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Molecular Characterization of *Tomato Yellow Leaf Curl Virus* in Korea and the Construction of an Infectious Clone

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Several tomato production regions in Korea were surveyed for tomato yellow leaf curl disease (TYLCD). Tomato leaf samples showing TYLCD-like symptoms were collected from Tongyeong (To), Geoje (Gi), and Gimhae (Gh) cities of the southern part of Korea. *Tomato yellow leaf curl virus* (TYLCV) was detected and the full-length genomes of the isolates were sequenced. The TYLCV isolates found in Korea shared high sequence identity (> 99%) with TYLCV-IL [JR:Omu:Ng] (AB110217). Phylogenetic relationship analysis revealed that they formed two groups (with little genetic variability), and the To, Gj, and Gh isolates belonged to the TYLCV-IL group. An infectious clone of TYLCV-To (JQ013089) was constructed and agroinoculated into *Nicotiana benthamiana*, *Nicotiana tabacum* var. Xanthi, *Petunia hybrida*, *Capsicum annuum*, and *Lycopersicon esculentum* cv. Hausumomotaro. Agroinfection with a dimeric infectious clone of TYLCV-To induced severe leaf curling and stunting symptoms in these plants, excluding *C. annuum*. Tomato plants then developed typical yellow leaf curl symptoms.

Keywords : Agroinfection, Infectious clone, Sequence, *Tomato yellow leaf curl virus*

Tomato yellow leaf curl disease (TYLCD) caused by *Tomato yellow leaf curl virus* (TYLCV; genus *Begomovirus*, family *Geminiviridae*) is one of the most devastating viral diseases affecting tomato plants worldwide. Infected tomato plants possess leaves exhibiting chlorotic and curled-up margins, with stunted plants and abscised flowers. TYLCV possesses a monopartite single-stranded DNA (ssDNA) genome component (DNA-A) encapsulated in geminated particles, which is transmitted by the whitefly *Bemisia tabaci* (Genn.). TYLCV was first identified in Israel, and the two viral genomic DNA populations represented a mixture of two TYLCV strains [TYLCV-Israel (TYLCV-IL) and TYLCV-Mild (TYLCV-Mld)], which differ in symptom severity. In Korea, TYLCD first occurred in 2008 in southeastern Tongyong City. It was later detected in several cities in the southeastern areas. Since then, TYLCD outbreaks have occurred in many regions of Korea and caused severe damage to tomato production. Therefore, in this report, we describe the molecular characterization of TYLCV isolates in Korea and constructed an infectious clone of the disease.

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We determined the complete nucleotide sequence of the novel TYLCV isolate (TYLCV-To, -Gj, -Gh) from Korea. The sequences were compared with TYLCV isolates already reported from Korea and Japan. In addition, we constructed an infectious clone derived from TYLCV-To to confirm that the monopartite genome induced typical TYLC disease symptoms on host plants after inoculation.

In 2008, leaf samples of naturally infected tomato plants showing yellow leaf curl and stunt symptoms were collected from tomato greenhouses (Fig. 1). Infected plants were collected in three regions (Tongyeong, Geoje, and Gimhae in the southeastern part of Korea) where tomatoes are commonly grown in Korea. Total plant DNA was extracted from leaves of naturally infected symptomatic and healthy tomato plants using a DNA extraction kit (Qiagen, Germany). The specific primers TYLNg1/TYLNg2 (Ueda *et al.*, 2004) were used to amplify the complete viral DNA genome. Detection and identification were performed by PCR from infected plants. DNA of the expected sizes (2.8 kb) from TYLCV could be amplified from symptomatic tomato plants using the primers TYLNg1/TYLNg2. Fig. 2 shows that expected sizes could be amplified from plants infected with TYLCV.

Fig. 1. Symptoms of *Tomato yellow leaf curl virus* of infected tomato in greenhouses. Infected tomato plants collected from three different areas (Tongyeong, Gimhae, Geoje) of Korea.

