Phytoprotection

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Le virus des feuilles jaunes en cuillère de la tomate favorise la tolérance au stress de la sécheresse de *Solanum lycopersicum* L.

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Article abstract

Plants develop defence mechanisms in response to abiotic and biotic stresses that can have both negative and positive effects. Tomatoes grown in the field are normally exposed to abiotic stresses such as high temperatures and water shortages, as well as biotic stresses such as tomato yellow leaf curl virus (TYLCV), which greatly reduces productivity in this crop. In this study, two TYLCV Korean isolates were used as molecular and physiological tools to identify interactions between TYLCV infection and drought tolerance in tomato. The tomatoes were inoculated by TYLCV-infectious clones and exposed to drought stress, which led to wilted leaves on plants in the mock group, while those on TYLCV-inoculated plants showed no significant drought symptoms. Moreover, the average relative water content (RWC) was higher in TYLCV-infected plants than in the mock group, and genes associated to drought tolerance were pre-activated in well-watered tomato plants. These results confirm that TYLCV infection enhance drought tolerance in tomato plants and pre-inoculation with symptomless TYLCV isolates can be applied to tomato plants before being cultivated in water-deficit regions.

Tomato yellow leaf curl virus infection promotes the tolerance against drought stress in *Solanum lycopersicum* L.

Phuong T. Ho[1]✉, Hee-Seong Byun[2], Thuy T. B. Vo[1], Aamir Lal[1], Young-Jae Jung[3], Eui-Joon Kil[4], and Sukchan Lee[1]

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Plants develop defence mechanisms in response to abiotic and biotic stresses that can have both negative and positive effects. Tomatoes grown in the field are normally exposed to abiotic stresses such as high temperatures and water shortages, as well as biotic stresses such as tomato yellow leaf curl virus (TYLCV), which greatly reduces productivity in this crop. In this study, two TYLCV Korean isolates were used as molecular and physiological tools to identify interactions between TYLCV infection and drought tolerance in tomato. The tomatoes were inoculated by TYLCV-infectious clones and exposed to drought stress, which led to wilted leaves on plants in the mock group, while those on TYLCV-inoculated plants showed no significant drought symptoms. Moreover, the average relative water content (RWC) was higher in TYLCV-infected plants than in the mock group, and genes associated to drought tolerance were pre-activated in well-watered tomato plants. These results confirm that TYLCV infection enhance drought tolerance in tomato plants and pre-inoculation with symptomless TYLCV isolates can be applied to tomato plants before being cultivated in water-deficit regions.

Keywords: tomato yellow leaf curl virus, infectious clone, drought stress, drought tolerance, plant-virus interaction.

[Le virus des feuilles jaunes en cuillère de la tomate favorise la tolérance au stress de la sécheresse de *Solanum lycopersicum* L.]

Les plantes développent des mécanismes de défense en réponse à des stress abiotiques et biotiques qui peuvent avoir des effets négatifs et positifs. Les tomates cultivées en champ sont habituellement exposées à des stress abiotiques tels que des températures élevées et des pénuries d'eau, ainsi qu'à des stress biotiques tels que le virus des feuilles jaunes en cuillère de la tomate (TYLCV), ce qui réduit grandement la productivité de cette culture. Dans cette étude, deux isolats coréens du TYLCV ont été utilisés comme outils moléculaires et physiologiques pour identifier les interactions entre l'infection au TYLCV et la tolérance à la sécheresse chez la tomate. Les tomates ont été inoculées par des clones infectés au TYLCV et exposées à un stress de sécheresse qui a conduit à des feuilles flétries sur les plantes du groupe simulé, alors que celles des plantes inoculées au TYLCV n'ont présenté aucun symptôme significatif de sécheresse. De plus, la teneur relative moyenne en eau était plus élevée chez les plants infectés par le TYLCV que chez les plants simulés, et les gènes associés à la tolérance à la sécheresse étaient préactivés chez les plants de tomates bien arrosés. Ces résultats confirment que l'infection par le TYLCV augmente la tolérance à la sécheresse chez les plants de tomates et que la préinoculation avec des isolats de TYLCV sans symptômes peut être appliquée aux plants de tomates ayant été cultivés dans des régions déficitaires en eau.

Mots-clés : virus des feuilles jaunes en cuillère de la tomate, clone infectieux, stress hydrique, tolérance à la sécheresse, interaction plante-virus.

1. Department of Integrative Biotechnology, Sungkyunkwan University, Suwon 16419, Korea. ✉ hophuongk59sinhhoc@gmail.com (P.T. Ho); bichthuy251188@gmail.com (T.T.B. Vo); aamirchaudhary43@gmail.com (A. Lal); cell4u@skku.edu (S. Lee)
2. Crop Protection Division, National Institute of Agricultural Sciences, Rural Development Administration, Wanju 55365, Korea. ✉ hsbyun73@korea.kr
3. Department of Life Science and Biotechnology, Shingyeong University, Hwaseong 18274, Korea. ✉ chenopodium@hanmail.net
4. Department of Plant Medicals, Andong National University, Andong 36729, Korea. ✉ viruskil@anu.ac.kr

INTRODUCTION

In nature, plants are simultaneously exposed to both abiotic and biotic stresses that significantly reduce crop production (Huang *et al.* 2013). Global warming and its associated extreme climate phenomena can increase the risk of biotic stress, which can negatively affect the productivity of crop plants. Abiotic stresses include extreme temperature, salt, heavy metals, drought, and intensive light, whereas animals, insects, nematodes, and pathogens such as fungi, bacteria, and viruses act as biotic stresses on plants (Gull *et al.* 2019; Jenks and Hasegawa 2005; Zhu 2016). Under stress stimuli, plants develop defence mechanisms that lead to the activation of complex signalling pathways. To cope with biotic stresses, phytohormones such as jasmonic acid (JA) and salicylic acid (SA) play pivotal roles in these defence mechanisms, while abscisic acid (ABA; Cao *et al.* 2017) contributes mostly to abiotic stress (Cohen and Leach 2019). Previous research has mainly focused on understanding plant responses induced by single abiotic or biotic stresses, but plants are confronted with a combination of stresses that require intricate crosstalk between different phytohormones. In recognition of stress to the final response in cells, plants dynamically combine defence pathways to adapt to different environmental challenges (Rejeb *et al.* 2014). The mechanisms that occur in plants in response to combined stresses are more complex and harder to predict than the responses triggered by each stimulus individually.

