Transmission of *Tomato chlorosis virus* (ToCV) by *Bemisia tabaci* Biotype Q and Evaluation of Four Weed Species as Viral Sources

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Abstract

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Tomato chlorosis virus (ToCV) is implicated in tomato yellows disease in many countries worldwide. It has a wide host range, including cultivated species as well as arable weeds, and it is transmitted in a semipersistent manner by at least five whitefly species or biotypes of the genera *Trialeurodes* and *Bemisia*. ToCV is not seed transmitted and more than 36 weed species have been recorded as natural reservoirs, acting as unique sources both for the virus and its vectors when susceptible crops are harvested. In this study, experiments were conducted to determine the transmission parameters of ToCV by biotype Q, the most abundant biotype of *Bemisia tabaci* in Greece. Results showed that biotype Q is an efficient

vector of ToCV and it is able to retain the virus for at least 6 days. This vector was then used for the evaluation of four widespread weed species (*Solanum nigrum*, *Sonchus oleraceus*, *Amaranthus retroflexus*, and *Chenopodium album*) as ToCV sources through transmission experiments. *Solanum nigrum* was shown to be the most significant viral source among the tested weeds, followed by *Sonchus oleraceus*, *A. retroflexus*, and, lastly, *C. album*. Nevertheless, none of them was as efficient a ToCV source as tomato. This variation could be attributed to differences in virus concentration in each plant species or possible host preference by the whitefly vector.

Tomato chlorosis virus (ToCV; genus *Crinivirus*, family *Closteroviridae*), along with *Tomato infectious chlorosis virus*, are the cause of Tomato yellows disease (TYD), a serious disease of tomato crops in more than 20 countries (Al Saleh et al. 2014; Dovas et al. 2002; Navas-Castillo et al. 2011; Wisler et al. 1998; Zhao et al. 2013). TYD induces interveinal yellowing and brittleness of leaves that initially appear on lower parts of the plant and gradually move toward upper leaves. Even though both viruses infect tomato crops in Greece, in the last decade, ToCV has expanded significantly and is responsible for serious epidemics, especially in the southern part of the country (Dovas et al. 2002; Orfanidou et al. 2014).

ToCV is transmitted semipersistently by at least five different whitefly species or biotypes: *Bemisia tabaci* biotypes A, B (Wintermantel and Wisler 2006), and Q (Navas-Castillo et al. 2000); *Trialeurodes vaporariorum*; and *T. abutilonea* (Wintermantel and Wisler 2006). Whitefly species or biotypes differ in their ability to transmit ToCV. Thus, *T. abutilonea* and *B. tabaci* biotype B are very efficient vectors, whereas *B. tabaci* biotype A and *T. vaporariorum* are inefficient vectors (Wintermantel and Wisler 2006). Moreover, ToCV persists in *T. abutilonea* up to 5 days, in *B. tabaci* biotype B 2 days, and in *B. tabaci* biotype A and *T. vaporariorum* only 1 day (Wintermantel and Wisler 2006).

B. tabaci is a complex whitefly species and, thus far, more than 24 morphologically indistinguishable biotypes exist (De Barro et al. 2011). They differ in several aspects such as geographical distribution, chemical resistance profile, host plant adaptivity, virus transmission efficiency, ability to induce phytotoxic disorders, and composition of endosymbiotic bacteria (Chiel et al. 2007; Perring 2001). *B. tabaci* biotype Q was observed almost 20 years ago in the Mediterranean basin (Guirao et al. 1997) and shows an impressive capacity to displace its

competitors (Brown 2007). Today, it has a worldwide distribution, including Greece (De Barro et al. 2011; Roditakis et al. 2009), where it prevails over biotype B (Orfanidou et al. 2014). Even though it has been reported that biotype Q transmits ToCV (Navas-Castillo et al. 2000), transmission parameters such as efficiency and persistence of the vector have not yet been determined.

ToCV has a rather wide host range which includes at least five cultivated crop species such as tomato (*Solanum lycopersicum*) (Wisler et al. 1998), sweet pepper (*Capsicum annuum*) (Lozano et al. 2004), potato (*S. tuberosum*) (Fortes and Navas-Castillo 2012), lettuce (*Lactuca sativa*) (Orfanidou et al. 2014), and tobacco (*Nicotiana tabacum*) (Fiallo-Olivé et al. 2014). In addition, more than 36 wild plant species from 19 different families (Alvarez-Ruiz et al. 2007; Arruabarrena et al. 2015; Fonseca et al. 2013; Font et al. 2004; Kil et al. 2015; Orfanidou et al. 2014; Solórzano-Morales et al. 2011; Trenado et al. 2007; Tsai et al. 2004) were found to be infected with ToCV, while it is able to infect 21 additional plant species (eight families) under experimental conditions (Morris et al. 2006; Wintermantel and Wisler 2006; Wisler et al. 1998).

