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Infectious cDNA clones of the crinivirus *Tomato chlorosis virus* are competent for systemic plant infection and whitefly-transmission

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ABSTRACT

Tomato chlorosis virus (ToCV) (genus *Crinivirus*, family *Closteroviridae*) causes important emergent diseases in tomato and other solanaceous crops. ToCV is not transmitted mechanically and is naturally transmitted by whiteflies. The ToCV genome consists of two molecules of linear, positive-sense RNA encapsidated into long flexuous virions. We present the construction of full-length cDNA clones of the ToCV genome (RNA1 and RNA2) fused to the SP6 RNA polymerase promoter and under the control of the CaMV 35S promoter. RNA1 replicated in the absence of RNA2 in *Nicotiana benthamiana* and tomato protoplasts after inoculation with cDNA-derived *in vitro* transcripts. Agroinfiltration of RNA1 and RNA2 under the 35S promoter resulted in systemic infection in *N. benthamiana* plants. In addition, tomato plants were infected by grafting with agroinfected *N. benthamiana* scions, showing the typical ToCV symptoms. The viral progeny generated in tomato was transmissible by the whitefly *Bemisia tabaci*.

Introduction

Tomato chlorosis virus (ToCV, genus *Crinivirus*, family *Closteroviridae*) causes a serious yellow leaf disorder syndrome in tomato plants. Symptoms of ToCV infection in tomato include interveinal yellow chlorotic areas that initially develop on lower leaves, and then progress towards the upper leaves of the plant. Bronzing and red patches also often occur within the yellow areas, and the leaves become thickened and crispy with the margins slightly curled upwards. In recent years, ToCV epidemics have emerged worldwide, causing severe damage to tomato production (Navas-Castillo et al., 2011; Tzanetakis et al., 2013). In addition to tomato, ToCV infects other economically important crops such as sweet pepper (*Capsicum annuum*) (Barbosa et al., 2010; Fortes et al., 2012; Lozano et al., 2004) and potato (*Solanum tuberosum*) (Fortes and Navas-Castillo, 2012; Freitas et al., 2012) and is able to naturally or experimentally infect about 37 plant species from 13 different families (Fonseca et al., 2013; Font et al., 2004; Louro et al., 2000, 2007; Morris et al., 2006; Solórzano-Morales et al., 2011; Trenado et al., 2007; Tsai et al., 2004; Wintermantel and Wisler, 2006). ToCV, like all criniviruses described till date, is phloem-limited and is not transmissible by mechanical inoculation. In nature, ToCV is transmitted by phloem-feeding whiteflies (Hemiptera: Aleyrodidae) belonging to two genera, *Bemisia* (*B. tabaci*) and *Trialeurodes* (*T. vaporariorum* and *T. abutiloneus*). The genome of ToCV is typical of the genus *Crinivirus*, composed of two molecules of single-stranded positive-sense RNA, RNA1 (8594–8595 nt) and RNA2 (8242–8247 nt) (Albuquerque et al., 2013; Kataya et al., 2008; Lozano et al., 2006, 2007; Wintermantel et al., 2005; Wisler et al., 1998; Zhao et al., 2014). Both RNA molecules are separately encapsidated in long and flexuous virions varying from 800 to 850 nm in length (Liu et al., 2000). RNA1 contains four open reading frames (ORFs), the two largest of which encode proteins associated with virus replication. RNA2 contains nine ORFs which encode proteins associated with functions including virus encapsidation, movement and whitefly transmission. Both genomic components encode proteins with RNA silencing suppression activity, the p22 protein in RNA1 and the major (CP) and minor (CPm) coat proteins in RNA2 (Cañizares et al., 2008).

The availability of infectious clones of plant RNA viruses makes it possible to manipulate their genomes for elucidating virus gene function by reverse-genetics, or for using them as vectors. For these reasons, considerable effort has been devoted to the development of efficient systems of plant infection using cloned versions of viral genomes. In the case of RNA plant viruses, cloned

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cDNA can be used to obtain either *in vitro* or *in vivo* infectious transcripts. Both strategies have been applied to members of the *Closteroviridae* family, which is probably the most complex group of plant viruses (Dolja and Koonin, 2013). Obtaining infectious clones of closterovirids has proven to be a difficult task, due to the low title of virions in plant tissues, the need for complex strategies of ligation to obtain full-length cDNA, problems of toxicity in *Escherichia coli* and difficulties in protoplast inoculation due to the large size of the *in vitro* transcripts (Satyanarayana et al., 1999, 2003). So far, full-length cDNA infectious clones of five species of closterovirids have been obtained, three in the monopartite genus *Closterovirus*: *Beet yellows virus* (BYV), *Citrus tristeza virus* (CTV) and *Grapevine leafroll-associated virus 2* (GLRaV-2) and two in the bipartite genus *Crinivirus*: *Lettuce infectious yellows virus* (LIYV) and *Lettuce chlorosis virus* (LCV). Initially, infections of *Nicotiana benthamiana* or *N. tabacum* protoplasts with *in vitro* transcripts using RNA polymerase promoters (SP6, T3) of LIYV (Klaassen et al., 1996), BYV (Peremyslov et al., 1998), CTV (Satyanarayana et al., 1999) and LCV (Mongkolsiriwattana et al., 2011) were achieved. Subsequently, the development of *Agrobacterium*-mediated inoculation systems has been described for BYV (Prokhnevsky et al., 2002), LIYV (Wang et al., 2009a), CTV (Ambrós et al., 2011; Gowda et al., 2005), GLRaV-2 (Kurth et al., 2012; Liu et al., 2009), and LCV (Chen et al., 2012). With the exception of GLRaV-2, for which systemic infection was shown to occur in grapevine (Kurth et al., 2012), these clones are not capable of producing systemic infection in their respective natural hosts by direct agroinoculation. Nevertheless, lettuce (*Lactuca sativa*), the natural host of LIYV and LCV, can be systemically infected by *B. tabaci*-mediated transmission of purified LIYV and LCV virions obtained from agroinfected *N. benthamiana* plants (Chen et al., 2012; Wang et al., 2009a). Also, several citrus species, the natural hosts of CTV, can be infected after stem-slash inoculation with virions from infected protoplasts and from agroinfected *N. benthamiana* leaves (Ambrós et al., 2011; Gowda et al., 2005 Satyanarayana et al., 2001).

