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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Development of a *Pepper yellow leaf curl Thailand virus* Infectious Clone and Identification of a Pathogenicity Factor of *Tomato yellow leaf curl Kanchanaburi virus* in Tomato

고추황화잎말림 Thailand 바이러스의 감염클론 개발 및 토마토황화잎말림 Kanchanaburi 바이러스의 병원성 인자 발견

AUGUST, 2019

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THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Development of a *Pepper yellow leaf curl Thailand virus* Infectious Clone and Identification of a Pathogenicity Factor of *Tomato yellow leaf curl Kanchanaburi virus* in Tomato

UNDER THE DIRECTION OF DR. BYOUNG-CHEORL KANG
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ABSRACT

Yellow leaf curl symptoms caused by *Pepper yellow leaf curl Thailand virus* (PYLCThV) and *Tomato yellow leaf curl Kanchanaburi virus* (TYLCKaV) are belonging to the begomovirus group in the geminiviridae family. begomoviruses, circular single-stranded DNA viruses cause severe crop losses in tropical and subtropical countries, particularly in tomato and pepper. In this study, PYLCThV were isolated from the yellow leaf curl symptomatic leaves of pepper. The viral genome sequence was confirmed and an infectious PYLCThV clone was developed. Both DNA-A and DNA-B of PYLCThV were inserted into a binary vector pICH86988. Agrobacteria containing each clone were infiltrated in to *N.*

benthamiana, tomato, and pepper to test infectivity of the infectious PYLCThV clone. *N.benthamiana* showed high infectivity to the infectious clone, whereas, no viral symptoms and no virus accumulation were observed in tomato and pepper. This result allowed us to conduct a genome swapping analysis by using TYLCKaV infectious clone that have strong infectivity in tomato. The genome swapping analysis revealed that the B genome of TYLCKaV was responsible for pathogenicity in tomato. Moreover, we mapped using a series of chimeric DNA-B genome. Among the chimeric clones, a chimeric clone containing PYLCThV-B with the TYLCKAV-B backbone showed infectivity in tomato. This result demonstrated that the TYLCKaV UTR region plays a role as a pathogenicity determinant in tomato. This study provides molecular basis of understanding TYLCKaV infectivity and the interaction between begomovirus and plants.

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LIST OF ABBREVIATIONS

AGO	ARGONAUTE protein
Avr	Avirulence
BLAST	Basic local alignment search tool
CP	Coat protein
DCLs	Dicer-like proteins
DPI	Days post inoculation
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
eIF4E	Eukaryotic translation initiation factor 4E
ER	Extreme resistance
GA	Gibson assembly
HR	Hypersensitive response
ICTV	International Committee of Taxonomy of Viruses
IR	Intergenic region
LRR	Leucine rich repeat
MP(BC1)	Movement protein
NB	Nucleotide binding

NCBI	National center for biotechnology information
NSs	Non-stuctural protein
NSP(BV1)	Nuclear shuttle protein
PRRs	Patter recognition receptors
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
PYLCThV	*Pepper yellow leaf curl Thailand virus*
PCR	Polymerase chain reaction
RCR	Rolling cycle replication
Rep	Replication initiator protein
Ren	Replication enhancer protein
TrAP	Transcription activator protein
TYLCKaV	*Tomato yellow leaf curl Kanchanaburi virus*
TYLCV	*Tomato yellow leaf curl virus*
UTR	Untranslated region
VSRs	Viral suppressor of RNA silencing
Vir	Virulence
YLCD	Yellow leaf curl disease

INTRODUCTION

The Geminiviridae are a family of plant DNA viruses characterized by single-stranded circular genomes encapsidated in twinned icosahedral particles that cause substantial crop losses around the world (Hanley-Bowdoin et al., 2013). Based on their genome structure and transmitting vectors, geminiviruses can be divided into four genera: *Begomovirus, Curtovirus, Mastrevirus, Topocuvirus* (Fondong VN 2013). According to the International Committee of Taxonomy of Viruses (ICTV), begomoviruses are the most diverse and geographically widespread viruses (Zerbini FM et al., 2017). Single-stranded DNA (ssDNA) is packaged in to virions for all begomoviruses, and replicated to double-stranded DNA (dsDNA) in plant cells. Their genomes are consisted of one (monopartite) or two (bipartite) DNA segments that encode 5-7 proteins responsible for viral replication, transmission, cell trafficking and pathogenesis (Rojas et al., 2005).

Over the past three decades, the emergence of begomoviruses has been associated with increase of whitefly population. The yellow leaf curl diseases cause severe yield losses in Solanaceous crops especially tomato (*S. lycopersicum)*, pepper (*Capsicum* spp.), and eggplant (*Solanum melongena* L.), in tropical and subtropical regions of the world (Kenyon et al., 2014). The YLCD of pepper has been observed since 1995 in Kanchanaburi province of Thailand, and TYLCKaV and PYLCThV genome sequences were characterized to be a distinct novel bipartite begomovirus

species from previous classification (Green et al., 2003; Chiemsombat et al., 2018).

The begomovirus are transmitted by a one white fly species, *Bemisia tabaci*. The species *B. tabaci* can be divided into three biotypes (A, B, Q) based on their ability to interbreed and divergence of the *mitochondrial cytochrome oxidase1* (*mtCO1*) gene (De Barro et al., 2011). To date, 360 begomovirus species and 41 morphologically indistinguishable *B.tabaci* species have been characterized however, very little is known about the whitefly determinants of begomovirus transmission (Patil et al., 2018).

To identify the fundamental knowledge of pepper-infecting begomoviruses, molecular phylogenic studies have been carried out. At least 37 ICTV species and 6 candidate species of begomovirus have been reported in *Capsicum* species south-east Asian countries (Kenyon et al., 2014). In Korea, tomato yellow leaf curl disease has first reported in 2008 and spread rapidly from southern to central areas (Lee et al., 2010). Recently, Kil et al (2014) developed an infectious clone for monopartite Korean TYLCV isolate and conducted successful whitefly-mediated tomato infection but failed to infect pepper.

There have been tremendous efforts to find resistance resources to TYLCV tomato. UP to date, six major resistance genes (From *Ty-1*- to *Ty-6*) have been reported in tomato (Lapidot et al., 2015). These genes have been deployed in tomato breeding and the identities of genes are known (Lapidot et al., 2002; Scott et al., 2015; Verlaan et al., 2013). Despite a high incidences of yellow leaf curl disease in peppers, there have been limited studies on resistance to this disease in pepper

(Chiemsombat P 2018; S.L. Shih et al., 2010; Koeda S. et al., 2016). In 2018, we developed TYLCKaV infectious clone and tested infectivity by agro-inoculation and whitefly-mediated inoculation in tomato and pepper. The agro-inoculation test was successful in tomato with obvious TYLCD symptoms. However, both whitefly-mediated and Agrobacterium-mediated inoculation methods were not successful in pepper (Choi et al., 2018). Thus, to identify the resistance resources of pepper plants to viruses, an efficient infection system is required**.**

There have been several studies on Agrobacterium-mediated infection using TYLCV infectious clones in pepper (Morilla, 2005; Kil et al., 2014). The first report using infectious clone demonstrated that peppers can be infected by TYLCV clone but did not show any visible symptoms. However, symptomless peppers harboring TYLCV can as a reservoir of TYLCV transmit the virus to tomato by whitefly (Morilla, 2005; Kil et al., 2014). Recently, an infectious clone were prepared from and Indonesian TYLCKaV isolate (KF446672). This clone had high infectivity in Tomato and *N. benthamiana.* Nevertheless, the clone was not able to infect pepper (Koeda et al., 2017).

The objectives of this study were to develop an infectious clone of *Pepper yellow leaf curl Thailand virus* for successful infection to *Capsicum* spp. and to map the pathogenicity factor of TYLCKaV through genome swapping experiment.

LITERATURE REVIEW

1. Plant immune system

Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system. Instead, they relying on the innate immunity of each cell and on systemic signals from infection sites (Venkatesh J and Kang 2019). Plants have interesting receptor called pattern recognition receptors (PRRs) to recognize pathogens through conserved and variable pathogen elicitors such as pathogen-associated molecular patterns (PAMPs) (Dodds et al., 2010). This PAMP-triggered immunity (PTI) can inhibit further pathogen colonization. On the other hand, for successful pathogen virulence, effectors can interfere with PTI by secretion of effectors proteins and this result in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). To overcome ETS, plants evolved to recognize the effectors by intracellular receptors such as (R) protein which derived from complex proteins motifs of nucleotide binding (NB) and leucine rich repeat (LRR) domain (Ting and Davis et al., 2005). This immunity system called effector-triggered immunity (ETI). Usually, ETI response shows more strong resistance than PTI, inducing hypersensitive response (HR) This model of recognition explain not only pathogen-host interaction obviously but it also provide evidences about co-evolution between plant and microbe (Jones and Dangl 2006).