Despite the need to understand simultaneous biotic and abiotic stress response in plants, few studies have been conducted to address how these responses interact to support plant growth and health. Recent data showed that the combination of biotic and abiotic stresses can cause either negative (susceptibility) or positive (tolerance) effects in plants depending on the nature, severity, and duration of the stresses (Ramegowda and Senthil-Kumar 2015). Indeed, the combination of pathogens and high temperatures indicated that high temperatures increased disease susceptibility in plants; in tobacco and pepper, high temperatures weakened plant defence mechanisms, making them susceptible to tobacco mosaic virus (TMV) and tomato spotted wilt virus (TSWV), respectively (Király *et al.* 2008). In contrast, resistance responses in tobacco, beet, and rice infected with cucumber mosaic virus (CMV) showed an increased tolerance to water deficit conditions (Xu *et al.* 2008). Barley treated with high salt concentrations showed resistance to powdery mildew (Xin *et al.* 2012), while tomato grown under drought stress had enhanced resistance to the fungus *Botrytis cinerea Pers.* (Achuo *et al.* 2006). Furthermore, rhizobacteria increased plant tolerance to salinity and drought (Yang *et al.* 2009) and the endophytic fungus *Piriformospora* improved barley resistance against disease and enhanced salt stress tolerance (Waller *et al.* 2005).

Plant viruses are obligate intracellular parasites which utilize host resources to support their own reproduction and dissemination; hence, viral infections are widely believed to be harmful to the host (Xu *et al.* 2008), especially under harsh environmental conditions such as water deficit. However, this concept represents an incomplete understanding of the virus-host relationship because the three-way interaction among plant-drought-virus can occur in various ways that could lead to positive or negative stress responses. For instance, viral infection may induce stomatal closure to interfere with a pathogen entry as well as limit the transpiration rate, improving plant resistance under drought stress (McElrone *et al.* 2003).

Tomato (*Solanum lycopersicum* L.) is one of the most important crops worldwide. Over the last decade, its production has increased continuously reaching almost 180 million tons of fresh fruit worldwide in 2020. Due to its excellent nutritional properties, tomato is widely consumed worldwide as a fresh and processed fruit. In subtropical and temperate countries such as South Korea, tomatoes grown in the field in the spring and summer are exposed to high temperatures and usually experience water shortages. In addition, tomato yellow leaf curl virus (TYLCV) is the largest constraint in tomato production because it greatly reduces the productivity of this cultivar.

Tomato yellow leaf curl virus (TYLCV) is a circular single-stranded DNA virus belonging to the *Begomovirus* genus, which is the largest genus in the family *Geminiviridae*. This virus was first isolated in Israel during the early 1960s, causing yellow leaf curl disease in tomato plants (Prasad *et al.* 2020). Since its first report, TYLCV has become the most devastating virus in the tropical and subtropical regions of the world, causing significant yield losses in crops. Plants infected by TYLCV present the typical symptoms of the virus, including yellowing, upward curling on the leaves, and severe stunting which eventually leads to reduced fruit production (Lapidot *et al.* 1997). The primary plant host of TYLCV is tomato, but TYLCV has been found to infect many other host plants, including cultivated vegetables, ornamentals, and weeds that belongs to 12 plant families (Pakkianathan and Ghanim 2014). TYLCV is whitefly and seed-transmittable (Kil *et al.* 2016), making it easily and globally dispersed. The TYLCV genome is approximately 2700-2800 nucleotides in length, comprising the six-overlapping transcribed open reading frames (ORFs), V1, V2, C1, C2, C3, C4, and one non-transcribed intergenic region (IR). According to many studies on TYLCV, C1, C2 and C3 proteins are involved in virus replication, and the C4 protein suppresses the defence mechanism of host plants by inhibiting TGS and PTGS (Hanley-Bowdoin *et al.* 2013; Settlage *et al.* 2005; Shivaprasad *et al.* 2005; Rodriguez-Negrete *et al.* 2013). The V1 protein is in charge of the encapsidation of TYLCV single-stranded DNA and the transport to the host while the V2 protein functions as the major component of systemic movement (Prasad *et al.* 2020). In Korea, TYLCV was first described in 2008 in Tongyeong, which is in the south region of the Korean peninsula before it quickly spreads to nearby regions (Kwak *et al.* 2008). In a previous study, TYLCV found in Korea was classified into two groups according to differences in their genomic sequences and named TY KG1 and TY KG2 (Lee *et al.* 2011).

Few studies have been conducted on the role of TYLCV in enhanced plant defence against abiotic stresses, such as heat or drought (Corrales-Gutierrez *et al.* 2020; Tsai *et al.* 2019). However, the mechanism that may render TYLCV infection good for plants exposed to water deficit conditions is still unknown. Therefore, we used TY KG1 and TY KG2 infectious clones to test their infection ability and used as physiological and molecular tools to identify the interaction between TYLCV viral infection and drought tolerance. We also evaluated whether TYLCV infection could be used as a potential strategy to limit the effect of drought stress in tomato plants.

MATERIALS AND METHODS

TYLCV sequences collection and distribution map

Complete sequences of all TYLCV Korean isolates were obtained from NCBI GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). The locations where each Korean isolate was sampled

were investigated and marked as distinguishable on the map of Korea (Kil *et al.* 2014; Kwak *et al.* 2008). Multiple alignment analysis was conducted with all obtained TYLCV sequences using MultAlin (multiple alignment program, http://multalin.toulouse.inra.fr/multalin/) and the representative isolate of each group was selected based on sequence consensus which are TY KG1 (JN680149.1 Goseong) and TY KG2 (GU325632.1 Nons).

Construction of the infectious clones of TY KG1 and TY KG2

To generate the infectious clones of each TYLCV group, primer sets (Table 1) were designed using the TY KG1 (TYLCV isolate Goseong, NCBI GenBank Accession No. JN680149), and TY KG2 (TYLCV isolate Nons, NCBI GenBank Accession No. GU325632) genomes. Two partial genomes (0.4 mer and 0.7 mer) containing restriction sites at the edge were amplified using primer sets (TY-IC1-F-SalI/TY-IC1-R-SphI and TY-IC2-F-SphI/TY-IC2-R-BglII; Table 1) were amplified by PCR and ligated into the pGEM®-T easy vector (Promega, Madison, WI, US), using the TA cloning technique according to the manufacturer's instructions (Fig. 1). The DNA fragments were sequenced (Macrogen, Seoul, South Korea), and then digested using specific restriction enzymes (Fig. 1). To produce an infectious 1.1-mer tandem repeat (Urbino *et al.* 2008), two partial genomes were introduced into pCAMBIA1303 vector (Abcam, Cambridge, UK) in an action called three-pieces ligation and then transformed into competent *Escherichia coli* strain DH5α using the heat shock method (Fig. 1). The transformed plasmids were extracted from *E. coli* using the AccuPrep® Nano-Plus Plasmid Mini Extraction Kit (Bioneer, Daejeon, South Korea) and cross-checked by digestion with three restriction enzymes *Sal*I, *Sph*I and *Bgl*II (TaKaRa, Shiga, Japan). The recombinant plasmids pCAMBIA1303-TY-KG1 and pCAMBIA1303-TY-KG2 were then transformed into the competent *Agrobacterium* strain GV3101 (Fig. 1). Accomplished infectious clones were confirmed by both enzyme digestion and colony polymerase chain reaction (PCR) using the 2 X AccuPower® PCR Master Mix (Bioneer) with the primers for IC1 and IC2 of TY KG1 and TY KG2 (Table 1).

