Short communication

Development of a graft inoculation method and a real-time RT-PCR assay for monitoring *Tomato chlorosis virus* infection in tomato

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ABSTRACT

A graft inoculation method coupled with RT-qPCR was developed for monitoring ToCV infection in tomato plants. Ten seed-grown tomato seedlings were graft inoculated with phloem tissue-containing stem segments from a ToCV-infected tomato plants. Another group of tomato seedling were grafted with similar stem segments from a healthy tomato plant as mock inoculated control. The CP gene of ToCV was cloned under the control of T7 promoter and *in vitro* synthesized RNA was used as a standard for quantification. Total RNA was isolated from leaf samples of ToCV-inoculated and mock-inoculated control plants before the inoculation and 1–60 days post inoculation (dpi). The presence and the titer of ToCV were determined from all ToCV-inoculated or mock-inoculated control plants by RT-qPCR. After 15 dpi, ToCV was detected in 20–30% of graft-inoculated plants. The infection rate then increased progressively and reached to 70–80% by 60 dpi. Titer of ToCV was at the detectable level at 15 dpi and increased and reached to maximum level by 40 dpi and then started to decrease. The results showed that patch grafting is a simple and efficient method for experimental inoculation of ToCV and can be used as an alternative and/or complementary to vector transmission in the laboratories. The patch grafting could be combined with RT-qPCR and used for infecting and quantitative monitoring of ToCV or other phloem-limited viruses in tomato or in other plants.

Tomato chlorosis virus (ToCV) causing severe chlorosis is considered one of the recently-emerged viruses of tomato (Hanssen et al., 2010). ToCV has been spreading in tomato production areas of the world since it was first reported in 1998 in Florida (Wisler et al., 1998b). While ToCV has been detected and currently spreading in most tomato production regions of the world, ToCV is more widespread in the Mediterranean countries (Navas-Castillo et al., 2000) including Turkey (Çevik and Erkış, 2008; Yeşilyurt and Çevik, 2018). Transmission of ToCV occurs in a semi-persistent manner by whiteflies including *Bemisia tabaci*, *Trialeurodes vaporariorum* and *T. abutilonea*, in a semi-persistent manner in the nature (Wintermantel and Wisler, 2006).

ToCV is a phloem-limited virus belonging to the genus *Crinivirus* within the family *Closteroviridae* (Wisler et al., 1998b). ToCV has a positive sense, single-stranded, bipartite genome denoted as RNA1 and RNA2. The genome is encapsidated into flexuous virions of 850 to 850 nm in length (Wisler et al., 1998a). RNA1 contains four ORFs primarily encoding replication-associated proteins, while RNA 2 contains nine different ORFs encoding variety of functions largely involved in encapsidation, movement, vector transmission and pathogenicity of the virus (Wintermantel et al., 2005; Dolja et al., 2006). The sequences of

the CP gene as well as the heat shock protein homolog (hsp70 h) gene have been used for detection of ToCV as well as determining genetic diversity and phylogenetic analysis of ToCV isolates from different regions (Wintermantel and Wisler, 2006; Barbosa et al., 2008, 2013; Albuquerque et al., 2013; Gharsallah et al., 2015).

Significant progress has been made for the detection and molecular characterization of ToCV isolates (Wisler et al., 1998a; Louro et al., 2000; Dovas and Katis, 2002; Trenado et al., 2007; Kataya et al., 2008; Jacquemond et al., 2009; Papayiannis et al., 2006), However, pathogenesis and evolution of the virus, as well as virus-host interactions have not been explored largely due to the absence of a simple and efficient inoculation method. ToCV is not transmitted mechanically (Wisler et al., 1998a; Dovas and Katis, 2002) but it is transmitted naturally by whitefly vectors in semi-persistent manner (Wintermantel and Wisler, 2006) Whitefly transmission is a natural and efficient inoculation system and is generally used for experimental inoculation of ToCV (Dalmon et al., 2005; Orfanidou et al., 2016). However, the time and labor required for rearing, maintaining, and handling the whitefly colonies needed for transmission makes the process relatively difficult. Therefore, alternative transmission systems, such as, inter-stock

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grafting (Garcia-Cano et al., 2010) is commonly used for ToCV inoculation. The inter-stock grafting method requires detaching the stem of the plants and placing an inoculated stem piece between the rootstock and the scion. This process can be difficult, tedious, requires skills for efficient grafting and is not always suitable for routine inoculations. Therefore, development of a more simple and efficient experimental inoculation system is needed for routine experimental transmission of ToCV. In this study. a simple and efficient grafting method using small phloem containing stem tissue, namely patch grafting, was developed for experimental transmission of ToCV. The patch grafting method was combined with a sensitive RT-qPCR assay for developing an efficient inoculation and quantitative monitoring system for ToCV infection in tomato.

MoneyMaker tomato cultivar, sensitive to ToCV and widely used for maintaining ToCV isolates, was used as the host plant in this study. Seeds were germinated in plastic vials containing peat, perlite and vermiculite mixture in a controlled plant growth chamber at 25 °C and 70% relative humidity and with 16/8 h photoperiod. The seedlings were transplanted into 1 L pots containing the same mixture, and grown in the controlled plant growth chamber at the same conditions until stem diameter reached about 0.5–1 cm. AKSU8 isolate of ToCV previously recovered from a greenhouse-grown tomato plants in Antalya (Akdura, 2011; Yeşilyurt and Çevik, 2018), maintained in MoneyMaker tomato cultivar by periodic inter-stock graft inoculation (Garcia-Cano et al., 2010) was used and source of virus inoculums.