It is well known that weeds, as important components of crop ecosystems, often are hosts for plant viruses and their arthropod vectors (Chatzivassiliou et al. 2007; Chen et al. 2013). Extensive research has revealed virus infection in a wide range of wild plant hosts in nature (Barreto et al. 2013; Chatzivassiliou et al. 2007; Duffus 1971; Seal et al. 2006; Thresh 1981; Webster et al. 2007). In the case of ToCV, weeds near or within tomato crops can provide alternative sources for whitefly vectors and for the virus between cultivating seasons (Tzanetakis et al. 2013). Therefore, the ability of ToCV to infect a large number of wild plant species in combination with the presence of whitefly vectors on these plants raises questions about the role of these weeds in ToCV epidemiology.

In the present study, in order to shed more light on ToCV epidemiology, we studied its transmission by the widely distributed *B. tabaci* biotype Q and then we used this vector to further investigate the role of four weed species (*S. nigrum*, *Sonchus oleraceus*, *Amaranthus retroflexus*, and *Chenopodium album*), known ToCV hosts under natural conditions (Orfanidou et al. 2014) and commonly found in tomato crops in Greece (I. Eleftherohorinos, personal communication) as virus sources.

Materials and Methods

Whitefly colony. *B. tabaci* biotype Q colony (kindly provided by A. Tsagarakou, Institute of Crop Protection, NAGREF, Heraklion)

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

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was maintained on cucumber (*Cucumis sativus* L.) plants in insect-proof cages in isolated growth chambers at temperatures ranging from 22 to 25°C. Identity of biotype status of the population in rearing was confirmed by determining the sequence of the cytochrome oxidase I mitochondrial gene (Tsagkarakou et al. 2007). No attempt was made to balance the sex ratio of insects used in the transmission experiments of this study.

Virus isolate. The isolate ToCV/Rh1835 (GenBank accession numbers HG380087 and HG380092) originated from a naturally infected tomato plant collected in 2012 from a greenhouse in Mandriko (Rhodes Island) and it was maintained in an insect-proof cage by serial passages onto tomato plants (*Solanum lycopersicum* L., hybrid Belladonna) using biotype Q.

Transmission parameters of *B. tabaci* biotype Q. ToCV-infected tomato plants (hybrid Belladonna) were used as viral sources 3 weeks postinoculation (wpi). Noninfected tomato plants (hybrid Belladonna) were also grown as negative controls. All tomato plants were infected in the fourth true leaf stage. Ten groups of 5, 10, 15, 25, 40, and 50 adult whiteflies were given 24- and 48-h acquisition access periods (AAP) while the inoculation access period (IAP) was 48 h in all experiments. Then, clip cages were removed and, at 1 wpi, the leaf on which the whiteflies fed was removed in order to prevent possible maturing of whitefly nymphs. Plants were transferred to different cages, where they were grown for 4 weeks.

In order to determine the retention period of ToCV by biotype Q, 50 adult whiteflies were given a 24-h AAP on five infected tomato plants. Whitefly density was selected to ascertain maximum transmission efficiency level that could be achieved by the 24-h AAP (Fig. 1). Whiteflies were then allowed to serially feed on the healthy plants: each batch of plants was changed every day for up to the seventh day. At 2 days postinoculation (dpi), clip cages were removed and plants were transferred to the greenhouse, treated with insecticide (imidacloprid), and observed for symptom development.