In this work, we developed full-length infectious cDNA clones for ToCV RNA1 and RNA2, which constitutes the third crinivirus for which this has been accomplished. First, capped *in vitro* transcripts for RNA1 were shown to replicate in *N. benthamiana* and tomato protoplasts. Also, agroinoculation with RNA1 and RNA2 constructs under the CaMV 35S promoter resulted in systemic infection in *N. benthamiana* plants. Tomato plants were successfully infected by grafting with agroinfected *N. benthamiana* scions and virions derived from systemically infected tomato plants were competent for transmission by *B. tabaci*.

Results

Construction of full-length cDNA clones of ToCV RNA1 and RNA2

Availability of the complete genome sequence of ToCV isolate AT80/99 (Lozano et al., 2006, 2007) facilitated the construction of full-length cDNA clones of RNA1 and RNA2 by RT-PCR amplification of dsRNA purified from infected tomato plants. First, we constructed clones under the control of the SP6 RNA polymerase promoter following the strategy outlined in Fig. 1. For this, three and two overlapping cDNA fragments corresponding to ToCV RNA1 and RNA2, respectively, were amplified and subsequently assembled in pUC18 by one- and two-step ligation, respectively. Viral inserts in the constructs were bordered by an upstream SP6 RNA polymerase promoter and a downstream unique restriction site (*Bam*HI for RNA1 and *Xma*I for RNA2) to allow linearization for *in vitro* transcription. *In vitro* transcripts generated using this strategy are predicted to have 5′-termini identical to ToCV RNA1 and RNA2, and four and two extra non-viral nucleotides at the 3′-termini of RNA1 and RNA2, respectively. ToCV genomic RNAs were successfully cloned and transformed in *E. coli* DH5α (RNA1 and RNA2) and SURE (RNA1). One clone of ToCV RNA1 (pToCV227) and two clones of ToCV RNA2 (pToCV230 and pToCV231), with the expected insert size, were sequenced for each insert end. The obtained sequences confirmed the identity of the insert with the original ToCV isolate and the correct insertion of cDNA fused to the SP6 RNA polymerase promoter. These clones were selected for *in vitro* transcription reactions and subsequent inoculation in protoplasts.

To construct ToCV full-length clones under the CaMV 35S promoter, a pCAMBIA vector was modified by adding a duplicated enhancer 35S promoter and the nopaline synthase termination signal (NOS), generating the pCAM2300-35SeNOS plasmid. Using primers corresponding to the 5′ and 3′ ends of both ToCV genomic RNAs, the clones described above (pToCV227 for RNA1 and pToCV230 for RNA2) and a DNA polymerase with proofreading and high-fidelity activity, full-length ToCV RNA1 and RNA2 were amplified. Subsequently, the amplified products were inserted into the binary plasmid pCAM2300-35SeNOS (Fig. 1). Repeated attempts to transform *E. coli* DH5α with the RNA1 construct were unsuccessful, yielding clones with inserts shorter than expected for a full-length RNA1. Therefore, *E. coli* ElectroMAX Stbl4, grown at 30 °C, were assayed for transformation. Two clones with the expected insert size and correct orientation in the binary plasmid were selected (pToCV-35S-RNA1-6 and *p*ToCV-35S-RNA1-20, hereinafter RNA1-6 and RNA1-20) and sequenced for each insert end. The obtained sequences confirmed the identity of the insert with the original ToCV isolate and the correct insertion of cDNA fused to 35S promoter. For ToCV RNA2, following a similar procedure, two clones were selected that contained the expected insert size and whose sequence was confirmed for both insert ends, pToCV-35S-RNA2-D3 (obtained from DH5α cells, hereinafter RNA2-D3) and pToCV-35S-RNA2-S8 (obtained from ElectroMAX Stbl4 cells, hereinafter RNA2-S8). Inserts of clones RNA1-20 (GenBank accession number KJ740256) and RNA2-D3 (KJ740257) were completely sequenced. Sequence analysis showed 10 nucleotides (3 amino acids) in RNA1 and 18 nucleotides (9 amino acids) in RNA2 differing from the published AT80/99 ToCV sequence.