Detection and quantification of ToCV in the inoculated plants is important for testing the efficiency of inoculation as well as monitoring the titer of virus in the inoculated plants. Absolute quantification of an RNA virus as copy number requires the use of RNA standards containing the target region with known copy numbers. Therefore, 774 bp CP gene of ToCV was amplified by RT-PCR using specific primers, BC85 and BC86 (Table 1) and cloned into a pGEM-Teasy vector (Promega, USA) under the control T7 promoter according to the manufacturer's instructions. An approximately 1000 bp fragment spanning ToCV CP gene and pGEM-Teasy vector cloning region containing T7 promoter site was amplified by PCR using ExTaq DNA polymerase (Takara, Japan) and M13 forward (MSC35) and M13 reverse (MSC36) universal primers (Table 1) to obtain the template for RNA synthesis. RNA transcripts of ToCV CP gene was synthesized from 100 ng of the purified PCR product used as the liner DNA template by *in vitro* transcription kit using T7 RNA polymerase (Roche, Germany). The *in vitro* synthesized RNA was purified by RNAeasy mini kit (Qiagen, Germany) and treated with DNAase on RNAeasy column to eliminate any template DNA. A total of 1.3 μg RNA in 1.3 ng/μl concentration was obtained and serially diluted to contain 10^8 to 10^3 copies of the CP gene to generate standards for absolute quantification of ToCV in tomato. An 80 bp region of the CP gene was amplified from the RNA standards and two ToCV-infected source plants by RT-qPCR along with uninoculated control plants. While no amplification observed from the negative controls, targets were amplified from all standards and two samples from infected source plants (Fig. 3 A). The copy number of ToCV in two

infected source plants was determined as 10^6 and 10^3 (Fig. 3). Standard curve revealed a R^2 value of 0.9720 indicating that the RNA standards were reliably used for ToCV quantification (Fig. 3B). The difference in the virus titer in two source plants was similar to difference of Ct values of these two plants in the previous experiment. The results showed that standards generated for quantification is reliable for ToCV quantification in tomato plants.

Then, ten seed-grown tomato seedlings were inoculated by patch grafting with phloem tissue containing stem segments from a tomato plants infected with AKSU8 isolate of ToCV (Fig. 1). Another group of tomato plant were patch grafted with similar stem segments from healthy tomato plants and used as mock-inoculated controls. Total RNA was extracted from leaf samples collected from ToCV-inoculated and mock-inoculated control plants before the inoculation (0 day) and 1, 2, 4, 7, 10, 15, 20, 25, 30, 35, 40, 45, and 60 day(s) post inoculation (dpi) using the same procedure as described above. Initially, two biological replications of graft inoculation for monitoring ToCV inoculations were conducted. Once the inoculation efficiency was determined at each dpi, two more graft inoculations with 20 plants were performed as described above, but grafted plants were tested only at either 45 or 60 dpi in these experiments. Total RNA was extracted from leaves collected from ToCV-inoculated and mock-inoculated tomato plants as mentioned before using RNeasy Plant Kit (Qiagen, Germany). Total RNA was quantified by SmartSpec Plus spectrophotometer (Bio-Rad, USA) and 100 ng total RNA from each sample at each time point from ToCV-inoculated and mock-inoculated tomato plants was used for the RT-qPCR assay. The presence and the titer of ToCV was determined for all ToCV-inoculated or mock-inoculated control plants by RT-qPCR methods using primers (BC173 and BC174) and a TaqMan probe (BC175) specific to the CP gene of ToCV (Table 1). In addition, cytochrome oxidase (CyOx) gene of tomato, used as an internal control, was amplified by RT-qPCR methods using gene specific primers (BC179 and BC180) and a TaqMan probe (BC181) to the CP gene of ToCV (Table 1). The CP gene of ToCV and CyOx gene of tomato were amplified together from total RNA samples of tomato plants from four different graft inoculation experiments. RT-qPCR was performed in 20 μl reaction containing 2X Master mix (Invitrogen, USA), Superscript II reverse transcriptase, platinum *Taq* DNA polymerase enzyme mix provided in One Step Real-Time RT-PCR Kit (Invitrogen, USA), 100 ng total RNA, 200 pmole of gene specific primers, FAM and Cy5 labeled florogenic TaqMan probes specific to ToCV and CyOx gene targets, respectively (Papayiannis et al., 2006). The amplification was conducted in CFX96 Real-Time Detection System (Bio-Rad, USA) programmed as 30 min RT at 42 °C followed by 40 cycles of 10 s for denaturation at 94 °C, 10 s annealing at 55 °C and 10 s for extension at 72 °C. RNA standards described above were amplified along with tomato samples and used for absolute quantification of ToCV in the graft-inoculated samples.