Agro-inoculation of tomato with the TY KG1 and TY KG2 infectious clone

Seeds of *S. lycopersicum* L. of the Seogwang cultivar were planted in sterilized soil and cultivated in one-litre volume pots in a growth chamber at Sungkyunkwan University, Suwon, South Korea. Four-week-old plants of similar size were selected and classified into mock, TY KG1- and TY KG2-inoculated groups. The *Agrobacterium* transformants as GV3101(pCAMBIA 1303-TY-KG1), GV3101(pCAMBIA1303-TY-KG2) and GV3101 (pCMABIA1303) were cultured in Luria broth media in the presence of selective antibiotics (kanamycin, rifampicin and gentamicin) at 28 °C for 30 h until the OD value at 600 nm was 0.8-1.0. The tomatoes were inoculated using a pin-picking method to the stem once with 1 mL agrobacteria culture.

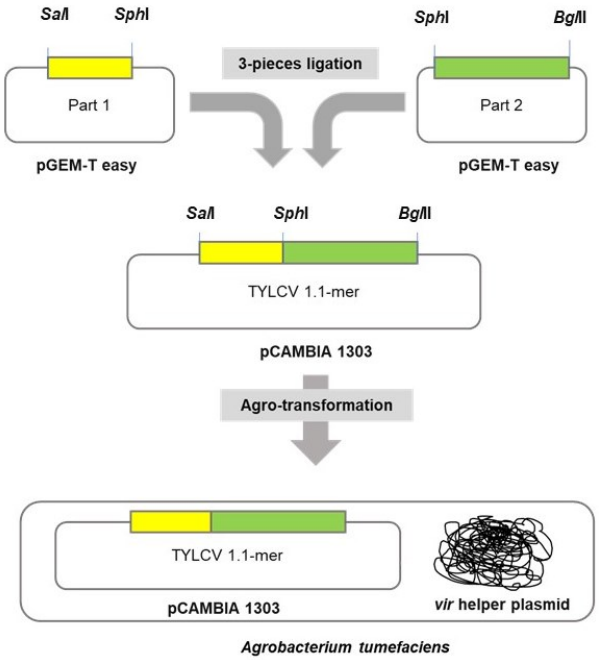

Figure 1. Scheme of 1.1-mer TYLCV infectious clone construction. TY-KG1 and TY-KG2 used the same procedure to generate infectious clones. Two purified separated fragments of each TYLCV strain were ligated into a plant expression vector (pCAMBIA1303) and transformed to *Agrobacteria tumifaciens* strain GV3101.

Table 1. Primers used for infectious clone construction

Purpose	Primer name	Primer sequence (5' - 3')	Target size
TY KG1 infectious clone	TY1-IC1-F-SalI	GTCGACGTTGAAATGAATCGGTGTCCCTC	1173 bp
	TY1-IC1-R-SphI	GCATGCGTACATGCCATATACA	
	TY1-IC2-F-SphI	GCATGCCTCTAATCCAGTGTAT	1901 bp
	TY1-IC2-R-BglII	AGATCTATTGCAAGACAAAAAACTTGGGGAC	
TY KG2 infectious clone	TY2-IC1-F-SalI	GTCGACGTTGAAATGAATTGGTGTCCCTC	1180 bp
	TY2-IC1-R-SphI	GCATGCGTACATGCCATATACAG	
	TY2-IC2-F-SphI	GCATGCGTACATGCCATATACAG	1914 bp
	TY2-IC2-R-BglII	AGATCTAGTGCAAGACAAATTACTTGGGG	

Drought treatment

Inoculated tomatoes were cultivated in sterilized soil in one-litre volume pots in a growth chamber at 60% humidity. The temperature range of the chamber was 20 °C (night) to 24 °C (day) with a 16 h daylength. For the first week after virus inoculation, all plants were watered equally with 250 mL. Then, five in each TY KG1-infected, TY KG2-infected, and mock treatment group were left un-watered for 7 days, while another five plants in each group (control treatment) were continued to water with 250 mL. After drought symptoms, such as wilted shoot tips, were induced, each group was photographed daily. Young leaves from each sample were collected at different time points before (7 dpi) and after drought stress (14 dpi) for additional analysis (Fig. 2).

DNA isolation

One and two weeks after agro-inoculation, samples from young leaves were collected from mock and inoculated tomato plants. Total DNA isolation from 100 mg tissues was conducted using STE method (Hosseinpour and Nematadeh 2013). The samples were ground with a mortar and pestle in liquid nitrogen, then dissolved in lysis buffer containing 470 µL STE buffer (0.4 M sucrose, 20 mM Tris-HCl, 20 mM EDTA), 30 µL of 20% SDS, 200 µL 8 M LiCl, 1 µL of 2-mercaptoethanol and 100 mg polyvinylpyrrolidone. The lysate was mixed well and incubated at 60 °C for 45 min. To separate DNA from other components, the same volume of chloroform: isoamyl alcohol (24:1) was added and centrifuge at 13000 rpm, 4 °C for 15 min. DNA was precipitated with 500 µL isopropanol, washed with cool 70% ethanol, and air-dried before being dissolved in 50 µL 1× TE buffer.

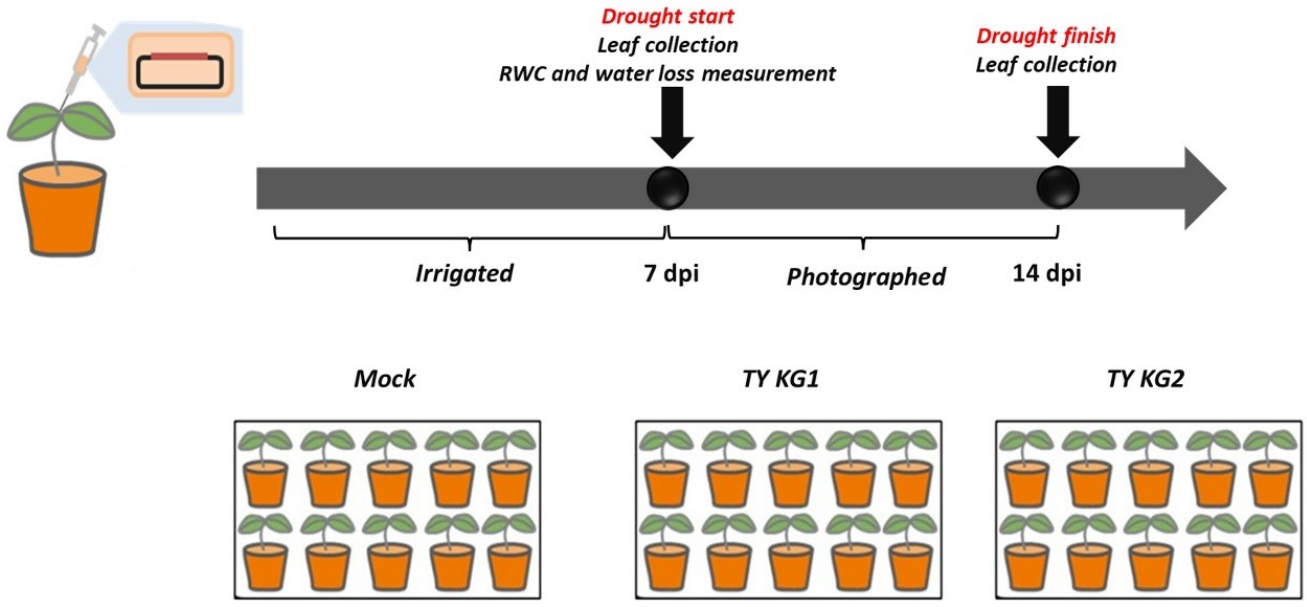

Figure 2. Scheme of the drought stress experiment. Ten tomato plants in each treatment group were inoculated and saturated with water for 7 days. From 8 to 14 days post inoculation (dpi), watering was stopped for five plants in each group.