2. Plant immunity against virus

Although (R) protein is well-characterized mechanism of plant antiviral defense, RNA silencing still plays a central role in plant immune response. Most plant viruses have conserved motif that contain imperfect regulatory hairpin and are copied in plant cell. (Pumplin and Voinnet 2013). When virus invade intracellular space, viral double stranded RNA is processed by dicer-like proteins (DCLs) and cleaved by ARGONAUTE protein (AGO) family (Hanley-Bowdoin et al., 2013). After primary siRNAs have been generated, induced virus small RNA can move to adjacent cell or elongated into long dsRNA by RNA-dependent RNA polymerase in order to trafficking resistant signal to nucleus or other plant cells (Csorba et al., 2009). To make successful infection, virus evolve to obtain viral suppressor of RNA silencing (VSRs) (Voinnet et al., 1999). For instance, Tomato spotted wilt virus nonstructural protein (NSs) binds to dsRNA or siRNA to inhibit dicer-mediated cleavage of long dsRNA and prevents siRNA binding to AGO protein (Schnettler et al., 2010). In recent studies revealed that miRNAs can also control the NB-LRR motif based on resistance mechanism (Li et al., 2012).

3. Genetics of plant virus resistance

Heritable resistance of plant virus can be controlled by dominant or recessive genes. In plant, dominant genes are usually (R) genes that provide full resistance to one or more pathogens (Kourelis et al., 2018). The most largely researched are single dominant resistance (R) genes. The R gene induces programed cell death

responses with rapid occurrence of necrotic lesions (HR) and invisible necrosis (extreme resistance, ER) (de Ronde et al., 2014). Recently, the research between R protein and microbial interaction is actively done. The plants and microbe proteins directly or indirectly interact to make plant resistance or susceptible in the intracellular space. If pathogen infects and replicates in the host to produce disease, the virus is described as virulent with compatible interaction, and when the virus molecule that specifically elicits R-protein-mediated responses, this call the avirulence (Avr) be termed an incompatible interaction (Soosaar et al., 2005).

The recessive resistance is more related with using host proteins. Since plant virus encodes limited number of genes, virus has evolved to use host factors. For achieving successful infection, the virus utilize host factors for viral replication, cell-to-cell movement and systemic development (Kang et al., 2005). Plant recessive resistance mechanism is derived by mutating host factors to prevent viral spreading. (Truniger and Aranda 2009). It can confer durable virus resistance for example, pepper eukaryotic translation initiation factor 4E (eIF4E) gene is required for several potyvirus infection however mutation in eIF4E results in disturbing in viral as multiplication and cell-to-cell movement of viral particle (Diaz-pendon et al., 2004).

4. Geminivirus

Geminiviruses threaten food security and agriculture, infecting major crop species, especially in tropical and subtropical regions (Ali et al., 2016). Geminiviruses have been classified into four genera: Begomovirus, Cutovirus, Topocuvirus and Mastrevirus based on host range, insect transmission vector and genome organization (Fondong et al., 2013). All geminivirus genomes consist of one (monopartite) or two (bipartite) circular single-stranded DNA (ssDNA) molecules that are encapsidated in icosahedral twinned particles (Fondong VN 2013). To maximize viral DNA replication, geminivirus obtained and evolved their mechanism to reprogram the plant cell life cycle by encoding multiple viral genes (Rojas et al., 2005). DNA-A has six open reading frames such as replication initiator protein (Rep), replication enhancer protein (Ren), the coat protein (CP, and Transcription activator protein (TrAP) (Fondong VN 2013). DNA-B has two ORFs that essential for intra- or inter-cellular trafficking by movement protein (BC1) and nuclear shuttle protein (BV1) interaction. (Gafni et al., 2002). Besides, geminivirus DNA-A and DNA-B share unique conserved region of ~200 nucleotide in their intergenic region (IR) (Harrison et al., 1985). The IR contains highly conserved motif of '5-TAATATTAC-3' and GC-rich region that essential sequence for viral DNA replication and transcription of both components (Hanley-Bowdoin et al., 2000).

In the plant cell, single stranded viral DNA is uncoated from virion and start to replication to form double stranded DNA with interaction of various host factors (Nagar et al., 1995). The initiation of replication occur when replication initiator protein (Rep) binds to hairpin structure with GC repeat sequence (Pradhan et al., 2017). Rep-hairpin structure then initiated rolling-cycle replication (RCR) by nicking the conserved motif of geminivirus ori at 5'-TAATATT↓AC-3' (↓: site of nicking) position and Rep protein binds to the nicking site covalently for RCR extension (Laufs et al., 1995). The replicated formed dsDNA can translated to mRNA to make viral symptoms or captured by BV1 to inter-cellular movement though BC1 derived tubular structures (Gafni et al., 2002).

Among the geminivirus, tomato yellow leaf curl virus (genus begomovirus) is one of the major viral diseases in tomato and ranked in the third most serious disease in worldwide (Scholthof et al., 2011). Recently, TYLCV broaden the host range to *Capsicum* spp. Pepper yellow leaf curl virus has been reported in 1995 at Kanchanaburi province in Thailand (Chiemsombat P 2018). Yellow leaf curl virus (YLCD) is naturally transmitted by the whitefly (*Bemisia tabaci*). Therefore, controlling whitefly and breeding resistance of YLCD cultivar is a key for successful pepper cultivation.

MATERIALS AND METHODS

Virus inocula and plant materials

Two different begomoviruses were used in this study. TYLCKaV (provided by East-West Seed Indonesia) and PYLCThV (provided by East-West Seed in Thailand) inoculum was used develop to full-length infectious clones. *N. benthamiana*, tomato (*S. lycopersicum* 'A39'), ECW30R (*C. annuum*) were tested infectivity of TYLCKaV (Choi et al., 2018), PYLCThV and chimeric clones.

Isolation and sequencing of viral genomes

Geminivirus DNA-A was extracted and amplified by polymerase chain reaction (PCR) using geminivirus universal primer pair PAL1v1978/PAR1c715 (Rojas et al., 1993) (Table 1). For viral DNA amplification, the following PCR condition were use: 30 cycles of denaturation at 94□ for 60 s, annealing at 50 □ for 60 s and extension, at 72□ for 3min. The amplicon of 1.4kb sized product were sequenced at Macrogen (Republic of Korea) and verified by BLAST search at NCBI (www.ncbi.nlm.nih.gov/). Based on the sequence of the 1.4kb DNA product, Additional primers were designed to complete the DNA-A sequence. Furthermore, DNA-B specific primer pairs were designed to amplify the full-length DNA-B sequence data based on the NCBI GenBank data.

Construction of PYLCThV infectious clone

To develop PYLCThV infectious clone, each construct carrying viral genome of 0.7 mer (1.8 kb) and 0.6 mer (1.5 kb) including hairpin region were initially cloned into pUC19b vector and then subcloned to pICH86988 expression vector. (Figure 1). Two amplicons from DNA-A which flanking Sph1, *Kpa*I and *Kpa*I, *Xma*I restriction enzyme sites are amplified by specific primers based on virus sequence. For DNA-B construction, flanking amplicons containing restriction enzyme sites of *Sbf*I*, Pst*I *Pst*I*,* and *Kpn*I were amplified and cloned into a pICH86988 expression vector.

Table 1. List of primers used for the construction of an infectious PYLCThV clone and virus detection.

Name	Sequence (5'→3')	Target	Purpose
PY-A-in1F	TTGCATGCCGTGGATATG TGAGGAAATAG	PYLCThV-A	PYLCThV-A Infectious clone
PY-A-in1R	TTGGTACCTATCAAGGTG CGAC	PYLCThV-A	
PY-A-in2F	AAGGTACCTGAGTAGAG TGGGC	PYLCThV-A	
PY-A-in2R	TTCCTGCAGGCCATAATC CCTAGCACGAATC	PYLCThV-A	
PY-B-in1F	TGCCTGCAGGAGAAGTG GTAACCCCAATCG	PYLCThV-B	PYLCThV-B Infectious clone
PY-B-in1R	GGTGCTGTACAGGGTGG AAG	PYLCThV-B	
PY-B-in2F	ATCTCGGAGGACTGTTTT GC	PYLCThV-B	
PY-B-in2R	TTGGTACCCACGTCGTTG TACTGGTTGG	PYLCThV-B	
PAL1v1978	GCATCTGCAGGCCCACAT YGTCTTYCCNGT	Universal	PYLCThV-A Isolation
PAR1c715	GATTTCTGCAGTTDATRT TYTCRTCCATCCA	Universal	
Bego-B-detec-F	TGGCATTTGTAGTGTATC TGGAAG	PYLCThV-BV1	PYLCThV-B Isolation
Bego-B-detec-R	CCTTCTCTCTAGACTTTC TCTCTCTA	PYLCThV-BC1	

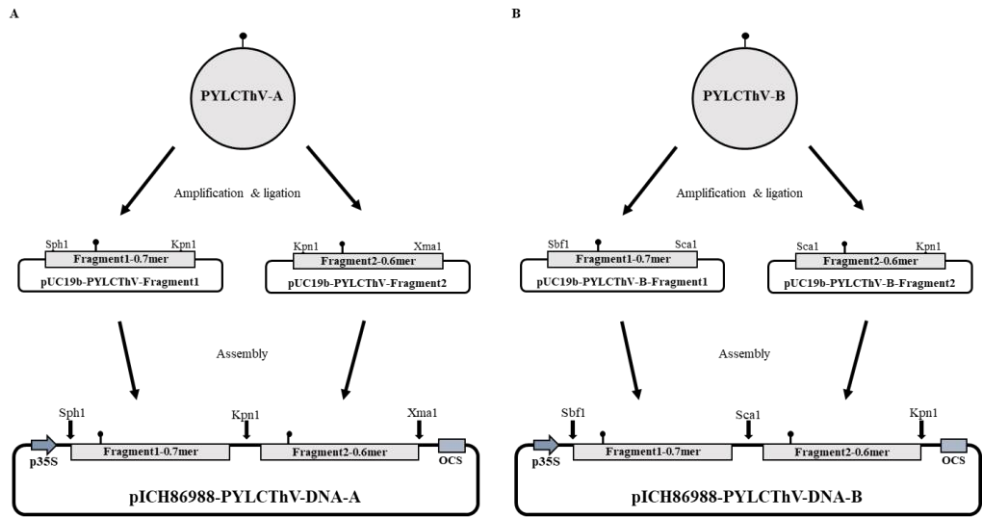


Figure 1. Schematic diagram of developing a PYLCThV infectious clone. (A) pICH86988-PYLCThV-DNA-A and (B) pICH86988-PYLCThV-DNA-B were constructed using recombinant DNA technology.