The success or failure of initial ToCV infection was first detected by amplification of ToCV CP gene from the source plants by RT-qPCR as described above. The results showed that ToCV was detected from two different source plants. No amplification from the un-inoculated tomato

Table 1
The list of primers and probes used in RT-qPCR assay for detection and quantification of ToCV in tomato.

Code	Target	Sequence (5' to 3')	Type/orientation	Annealing Temp. (°C)	Amplicon size (bp)
BC 85	ToCV CP Gene	ATGGAGAACAGTGCTGTTGC	Primer/sense	57	774
BC 86		TTAGCAACC AGTTATCGATGC	Primer/antisense		
MSC 35	pGEM-Teasy	GTTTTCCCAGTCACGAC	Primer/sense	50	1021*
MSC 36	Vector	CAGGAAACAGCTATGAC	Primer/antisense		
BC173	ToCV CP Gene	TCTCGAACCTGCTTATGAAAAGAAA	Primer/sense	55	80
BC174		ATGCAAGTTGGTTAACGTTGTACAGT	Primer/antisense		
BC175		FAM-TTTGTGCAAGGGTAACGAGGGCAAGG-BHQ1	Probe/sense		
BC179	Tomato	TGGTAATTGGTCTGTTCCGATT	Primer/sense	55	87
BC180	mtCytOx gene	TGGAGGCAACAACCAGAATG	Primer/antisense		
BC181		CY5-ATAGGTGCGGCCTGACATGGCATTTCCACA-BHQ1	Probe/sense		

* Amplicon size with 774 bp ToCV CP gene cloned into the pGEM-Teasy plasmid vector.

Fig. 1. Patch graft inoculation of tomato seedlings with ToCV.

plants used as negative control and graft source for mock inoculation were observed. While one of the source plants was infected with ToCV with very high relative fluorescence units, RFU value (more than 2500 RFUs) and very low Ct value (7), the other source plants were infected with ToCV with relatively lower RFU value (less than 2000 RFUs) and Ct value (17) was relatively higher than the first source plant. The results showed that both source plants were infected with ToCV and the virus was readily detected from the source plant using RT-qPCR (Fig. 2A). The presence of ToCV in the source plants was also confirmed by amplification of about 774 bp fragment by RT-PCR endpoint assay using primers (BC86 and BC87, Table 1) specific to the CP gene.

To exclude the possibility of an unsuccessful RNA extraction leading to a false negative the cytochrome oxidase (CyOx) gene of tomato was also amplified as internal control using gene specific primers (BC179 and BC180) and a probe (BC181) (Table 1) along with ToCV CP gene. When viral CP target and CyOx internal control were amplified within the same cDNA preparation by RT-qPCR, both targets were simultaneously amplified from the ToCV-infected source plants. However, only plant CyOx gene was amplified from the healthy control plants. While the RFU value of ToCV CP gene was over 10^3, that of CyOx gene was only 10^2 in the RT-qPCR. Analysis revealed that the viral CP gene was amplified more efficiently than CyOx gene of tomato plants (Fig. 2B)
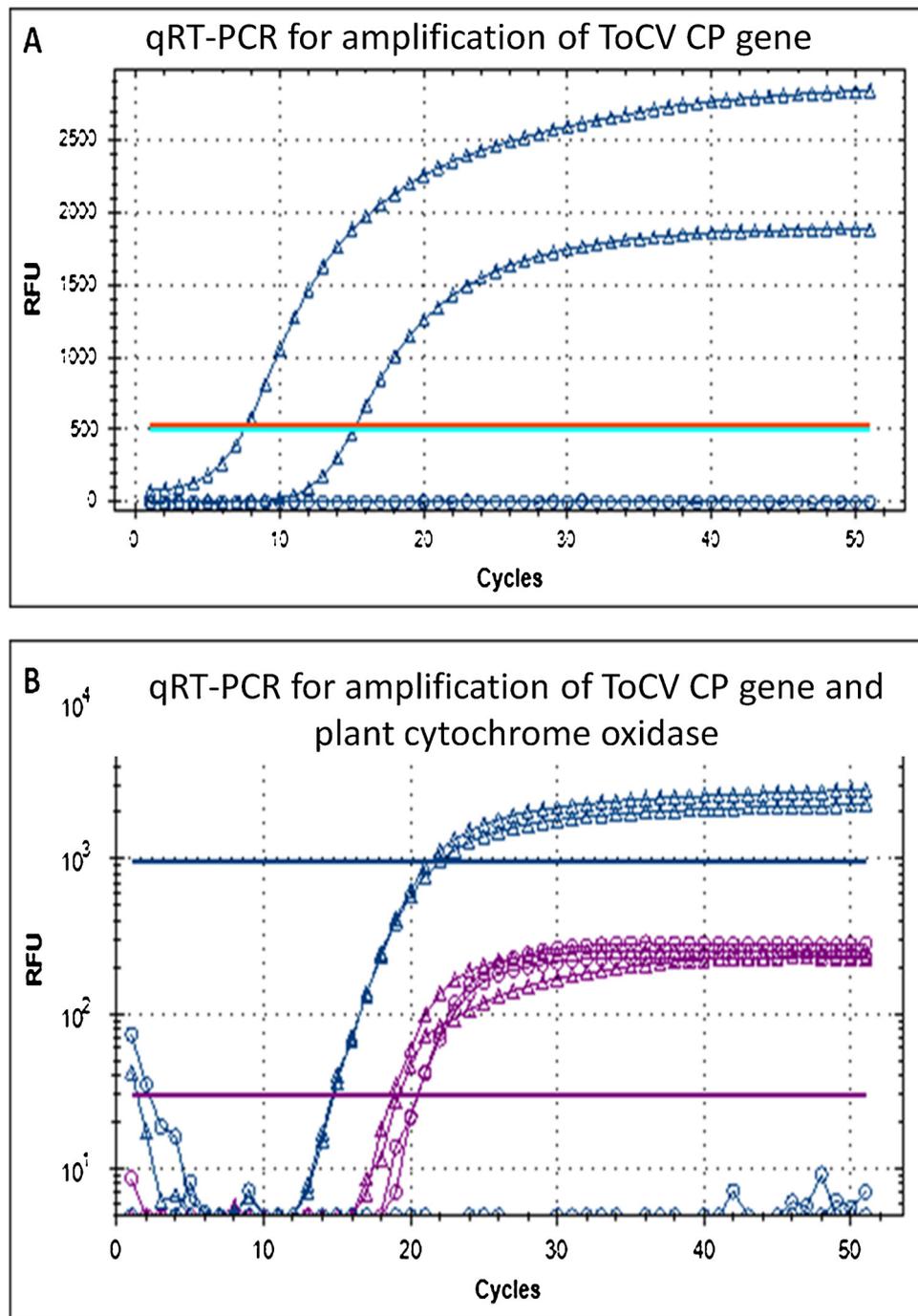
Fig. 2. Amplification of ToCV CP gene alone (A) and ToCV CP gene with cytochrome oxidase gene (B) in tomato by RT-qPCR.