Measurement of relative water content and water loss

Leaf samples were collected 7 days after virus inoculation and before water withholding. To measure the water relative content, topmost fully expanded leaves from each plant were detached and weighed immediately to obtain the leaf sample weight (W), and the samples were hydrated to full turgidity by floating the leaves on deionized water in a closed Petri dish for 3-4 h at 25 °C in the dark. After hydration, the leaves were removed from the water, and excess water was removed with tissue paper. Leaves were weighed to obtain fully turgid weight (TW). Samples were then oven-dried at 55 °C for 48 h and weighed to determine the dry weight (González and González-Vilar 2001). The relative water content was calculated using the following formula:

$$RWC\ (\%) = [(W - DW) / (TW - DW)] \times 100$$

W = sample fresh weight;
TW = sample turgid weight;
DW = sample dry weight).

For water loss determination, we collected the leaves from individual plants and placed them in a Petri dish. Leaves were kept in a growth chamber at 28 °C. The weight of the leaves was measured at 0, 30, 60, 120, 180, 240, 360, 420, 480, and 600 min after detachment. The ratio of water loss for each plant was calculated by dividing the weight loss at each time point by the initial leaf weight. An unpaired Student's t-test was used to determine significant difference with the data expressed as mean ± SE, and statistical difference was defined by $P < 0.05$.

Table 2. Primers used for qPCR analyses

Gene	Primer sequence (5' - 3')	Function	Target size
SlACTIN7	F: CCAAGCAGCATGAAAATTAAGG R: CCTTTGAAATCCACATCTGCTG	Essential component of the cytoskeleton Housekeeping gene	114 bp
JA2	F: CAGCCATGGTTCGTCGACTT R: TTGAGCCCAGCGAGAATTGC	An NAC transcription factor that promotes stomatal closure by binding to the NAC core regions of *NCED1*	176 bp
ER5	F:AAGGTGGAGAAACCGGAGGC R: AACCCTGCCGGAGCATTTGA	Belongs to the LEA group that helps increase the water binding capacity and create a protective environment for other proteins. Protects the partner protein from degradation and proteinases that function to remove denatured and damaged proteins	158 bp
AOS1	F: GCTGGGCTCAATGCAGCAAA R: TGAAGCTGGAACAGCACCCA	A key gene in the JA biosynthetic pathway	136 bp
SlNCED1	F: CTTATTTGGCTATCGCTGAACC R: CCTCCAACTTCAAACTCATTGC	A rate-limiting enzyme in ABA biosynthesis in tomato	242 bp
C1-TYLCV	F: GCTCGTAGAGGGTGACGAAG R: ACACAAAGTACGGGAAGCCCAT	A gene encodes for coat protein of TYLCV	164 bp

Southern hybridization blotting

Southern hybridization blotting is used widely to identify geminivirus replication (viral DNA) from the extracted plant tissue. Genomic DNA (15-20 µg) was loaded in a 1.0% agarose gel and separated by electrophoresis at 30 V for 8 h. After electrophoresis, the gel was depurinated in 0.2 N HCl for 10 min, denatured in 0.4 N NaOH for 15 min, and neutralized in 0.5 M NaCl solution for 30 min. DNA was then transferred to a positively charged nylon membrane (Hybond-N+ membrane, GE Healthcare, UK) for 14 h using the capillary transfer method. After the transfer, the nylon membrane was exposed to ultraviolet radiation for 2 min using ultraviolet crosslinker machine (UVC 500 crosslinker, GE Healthcare) to permanently attach the transferred DNA to the membrane. The polymerase chain reaction (PCR) products for the conserved TYLCV coat protein coding sequence (C1-TYLCV) (Table 2) were gel purified and labeled with [^{32}P]-dCTP using the Rediprime II Random primer Labeling System (Cytiva) and used as a probe. Probe was prepared by incubating at 37 °C for 15 min, at 100 °C for 3 min and then at -20 °C for 5 min. Hybridization was performed at 65 °C for 12 h in hybridization buffer. The nylon membrane was washed with 2× SCC (saline-sodium citrate) and 1× SCC buffers each for 30 min, respectively. After washing, the membrane was exposed to X-ray film (Agfa-Gevaert N.V, Belgium) for at least 24 h in a -80 °C freezer.

Quantitative real-time PCR

Primer sets for quantitative real-time PCR (qPCR) analysis of drought-responsive genes were designed (Table 2). Total RNA was extracted from the 100 mg tissue samples at two-time points: immediately before water withdrawal and 7 days after water withdrawal, using RNeasy Mini Kit (QIAGEN). Total RNA concentration was measured, and 1 µg of RNA was used to synthesized cDNA using MMLV reverse transcriptase (Bioneer). qPCR was performed using 1 X TB Green™ Premix Ex Taq™ II (TaKaRa) in triplicate for each sample with the template diluted three times from synthesized cDNA. Temperature control and fluorescence measurement were conducted using a Rotor Gene Q thermocycler (QIAGEN). *SlACTIN7* was used as internal control data normalization. PCR conditions were set up as follows: initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s, elongation at 72 °C for 20 s. Relative target gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The statistical comparison was performed by the two-way ANOVA test together with the Dunn's multiple comparisons test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns: not significant.

RESULTS

Korean TYLCVs were classified as two groups by genome differences

After TYLCV spread throughout Korea, many TYLCV sequences have been reported in various regions (Fig. 3a). The sequences of all TYLCV Korean isolates were divided into two groups: the TYLCV Korea Group I (TY KG1) and the TYLCV Korea Group II (TY KG2). TY KG1 was mainly discovered in the east and south of the Korean peninsula (Fig. 3a), and has a genome size of 2774 bp. TY KG2 was found in the west of the Korean peninsula and Jeju Island (Fig. 3a), and has a genome size of 2781 bp (Lee *et al.* 2011). Additional alignment analysis of the whole genome was conducted to compare the genomic sequence difference between these isolates and revealed that the total genome difference was approximately 2.8%. The intergenic region was compared to the nucleotide level because it is a noncoding gene, and the remaining genes were compared to the amino acid level. Protein differences of each coding gene were 2~8 amino acids and nucleotide differences of intergenic region is 26 nts with the deletion of seven nucleotides of TY KG1 (Fig. 3b).