ToCV RNA1 replicates in N. benthamiana and tomato protoplasts inoculated with transcripts obtained in vitro

Replication and time course accumulation of ToCV RNA1 in *N. benthamiana* protoplasts inoculated with capped transcripts synthesized *in vitro* from pToCV227 was evaluated. Total RNA extracted from protoplasts at 0–3 days post-inoculation (dpi) was analyzed by northern blot hybridization using positive and negative sense DIG-labeled RNA single stranded probes that were complementary to the p22 gene of ToCV RNA1. This analysis showed that RNA1 replicates efficiently in *N. benthamiana* protoplasts in the absence of RNA2 and can be detected after 1 dpi (Fig. 2A). Both positive and negative strands of RNA1 showed a similar temporal pattern of accumulation. Furthermore, RNA1 replication in tomato (cv. Moneymaker) protoplasts inoculated with *in vitro* transcripts was observed at 1 dpi by northern blot analysis (Fig. 2C). We were not able to detect the accumulation of ToCV RNA2 in *N. benthamiana* protoplasts inoculated with *in vitro* transcripts from pToCV230 or pToCV231 (RNA2) co-inoculated with *in vitro* transcripts from pToCV227 (RNA1) after northern blot analysis (not shown).

ToCV replicates and infects systemically N. benthamiana, but not tomato, after agroinoculation with RNA1 and RNA2 clones

To analyze the biological activity of the full-length ToCV cDNA clones *in planta*, we first tested the ability of ToCV RNA1 clones to

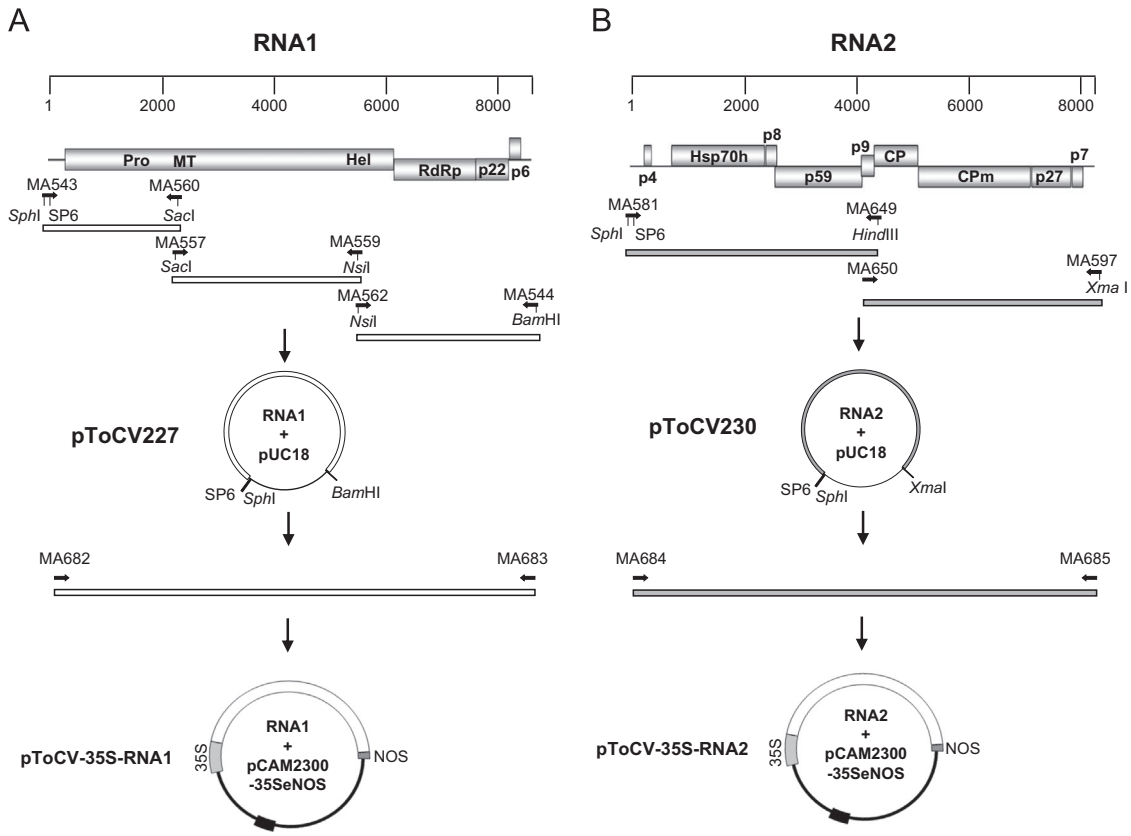

Fig. 1. Genome organization and construction of full-length cDNAs clones of *Tomato chlorosis virus* (ToCV) RNA 1 (A) and 2 (B) under the control of SP6 RNA polymerase promoter (pToCV227 and pToCV230) or 35S promoter (pToCV-35S-RNA1 and pToCV-35S-RNA2). ORFs are represented by boxes in the schematic representation of the genome. Bars below the genome represent PCR products used for cloning. Primers used in RT-PCR are represented by horizontal arrows. The restriction endonuclease sites used in cloning are indicated. SP6, bacteriophage SP6 RNA polymerase promoter sequence; 35S, duplicated enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter; NOS, nopaline synthase polyadenylation signal.

agroinfect *N. benthamiana* plants. *A. tumefaciens* strain GV3101 carrying RNA 1 clones (RNA1-6 or RNA1-20) were infiltrated into *N. benthamiana* leaves. Analysis of infiltrated leaves by northern blot showed that these clones were transcribed *in vivo* and ToCV RNA 1 was locally replicated (Fig. 2B). Accumulation of positive strand RNA1 was detected at 1–2 dpi and the negative strand at 3 dpi.