indicating that virus titer was higher than that of CyOx. The results showed that ToCV CP gene and tomato CyOx gene are efficiently amplified by RT-qPCR and this assay could be used for testing ToCV inoculation with the CyOx as an internal control.

The efficiency of patch graft inoculation into ToCV-sensitive MoneyMaker tomato seedlings was determined by detection of ToCV in infected plants by RT-qPCR. ToCV CP gene was detected from total RNA of ToCV-inoculated seedlings and RNA standards with known copy number of ToCV CP gene to monitor ToCV infection in tomato plants. Samples from all patch graft-inoculated and mock-inoculated control plants were tested for the presence of ToCV at 0, 1, 2, 4, 7, 10, 15, 20, 25, 30, 35, 45 and 60 dpi. Since more than 96 samples were tested, they were divided into two groups as 0–20 dpi (Fig. 4A–C) and 21–60 dpi (Fig. 4D–F). RT-qPCR analysis showed that while plant internal control

CyOx gene was amplified in all tested samples, only 2–3 samples from 0 to 20 dpi were ToCV positive (Fig. 4). The results indicated that only 20–30% of inoculated plants were infected with ToCV in the 0–20 dpi; however, the number of ToCV positive samples increased as the time progressed and reached to 70–80% in 60 dpi. Similar results were observed in RT-qPCR analysis of two biological and two technical replications. The results of graft inoculation are summarized in Table 2. These results showed that the earliest ToCV infection was detected at 15 dpi and the infection rate increased as time progressed and more than 75% of graft inoculated plants were eventually infected with ToCV. The results also demonstrated that ToCV was readily transmitted by grafting in tomato and that ToCV was not detected until 15 days after inoculation (Table 2).