At 3 wpi, total RNA was extracted from all plants by using a modification of the in-house extraction protocol A developed by Chatzinasiou et al. (2010). In detail, plant tissue was grounded 1/10 (wt/vol) in lysis buffer (8 M GuHCl, 25 mM EDTA, 1% Sarcosyl, 2% Triton X-100, 25 mM sodium citrate, and 0.2 M sodium acetate, with pH adjusted to 5.2 with acetic acid). The lysate was centrifuged at $16,000 \times g$ for 10 min and 500 μ l of the upper phase was transferred to a new tube and mixed with 625 μ l of absolute ethanol. Then, the mixture was passed through a silica column (FT-2.0 Filter-Tube Spin-Column System; G. Kisker GbR) by centrifugation at $4,000 \times g$ for 3 min. The column was washed once with 700 μ l of wash buffer 1 (4 M GuHCl, 25 mM Tris-HCl [pH 6.6], and 60% ethanol) and twice (700 and 400 μ l, respectively) with wash buffer 2 (2 mM Tris-HCl [pH 7.0], 20 mM NaCl, and 80% ethanol) by centrifugation at $5,000 \times g$ for 3 min. RNA was finally recovered in 80 μ l of preheated (80°C)

nuclease-free elution buffer (10 mM Tris-HCl, pH 8.0). Two microliters of total RNA were used in nested RT-PCR as described by Dovas et al. (2002).

Evaluation of weeds as sources of ToCV. Four weed species (namely, *Solanum nigrum*, *Sonchus oleraceus*, *A. retroflexus*, and *Chenopodium album*; Herbiseed) were used in the experiments including the main host of the virus, tomato (hybrid Belladonna). All plants were inoculated at the fourth leaf stage and were used as virus sources 3 wpi. In order to obtain a high number of infected weeds for the oncoming experiments, a large number of whiteflies was used (40 adults/plant) in 48 h of AAP and IAP (10 plants/replicate, five replicates in total). At 2 dpi, clip cages were removed and, at 1 wpi, the leaf on which the whiteflies fed was also removed. Plants were transferred to different cages, where they were grown for 6 weeks at temperatures of 22 to 25°C. Noninfected tomato and weed plants were also grown as negative controls.

In order to estimate the titer of ToCV in plant tissues of weeds and tomato, an absolute quantification was conducted using a Taq-Man reverse-transcription polymerase chain reaction (RT-PCR) (Papayiannis et al. 2011). For this purpose, the quantification limits of the assay were determined herein using a standard curve that was developed based on serial dilutions of in vitro synthesized RNA transcripts. More specifically, a 944-bp fragment of the coat protein gene was amplified using the primer set TOC UP (5'-ATTCTTGAGGT GTTTGAATCTTTTAG-3') and TOC DO (5'-GCTAAATCAAC GGGAAACACATC-3') and subsequently cloned into the pDrive Cloning Vector (Qiagen). The recombinant plasmid was purified using the NucleoSpin Plasmid kit (Macherey-Nagel), linearized using the restriction enzyme *Pst*I (New England Biolabs), and gel purified using the NucleoTrap extraction II kit (Macherey-Nagel). SP6 RNA polymerase (New England Biolabs) was used for production of the RNA transcripts, which were treated with DNase I (RNase-free; New England Biolabs). The residual RNA was purified according to the RNA extraction method described above and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies). Ten-fold serial dilutions (10^8 down to 10 transcripts/ μ l) were prepared and, finally, 2 μ l from each dilution was used in Taq-Man RT-PCR in three replicates for the determination of the amplification efficiency, the dynamic range of quantification, and the detection limit of the assay.

Briefly, Taq-Man RT-PCR was performed in a Stratagene Mx3005p qPCR System in a 25- μ l reaction mix containing 10 $\times$ buffer consisting of F-517 Optimized Detergent-free DyNAzyme EXT Buffer (50 mM Tris-HCl, 1.5 mM MgCl₂, and 15 mM (NH₄)₂SO₄) (Thermo Scientific) 0.2 mM each dNTP, 4 mM dithiothreitol, 1 U of Superscript III RNaseH⁻ reverse transcription (Invitrogen-Life Technologies), 1.5 mM additional MgSO₄ (New England Biolabs), 3 U of HotStartTaq DNA polymerase (Qiagen), 0.3 μ M each primer (ToCVF/ToCVR), and 0.1 μ M TaqMan probe ToCVTAQ (Papayiannis et al. 2011). The thermal cycling conditions were 50°C for 30 min, followed by 95°C for 15 min and 50 cycles in two steps: 30 s at 95°C and 30 s at 60°C. Fluorescence levels were measured at the end of each cycle and the analysis of fluorescence data was conducted using MxPro-Mx3005P software (version 4.00; Agilent Technologies).