Subsequently, plants of *N. benthamiana* were agroinfiltrated with a mix of *A. tumefaciens* GV3101 carrying ToCV RNA1 (RNA1-6 or RNA1-20) and RNA2 (RNA2-D3 or RNA2-S8). Molecular hybridization of infiltrated and non-infiltrated apical leaves using positive and negative sense p22 (RNA 1) and CP (RNA2) probes showed that the progeny virus is able to replicate and cause systemic infection in *N. benthamiana* plants (Fig. 3). When the accumulation of both genomic RNAs was analyzed in the infiltrated leaves, replication of RNA1 was detected at 2–3 dpi, while accumulation of RNA2 was detected at 5 dpi at a lower level (Fig. 3A). Systemic ToCV infection was confirmed in non-infiltrated apical leaves of *N. benthamiana* plants by tissue printing hybridization using an RNA2 probe (Fig. 3B), immunodetection of petiole and stem printing using an antiserum against ToCV CP protein (Fig. 3C), and western blot analysis using the same antiserum (Fig. 3D). Additionally, infected *N. benthamiana* plants showed mild chlorosis symptoms on lower leaves (Fig. 3E). Co-inoculation experiments of RNA1-20 and RNA2-D3 clones with the RNA silencing suppressor proteins ToCV p22 (Cañizares et al., 2008) and PVY HC-Pro (Hamilton et al., 2002) did not reveal any significant difference, neither in the number of infected plants (Table 1) nor in the level of virus accumulation (not shown), when compared to the controls.

After verifying that the RNA1 and RNA2 clones were infectious in *N. benthamiana*, we performed agroinoculation experiments in the natural host, tomato cv. Moneymaker, and in the plant model *Arabidopsis thaliana*. In none of the cases, using all possible combinations of available RNA1 and RNA2 clones, was ToCV RNA accumulation detected in non-infiltrated apical leaves by tissue printing, northern blot or RT-PCR (Table 1). In all experiments, *N. benthamiana* plants were used as a control and were systemically infected.

ToCV RNAs produced in agroinoculated *N. benthamiana* plants were expected to contain the exact 5′ and 3′ termini present in the cloned ToCV isolate. To confirm this, total RNA and dsRNA from systemically-infected leaves of two agroinoculated *N. benthamiana* plants were extracted and the 5′ and 3′ termini of viral RNA progeny were analyzed by Rapid Amplification of cDNA Ends (RACE) and polyadenylation. All of the clones analyzed contained the expected 5′ and 3′ terminal sequences.

ToCV infects tomato plants systemically and causes typical yellowing symptoms after grafting with N. benthamiana scions infected by agroinoculation

We hypothesized that the *in vivo* transcripts synthesized in *N. benthamiana* plants from RNA1-20 and RNA2-30 clones from *A. tumefaciens* contained all of the genomic information required for the complete biological activity of ToCV. To confirm this, we assessed the ability of the virus progeny generated in *N. benthamiana* plants to infect tomato by grafting. Tissue printing hybridization with a negative sense CP RNA probe of apical leaves from grafted tomato plants showed that successful infection was achieved (Fig. 4A). Additionally, immunoblot analysis of petiole