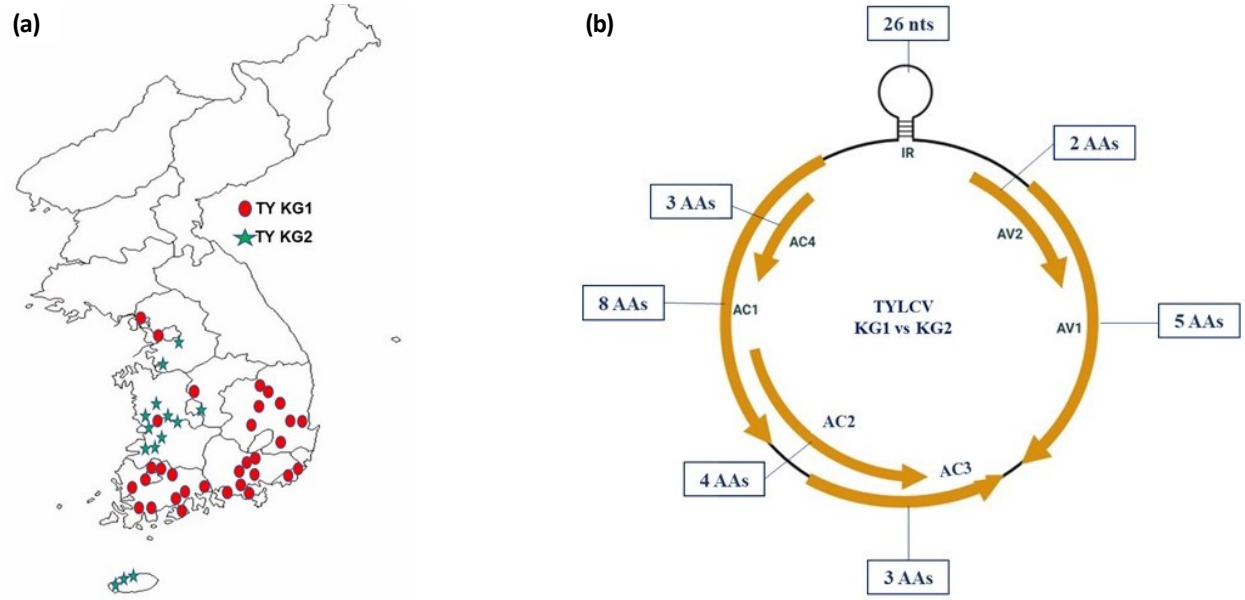

Figure 3. Geographic distribution of the two Korean TLCV strains TY KG1 and TY KG2. (a): TY KG1 and TY KG2 are marked as red circles and green stars on the distribution map, respectively. **(b):** The genome difference is compared between the representative sequence of TY KG1(JN680149.1 Goseong) and TY KG2(GU325632.1 Nons). Each orange arrow represents one viral ORF and the number of amino acids (AA) and nucleotide (nt) differences between the two groups are indicated.

Figure 4. TYLCV-infected tomatoes in the field that show the typical symptoms of yellow leaf curl disease. (a): TY KG1. **(b):** TY KG2.

TY KG2-infected tomato showed late and milder symptoms compared to TY KG1

In the field, tomato plants infected by TY KG1 and TY KG2 showed typical symptoms of yellow leaf curling, but the severity was not similar between the two groups (Fig. 4). To investigate the pathogenicity of the two viruses in more detail, infectious clones of TY KG1 and TY KG2 were constructed using a plant expression vector expressed in *Agrobacterium tumefaciens* (Fig. 1). Four-week-old tomato plants were inoculated with *Agrobacterium* transformants GV3101(pCAMBIA1303-TY-KG1), GV3101(pCAMBIA1303-TY-KG2) and GV3101(pCAMBIA1303) which act as the mock-inoculation treatment. Two weeks after infection, TY KG1-infected tomatoes showed leaf curling and yellowing in young leaves, while TY KG2-infected tomatoes did not show

any symptoms (data not shown). While the TY KG1-infected tomatoes symptoms became severe and the growth rate was noticeably slowed over time, TY KG2-infected tomatoes showed very mild leaf curling and yellowing at 8 weeks post inoculation (wpi; Fig. 5a). Despite the similar genome sequences of the two viral groups, their patterns of symptom development were very different. Total DNA from each virus was extracted from young leaves every week, and TYLCV inoculation was confirmed by Southern blot hybridization using V1 probe. The results showed that most bands from TY KG1 were thick and clear, and a faint band was only seen at 1 wpi. In contrast, the bands from TY KG2 were relatively faint and no bands were observed until 4 wpi indicating the low virus titer (Fig. 5b). A similar expression pattern was obtained using time-course qPCR analysis with C1-TYLCV primer sets (Table 2). The virus titer of TY KG1 steadily increased over the course of 8 weeks after inoculation, meanwhile, the virus titer of TY KG2 was extremely low until 4 wpi and then sharply increased (Fig. 5c). These data indicated that TY KG1 can quickly induce various host mechanisms that are beneficial to geminivirus such as cell cycle regulation, DNA replication and suppression of silencing. In contrast, TY KG2 did not induce these responses, and hence, the self-replication of TY KG2 was suppressed over time.