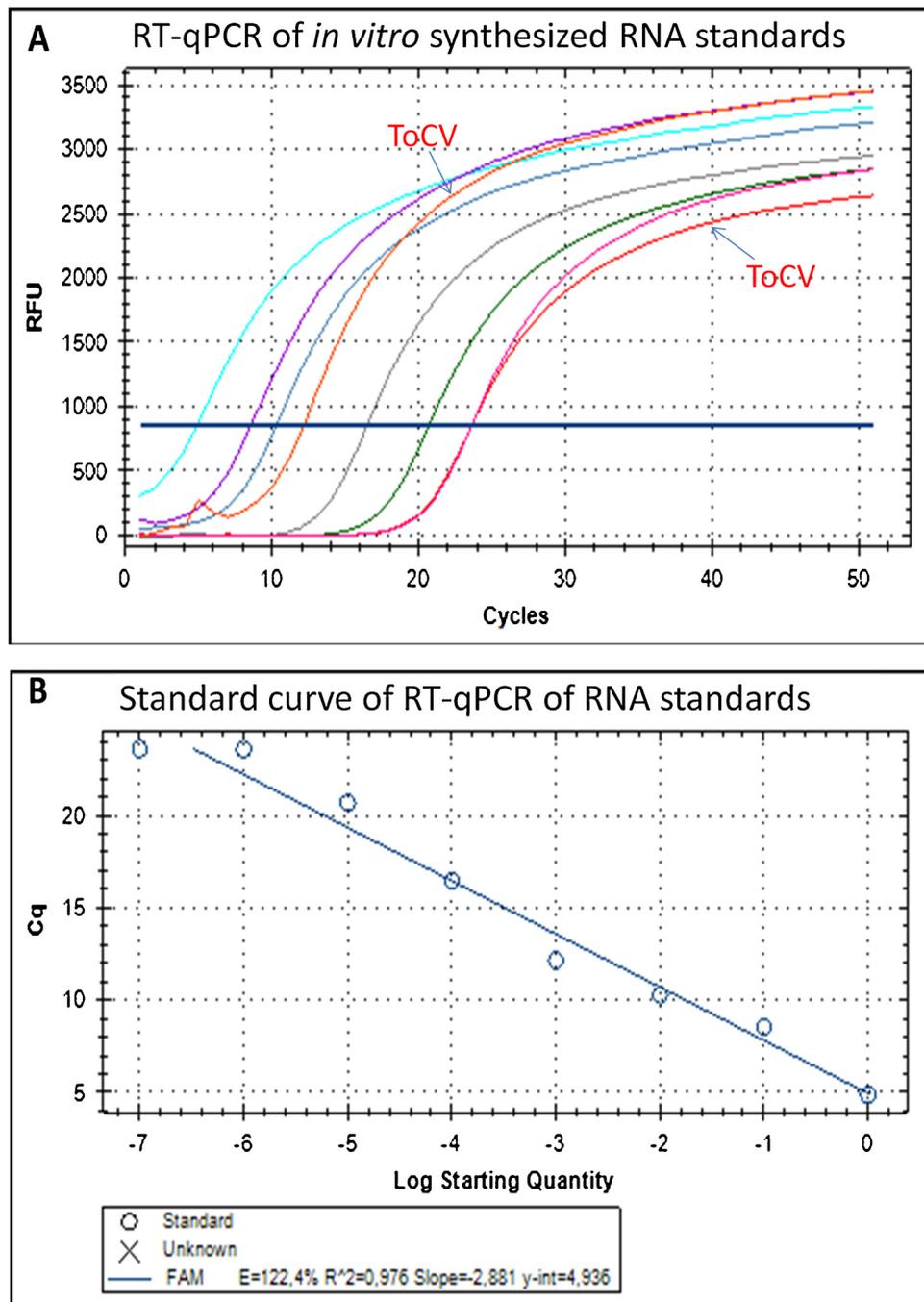The quantity of plant internal control CyOx gene was determined

Fig. 3. Amplification and standard curve analysis of RT-qPCR of *in vitro* synthesized RNA standards of the CP gene of ToCV.

using threshold cycle (Ct) values for all graft-inoculated plants. Quantity of ToCV CP target gene was determined using Ct values for only ToCV positive plants. The average Ct values of CyOx gene ranged from 18 ± 2 to 22 ± 2 for all graft-inoculated plants and it did not show significant change at each time point (Fig. 5A). In ToCV inoculated plants, the average Ct values of ToCV CP target gene ranged from 17 ± 2 to 22 ± 2. Ct values of ToCV infected plants were different and changes were significant at some time points (Fig. 5B). While Ct values were significantly lower at 20, 25, 30, 35 and 40 dpi indicating high virus titer, it was higher at 15 dpi; at the beginning of the infection and 45 and 60 dpi; close to the end of the infection period studied.

