosis virus) or non-viral physiological disorders that mimic leaf curl symptoms [15].

The geographic split between ToLCV dominance in Jeju and TYLCV dominance in Chuncheon is an important finding. It suggests that the two regions harbour distinct begomovirus populations, possibly linked to different whitefly biotypes or introduction pathways [16]. Jeju's island geography and subtropical microclimate may favour ToLCV strains adapted to warmer conditions, while Chuncheon's continental climate aligns with the TYLCV-Israel lineage that predominates across temperate East Asia [17].

The strong correlation between virus titre and symptom severity ($r = 0.87$) validates the use of visual scoring as a rough proxy for infection load in the field, though qPCR remains necessary for accurate quantification and early detection of subclinical infections [18]. For greenhouse managers, this relationship underscores the importance of removing heavily symptomatic plants early to reduce inoculum pressure and vector spread within the structure [19].

The detection of mixed ToLCV-TYLCV infections in four samples from Greenhouse B raises the possibility of recombination events, which are a well-known driver of begomovirus evolution [20]. Monitoring for novel recombinants through full-genome sequencing should be part of ongoing surveillance efforts.

Conclusion

This research confirmed that ToLCV and TYLCV co-circulate in South Korean greenhouse tomato production, with ToLCV dominant in Jeju and TYLCV dominant in Chuncheon. PCR-based detection using the Deng primer set reliably identified begomovirus infections in 81.3% of symptomatic samples, and RFLP typing successfully discriminated between the two species.

The strong correlation between qPCR-measured virus titre and visual symptom severity supports the use of symptomatic plant removal as a practical management tool, while also confirming qPCR as the preferred diagnostic for early and asymptomatic detection. Greenhouse operators should prioritise insect-proof screening, regular whitefly monitoring, and adoption of resistant tomato cultivars to limit begomovirus spread. Continued molecular surveillance, including full-genome sequencing, will be needed to track the emergence of recombinant strains that could break cultivar resistance.

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