In order to test for the putative presence of RNA extraction inhibitors in the plant tissue of weeds, we performed a total RNA isolation using extract from healthy plant tissue from each weed species as well as from tomato, in which a known amount (10^4) of ToCV RNA copies had been added. The extraction and Taq-Man RT-PCR were done in three replicates/sample according to the protocols described above. Each treatment (2 μ l) was added to the RT-PCR.

For the evaluation of virus concentration in ToCV-infected weeds and tomato, total RNA was extracted from 0.3 g of a pool of tissue which was cut from the tip of the leaves from the fourth to the eighth leaf stage, according to the protocol described above. RNA concentration was calculated using a NanoDrop spectrophotometer and was adjusted to 30 ng/ μ l. The concentration was used at 2 μ l/sample in real-time Taq-Man RT-PCR. Two replicates per ToCV-infected sample were included in the analysis as well as two replicates from one healthy tomato, one healthy plant of each of the four weed species tested, and, finally, two replicates of nontemplate (reaction mix)

Fig. 1. Transmission efficiency of *Tomato chlorosis virus* by biotype Q using 24-h (white bars) and 48-h (gray bars) acquisition access periods (AAP) and different number of whiteflies (5, 10, 15, 25, 40, and 50) among the two AAP. Error bars indicate the standard deviation.

control. ToCV-infected weeds and tomato plants showing similar cycle threshold (Ct) values were selected as virus sources (Supplementary Table S1) for transmission to tomato. In order to reveal possible differences in transmission, it was necessary to use parameters that would give transmission efficiency to a medium level. Therefore, 15 adult whiteflies were used per plant (10 plants/replicate, five replicates in total) in a 48-h AAP and 48-h IAP.

The titer of ToCV was estimated again on the four weed species at 45 dpi. Total RNA was extracted at that time from the plants of the first replicate and real time RT-PCR (Papayiannis et al. 2011) was used for calculation of the titer. In all replicates, weeds and tomato plants were examined and recorded for yellowing symptoms for 6 wpi.

Data analysis. Statistical analysis was conducted by one-way analysis of variance and Tukey's honestly significant difference was employed to compare the mean values at $P \leq 0.05$ using the statistical package SPSS (version 17.0; SPSS, Inc.). In the case of evaluation of two AAP in transmission efficiency, an independent samples *t* test was used.

Results

Transmission efficiency of ToCV by biotype Q. Efficiency of transmission was statistically different ($P \leq 0.05$) between 24- and 48-h AAP. In 24-h AAP experiments, biotype Q was unable to transmit ToCV when 5 and 10 adults/plant were used and transmission efficiency was low using 15 and 25 adults (Fig. 1). However, when 50 whiteflies were fed on tomato plants, transmission efficiency was 100% (Fig. 1). When AAP was raised to 48 h, transmission efficiency was significantly higher ($P \leq 0.05$) in both low and high whitefly densities, and it reached 100% using 40 whiteflies/plant (Fig. 1). In both experiments, transmission efficiency had a positive correlation with the number of whiteflies used (Fig. 1).

Persistence of ToCV in the whitefly vector. ToCV persisted in *B. tabaci* biotype Q for at least 6 days (Fig. 2). Transmission efficiency ranged from 96 to 98% within the first 48 h following virus acquisition (Fig. 2) and remained high up to 4 days postacquisition (76%). Then, efficiency decreased rapidly by the fifth day (28%) ($P \leq 0.05$) and no transmission was observed 7 days postacquisition (Fig. 2).

Evaluation of weeds as sources of ToCV. The Taq-Man RT-PCR amplification efficiency of the optimized assay was 98.1%, revealing optimal amplification conditions, while the linear range of quantification was from 20 to 2×10^8 copies (Fig. 3). Therefore, 20 copies, corresponding to a Ct value of 43.3, was set as the limit of positive ToCV detection.