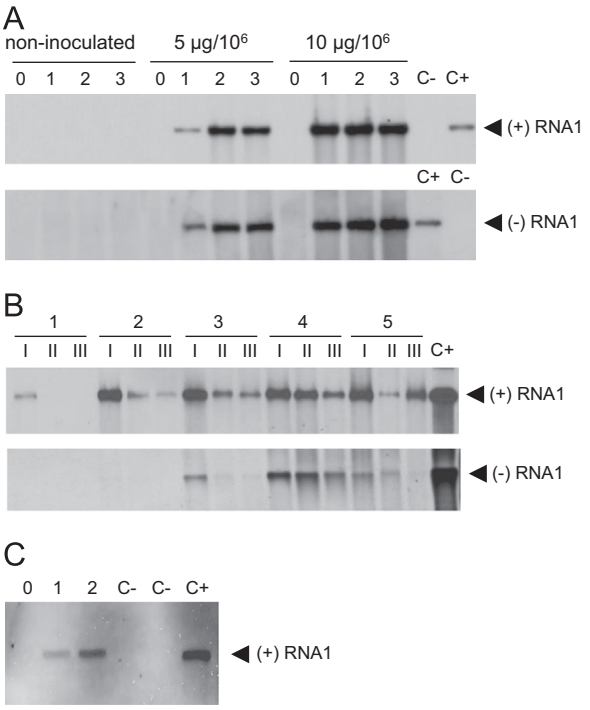

Fig. 2. Northern blot hybridization analysis using DIG-labeled RNA probes, complementary to p22 gene, to detect positive (+) or negative (−) sense of genomic RNA1 (RNA1) of *Tomato chlorosis virus* (ToCV). (A) Viral RNA1 accumulation in *N. benthamiana* protoplasts inoculated with *in vitro* transcripts of pToCV227 clone (5 or 10 μg per 10^6 protoplasts) at 0, 1, 2 and 3 days post-inoculation (dpi). Non-inoculated protoplasts were included as negative control. (B) Local RNA1 accumulation in leaves of *N. benthamiana* agroinoculated with RNA1-20 clone. Three agroinoculated leaves per plant (I, II, III) were analyzed at 0, 1, 2, 3, 4 and 5 dpi. (C) Viral RNA1 accumulation in tomato protoplasts inoculated with *in vitro* transcripts of pToCV227 (5 μg per 10^6 protoplasts). Total RNA at 0, 1 and 2 dpi were analyzed. Double-stranded RNA extracted from healthy or ToCV-infected *N. benthamiana* plants inoculated by *B. tabaci* transmission were included as negative (C−) and positive (C+) controls, respectively.

and stem printing of grafted tomato plants, using a polyclonal anti-CP ToCV antiserum, revealed CP protein accumulation in the phloem (Fig. 4B). Furthermore, immunoelectron microscopy of extracts from tomato plants showed the presence of ToCV virions (Fig. 4C).

ToCV virions generated in graft-inoculated tomato plants are competent for transmission by the whitefly B. tabaci

To fully confirm the biological activity of ToCV clones, we tested the transmissibility by *B. tabaci* of the viral progeny generated in the infected tomato plants. Non-viruliferous adults of *B. tabaci* Mediterranean (MED, formerly Q biotype) and Middle East-Asia Minor 1 (MEAM1, formerly B biotype) were allowed to feed on tomato plants grafted with scions of agroinfected *N. benthamiana* plants and were then transferred to uninfected tomato plants. ToCV was efficiently transmitted by both *B. tabaci* genotypes, at a rate of 83% (10/12) for MED and 100% (12/12) for MEAM1, as shown by tissue printing hybridization using a negative sense CP RNA probe (Fig. 5). Also, typical yellow disease symptoms were observed, consisting of interveinal yellowing and basal leaves rolled longitudinally (not shown).

Discussion

ToCV, a typical bipartite crinivirus, is not only a major concern for tomato cultivation worldwide (Navas-Castillo et al., 2011), but an emerging threat for other important solanaceous crops such as pepper and potato (Barbosa et al., 2010; Fortes et al., 2012; Fortes and Navas-Castillo, 2012; Freitas et al., 2012; Lozano et al., 2004). ToCV, like most members of the family *Closteroviridae*, is not transmitted by mechanical inoculation and the only experimental transmission method available to date is the use of the insect vectors, with several species of whiteflies belonging to two genera. This greatly hinders basic biological studies such as determining host range or searching for potential sources of genetic resistance. The availability of ToCV infectious clones, in addition to benefitting these studies, would allow reverse genetics experiments to be carried out to determine the role of the encoded proteins in the virus–host plant–insect vector interactions, with emphasis on host range and specificity of transmission by different whitefly species.

In this work, we have constructed two sets of full-length ToCV cDNA clones, under the control of the SP6 RNA polymerase promoter and CaMV 35S promoter, respectively. cDNA clones of RNA viruses are not always stable in *E. coli* (Jakab et al., 1997; Maiss et al., 1992). Introduction of mutations that alter the reading frame in regions allegedly encoding toxic proteins can reduce this toxicity, as has been observed for CTV clones (Satyanarayana et al., 2003). Also, expression of toxic protein may be prevented by inserting one or more introns in different regions of the cDNA. For CTV, to reduce toxicity produced at the 5′ genome end, a potato intron was inserted and a hybrid vector of binary plasmid and BAC was successfully employed (Ambrós et al., 2011). In some cases, the stability of viral cDNAs in *E. coli* has been improved by changing the bacterial strain or using a low copy number plasmid (Boyer and Haenni, 1994). Although serious difficulties have arisen in ToCV RNA1 cloning, insert instability was solved simply by using SURE (for SP6 construct) and ElectroMAX Stbl4 (for 35S construct) *E. coli* strains. Another key factor was to reduce the temperature for *E. coli* incubation to 30 °C. Incubation at temperatures lower than 37 °C reduces the risk of undesired deletions and minimizes the instability of plasmids (Joshi and Jeang, 1993; Kanahan et al., 1991; Singh and Singh, 1995).

Sequence analysis of clones RNA1-20 and RNA2-D3 revealed the presence of 10 and 18 nucleotides that differed from the sequence of the AT80/99 isolate. However, these changes did not abolish the biological activity of the RNA1-20- and RNA2-D3-derived transcripts, which is in agreement with the complex genetic structure that was previously shown for ToCV within a host plant (Lozano et al., 2009).