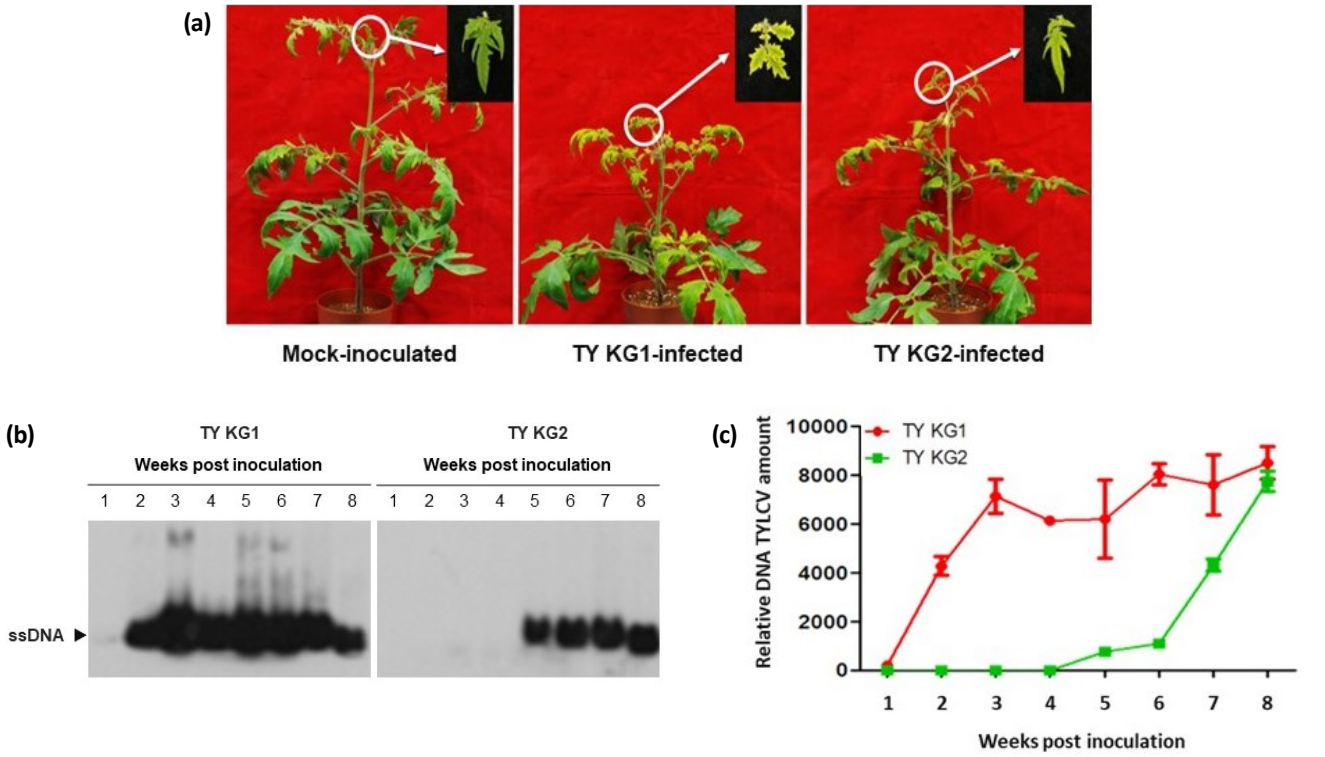

Figure 5. Symptom differences in three groups 8 weeks post inoculation (wpi) and the confirmation of the functional infectious clone by Southern hybridization blotting and qPCR. (a): Three groups of five tomato plants each were infected by *Agrobacterium* transformants GV3101(pCAMBIA1303-TY-KG1), GV3101(pCAMBIA1303-TY-KG2) or GV3101(pCMABIA1303) and grown for eight weeks. Mock-inoculated tomato was healthy while TY KG1-infected tomato showed stunting, leaf yellowing and severe leaf curling; TY KG2-infected tomato had mild leaf curling and stunting. The enlarged leaves were sampled from shoot tips (white circle) from each group. **(b):** TY KG1 was detected in tomato leaves 1 week post inoculation (wpi), while TY KG2 was not detected until 4 wpi; ssDNA means single-stranded DNA. **(c):** Real-time PCR analysis indicated a greater increase in TY KG1 than in TY KG2 2 wpi.

TYLCV infection improved drought tolerance in tomato

Ten tomato plants were inoculated in each TYLCV group. After water withdrawal, most leaves of mock plants wilted, whereas in TYLCV-infected plants, the leaves were still fresh and green. In addition, the stems of TYLCV-infected plants were thicker than those of mock plants (Fig. 6a). To confirm the effects of TYLCV infection on drought tolerance, the relative water content (RWC) and water loss were measured and compared among the three groups. RWC is a parameter of water status indicating the balance between the water supply to the leaf tissue and the transpiration rate. Our results showed that the RWC in TYLCV-infected plants was higher than in mock plants, especially in the TY KG2 group (Fig. 6b), indicating that TYLCV-infected plants can hold more water. To measure water loss as an indicator of leaf transpiration rate, leaves were detached from the plant stems and measured after 600 min. We found that the water loss in the mock group was approximately 30%, whereas in both TYLCV-infected groups, it was approximately 20% (Fig. 6c). These results indicated that TYLCV infection could reduce water loss in tomato grown under drought stress.

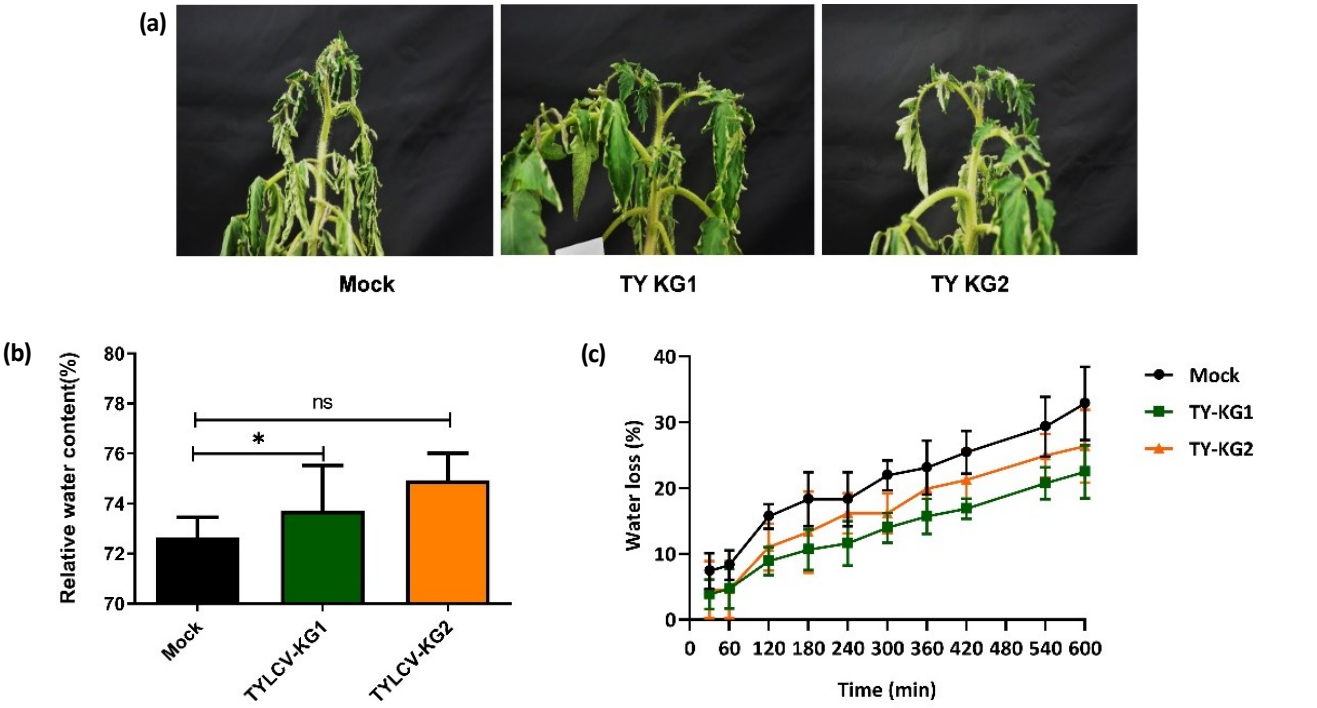

Figure 6. Comparison of drought symptoms, relative water content and water loss in the leaves of mock and TY-LCV-inoculated tomato plants. (a): After 7 days without water, while most leaves of the mock-inoculated group were wilted, the leaves of the TYLCV-infected groups seemed fresher and greener. **(b):** Relative water content in TYLCV-infected plants was higher than in mock plants. **(c):** Water loss was significantly higher in the mock group compared to that in TYLCV-infected plants. The bar graphs indicate the mean ± SD (n = 5). The statistical comparison was performed by the unpaired t-test: * $P < 0.05$, ns: not significant.