Furthermore, no significant differences were observed on the Ct values (34 to 36) obtained from the RNA extracts originating from healthy *Solanum nigrum*, *Sonchus oleraceus*, *A. retroflexus*, *C. album*, and tomato plants in which a known quantity of ToCV RNA transcripts was added (data not shown), thus indicating that the plant species probably is not affecting the recovery of RNA. Transmission efficiency of ToCV from tomato to weed species varied ($P \leq 0.05$) (Fig. 4), with *Solanum nigrum* reaching 96%, followed by *Sonchus oleraceus* (86%), *A. retroflexus* (68%), and *C. album* (56%). As expected, all tomato plants were infected (Fig. 4). All ToCV-infected weed species revealed higher Ct values (thus, lower virus concentration) when compared with tomato plants (Table 1). In tomato, Ct values ranged from 21.54 to 26.23 (Table 1), whereas they were 24.07 to 41.99 in *Solanum nigrum*, 29.37 to 41.55 in *A. retroflexus*, 21.19 to 42.55 in *Sonchus oleraceus*, and 29.87 to 42.55 in *C. album* (Table 1). Moreover, Ct values in tomato plants showed limited variation compared with Ct values in weeds (Table 1). It should be mentioned that no amplification was obtained from healthy plants of tomato or any of the weed species tested. ToCV titer increased in both tomato and weeds 4 weeks after its first estimation; however, it still remained higher in tomato and lower in weed species (Fig. 5).

Transmission studies indicated that weeds differed considerably ($P \leq 0.05$) as ToCV sources compared with tomato plants (Fig. 6) when 15 adult whiteflies were used. *Solanum lycopersicum* plants appeared to be the best source for the virus, because efficiency of transmission reached 78%. When weed species were used as virus

sources, transmission efficiency decreased in a species-specific way. Whitefly transmission rates from *S. nigrum*, *Sonchus oleraceus*, *A. retroflexus*, and *C. album* to *Solanum lycopersicum* plants were variable and reached 68, 38, 32, and 22%, respectively (Fig. 6).

Yellowing symptoms and mild leaf curl appeared in tomato plants 18 to 22 dpi, initially in the lower (second to third true leaf) and gradually on the upper leaves. In the case of weeds, most of the infected *S. nigrum* (41/48) and *Sonchus oleraceus* (32/41) plants showed mild interveinal yellowing 22 to 25 dpi. A few infected *A. retroflexus* plants showed symptoms (6/34), while only 1 of 28 *C. album* infected plants showed mild interveinal yellowing. In the last two species, symptoms appeared 26 to 28 dpi.

Discussion

Whitefly-transmitted viruses cause serious losses in many economically important crops worldwide, especially in tropical and subtropical areas where their whitefly vectors usually occur in high numbers (Tzanetakis et al. 2013). Almost all whitefly-transmitted viruses such as begomoviruses, ipomoviruses, and torradoviruses are transmitted by whitefly species belonging to the genus *Bemisia* (Polston et al. 2014). On the other hand, transmission of criniviruses is achieved by either different biotypes of *B. tabaci* or *Trialeurodes* spp. (*T. abutilonea* and *T. vaporariorum*), usually not by both. ToCV possesses unique biological properties among members of the genus *Crinivirus*, due to its transmission by at least five different whitefly species or biotypes (*B. tabaci* biotype A, B, and Q; *T. vaporariorum*; and *T. abutilonea*) (Navas-Castillo et al. 2000; Wintermantel and Wisler 2006). Although *B. tabaci* biotype Q is a known vector of ToCV, transmission parameters are not known.

Biotypes of *Bemisia* reveal interesting features such as different levels of fitness on host plants, insecticide resistance, endosymbiotic communities, and transmission efficiency of criniviruses such as ToCV (Wintermantel and Wisler 2006) and Lettuce infectious yellows virus (Cohen et al. 1992) and begomoviruses such as Tomato yellow leaf curl virus (Pan et al. 2012). *B. tabaci* biotype Q has a wide distribution attributed to its polyphagous nature, great dispersal capacity, and high population growth rate as well as its broad-spectrum resistance to insecticides. Moreover, there are recent examples of replacement of biotype B by Q in many countries, such as China and Israel (Chu et al. 2010; Horowitz and Ishaaya 2014). In Greece, both biotypes B and Q are endemic, with B mainly restricted to southeastern islands and Q having a broader distribution in mainland Greece and the southern islands, where tomato crops are suffering from ToCV epidemics (Orfanidou et al. 2014). For these reasons, it was considered necessary to study transmission parameters of ToCV by *B. tabaci* biotype Q.

Transmission efficiency of criniviruses may vary among *Bemisia* vector species and it can be affected by factors such as the number of individuals and the host plant used in the study (Ng and Falk 2006;

Fig. 2. Persistence of *Tomato chlorosis virus* in *Bemisia tabaci* biotype Q adults. Percent transmission is compared by the number of days after virus acquisition during seven serial passages. Error bars indicate the standard deviation. Means followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's honestly significant difference.