Analysis of infectivity for ToCV clones was performed by inoculation of *in vitro* transcripts in *N. benthamiana* and tomato protoplasts and by *Agrobacterium*-mediated inoculation. ToCV RNA1 replication was observed in *N. benthamiana* and tomato protoplasts 1 dpi using *in vitro* transcripts and in *N. benthamiana* leaves 2 dpi using agroinoculation. As in the case of LIYV (Klaassen et al., 1996; Wang et al., 2009a) and LCV (Mongkolsiriwattana et al., 2011), ToCV RNA1 can replicate in the absence of RNA2, as expected from the sequence analysis showing that RNA1 possesses genes encoding proteins involved in viral replication. In the case of LIYV, RNA1 replication was observed at 3–5 dpi using a mutant expressing GFP (Wang et al., 2009b). In our study, it was not possible to detect the replication of RNA2 in protoplasts using *in vitro* transcripts. This may be a result of the inability to detect RNA2 due the presumed low number of protoplasts doubly infected with RNA1 and RNA2. In contrast, analysis of leaves agroinfiltrated with RNA1 and RNA2 revealed that both RNAs accumulated. RNA2 accumulation suffered a delay in comparison with RNA1, which is in agreement with the asynchronous accumulation in protoplasts shown for LIYV (Yeh et al., 2000).

Furthermore, this work has demonstrated that the obtained clones lead to a systemic infection in *N. benthamiana* plants, accumulating both RNA1 and RNA2 in non-infiltrated apical leaves