Virus replication is reduced by drought in TYLCV-infected tomato plants

To test whether drought stress weakens the plant immune system to aid viral invasion, we used quantitative real-time PCR to measure virus titer and Southern hybridization blotting to monitor virus replication. The qPCR result showed that the TYLCV titer in the drought-treated group was significantly lower than that in the well-watered plants, and the virus titer was extremely low in the TY KG2 group two weeks after inoculation compared to that in TY KG1 (Fig. 7a). Southern blotting analysis indicated that in TY KG1-inoculated plants, DNA bands in well-watered group were thicker than those in the drought-treated group; however, we could not confirm TY KG2 replication by this method (Fig. 7b). From these data, we found that drought stress did not stimulate viral infection but rather suppressed virus replication.

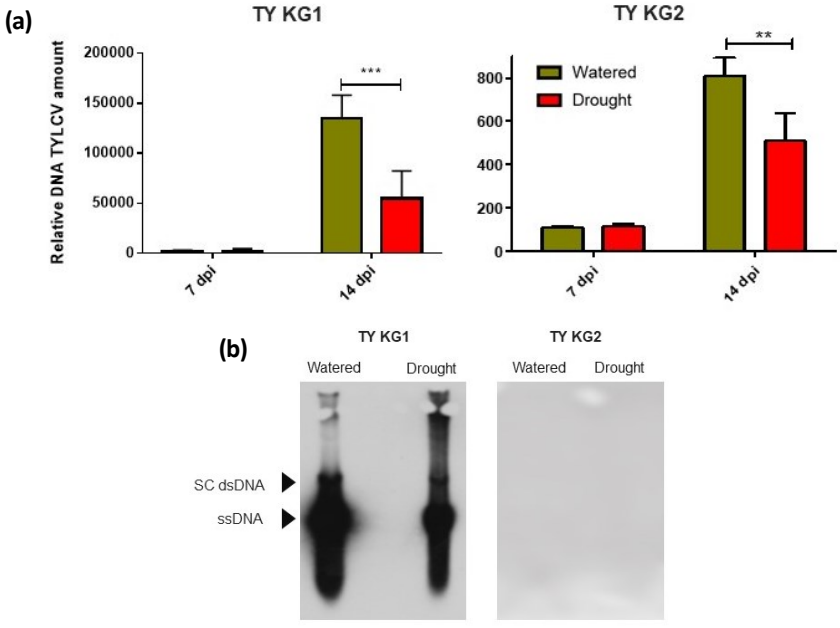

Figure 7. Viral replication in non-watered plants was less efficient compared to that in well-watered plants. (a): Quantitative real-time PCR to show the virus titers of TYLCV. The bar graphs indicate the mean ± SD (n = 5). The statistical comparison was performed by the two-way ANOVA test together with the Dunn's multiple comparisons test: ** $P < 0.01$, *** $P < 0.001$. **(b):** Southern hybridization confirmed TY KG1 replication in drought and well-watered tomatoes at 14 dpi, but not in TY KG2.

Quantification of drought tolerance gene expression in TYLCV-infected hosts

To analyze the effect of TYLCV infection on drought stress, some drought-related genes were selected (*AOS1, JA2, ER5* and *NCED1*) for qPCR analysis (Arbona *et al.* 2020; He *et al.* 2018; Xiong *et al.* 2020; Zegzouti *et al.* 1997). In plant abiotic stress studies, commonly used housekeeping genes such as *GADPH* are not recommended because the expression level of *GADPH* is not stable between treatment conditions. Therefore, we used *SlACTIN7* which has a stable gene expression under different environmental conditions as a reference gene (Feng *et al.* 2019). Genetic analysis indicated that *AOS1*, a pivotal gene in the JA biosynthesis pathway, and genes associated with drought tolerance such as *ER5* and *JA2*, were pre-activated even when infected tomato plants were not exposed to drought. *NCED1*, which encodes a key enzyme in ABA biosynthesis, increased its expression in plants inoculated with TYLCV before drought stress was introduced; however, its expression level was reduced when plants experienced prolonged water deficit conditions (Fig. 8). In general, the expression level of drought-related genes in TY KG1 group was higher than that in the TY KG2 group. These results indicate that TYLCV infection primes the tomato plants for drought tolerance by altering plant gene expression and the two viral isolates have different effects on tomato plants.

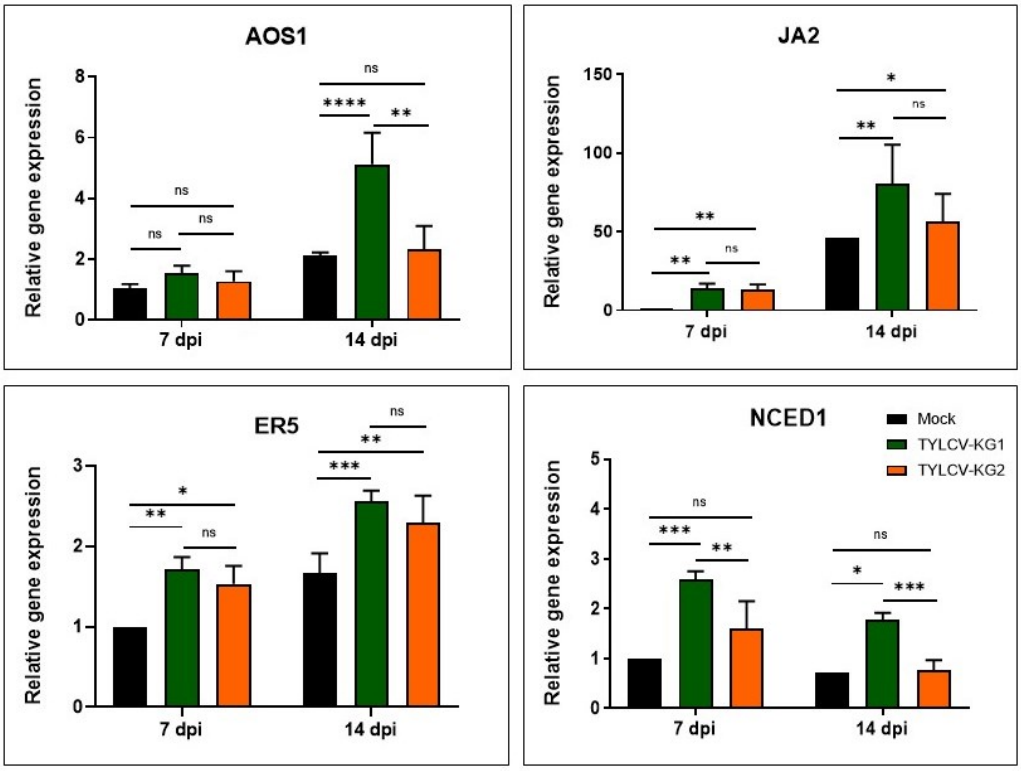

Figure 8. Quantitative real-time PCR of some drought-related genes with TYLCV-infected and mock-treatment groups before and after drought stress. The bar graphs indicate the mean ± SD ($n = 5$). The statistical comparison was performed by the two-way ANOVA test together with the Dunn's multiple comparisons test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant.