Construction of TYLCKaV/PYLCThV chimeric clone

Previously, the complete TYLCKaV-A and TYLCKaV-B were cloned into the pICH86988 vector by golden-gate cloning method (Engler et al., 2008) in our laboratory (Choi 2018). Based on the virus sequence, total of four YLCKaV/PTLCThV chimeric clones were made by swapping sequences of the TYLCKaV and PYLCThV by Gibson Assembly (GA) method (Gibson et al., 2009). To construct chimeric infectious clones of TYLCKaV-B and PYLCThV-B, empty pICH86988 vector digested with *Bsa*I (Figure 2) and three segments were amplified with a 20-22 nucleotide overlap region between genome segments using GA specific primer pairs (Table 2). Each geminivirus DNA-B genome segments were assembled using Gibson Assembly Master Mix (Transgen Biotech, China) as described in the provided manual. Briefly, 1μl of the linearized vector (40ng/ μl), 1μl of the amplified fragments (33ng/ μl), 1 μl of ultrapure H2O and 5μl of 2X Assembly master mix were mixed and incubated at 50□ for 15 min. The reaction product were immediately transformed into *Trans*1-T1 Phage Resistant Competent *E.coli* Cell (Transgen Biotech, China). Selected colonies were grown at 37□ for 18 h, and plasmid DNA was extract using the AccuPrep Plasmid Mini Extraction Kit (Bioneer Korea).

Table 2. List of primers used for construction of the PYLCThV/TYLCKaV chimera clones**.**

Name	Sequence (5'→3')	Target	Purpose
PBC1c_Frag1_F	TACATTTACAATTATCGATACAATGAGAAGTG GTAACCCCAATCGT	PYLCV BV1	PYLCThV BC1 chimera
PBC1c_Frag1_R	TTATCCAATGTAGTTCAAATCATATTGCACGT GCACTTCG	PYLCV BV1	
PBC1c_Frag2_F	GATTTGAACTACATTGGATAAATAAAATCATA TTTTTTTTACATTTGAAG	TYLCV BC1	
PBC1c_Frag2_R	ATGGAGTCCAGCAATAGCAGTATTGCCTATAC	TYLCV BC1	
PBC1c_Frag3_F	CTGCTATTGCTGGACTCCATTAAAATAATAAT AACTCTTTATAG	PYLCV UTR	
PBC1c_Frag3_R	CTCATTAAAGCAGGACAAGCCTGCAGACTAG TGTCGACGGATTTCTGCATATCT	PYLCV UTR	
TBC1c_Frag1_F	TACATTTACAATTATCGATACAATGAGAAGTG TTAACCCCATTTA	TYLCV BV1	TYLCKaV BC1 chimera
TBC1c_Frag1_R	TTATCCAATATAGTTCAAATCATACTGTACGT GCACTTCG	TYLCV BV1	
TBC1c_Frag2_F	GATTTGAACTATATTGGATAAATAAAATTATAT TTTTTTTACATTCGAAG	PYLCV BC1	
TBC1c_Frag2_R	ATGCCATCATCAATAATGGAGTCCAGAAATA AC	PYLCV BC1	
TBC1c_Frag3_F	CTCCATTATTGATGATGGCATTATTGCCGATGT CAATATAATAATG	TYLCV UTR	
TBC1c_Frag3_R	CTCATTAAAGCAGGACAAGCCACGTCTTTGG ACTTG	TYLCV UTR	
PBV1c_Frag1_F	TACATTTACAATTATCGATACAATGTAAAATA ATAATAACTCTTTA	PYLCV UTR	PYLCThV BV1 chimera
PBV1c_Frag1_R	TGTTTGTTACAATGAGAGAACTACATACATA	PYLCV UTR	
PBV1c_Frag2_F	TTCTCTCATTGTAACAAACAATGAGAGTTCC AATCCGGA	TYLCV BV1	
PBV1c_Frag2_R	TTATCCAATATAGTTCAAATCATACTGTACGT GCAC	TYLCV BV1	
PBV1c_Frag3_F	GTATGATTTGAACTATATTGGATAA ATAAAAT TA TATTTTTTTTACAT	PYLCV BC1	
PBV1c_Frag3_R	CTCATTAAAGCAGGACAAGCTGTTTGTTACA ATGAGAG	PYLCV BC1	
TBV1c_Frag1_F	TACATTTACAATTATCGATACAATGTATTGCCG ATGTCAATATAAT	TYLCV UTR	TYLCKaV BV1 chimera
TBV1c_Frag1_R	TTTTAGATACAAATGAGATGAAAAATAAATA ACACCAATATATAG	TYLCV UTR	
TBV1c_Frag2_F	CATCTCATTTGTATCTAAAAATGAGAGTTCCA ATCCGG	PYLCV BV1	
TBV1c_Frag2_R	TTATCCAATGTAGTTCAAATCATATTGCACG	PYLCV BV1	
TBV1c_Frag3_F	TGATTTGAACTACATTGGATAAATAAAATCAT ATTTTTTTTACA	TYLCV BC1	
TBV1c_Frag3_R	CTCATTAAAGCAGGACAAGCTTTTAGATACA AATGAGAT	TYLCV BC1	

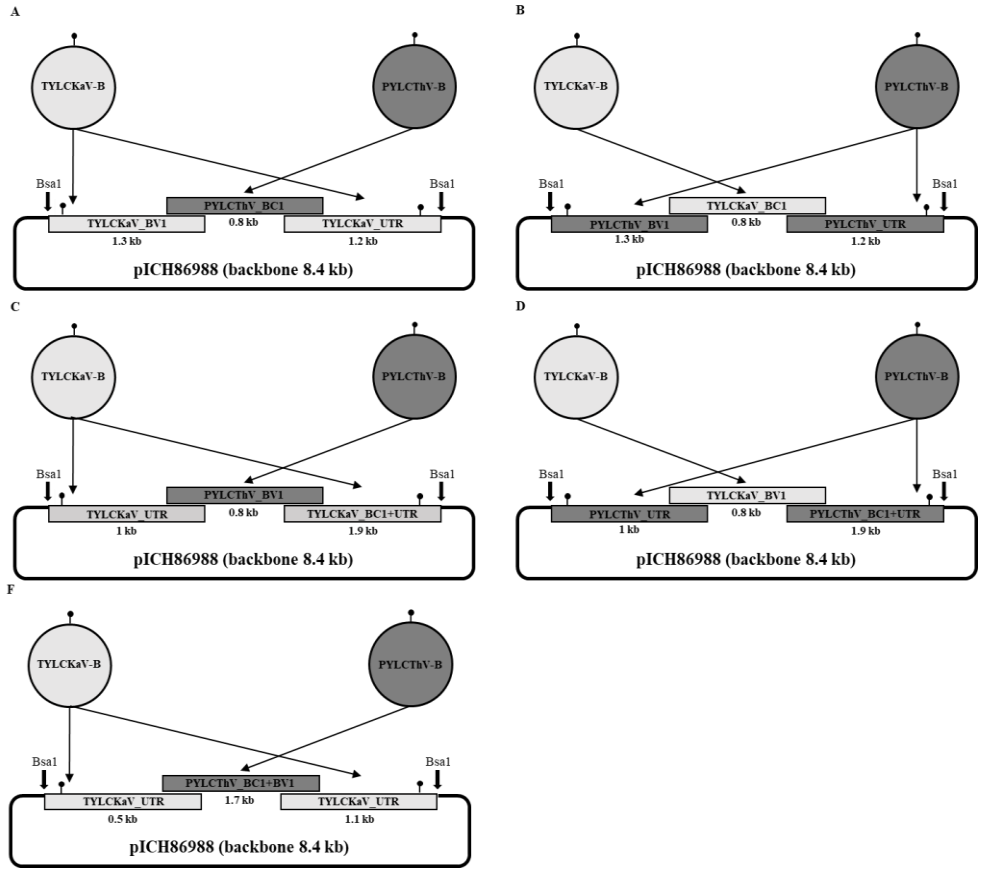


Figure 2. Diagram showing construction of chimeric clones of TYLCKaV-B and PYLCThV-B. (A) pICH86988 vector which is cleaved by Bsa1 is used for backbone. The BC1 protein, a part of TYLCKaV-B is replaced with PYLCThV-B-BC1 protein by Gibson assembly cloning. (B) pICH86988 vector which is cleaved by Bsa1 is used for backbone. The BC1 protein, a part of PYLCThV-B is replaced with TYLCKaV-B-BC1 protein by Gibson assembly cloning.