Tzanetakis et al. 2013; Wintermantel and Wisler 2006). Experiments presented herein confirm the effect of the number of whiteflies as well as the role of AAP on ToCV transmission. By increasing the density of whiteflies and the feeding time period on inoculated leaves, transmission efficiency was also increased significantly ($P \leq 0.05$; Fig. 1). Biotype Q transmitted ToCV more efficiently after a 48-h AAP compared with a 24-h AAP in both low and high whitefly densities (Fig. 1). Therefore, biotype Q seems to benefit from a prolonged feeding period in order to acquire ToCV. This observation agrees with the results of studies of other semipersistent viruses, in which transmission efficiency increased during enhanced AAP (Ng and Falk 2006).

Moreover, ToCV persisted on biotype Q for at least 6 days, whereas it has been reported on *T. abutilonea* for 5 days, on *B. tabaci* biotype B for 2 days, and for only 1 day on biotype A and *T. vaporariorum* (Wintermantel and Wisler 2006). In addition, biotype Q remained an efficient vector (78% transmission) for 4 days whereas, in *T. abutilonea*, high efficiency lasted 2 days postacquisition (89%) and began to decrease by day 3 (47%). The present data highlight the importance of biotype Q as a vector of ToCV due to its ability to transmit the virus even at low density (five adults resulted in 17%

Fig. 4. Transmission efficiency of *Tomato chlorosis virus* onto *Solanum lycopersicum*, *S. nigrum*, *Amaranthus retroflexus*, *Sonchus oleraceus*, and *Chenopodium album* by using tomato as a donor plant. Error bars give the standard deviation. Means followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's honestly significant difference.

Fig. 3. Amplification plots of *Tomato chlorosis virus* (ToCV) RNA detection. **A**, FAM fluorescent signals generated from a dilution series of ToCV RNA transcripts. From left to right, the curves represent RNA 10-fold serial dilutions, from 2×10^8 to 20 copies per reaction, performed in three replicates. **B**, Corresponding standard curve.

transmission) and its capacity to remain viruliferous for a longer period, in contrast to the other four reported vectors.

ToCV is not transmitted by seed; as a result, older tomato plants play a very important role in its epidemiology and they represent efficient virus sources for younger plants. In addition, a number of weed species have been reported as ToCV hosts (Kil et al. 2015; Orfanidou et al. 2014) and they are probably involved in its epidemiology because they are the only viral sources when tomato, the major

crop host, is absent. However, their importance as viral sources has still to be demonstrated. In this study, we evaluated four weed species as ToCV sources. In agreement with our results, crinivirus transmission efficiency and persistence in the vector has also been shown to depend upon factors such as the virus titer in source plants (Ng and Falk 2006; Wintermantel and Wisler 2006). ToCV titer was lower in weeds than in tomato plants, based on calculations at 2 and 6 wpi (Fig. 5). Moreover, Ct values, which correspond to ToCV concentration, appear more uniform in tomato plants (standard deviation [SD] = 1.02) than in weeds (SD = 3.44 to 5.39) (Table 1). It is well known that genetic variability and plasticity of weeds contribute to their successful establishment in agroecosystems (Green et al. 2001), in which they infest a wide range of diverse habitats. Therefore, variation of Ct values in weeds possibly could be attributed to their genetic diversity, as a result of their interaction with the ToCV infection cycle.

Furthermore, transmission efficiency of criniviruses appears, in some cases, to be strongly influenced by host plant (Wintermantel et al. 2008, 2016). This was also confirmed in this study because ToCV transmission onto weed species differed considerably when tomato was used as inoculum source. More specifically, transmission efficiency of ToCV to Solanaceae spp. (*Solanum lycopersicum* and *S. nigrum*) was higher than onto nonsolanaceous species. In addition, *S. nigrum* was a better source of ToCV compared with the other three weeds when they were used as viral sources, and gave higher transmission rates (Fig. 6). Subsequently, *Sonchus oleraceus*, *A. retroflexus*, and, finally, *C. album* appeared to be less favorable sources of the virus (Fig. 6). Previous studies on biotype Q suggested that Solanaceae weeds are more suitable hosts of this biotype than species belonging to other taxonomic families (Muñiz 2000). A comprehensive study of the reproductive parameters of biotype Q such as female oviposition and pupal and adult production on *Solanum nigrum*, *A. retroflexus*, and *C. album* suggested that they were significantly higher on *S. nigrum* (Muñiz 2000). In addition, in our study, oviposition and pupal development was observed on *S. nigrum* and *Sonchus oleraceus* plants, in contrast to *A. retroflexus* and *C. album*, where oviposition was never observed (data not shown). Therefore, host preference of biotype Q seems to be an important factor in virus transmission of ToCV from the arable weeds.