DISCUSSION

We used two TYLCV strains to test drought tolerance. The infection test showed that TY KG1 could induce typical symptoms such as yellowing, leaf curling, and stunted growth, whereas TY KG2 appeared to be non-virulent at early infection stages and showed milder symptoms at the late stages of plant growth. Moreover, these strains showed different effects in tomatoes grown in drought conditions.

Viruses are obligatory parasites that utilize host materials for their survival and replication and are normally believed to be harmful to the host. However, we observed that TY KG1 and TY KG2 infection slightly reduced the effects of drought stress on tomato plants, as evidenced by the delayed leaf wilting, lower water loss, higher water accumulation, and up-regulation of some drought-related genes. In addition, we found that under drought stress conditions, virus replication was remarkably decreased, which was contrary to other researches that reported drought conditions enhancing the susceptibility of plants to viruses (Atkinson *et al.* 2013; Kissoudis *et al.* 2015). The resistance to viral multiplication observed in this study might be a result of the production of reactive oxygen species, which can stimulate cell wall modifications (e.g., callose synthesis at the plasmodesmata), which could limit pathogen penetration (Alazem and Lin 2017).

Jasmonic acid (JA) is a plant hormone that accumulates in response to biotic and abiotic stresses (Golldack *et al.* 2014). JA is a central hormone in the plant defence system, and therefore, we examined its change of transcription level during the interaction between TYLCV infection and drought stress in tomato. Based on qPCR results with drought-related genes, we hypothesized that virus infection would lead to the induction of JA and activate NAC family transcriptional factors. Up-regulation of NAC transcriptional factors could activate genes related to ABA biosynthesis and accumulation, which is another well-known abiotic stress hormone. *Allene oxide synthase 1* (AOS1) is a key gene in the JA biosynthetic pathway. When exposed to viral infection, JA accumulates inside plants (Seo *et al.* 2018). We observed an increase in *AOS1* expression in response to TYLCV infection and water deficit conditions compared to that with the mock control, suggesting that JA plays an important role in both biotic and abiotic stresses. The up-regulation of JA activates NAC transcription factors such as *JA2* (Muñoz-Espinoza *et al.* 2015; Seo *et al.* 2018) which promotes stomatal closure (Du *et al.* 2014). In our study, we observed an increase in *JA2* transcription levels in response to TYLCV infection, indicating that it was induced during the early phase of virus-tomato interactions. After drought stress, the expression level of *JA2* was up-regulated in both inoculated and control groups but was more significant in TY KG1-infected plants. In addition to inducing stomatal closure, *JA2* plays a pivotal role in ABA biosynthesis because it selectively binds to the NAC core region of the nine-cis epoxycarotenoid dioxygenases (NCED1), which is a rate-limiting enzyme of ABA biosynthesis in tomato plants (Du *et al.* 2014). It was clear that *NCED1* expression was stimulated after the viral infection; however, after drought, the transcript level of *NCED1* showed a slight decrease in both healthy and infected groups. This can be explained by the negative feedback signalling used in ABA biosynthesis when plants face prolonged water deficit conditions (Long *et al.* 2019). During drought stress, ABA is highly expressed and leads to the closure of stomata to limit water loss and photosynthesis rate. However, to achieve the ABA homeostasis and physiological balance, the ABA concentration decreases over time and can drop even lower than that in the pre-drought mock group (due to intracellular damage caused by drought stress), leading to the down-regulation of ABA-related genes (Long *et al.* 2019; Muñoz-Espinoza *et al.* 2015).

The *ER5* is in the late embryogenesis abundant protein family, which increases the water-binding capacity by creating a protective environment for other proteins (Cohen and Leach 2019). It also protects the partner protein from degradation and proteinases that function to remove denatured and damaged proteins (Olvera-Carrillo *et al.* 2011). *ER5* is highly induced by drought stress and ABA (Zegzouti *et al.* 1997). In this study, we observed that before drought stress, the expression level of *ER5* in the two TYLCV-infected groups was higher than that in the mock group. After drought stress, in the mock-inoculated group, *ER5* was also upregulated, but the expression level was lower than that in the virus-infected groups, indicating a higher expression level of ABA in virus-infected plants.

Can all pathogens induce drought tolerance in plants? Results from many studies have shown that not all pathogens can perform this function. To enter the intercellular spaces of internal leaf tissues, most fungi directly penetrate the epidermis using special enzymes that degrade the cuticle layer and cell wall or with mechanical force (Melotto *et al.* 2008). Bacteria invade plants via natural openings such as stomata or wounds on the leaf, eventually creating holes on the leaf surface, which stimulates the water loss; hence, they are not a good candidate for enhancing drought tolerance (Zeng *et al.* 2010). Conversely, virus particles enter plants, insects and mites that feed on plants and cause little damage to the leaves themselves.

Our results showed that both TY KG1 and KG2 can induce drought tolerance in tomato plants even though TY KG2 titer was low in the first two weeks after virus inoculation. A high throughput sequencing method needs to be performed to elucidate the gene expression pattern of host plants when being infected by two strains of TYLCV. In here, we did not observe a significant difference in physiology between these two groups although the expression level of drought-related genes revealed TY KG1 as a better candidate for drought tolerance induction. The TY KG1 infection damaged tomato plants more severely than drought stress alone, suggesting that TY KG2 could be a better choice for inducing drought tolerance in tomatoes.

In the crop field, delaying drought-stress symptoms in plants for just a few days can be very significant to the plant's overall productivity. Thus, understanding the mechanisms by which plants respond to combined biotic and abiotic stresses can shed new light on some potential agricultural applications. The demand for plant crop production, on the one hand, and the effects of climate change, on the other, require the development of cultivars with multi-stress resistance, which cannot be obtained by producing single stress resistance traits in isolation. Here, we proposed an approach that exploits the use of viral infection to protect plants from drought stress, which is one of the most limiting factors for crop production worldwide. We show that TYLCV infection has a positive effect on drought tolerance by slowing the transpiration rate and inducing drought-related genes. Therefore, it may be possible to use symptomless TYLCV pre-inoculated tomatoes in low precipitation regions to reduce the negative effects of drought stress on crop yield.

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