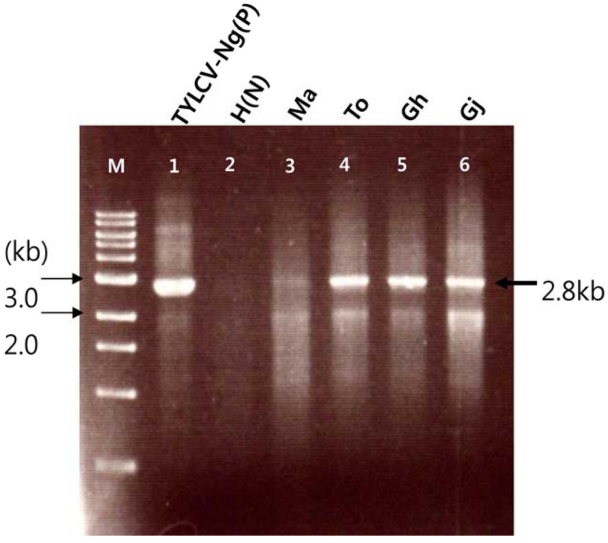

Fig. 2. Agarose gel (1%) electrophoresis of PCR amplified products of the full-length genome. Lanes 1–6 represent TYLCV-Ng (Nagasaki), H (Healthy), Sa (Sacheon), To (Tongyeong) , Gh (Gimhae), and Gj (Geoje) collected from different locations, respectively. Lane M represents the 1-kb DNA Ladder. TYLCV-Ng(P) is the positive control and H(N) is negative control .

Table 1. TYLCV isolates used for phylogenetic analyses

Isolate	Abbreviation	Accession No.
Tomato yellow leaf curl virus		
Boseong, Korea	Bos	GU325634
Geoje, Korea	Gj	JQ013091
Tongyeong, Korea	To	JQ013089
Busan, Korea	Bus	GQ141873
Gimhae, Korea	Gh	JQ013090
Jejudo, Korea	Jeju	GU325633
Nons, Korea	Nons	GU325632
Misumi, Japan	Mis	AB116631
Miyazaki, Japan	Miy	AB116629
Nagasaki, Japan	Ng	AB110217
Mild[Aic], Japan	Mld[Aic]	AB014347
Mild[Kisozaki], Japan	Mld[Kis]	AB116634
Mild[Daito], Japan	Mld[SzD]	AB116635
Tosa, Japan	Tos	AB192965
Mild[Sz], Japan	Mld[Sz]	AB110218
Mild [Shizuoka], Japan	Mld[Shi]	AB014346
Mild[Portugal], Portugal	Mld[PT]	AF105975
Nobaria, Egypt	Nob	EF107520
Mild[Spain]	Mld[Spain]	AJ519441
Turkey	Mersin 1	AJ812277
Mexico	Guasave	FJ609655
Iran	IR	AJ132711
Jordan	Jordanian	EF054894
Tomato yellow leaf curl Malaga virus, Spain	TYLCMalV	AF271234

Amplified PCR products (~2.8 kb) were used to determine viral genome sequences directly using the ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, USA). Sequence data were assembled and analyzed with Genious software (www.geneious.com) and the BLAST program. Reported TYLCV sequences from Korea (which were used for comparison) included TYLCV-Bus (GQ141873), TYLCV-Bos (GU325634), TYLCV-Jeju (GU325633), and TYLCV-Nons (GU325632) isolates from naturally infected tomato plants. Sequencing alignments were generated using Clustal W or X (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1994). Their phylogenetic relationships were determined using the neighbor-joining (NJ) and maximum-parsimony (MP) algorithms with Genious software. TYLCV isolates for comparison and phylogenetic analyses are shown in Table 1. DNA-A complete nucleotide sequences of three TYLCV isolates obtained from tomato were 2,774 bp in length. The database accession numbers of the determined sequences were as follows: Tongyong (TYLCV-To: JQ013089), Gejae (TYLCV-