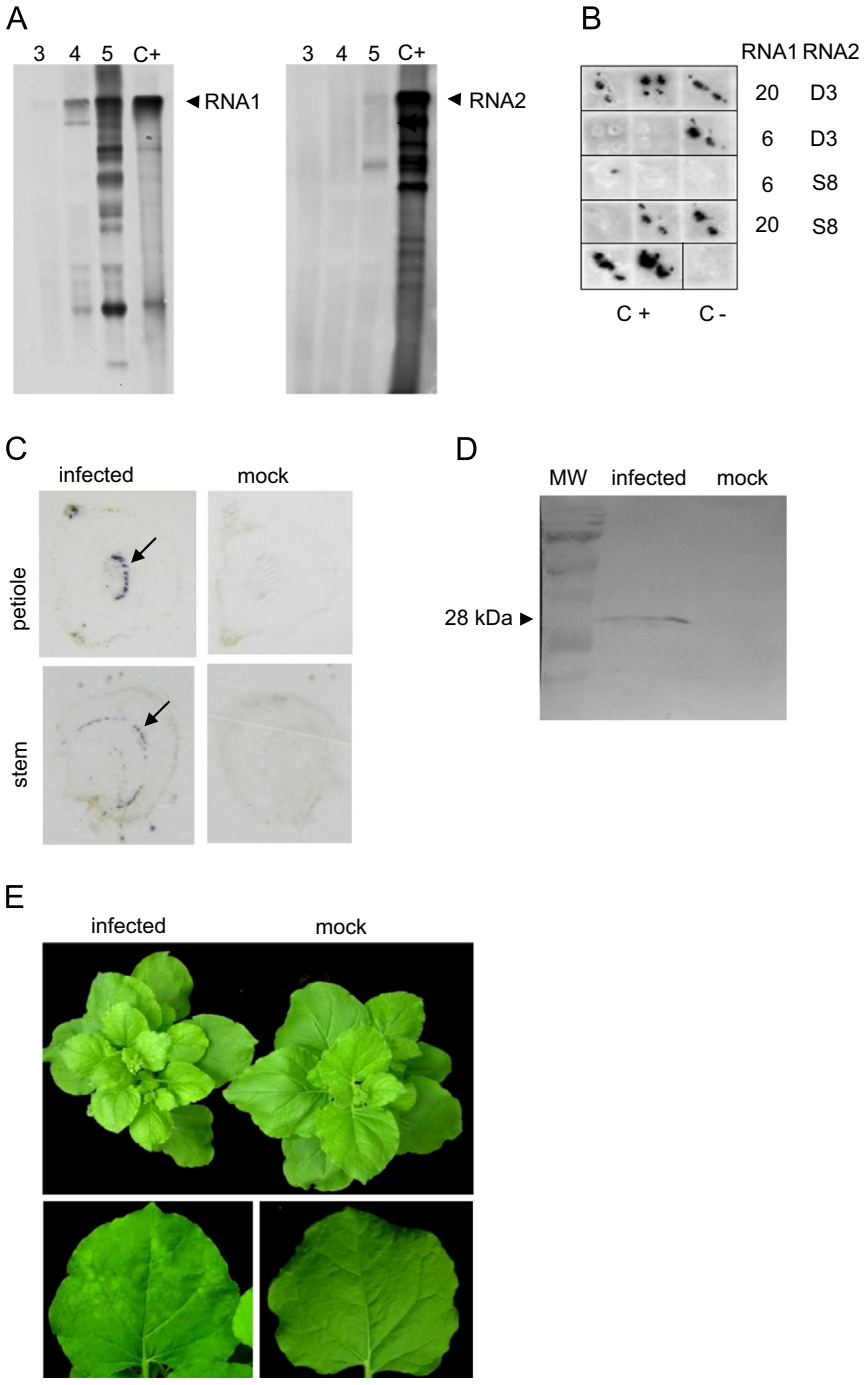

Fig. 3. ToCV infection following *Agrobacterium*-mediated inoculation of *N. benthamiana* plants with a mixture of *Agrobacterium* culture containing the pToCV-35S-RNA1 and pToCV-35S-RNA2 clones. Systemic plant infection was determined by molecular hybridization, immunoblot and symptom development. (A) Northern blot analysis of ToCV RNA1 and RNA 2 replication in local agroinfiltrated *N. benthamiana* leaves with RNA1-20 and RNA2-D3 clones at 3, 4 and 5 days post-agroinoculation (lines 3, 4 and 5) using negative sense DIG-labeled RNA probes of p22 (RNA1) and CP (RNA2) genes. C+: dsRNA from ToCV infected *N. benthamiana* after *B. tabaci* transmission. Arrowheads indicate genomic RNA1 and RNA2. (B) Tissue printing hybridization of non-infiltrated upper *N. benthamiana* leaves at 15 dpi with different ToCV RNA1 and RNA2 full-length clone combinations using a negative sense DIG-labeled RNA CP probe. C+: positive control, ToCV AT80/99 tomato infected; C-: negative control, healthy tomato. 20: plasmid pToCV-35S-RNA1-20; 6: plasmid pToCV-35 S-RNA1-6; D3: plasmid pToCV-35S-RNA2-D3; S8: plasmid pToCV-35S-RNA2-S8. (C) Tissue-printing immunoblot of petioles and stems of agroinfected and uninfected *N. benthamiana* plants using ToCV CP antiserum. Purple-stained foci indicate the presence of ToCV CP, which was restricted to the vascular tissue. (D) Western immunoblot analysis for ToCV in *N. benthamiana* plants agroinfected and uninfected (mock) using a ToCV CP antiserum. Arrowhead indicates the position and size of ToCV CP protein. MW: protein molecular weight marker. (E) Yellowing symptoms induced by systemic ToCV infection in *N. benthamiana* plants at 21 dpi. Mock: uninfected control plant.

of agroinoculated plants. Moreover, CP accumulation in phloem tissue of these plants was detected and typical ToCV symptoms were observed. ToCV is only the third crinivirus for which an agroinoculation system has been developed to accomplish systemic infection of *N. benthamiana* plants. Systemic agroinfection of *N. benthamiana* by closterovirids has been described for BYV (Chiba et al., 2006; Prokhnevsky et al., 2002), GLRaV-2 (Liu et al., 2009), LIYV (Wang et al., 2009a), CTV (Ambrós et al., 2011; Gowda et al., 2005) and LCV (Chen et al., 2012). It is worth mentioning that direct agroinfection in the natural host, tomato, or in the plant model *A. thaliana*, was not achieved with these clones, despite repeated attempts using different modes of agroinoculation.

However, tomato plants were successfully infected by grafting with scions from *N. benthamiana* plants previously agroinfected using RNA1 and RNA2 cDNA clones. This confirms that viral progeny of the obtained clones are competent for replication and systemic spread in the natural host of ToCV, tomato. The functional integrity of the virus progeny was confirmed by the transmission to healthy tomato plants by the insect vector *B. tabaci* MED and MEAM1 and by the development of typical yellowing symptoms. The two other criniviruses for which agroinfectious clones have been obtained, LIYV and LCV, were also unable to directly accumulate in the natural host, lettuce, after agroinoculation. To circumvent this problem, partially purified LIYV and LCV virions from agroinoculated *N. benthamiana* plants were successfully acquired via membrane-feeding and transmitted to lettuce plants by *B. tabaci* (Chen et al., 2012; Wang et al., 2009a). Also, small citrus trees, the natural hosts of CTV, became infected by slashing stems in the presence of virions partially purified from CTV-agroinfiltrated *N. benthamiana* leaves (Ambrós et al., 2011; Gowda et al., 2005). An inability to achieve systemic infection in species that are natural hosts of a virus can be caused by factors such as inefficient transcription, lack of virulence of some *A. tumefaciens* strains on specific hosts, defense responses of the plant caused by certain combinations of *A. tumefaciens* strains and viruses, or the inaccessibility of phloem cells (Birch, 1997; Pruss et al., 2008; Saeed, 2008; Stephan and Maiss, 2006; Voinnet et al., 2003; Wroblewski et al., 2005). Nevertheless, agroinfiltration of mini-replicons of GLRaV-2 in grapevine resulted in epidermal and mesophyll cell infection, but not in the phloem (Liu et al., 2009). As this first full-length infectious clone of GLRaV-2 was constructed from an isolate infecting *N. benthamiana* plants, the authors reasoned that the inability of a grapevine virus to systemically infect its natural host may be due to rapid adaptive evolution in a new host, which is typical of RNA viruses. Thus, a new full-length cDNA from infected grapevine was constructed and the agroinoculated grapevine plants became systemically infected (Kurth et al., 2012).