Virus inoculation

Agrobacterium-mediated infection of infectious clones was tested using tomato and *N.benthamiana.* The TYLCKaV, PYLCThV, chimeric constructs were introduced into *Agrobacterium* strain GV3101 by electroporation. *A. tumefaciens* harboring begomovirus constructs were grown in LB media containing kanamycin (50 μg/ml) and rifampicin (50 μg/ml) at 30 □ shaking incubator. The culture were centrifuged at 4500 rpm, 5min under 4 □. Cells were pelleted and resuspended in infiltration medium (10mM MGCl2, 10mM MES, 200μM acetosyringone). A suspension with a final density at 600nm (OD600nm) of 1.0 was incubated at room temperature for 2-3 hours. *A. tumefaciens* containing pICH86988-TYLCKaV-A and TYLCKAV-B were mixed 1:1, and identically PYLCThV-A with PYLCThV-B, TYLCKaV-A with TYLCKaV-chimera-B, PYLCThV-A with PYLCThV-B in equal proportions. The TYLCV (monopartite) is solely used for positive control. The cell suspension was inoculated into *N. benthamiana* and tomato using a 1-ml syringe at four-leaf stage. Inoculated plants were grown in a growth chamber with 16 h light/8h dark cycle ay 23 □.

Detection of TYLCKaV and PYLCThV accumulation

In order to confirm viral accumulation by PCR, systemically infected leaf samples of tomato and *N.benthamiana* were collected at 30 days post inoculation (dpi). Total genomic DNA was isolated from plant tissues according to CTAB

method (Choi 2018). For the viral detection, same primer pairs of Gibson assembly construction were used and expected size of fragments were observed only from symptomatic plants.

Infectivity test of infectious clones in pepper

ECW30R were grown to 3-4 weeks old stage in grow chamber and used for inoculation of PYLCThV and PYLCThV/TYLCKaV chimeric clones with two methods. The first, pICH86988-NSs clone was co-suspended in 1:1:1 portion with PYLCThV-A+B, TYLCKaV-A + TYLCKaV chimeric DNA-B and PYLCThV-A + PYLCThV chimeric DNA-B. The suspension containing pICH86988-NSs was infiltrated in same condition as described previously. The second, Sap-inoculum were used with pICH86988-NSs. The 3-4 leaves stage of ECW30R was infiltrated by 1mL of agrobacterium harboring pICH86988-NSs. The infected *N. benthamiana* by agro-inoculation of PYLCThV-A+B and infected *S. lycopersicum* by TYLCKaV-A+B, TYLCKaV-A + TYLCKaV chimeric DNA-B were then ground in 0.1M potassium phosphate buffer, pH 7.0, mixed with 400-grit carborundum, and rubbed on the pICH86988-NSs infiltrated leaves of ECW30R. After incubate for 30 minutes in the room temperature, carborundum were washed with distilled water.

Phylogenetic analysis

To infer phylogenetic relationships of PYLCThV among begomoviruses, multiple sequence alignment of BC1 was performed using Molecular Evolutionary Genetics Analysis version 5 (MEGA5). For establishing demarcation of begomoviruses, the International Committee on Taxonomy of Viruses (ICTV) threshold of 89% identified nucleotide sequences were obtained from NCBI data. The final dataset of 274 amino acid positions of begomoviruses were aligned using CLUSTALW and MUSCLE program to optimize the sequence rectangular region. The sequence were then subjected to UPGMA analyses for computing evolutionary distance with 1,000 bootstrap replications (Tumura et al., 2011).

RESULTS

Characterization of PYLCThV

To obtain full-length viral PYLCThV DNA, DNA prepared from yellow leaf curl symptomatic pepper leaf samples and begomovirus universal primers PAL1v1978/PAR1c715 (Rojas et al., 1993) were applied for polymerase chain reaction (PCR). To confirm the identity of begomovirus causing symptoms of leaf samples, an expected size of 1.4 kb amplicon containing geminiviral conserved hairpin sequence (5'-TAATATTAC-3') in the intergenic-region were obtained (Figure 3A). The amplicon sequence showed 99.5% identity with *Pepper yellow leaf curl Thailand virus* (PYLCThV, Genbank Accession No. KX943290.1). PYLCV-A01F/PYLCV-A01R primers were additionally used for full-length cloning and 2.7 kb size of a DNA-A fragment was obtained. Furthermore, PYLCV-B01F/PYLCVB-01R primers were used to obtain a 2.7 kb DNA-B fragment. Based on the NCBI data, the proteins of PYLCThV was analyzed. A Total six proteins were encoded in DNA-A and two proteins were encoded in DNA-B (Figure 3B).

To confirm identity of PYLCThV DNA-B, a phylogenetic tree was constructed using the begomoviral DNA-B BC1 sequences (Figure 4). For establishing demarcation of virus species, the International Committee on Taxonomy of Viruses (ICTV) threshold of 89% identified nucleotide sequences were used (Fauquet et al., 2008). The PYLCThV DNA-B sequence obtained in this study was located in

PYLCThV group and had the highest nucleotide sequence identity with PYLCThV isolate (KX943291, 99%) from Thailand.

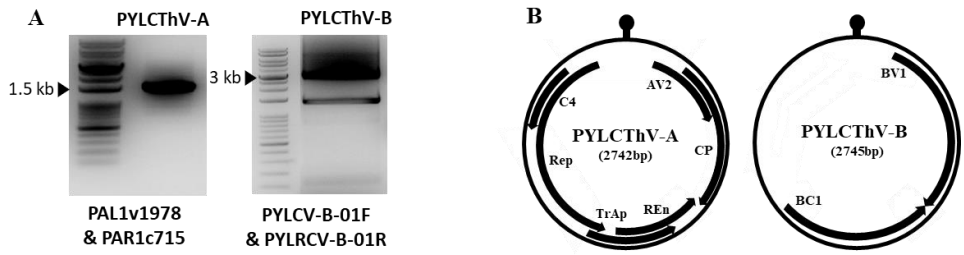


Figure 3. Detection and sequence analysis of viral genome. (A) Using the geminivirus universal primer, ±1.4 kb of PYLCThV DNA-A was detected. PYLCV DNA-B was detected by viral genome specific primer based on NCBI database. (B) Genome organization of PYLCThV containing genimivirus common intergenic region.

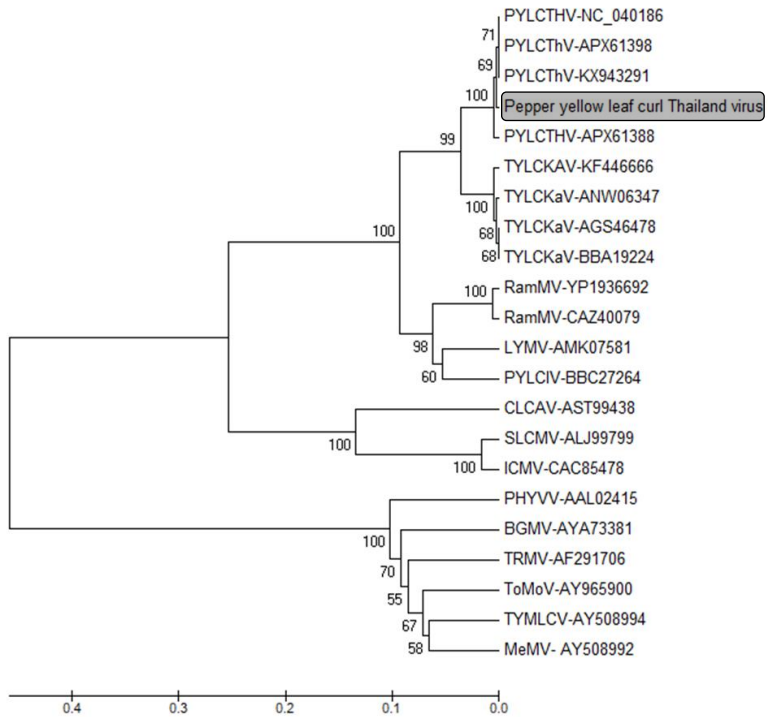


Figure 4. Phylogenetic tree based on begomovirus DNA-B BC1 region. The placement of the PYLCThV isolates in the present study is marked with a box. The sequences of DNA-B BC1 protein were obtained from GenBank and used for UPGMA analysis. The number at the phylogenetic tree represents the bootstrap value (1000 replicates).