When the absolute quantity of the virus titer was determined as the average copy number in ToCV-infected plants, virus titer was significantly different at some time points. The average copy number of ToCV ranged from 5×10^5 at 60 dpi, to 5×10^8 at 40 dpi in ToCV positive plants. The titer was at detectable level at 15 dpi and it increased by 40 dpi and then decreased thereafter. Differences in virus titer was not significant among 15, 45 and 60 dpi with lower values, and among 20, 25, 30, 35 and 40 dpi with higher values. However, differences between these two groups were statistically significant. The results showed that virus titer steadily increased from 15 to 40 dpi, then decreased (Fig. 5C). The RT-qPCR results also demonstrated that the virus titer was reached to the maximum level 40 days after inoculation.RT-qPCR results were analyzed and displayed in graphical and tabular format using CFX Manager program (Bio-Rad, USA). The RT-qPCR data was used for calculation of graft inoculation efficiency, which was determined based on the number and percentage of
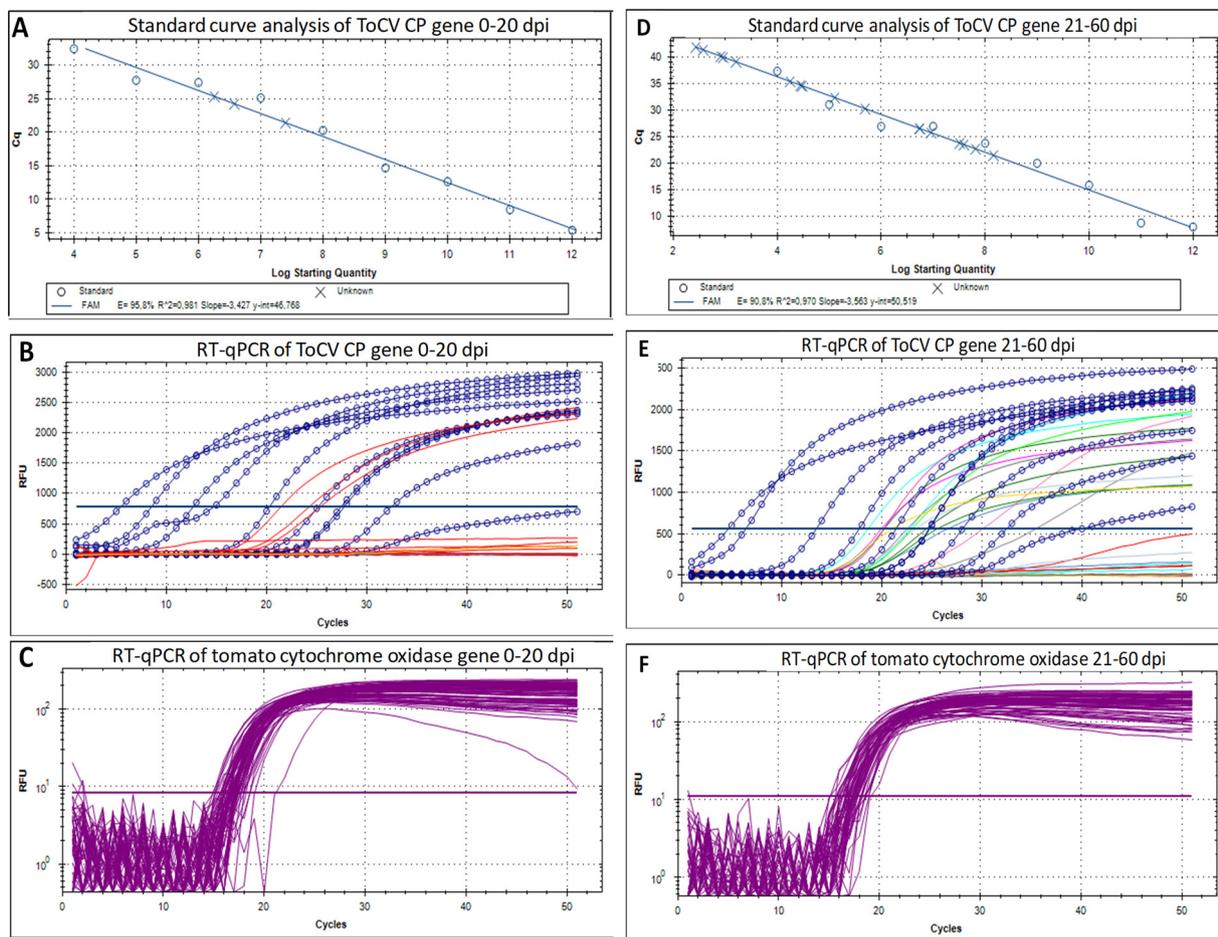
Fig. 4. Detection of ToCV CP gene and tomato cytochrome oxidase gene in graft inoculated tomato plants by RT-qPCR assay. **A–C**: Detection and quantification of ToCV CP gene and tomato cytocrome oxidase gene in graft inoculated tomato plants at 0–20 dpi **D–F**: Detection and quantification of ToCV CP gene and tomato cytochrome oxidase gene in graft inoculated tomato plants at 21–60 dpi.

successful ToCV inoculations in all graft inoculated plants at each time points for each biological replication. The average inoculation efficiency was calculated as number and percentage at each time point for all biological replications. The kinetic RT-qPCR data was used for determination of threshold cycle (Ct) values for CyOx internal control and ToCV CP genes for the virus in all ToCV positive graft-inoculated plants. Then, average Ct values for ToCV CP genes were calculated in ToCV infected plants and results were graphically displayed. Finally, the absolute quantity of virus titer as copy number was determined in each ToCV-infected plant at each time point for each biological replication using the serial dilution of the RNA standards with known copy number of the CP gene of ToCV. Graft-inoculated, ToCV negative plants were not included in the quantification analysis. The average virus titer was calculated for all graft-inoculated plants at each time point for all technical and biological replications. The average virus titer was graphically displayed for each time point.

Table 2
Summary of ToCV infection efficiency in different graft inoculation experiments.