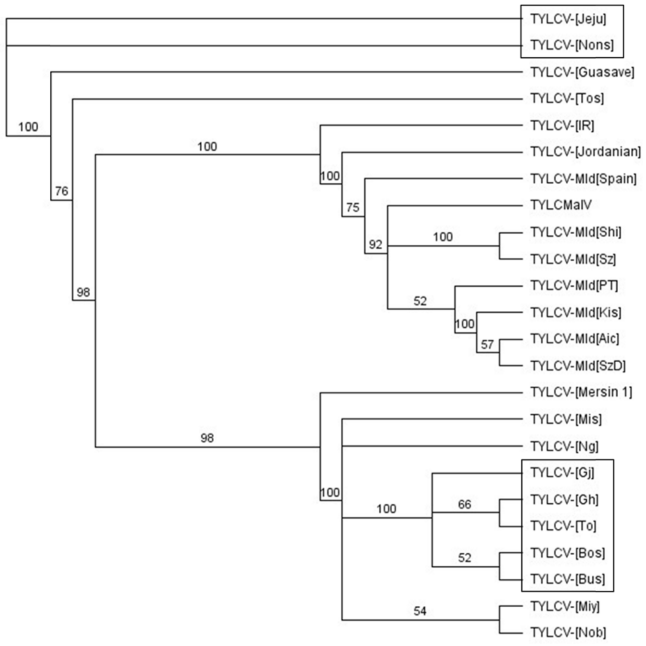

Fig. 3. Phylogenetic tree of complete nucleotide sequences of 27 isolates of TYLCV and TYLCMalV. TYLCV-To, -Gh, -Gj with reported begomoviruses listed in Table 1. Isolates reported from Korea are boxed. The tree was generated using the neighbor-joining method in Genious. The numbers at each branch represent the percentage of 1,000 bootstraps.

Gj: JQ013090), and Gimhae (TYLCV-Gh: JQ013091). Sequence homology indicated that these isolates were more related to TYLCV-IL [JR:Omu:Ng]. Their sequences had 99% identity with each other, and 99% identity when compared with TYLCV-IL [JR:Omu:Ng]. They also had 96–97% identity when compared with TYLCV-Jeju and -Nons, which had been sequenced from Korea. Comparison of the reported TYLCV sequences from Korea and Japan showed that they could be separated into two groups (Fig. 3). One group, which included TYLCV-Gj, Bos, Bus, Gh (from Korea), Ng, and Mis isolates (from Japan), had sequences 2,774 bp in length. The other group contained TYLCV-Jeju, Nons (from Korea) and Tos isolates (from Japan), which were 2,781 bp in length. TYLCV-To was more closely related to TYLCV-Almeria than TYLCV-Ng, Sz, as reported in Japan (Ueda, 2005). Group I and group II also included some isolates of TYLCV-IR, -Mersin 1, -PT, -Kis, and TYLCMaV.

Based on the sequencing data obtained in this study, infectious clones were constructed from TYLCV-To. Total DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Germany). Virus genomes were amplified using the Phi29 DNA polymerase and the rolling-circle amplification strategy (TempliPhi™, GE Healthcare, USA) (Inoue-Nagata *et al.*, 2004). After partial digestion with *Eco*RI, the full-length dimer genome (~5.6 kb in length) was ligated into the binary vector Pcambia 0380. *Agrobacterium tumefaciens* strain LBA4404 was transformed with