Pathogenicity test of the PYLCThV infectious clone in *N. benthamiana* and tomato

To confirm the infectivity of the PYLCThV clone, the DNA-A and DNA-B were subcloned into a binary vector pICH86988 and named PYLCThV-A and pICH86988-PYLCTHV-B. The inoculum of *A. tumefaciense* strain GV3101 containing each clone were mixed at the proportion of 1:1 and infiltrated to true leaves of *S.lycopersicum* 'A39' and *N. benthamiana*. The infectious clone TYLCV was used as a positive control. At 15 dpi, *N. benthamiana* and *S. lycopersicum* inoculated vector only showed no symptoms (Figure 4A-a), whereas plants with the positive control TYLCV started to show typical leaf curling symptoms (Figure 4A-b,f). At 20 dpi, PYLCThV-A + PYLCThV-B infected *N. benthamiana* started to show leaf curling symptoms and excessive leaf curling with yellow mottling were observed at 25 dpi (figure 4A c). At 45 dpi, acute stunting and chlorosis symptoms were observed (figure 4A d). On the other hand, at 25 dpi, PYLCThV-A + PYLCThV-B inoculated *S. lycopersicum* showed no symptoms until 45 dpi (Figure 5A g,f)

To confirm that observed symptoms were caused by PTYLCThV, virus genome in systemic leaves was detected by PCR. DNA-A and DNA-B genome segments were amplified in infected *N. benthamiana* (Figure 5B c). However, PYLCThV inoculated *S. lycopersicum* showed no PCR detection until 45 dpi (Figure 5B g).

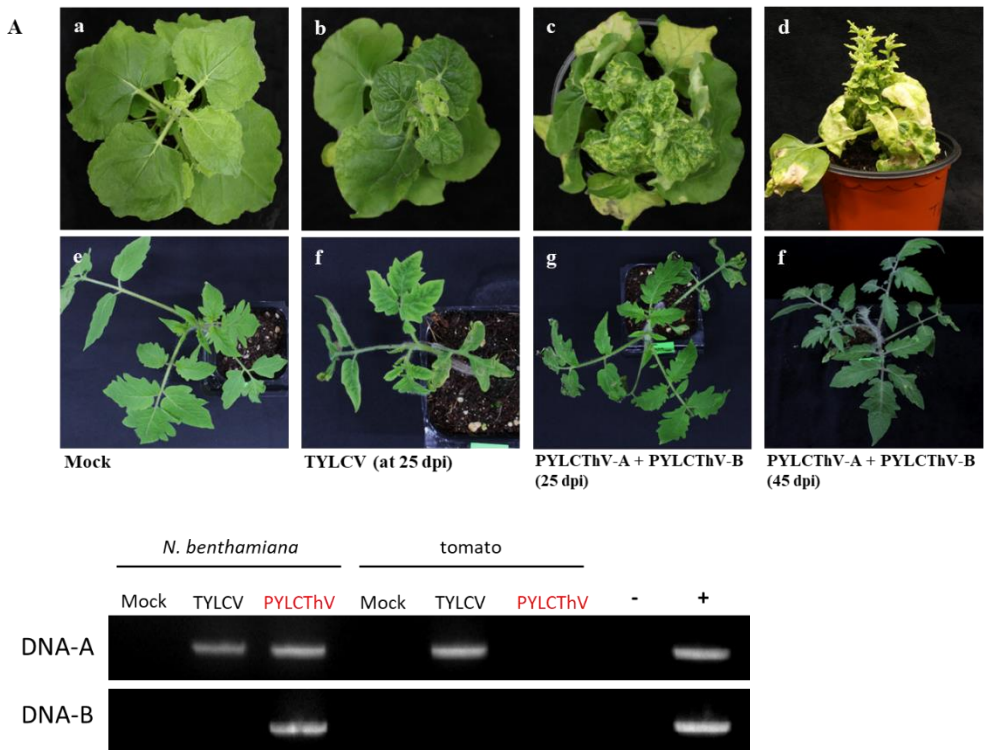


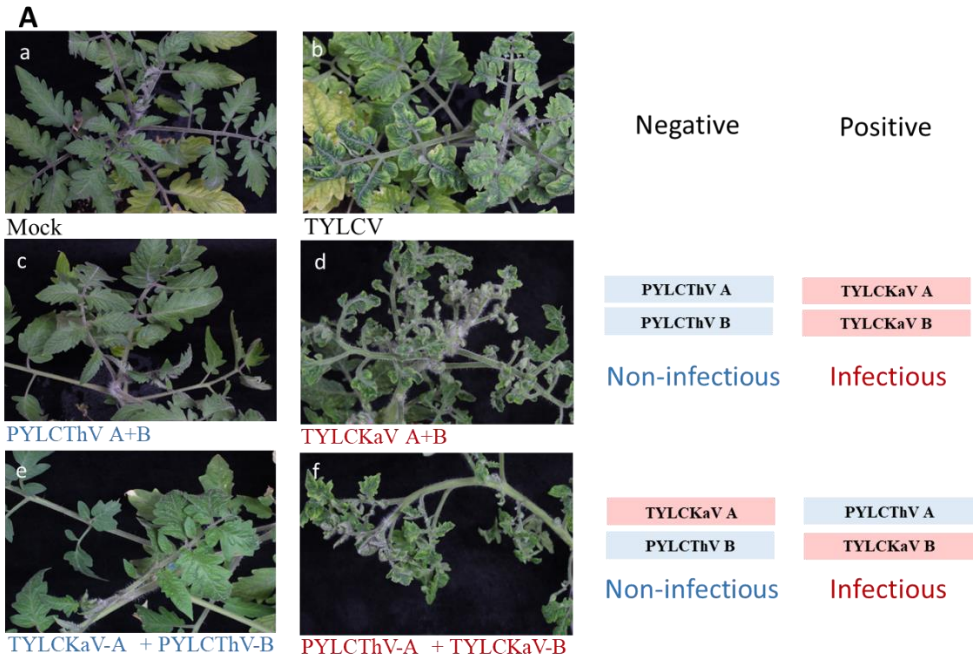
Figure 5. Symptoms of *N. benthamiana* and *S .lycopersicum* 'A39' infected with PYLCThV. (a)Mock, (b)TYLCV (c) PYLCThV-A+PYLCThV-B (at 25 dpi), (d) PYLCThV-A+PYLCThV-B (at 45 dpi). (e)Mock (f) TYLCV (g) PYLCThV-A+PYLCThV-B (at 25 dpi) (h) PYLCThV-A+PYLCThV-B (at 45 dpi). **(B)** PYLCThV genome detection by PCR in the systemic leaves of inoculated *N. benthamiana* (a) Mock (b) TYLCV (c) PYLCThV-A+PYLCThV and inoculated *S. lycopersicum* 'A39' (e)Mock (f) TYLCV (g) PYLCThV-A+PYLCThV-B. (+) pICH86988-PYLCThV-A and B.

Table 3. Infectivity test of PYLCThV infectious clone in *N. benthamiana* and tomato.

Plant	Number of plants [infected/inoculated]		
	Mock	TYLCV	PYLCThV
N. benthamiana	[0/3]	[3/3]	[6/6]
S. Lycopersicum 'A39'	[0/3]	[3/3]	[0/6]

Genome swapping analysis of PYLCThV and TYLCKaV DNA-B

Previously, Choi showed that TYLCKaV had a high infectivity in *S. lycopersicum* 'A39', which is contrasting to the infectivity of PYLCThV in this study. To reveal the genomic region responsible for infectivity in tomato, TYLCKaV and PYLCThV genomes were swapped by mixing subgenomes of each virus: TYLCKaV-DNA-A + PYLCThV-DNA-B and PYLCThV-DNA-A + TYLCKaV-DNA-B. The symptom development of inoculated tomatoes are monitored until 60 dpi. Fifteen to twenty dpi, TYLCKaV-A+B and swapped combination of PYLCThV-A + TYLCKaV-B started to develop leaf curling symptoms (Figure 6A d,f). However, PYLCThV-A+B and TYLCKaV-A + PYLCThV-B showed no viral symptoms (Figure 6A c,e). Until at 60 dpi, only TYLCKaV-A+B and PYLCThV-A + TYLCKaV-B combination showed leaf curling and retarded plant growth symptoms (Figure 6A d,f). To test virus accumulation, strain specific primer sets were designed and used for PCR detection of each virus. The viral DNA segments were detected in all symptomatic plants (Figure 6B a,d). However, viral DNA was not detected in symptomless plants (Figure 6B b,c). This virus genome swapping experiment result revealed that TYLCKaV-DNA-B genome is a responsible for the infectivity in tomato.