| Sampling time	1st Experiment		2nd Experiment		3rd Experiment		4th Experiment		Average	
	ToCV + /Grafted		ToCV + /Grafted		ToCV + /Grafted		ToCV + /Grafted		ToCV + /Grafted	
	Number	%	Number	%	Number	%	Number	%	Number	%
0 dpi	0/10	0 %	0/10	0 %	0/10	0 %	0/10	0 %	0/40	0 %
1 dpi	0/10	0 %	0/10	0 %	NT	NT	NT	NT	0/10	0 %
2 dpi	0/10	0 %	0/10	0 %	NT	NT	NT	NT	0/10	0 %
4 dpi	0/10	0 %	0/10	0 %	NT	NT	NT	NT	0/10	0 %
7 dpi	0/10	0 %	0/10	10 %	NT	NT	NT	NT	0/10	0 %
15 dpi	1/10	10 %	2/10	20 %	NT	NT	NT	NT	3/20	15%
20 dpi	2/10	20 %	3/10	30 %	NT	NT	NT	NT	5/20	25 %
25 dpi	3/10	30 %	4/10	50 %	NT	NT	NT	NT	7/20	35 %
30 dpi	5/10	50 %	4/10	60%	NT	NT	NT	NT	9/20	45%
35 dpi	6/10	60%	6/10	70 %	NT	NT	NT	NT	12/20	60%
40 dpi	7/10	70 %	7/10	70 %	NT	NT	NT	NT	14/20	70 %
45 dpi	7/10	70 %	8/10	70 %	13/20	65 %	NT	NT	28/40	68.3 %
60 dpi	7/10	70 %	8/10	75 %	NT	NT	16/20	80%	31/40	77.5 %
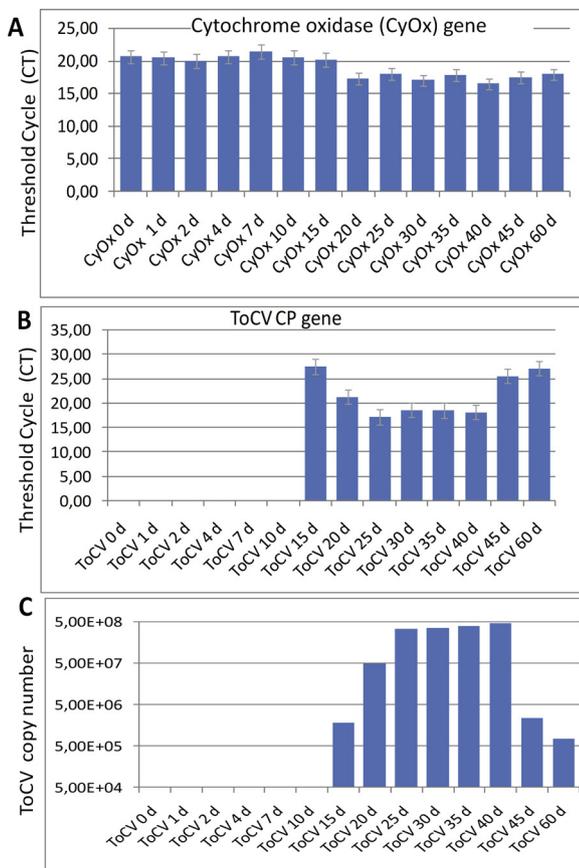
Fig. 5. Temporal expression analysis of cytochrome oxidase and ToCV CP genes and copy number determination of ToCV based on the CP gene by RT-qPCR analysis in tomato plants graft inoculated with ToCV. **A)** Expression level of ToCV CP gene **B)** Expression level tomato cytochrome oxidase **C)** Copy number of ToCV based on the CP gene standards.

Currently studies on ToCV are largely limited to detection and identification isolates in various regions mainly due to the absence of a simple and efficient experimental inoculation system. This limitation significantly hinders the progress on pathogenesis and evolution of ToCV as well as virus-host interaction and resistance studies. An infectious clone of ToCV was developed to overcome this limitation (Orílio et al., 2014). However, the infectious clone was not very efficient for inoculation of the natural host, tomato plants, and was only effective for tobacco plants and for tobacco and tomato protoplasts (Orílio et al., 2014). Therefore, development of this infections clone was not able to overcome difficulties associated with ToCV. Thus, experimental studies related with understanding the mechanism of disease development process and exploring the resistance against ToCV still depends solely on whitefly transmission. Whiteflies are the natural vectors of the virus and whitefly transmission was has been used for studying several parameters related to ToCV (Wisler et al., 1998a, b; Wintermantel and Wisler, 2006; Dalmon et al., 2005; Garcia-Cano et al., 2010; Orfanidou et al., 2016), However, rearing, maintaining, and handling the whitefly colonies can be difficult, time consuming and requires valuable resources and labor (Polston and Capobianco, 2013). In addition, whitefly transmission can be challenging due to experimental variation caused by gender, age, number or feeding habit of whitefly (Lapidot, 2007). Furthermore, acquisition feeding time and transmission feeding time is difficult to control and insect collection method such as aspiration and release procedures may damage whiteflies and adversely affect virus transmission. Finally, virus titer in an individual whitefly or a population of whitefly may differ and difficult to control (Lapidot, 2007; Polston and Capobianco, 2013). Therefore,

development of a simple, rapid, more efficient and controllable experimental transmission system is needed to facilitate and accelerate studies requiring ToCV inoculations. Thus, we have developed a simple and efficient patch grafting method for ToCV inoculation coupled with a RT-qPCR detection assay for quantitatively monitoring ToCV infection process in tomato plants.

The results of graft inoculation showed that ToCV was readily transmitted by patch grafting in tomato and average transmission efficiency was 20–75% starting from 15 dpi to 60 dpi. This transmission efficiency was higher than vector transmission with single *T. vaporiaroum* and was comparable with vector transmission with 40–60 individual *T. vaporioroum* (Dalmon et al., 2005). In addition, ToCV transmission efficiency was higher than vector transmission with single *B. tabaci* and it was comparable with vector transmission with 40 individual *B. tabaci* (Wisler et al., 1998a, b). Furthermore, transmission efficiency was higher than inter-stock grafting method commonly used for ToCV (Garcia-Cano et al., 2010) or other graft transmissible viruses (Galipienso et al., 2000; Matic et al., 2010; Milosevic et al., 2011). The simplicity and comparable transmission efficiency of patch grafting reported in this study demonstrated that it could be an alternative and/ or complementary experimental system to currently used inoculation methods.