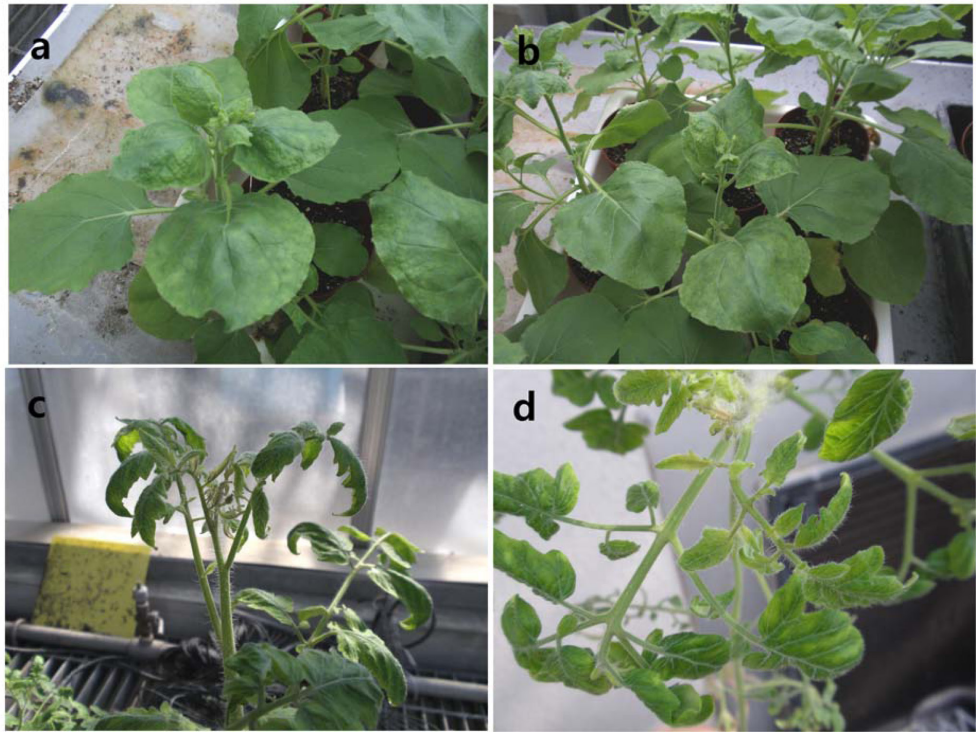

Fig. 4. Symptoms of plant inoculated with *Agrobacterium tumefaciens* strain LBA4404, which was transformed with the TYLCV-To dimer clone (pCTYTo). **a**, **b**: *Nicotiana benthamiana* at 20 days after agroinoculation **c**, **d**: Tomato (*Lycopersicon esculentum* cv. Hausumomotaro) at 30 days after agroinoculation.

plasmid TYLCV-To infectious clone (pCTYTo) by electroporation. *A. tumefaciens* culture was grown in YEP medium at 28°C, after which the bacterial culture was injected into petioles of test plants (*Lycopersicon esculentum* cv. Hausumomotaro, *Nicotiana benthamiana*, *Nicotiana tabacum* Xanthi, *Petunia hybrida*, *Capsicum annuum*) at the four- to six-leaf stage after addition of 3,5′-dimethoxy-4′-hydroxyacetophenone (10 μg/ml) with a 1-ml syringe. Plants were kept at 26°C with 16 hour/day light in an insect-free greenhouse, and symptom development was examined daily. The TYLCV dimer clone of pCTYTo was shown to be infectious in all inoculated *N. benthamiana*, *N. tabacum* Xanthi, *L. esculentum*, and *P. hybrida* plants. In *N. benthamiana*, inoculated plants displayed downward leaf curling, as well as stunting symptoms at 21 days postinoculation (dpi) (Fig. 4). In *L. esculentum*, typical downward yellow leaf curl was observed at 30 dpi, which was indistinguishable from that associated with natural infection (Fig. 4). Approximately 2.8-kb PCR products were successfully amplified when total DNA was extracted from the systemically symptomatic leaves of *L. esculentum*, *N. benthamiana*, *N. tabacum* Xanthi, and *P. hybrida* plants (data not shown).

In 2008, an outbreak of TYLCD occurred in the southeastern part of Korea. Thereafter, TYLCD spread to the middle part of Korea in 2009 and 2010. TYLCV is known to have been accidentally introduced during the mid-1990s to the Caribbean islands and to the United States (Nakhla *et al*., 1994; Polston and Anderson, 1997).

In Japan, the first evidence of economic damage to tomato caused by TYLCV was recorded in 1996 in Shizuoka and Aichi prefectures in central Japan and Nagasaki Prefecture in southwest Japan (Kato *et al*., 1998). Since then, TYLCV has been isolated from many prefectures in Tokai district and Kyushu (Haga and Doi, 2002). TYLCV may have been recently introduced to Korea from overseas, although it likely arrived through the international plant trade in certain plant hosts or with viruliferous *B. tabaci*. The Korean isolates closely related to TYLCV-IL have spread worldwide. The complete DNA-A sequences of three field isolates of TYLCV in Korea were determined in this study. All three had high sequence identity with previously reported TYLCV-IL [JR:Omu:Ng]. However, previously a reported Korean isolate (TYLCV-Jeju) was very similar to TYLCV-To. Until now, TYLCV-Mld had not been identified in Korea. TYLCV-To, which was used to construct the infectious clone of pCTYTo, belonged to the group Ng. Inoculation of *N. benthamiana* and tomato plants with pCTYTo resulted in systemic infection of these plants. *N. benthamiana* plants developed severe leaf curling. Symptoms on tomato plants inoculated with clone pCTYTo were similar to those of naturally infected tomato plants, and also resembled those reported in Shizuoka isolates (Thompson *et al*., 1994).

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