Host preference by the vector is also supported by the fact that transmission efficiency of ToCV seems to be affected by the weed species on which the whitefly feeds, regardless of the concentration of the virus. Different weed species with very similar Ct differed in

Table 1. Range, mean average, and standard deviation (SD) of cycle threshold (Ct) values of *Tomato chlorosis virus* in five infected plant species (*Solanum lycopersicum*, *S. nigrum*, *Amaranthus retroflexus*, *Sonchus oleraceus*, and *Chenopodium album*) 2 weeks postinoculation

Plant species	Ct values ^a		
	Range	Mean average	SD
<i>Solanum lycopersicum</i>	21.54–26.23	23.23	1.02
<i>S. nigrum</i>	24.07–41.99	31.48	4.09
<i>A. retroflexus</i>	29.37–41.55	36.74	3.44
<i>Sonchus oleraceus</i>	21.19–42.55	35.47	5.39
<i>C. album</i>	29.87–42.55	36.18	5.27

^a All five replicates included.

Fig. 5. Mean average of cycle threshold (Ct) values of *Tomato chlorosis virus* in five plant species (*Solanum lycopersicum*, *S. nigrum*, *Amaranthus retroflexus*, *Sonchus oleraceus*, and *Chenopodium album*) calculated 2 and 6 weeks postinoculation (wpi). Plants used for the titer calculation originated from the first replicate. Error bars give the standard deviation.

Fig. 6. Transmission efficiency of *Tomato chlorosis virus* onto tomato by using *Solanum lycopersicum*, *S. nigrum*, *Amaranthus retroflexus*, *Sonchus oleraceus*, and *Chenopodium album* as source plants. Numbers inside bars indicate the cycle threshold (Ct) values of each source plant used for transmission. In the case of two Ct values, two different plants were used as sources. Means followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's honestly significant difference.

transmission efficiency and, on the other hand, transmission rates within the same species were similar, even with Ct values ranging from 29.37 to 37.00 (Fig. 6, *A. retroflexus*). It should be noted that, in semipersistently transmitted viruses, a preferable host for the vector seems to be more critical for virus acquisition than for inoculation (Ng and Falk 2006). Recent studies in other whitefly-transmitted viruses, such as begomoviruses, have revealed that virus infection can change the composition and percentage of free amino acids in the phloem or the nutritional composition of the plant tissue (Chen et al. 2013; Mauck et al. 2010; Stout et al. 2006) and thereby affect the fitness of the vector (Chen et al. 2013; Mauck et al. 2010). Based on such observations, it would be worthwhile to study whether there is a similar mechanism that, indirectly, enhances crinivirus transmission as well.

In natural conditions, virus-infected arable weeds do not usually develop symptoms (Chatzivassiliou et al. 2001; Duffus 1971; Salati et al. 2002) and virus titer is not always related to symptom intensity. Therefore, symptomless infection of weeds potentially could be very important for virus epidemiology. In particular, ToCV in weeds appears either symptomless or associated with slight interveinal chlorosis (Kil et al. 2015; Orfanidou et al. 2014). Indeed, in the present study, in contrast to *Solanum nigrum* and *Sonchus oleraceus*, ToCV infection was asymptomatic in infected *C. album* and *A. retroflexus* plants.

The major goal of this study was to investigate (demonstrate) the importance of ToCV-susceptible weeds as viral sources under natural conditions. We have shown that, although *Solanum nigrum*, *Sonchus oleraceus*, *A. retroflexus*, and *C. album* differ as ToCV sources, they all may affect virus epidemiology in the field. Also, all of them were less efficient sources compared with tomato. Although we cannot exclude the role of other weed species or volunteer tomato plants, this study indicates the vital role of four weed species in maintenance of ToCV in the field in the absence of tomato because they facilitate the introduction of the virus to the tomato crops. The results of this study underline the need to control the weeds in and around infected tomato crops, not only for managing alternative inoculum sources of ToCV but also for eliminating those which constitute sufficient hosts for whitefly vectors such as *B. tabaci*.

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