Monitoring virus infection in plants depends on the time of detection after inoculation. The time of detection is affected by efficiency of transmission as well as titer of the virus in the source plants. It was reported that ToCV was detected 4–8 weeks after inter-stock graft inoculation in which a 1–3 cm of infected inter-stock was used as inoculum (Garcia-Cano et al., 2010). Similarly, ToCV was detected by ELISA 4–6 weeks after whitefly transmission in which whiteflies fed on entire ToCV-infected plants were used as source of inoculum. In patch graft inoculation used in this study, ToCV was detected as early as 15 dpi by RT-qPCR. The results suggested that ToCV infection process is completed between 15 and 40 dpi. Considering the size of tissue used for graft inoculation, the patch grafting method reported here is not only an efficient method, but also enable detection of ToCV infection two weeks earlier than other transmission methods when it combined with RT-qPCR.

Quantification of virus titer is important for monitoring virus infection process as well as host response to virus infection. Therefore, we have developed an absolute quantification method for ToCV inoculated plants. We have cloned the CP gene of ToCV and synthesized full-length CP transcript by *in vitro* transcription to be used as standards with known copy numbers of ToCV CP gene for determination of virus titer. The copy number of the *in vitro* synthesized ToCV CP RNA was calculated as previously described (Ruiz-Ruiz et al., 2009; Debreczeni et al., 2011; Pacifico et al., 2011; Ferriol et al., 2011) and standards ranging from 10^3 to 10^8 copies of the CP gene were used as RNA standards for quantification of ToCV in inoculated tomato plants. Detection of as little as 10^3 virus copies by the RT-qPCR assay and regression analysis with R scores of 0.98-0.99 indicated that the standards were suitable for detection and reliable quantification of ToCV in tomato plants. The detection limit of the RT-qPCR assay and the virus titer of the standards were correlated with similar results from previous studies (Ruiz-Ruiz et al., 2009; Debreczeni et al., 2011; Pacifico et al., 2011; Ferriol et al., 2011) with other viruses.

Monitoring virus infections within the host plant is important for determining viral titer, disease development process, testing for resistance, transmission and virus plant-virus interaction at different stages. Although grafting, as inter-stock grafting, (Garcia-Cano et al., 2010 and RT-qPCR (Papayiannis et al., 2011) were previously used independently for inoculation and detection of ToCV, respectively, we have modified and combined two methods for developing an alternative experimental system for monitoring ToCV infection in tomato. This system can be used for qualitative and quantitative assays for determination of virus titer at different stages of infection and accumulation of virus in different tissue or organs. The spread of virus
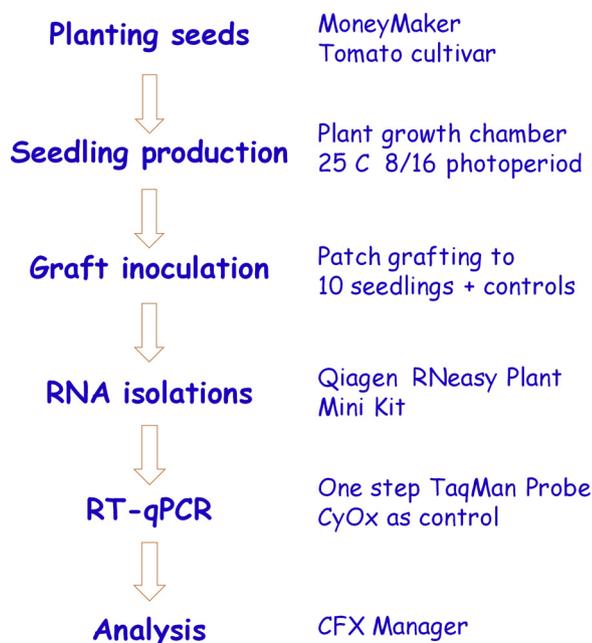
Fig. 6. Flowchart of monitoring ToCV inoculation in tomato seedling by patch graft. inoculation and RT-qPCR assay.

within a ToCV-infected plant in tomato or other newly described host, as well as similarly transmitted viruses including but not limited to other *Criniviruses* can be monitored using this method. In addition, combination of patch graft inoculation and RT-qPCR methods reported here could be used for studying virus-host interactions, including testing host resistance, host response at different stages of infection as well as host response to different virus titer (Fig. 6). ToCV infection started at 15 dpi and the virus titer was gradually increased until 40 dpi then it was started to drop. Based on this data, when the patch graft inoculation reported in this study is used for studying virus host-interaction analysis should be done between 20 and 40 dpi. We also used patch graft inoculation for testing different commercial tomato varieties to ToCV infection and determination of tomato's response to ToCV infection by suppression subtractive hybridization at 20 and 30 dpi. Since graft inoculation provides continuous exposure of a test plant to high levels of viral inoculum (Friedmann et al.,1998; Lapidot, 2007) and the patch grafting might be used for screening for resistance to ToCV in tomato or other hosts. The results reported here regarding the development of ToCV inoculation and monitoring method and its application to study virus-tomato interaction showed that it could be used as an efficient alternative and/or complementary experimental inoculation system to the currently used methods for ToCV and similar viruses.

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