A
a
b
Mock
TYLCV
Negative
Positive
c
d
PYLCThV A
PYLCThV B
TYLCKaV A
TYLCKaV B
PYLCThV A+B
TYLCKaV A+B
Non-infectious
Infectious
e
f
TYLCKaV A
PYLCThV B
PYLCThV A
TYLCKaV B
TYLCKaV-A + PYLCThV-B
PYLCThV-A + TYLCKaV-B
Non-infectious
Infectious

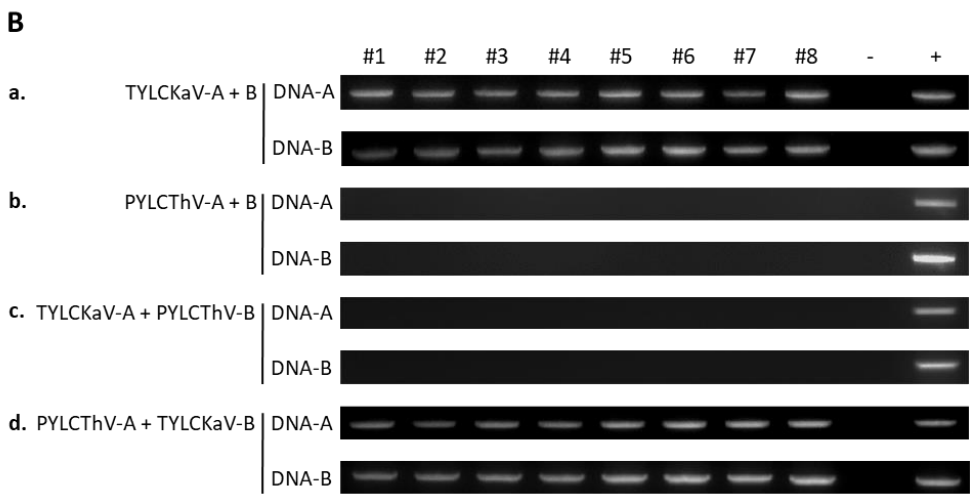

B
#1
#2
#3
#4
#5
#6
#7
#8
-
+
a.
TYLCKaV-A + B
DNA-A
DNA-B
b.
PYLCThV-A + B
DNA-A
DNA-B
c.
TYLCKaV-A + PYLCThV-B
DNA-A
DNA-B
d.
PYLCThV-A + TYLCKaV-B
DNA-A
DNA-B

Figure 6. (A) Inoculation of PYLCThV and TYLCKaV DNA-B swapping clones. (at 60 dpi) (a) Mock, (b) TYLCV, (c) PYLCThV-A+PYLCThV-B, (d) TYLCKaV-A+TYLCKaV-B, (e) TYLCKaV-A+PYLCThV-B, (f) PYLCThV-A+TYLCKaV-B, **(B) viral genome detection by PCR of TYLCKaV-A,B and PYLCThV-A,B in systemic leaves from infected *S. lycopersicum* 'A39'**. (a) TYLCKaV-A+B (b) PYLCThV-A+B (c) TYLCKaV-A+PYLCThV-B (d) PYLCThV-A + TYLCKaV-B. Geminivirus genome A and B specific detection primer sets were used**.**

Table 4. Infectivity test of TYLCKaV and PYLCThV DNA-B swapping.

Plant	Number of plants [infected/inoculated]					
	Mock	TYLCV	TYLCKaV-A TYLCKaV-B	PYLCThV-A PYLCThV-B	TYLCKaV-A PYLCThV-B	PYLCThV-A TYLCKaV-B
S. Lycopersicum 'A39'	[0/3]	[3/3]	[8/8]	[0/8]	[0/8]	[8/8]

Mapping the pathogenicity factor of TYLCKaV in tomato.

To reveal the pathogenicity factor of TYLCKaV in tomato, the sequence of TYLCKaV and PYLCThV ORFs were compared. TYLCKaV and PYLCThV were consisted of untranslated region (UTR) containing hairpin structure which is important for virus replication in plant cell. Furthermore, two ORFs, BV1 and BC1 which are essential for cell to cell movement and nuclear shuttling were encoded respectively. Sequence analyses showed 80% nucleotide identity and 89% amino acid identity. To delimit the ORFs in DNA-B controlling tomato infection, four chimeric constructs between TYLCKaV and PYLCThV were constructed. Each construct was generated by substitution of BC1 and BV1 proteins of TYLCKaV with PYLCThV (Figure 7). To facilitate the whole protein replacement and to overcome six nucleotides of intergenic region (IR), Gibson Assemby (GA) method was used. Compared to conventional restriction enzyme cloning methods, this cloning method permit us to clone the chimeric clones under limited selectable restriction enzyme condition. The construction of chimeric clone was simultaneously combined up to 20 DNA fragments based on sequence identity with adjacent DNA fragments. A total 4 fragments of 13000 bp were assembled in one 2X master mix (contains exonuclease, DNA polymerase and DNA ligase). These chimeric viruses were mixed with the TYLCKaV DNA-A or PYLCThV DNA-A and inoculated to tomato. At 10-15 dpi, positive control and native TYLCKaV-A+B started to develop symptoms. At the same time, TYLCKaV backbone chimeric constructs replaced with BC1 or BV1 of PYLCThV showed leaf curling and yellowing. Whereas PYLCThV backbone

chimeric construct replaced with BC1 or BV1 of TYLCKaV caused no visible symptoms in tomato (Figure 8A g,i) However, unlike the PYLCThV-A+B, The PYLCThV with TYLCKaV backbone showed clear symptoms (Figure 8A e). To confirm virus accumulation, symptomatic leaves and symptomless leaves were collected and virus genome was detected by PCR. The primers of GA were used to verify contamination of original construct infection and as can be seen in figure 8B-c,e,f,h and proper chimeric DNA segment were detected. However no viral DNA were detected in symptomless leaves (Figure 8B d,g,i). Taken together, these results suggest that TYLCKaV UTR region is responsible for pathogenicity in tomato (Figure 8C).

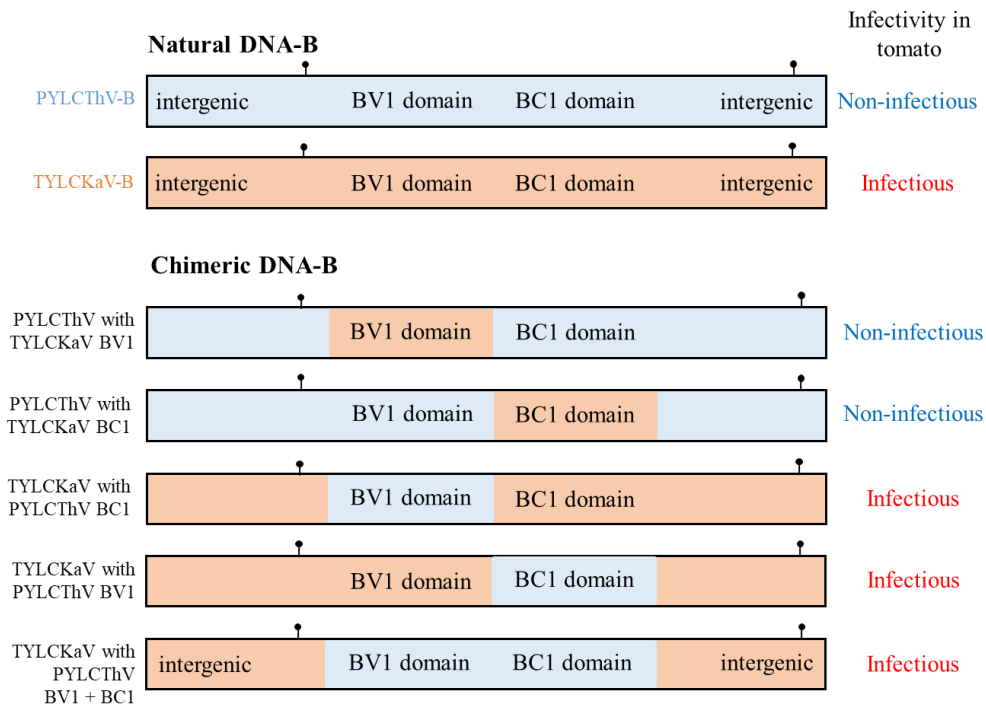


Figure 7. Schematic diagram of PYLCThV/TYLCKaV DNA-B chimera clones. BV1 and BC1 domains were exchanged by Gibson assembly between TYLCKaV and PYLCThV.

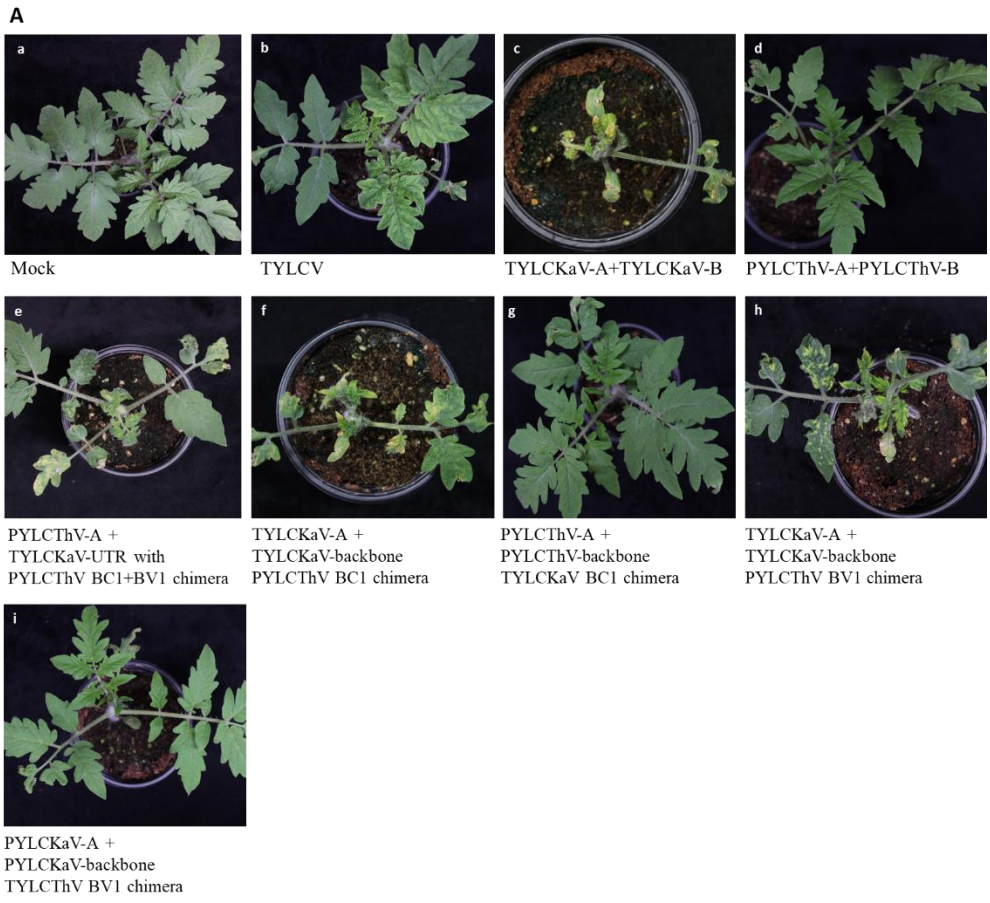

A
a
b
c
d
Mock
TYLCV
TYLCKaV-A+TYLCKaV-B
PYLCThV-A+PYLCThV-B
e
f
g
h
PYLCThV-A +
TYLCKaV-UTR with
PYLCThV BC1+BV1 chimera
TYLCKaV-A +
TYLCKaV-backbone
PYLCThV BC1 chimera
PYLCThV-A +
PYLCThV-backbone
TYLCKaV BC1 chimera
TYLCKaV-A +
TYLCKaV-backbone
PYLCThV BV1 chimera
i
PYLCKaV-A +
PYLCKaV-backbone
TYLCThV BV1 chimera
B
#1 #2 #3 - +
a. Negative control
Mock
b. Positive control
TYLCV
c. TYLCKaV-A + B
DNA-A
DNA-B
d. PYLCThV-A + B
DNA-A
DNA-B
e. PYLCThV-A +
TYLCkaV backbone with
PYLCThV-BC1+BV1
DNA-A
DNA-B
#1 #2 #3 #4 #5 - +
f. TYLCKaV-A +
TYLCKaV-backbone
PYLCThV BC1 chimera
DNA-A
DNA-B
g. PYLCThV-A +
PYLCThV-backbone
TYLCKaV BC1 chimera
DNA-A
DNA-B
h. TYLCKaV-A +
TYLCKaV-backbone
PYLCThV BV1 chimera
DNA-A
DNA-B
i. PYLCThV-A +
PYLCThV-backbone
TYLCkaV BV1 chimera
DNA-A
DNA-B
C
Natural combination
PYLCThV-DNA-A
PYLCThV-DNA-B
TYLCKaV-DNA-A
TYLCKaV-DNA-B
Non-
infectious
Infectious
Significant chimeric combination
PYLCThV-DNA-A
BV1 domain BC1 domain
Infectious
Pathogenicity determinant

Figure 8. (A) Infectivity test of PYLCThV/TYLCKaV chimeric clones. (B) Viral genome detection by PCR of TYLCKaV-A, PYLCThV-A, and chimeric DNA-B in systemic leaves from infected *S. lycopersicum* 'A39'. (C) Schematic summary of mapping of TYLCkaV-B pathogenicity determinant. (a) Mock, (b) TYLCV (c) TYLCKaV-A+TYLCKaV-B, (d) PYLCThV-A+PYLCThV-B, (e) PYLCThV-A + TYLCKaV backbone with PYLCThV BV1+BC1 chimera, (f) TYLCKaV-A + TYLCKaV-backbone PYLCThV BC1 chimera, (g) PYLCThV-A + PYLCThV-backbone TYLCKaV BC1 chimera, (h) TYLCkaV-A + TYLCKaV-backbone PYLCThV BV1 chimera (i) PYLCThV-A + PYLCThV-backbone TYLCKaV BV1 chimera.

Table 5. Infectivity test of TYLCKaV/PYLCThV chimeric clone in tomato.

Plant	Number of plants [infected/inoculated]								
	Agro-inoculation construct								
	Mock	TYLCV	TYLCKaV-A TYLCKaV-B	PYLCThV-A PYLCThV-B	TYLCKaV-A TYLCKaV backbone PYLCThV BC1 chimera	PYLCThV-A PYLCThV backbone TYLCKaV BC1 chimera	TYLCKaV-A TYLCKaV backbone PYLCThV BV1 chimera	PYLCThV-A PYLCThV backbone TYLCKaV BC1 chimera	PYLCThv-A TYLCKaV backbone PYLCThV BC1+BV1 chimera
S. Lycopersicum 'A39'	[0/3]	[3/3]	[3/3]	[0/3]	[5/5]	[0/5]	[5/5]	[0/5]	[3/3]

Sequence analyses between PYLCThV and TYLCKaV of intergenic region.

To compare the variation between putative pathogenicity determinant of TYLCKaV and PYLCThV. The sequence of PYLCThV/TYLCKaV intergenic region containing geminivirus conserved stem-loop structure (Figure 9A) are aligned (Figure 9B). The total IR region of 1078nt (PYLCThV) and 1074nt (TYLCKaV) showed 71.92% identity with 292 nt mismatching. The putative loop forming known as viral replication origin of 9 bp 5'-TAATATTAC-3' was observed in both PYLCThV and TYLCKaV. The GC-rich region that forming stretch of complementary stem region showed 3 SNPs and 1 nucleotide deletion. The minimal DNA binding domain were detected at upstream of hairpin structure. The common 'GTGTC' iteron in PYLCThV and TYLCKaV were in different location. Besides, PYLCThV has additional 'GAATGGGGG' motif that TYLCKaV is no found in TYLCKaV (Figure 9B).

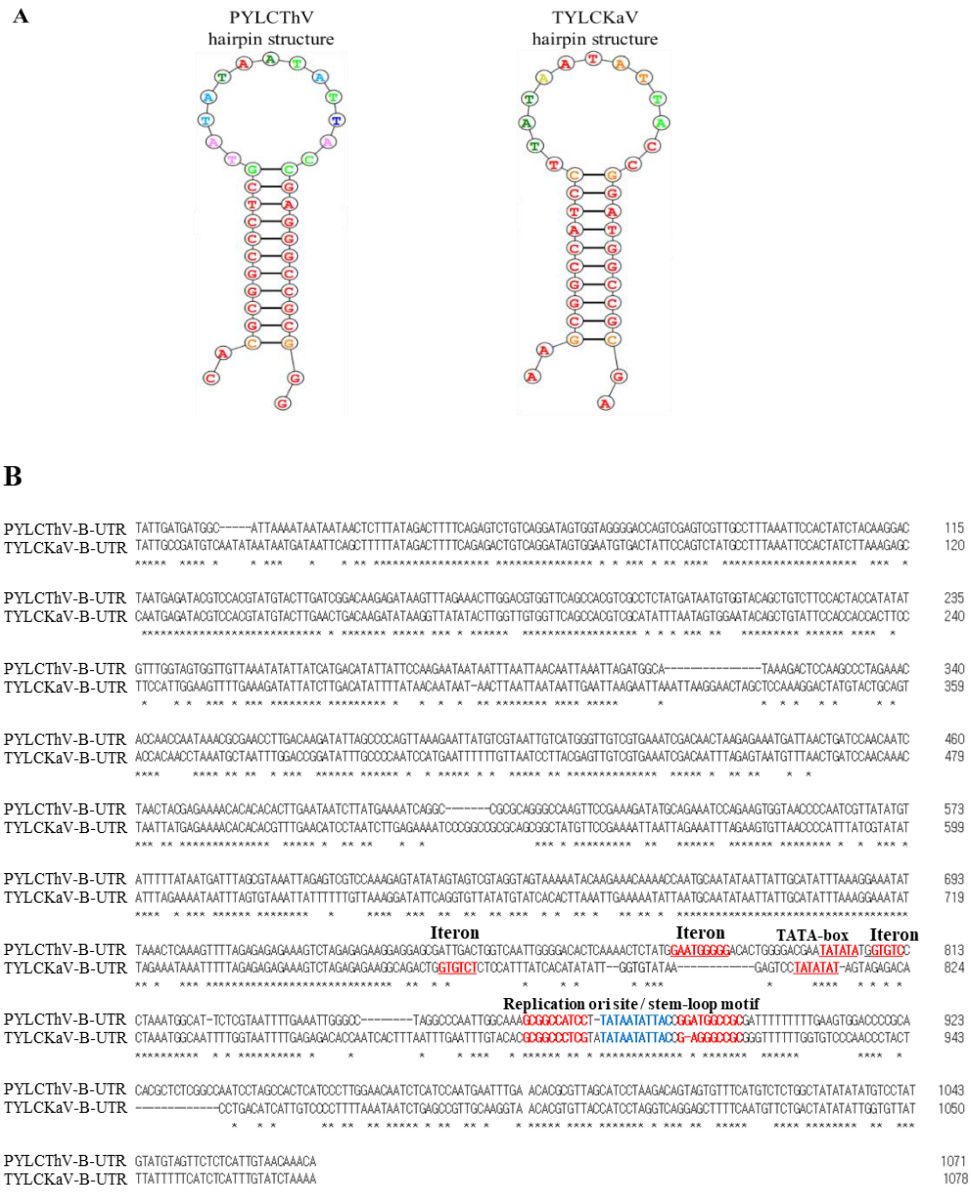


Figure 9. Comparison of PYLCThV and TYLCKaV intergenic region. (A) The geminivirus conserved 'TATAATATTAC' stem-loop motif is flanked on side by a short stretch of complementary GC-rich motifs. (B) Elements relevant for replication such as TATA-box and iteron are shown in upstream of hairpin structure.

DISCUSSION

In this study, PYLCThV infected pepper leaves were obtained from Thailand and an infectious clones was constructed. The infectivity tests were conducted using the developed PYLCThV infectious clone and TYLCKaV/PYLCThV chimeric clones in tomato, pepper and *N. benthamiana*. The pathogenicity of PYLCTHV was obvious in *N. benthamiana* with the rapid deveopment of typical YLCD symptoms. By constrast, tomato and pepper did not show any symptoms and no viral accumulation.

To date, several viral factors that responsible for pathogenicity in Geminivirus have been reported. Zhou et al (2007) verified that nuclear shuttle protein (BV1) determines virulence or avirulence in common bean by using a series of hybrid DNA-B components expressing chimeric *Bean dwarf mosaic virus* (BDMV) and *Bean golden yellow mosaic virus* (BGYMV) NSP. The BDMV with BGYMV NSP and BDMV with BGYMV N-terminal 1 to 42 amino acids can overcome the BDMV resistance of UI 114. Jupin et al (1995) identified the determinant of DNA replication specificity in monopartite TYLCV. The Rep with 146 nt sequence encompassing the left half of the UTR was strain specifically interacted and replicated.

In our study, contrary to PYLCThV, TYLCKaV showed clear symptoms in both *N. benthamiana* and tomato (Choi 2018). The availability of infectious clones allowed us to conduct a genome swapping analysis using PYLCThV and TYLCKaV.

The genome swapping analysis revealed that the B genome of TYLCKaV was responsible for pathogenicity in tomato. Moreover, by mapping analysis, the agro-inoculation harboring five chimeric constructs reveals that the UTR region of TYLCKaV was responsible for tomato infection. In plant cell, once single stranded viral DNA succeeds to invade, the virus starts to form a replication complex by combining DNA with various host factors (Nagar et al., 1995). Then, the Rep protein-host factor complex initiate rolling-cycle replication by nicking the 7th sequence of conserved motif for use as a replication origin (Laufs et al., 1995). By comparing hairpin structures of TYLCKaV and PYLCThV, we found variations including one deletion and three SNPs in GC-rich region. Furthermore common geminiviral iteron of 'GTGTC' was positioned in different location, and PYLCThV has additional 'GAATGGGGG' motif that is no found in TYLCKaV. Taken together, we speculated that the difference of pathogenicity between TYLCKaV and PYLCThV may be derived from the variation of these motifs.

Even though the putative pathogenicity factor of TYLCkaV for tomato infection was is identified in this study, the successful infection of pepper by infectious clone is still elusive. A recent study showed that the pepper infection can be caused by acquisition of satellite DNA molecule that resulted in more aggressive crop-adaptation (Patil et al., 2018). Additionally, the beta satellite usually related with transcriptional and post-transcriptional gene silencing (Yang et al., 2011). Based on these observation, we used TSWV-NSs protein as RNAi suppressor (Schnettler et al., 2010) when sap-inoculation. Finally, we could infect *C. annuum* 'ECW30R' but

unfortunately only one out of fifty plants was showed clear symptoms in the plant inoculated with TLYCKaV-A + TYLCKaV with PYLCThV BC1 (Figure 10). Although this method could not be repeated, suppression of the nene silencing may be helpful for successful Agrobacterium-mediated infection. Additionally previous reports showed that co-infection of *helper component protein* (*HCpro*) of *Plum pox virus* (PPV) with *Cucumber vein yellowing ipomovirus* (CVYV) can suppress the resistance conferred by RNAi defense mechanism (Mbanzibwa et al., 2009; Hanley-Bowdoin et al., 2013, Valli et al., 2006). For the further study, we are testing transmission using Korean Q type of *B. tabaci.* The Figure 11 shows leaf curling symptoms observed at 15 dpi in EXW30R inoculated with a whitefly-mediated method.

In South Korea, there has been no report on the incidence of TYLCD in pepper. TYLCV incidence in tomato was first reported in 2008 (Ko et al., 2014; Kwak et al., 2008). Owing to global warming the average temperature of Korea is continuously rising and the whitefly population could be rapidly increase. This study provides molecular basis of understanding TYLCV and PYLCV infectivity and the interaction between virus and plants.

Figure 10. (A) *C. annuum* 'ECW30R' infected by TYLCKaV + TYLCKaV backbone chimeric constructs with PYLCThV BC1. (a) mock, (b) leaf curling symptoms at 14 dpi (c) severe leaf curling with yellow vein mosaic and shortened internodes symptom at 60 dpi. (**B) PCR detection by virus genome specific primers**.

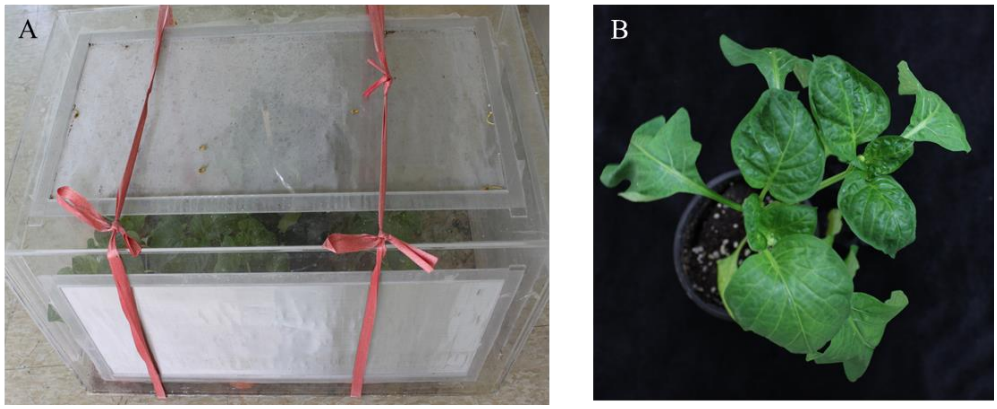


Figure 11 Confirmation of *C. annuum* 'ECW30R' as a natural host using insect vectors. (A): TYLCKaV-viruliferous Bemisia tabaci were released in the insect cage. (B): leaf curling-like symptoms in ECW30R at 15 dpi.

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ABSTRACT IN KOREAN

황화잎말림바이러스 (Yellow leaf curl virus)는 Geminivirus 계통의 Begomovirus 그룹에 속하는 원형의 단일가닥 DNA 바이러스로 열대 및 아열대 국 특히 토마토와 고추에서 심각한 손실을 일으킨다. 본 실험에서는 고추에 감염된 PYLCThV를 분리하여 유전체를 확인 및 분석했다. PYLCThV 유전체 정보를 바탕으로 recombinant DNA technology를 이용하여 바이러스 감염 클론을 개발했다. PYLCThV 감염클론은 agroin filtration법으로 담배, 토마토, 고추에 접종했다. 접종된 담배는 바이러스 증상이 확인된 반면, 토마토와 고추에서는 PYLCThV의 감염 증상이 나타나지 않았다. 바이러스의 감염 여부는 중합효소 연쇄반응(PCR법을 이용하여 재 확인 하였다. 이 결과를 바탕으로, 실험실에 보유하고 있던 병원성이 높은 TYLCKaV 감염클론을 사용하여 게놈 교환 실험을 수행했다. 그 결과 TYLCKaV-B 게놈이 토마토의 병원성을 일으킨다는 것을 밝혔다. 또한 TYLCKaV-B 게놈의 병원성 인자를 매핑하기 위하여 5개의 키메라 클론을 제작하였다. 그 결과 TYLCKaV의 유전자간부위 (intergenic region)가 토마토의 병원성을 결정하는데 핵심적인 역할을 한다는 것이 밝혀 졌다. PYLCThV와 TYLCKaV간의 병원성인자인 intergenic region간 변이를 확인해 본 결과 바이러스 유전자 복제 개시와 관련된 조절 유전자 (regulatory gene)들의 변이를 발견할 수 있었다. 본 연구에서 개발된 PYLCThV 감염 클론은 앞으로 병저항성

연구에 유용한 도구로 이용될 것이다. 또한 토마토의 병원성을 결정하는 인자는 앞으로 Begomovirus와 식물간의 상호작용을 이해하는데 분자적 기초를 제공할 것이다.

주요어: Pepper yellow leaf curl Thailand virus (PYLCTV), Tomato yellow leaf curl Kanchanaburi virus TYLCKaV), Pathogenicity factor, Intergenic region (IR)

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