It has been previously reported that ectopic expression of homologous or heterologous viral gene silencing suppressors can enhance the infectivity of infectious clones of several viruses in *Nicotiana* species (Chiba et al., 2006; Liu and Kearney, 2010; Yoon et al., 2011). We checked whether the agroinfection efficiency of ToCV could be increased by co-infiltration of *A. tumefaciens* harboring the ToCV clones and an RNA silencing suppressor (ToCV

Table 1
Summary of agroinoculation experiments carried out in *N. benthamiana*, tomato cv. Moneymaker and *A. thaliana* Columbia plants with various combinations of RNA1 and RNA2 ToCV binary plasmid clones (in the presence or absence of silencing suppressor proteins, ToCV p22 and PVY HC-Pro). Systemic infection was determined by tissue printing, northern blot or RT-PCR.

Host	Clones	Systemically infected plants/ Inoculated plants
N.	*benthamiana*	RNA1-6+RNA2-D3
1/3		
	RNA1-6+RNA2-S8	0/3
	RNA1-20+RNA2-S8	2/3
	RNA1-20+RNA2-D3	32/37
	RNA1-20+RNA2-D3+p22	17/20
	RNA1-20+RNA2-D3+HC-Pro	19/20
Tomato	RNA1-6+RNA2-D3	0/5
	RNA1-6+RNA2-S8	0/5
	RNA1-20+RNA2-S8	0/5
	RNA1-20+RNA2-D3	0/143
	RNA1-20+RNA2-D3+p22	0/20
	RNA1-20+RNA2-D3+HC-Pro	0/20
A. thaliana	RNA1-6+RNA2-D3	0/5
	RNA1-6+RNA2-S8	0/5
	RNA1-20+RNA2-S8	0/5
	RNA1-20+RNA2-D3	0/33

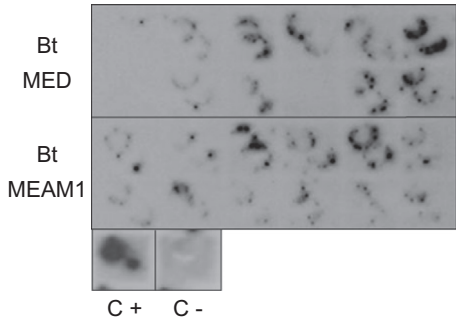

Fig. 5. ToCV systemic infection in tomato plants inoculated by *B. tabaci* MED and MEAM1 with viral progeny from agroinfectious clones. Tissue printing hybridization of apical tomato leaves at 30 dpi using a negative sense labeled RNA CP probe. C+, agroinfected *N. benthamiana* leaf; C−, healthy tomato leaf.

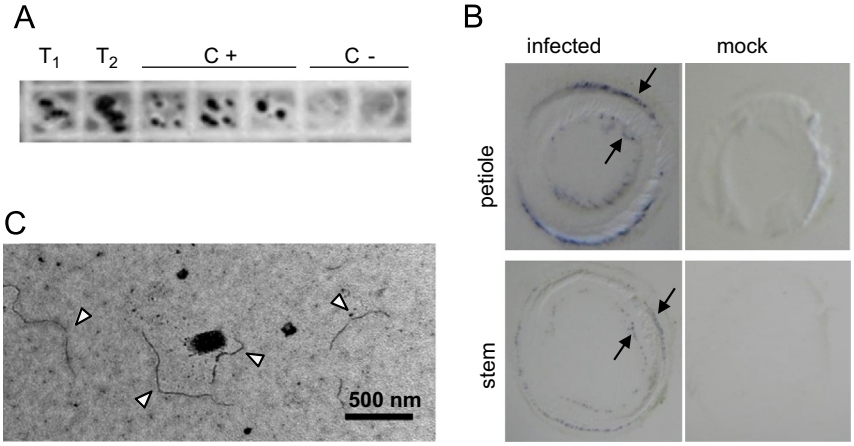

Fig. 4. ToCV infection of tomato plants inoculated by grafting with agroinfected *N. benthamiana* plants. (A) Tissue printing molecular hybridization of apical leaves from grafted tomato plants (T1 and T2) at 21 dpi using a negative sense RNA CP probe. C+, agroinfected *N. benthamiana* leaf; C−, healthy tomato leaf. (B) Tissue-printing immunoblot of petioles and stems of graft-inoculated (infected) and non-inoculated (mock) tomato plants using a ToCV CP antiserum. Blue-stained areas indicate the presence of ToCV CP in phloem tissues. (C) Immunoelectron microscopy image of ToCV virions prepared from the infected apical tomato leaves.

p22 [Cañizares et al., 2008] or PVY HC-Pro [Hamilton et al., 2002]). In no case was infection achieved in tomato and no infection differences were observed in *N. benthamiana*. A similar result was obtained when LIYV clones were tested in the presence of *Tomato bushy stunt virus* (TBSV) on *N. benthamiana*, lettuce, zucchini and melon (Wang et al., 2009a). In contrast, the systemic infection of *N. benthamiana* with a CTV clone of CTV was facilitated by the presence of five different silencing suppressors, TBSV p19, ToCV p22, CTV p23, GLRaV-2 p24 and *Tobacco etch virus* or *Turnip mosaic virus* HC-Pro (Ambrós et al., 2011). Also, increased infection was achieved in *N. benthamiana* by a BYV mini-replicon co-expressed with the homologous p21 suppressor (Chiba et al., 2006).

The infectious clones described in this work, together with directed mutagenesis, will allow reverse genetics studies to be carried out to characterize functions of ToCV genes, with emphasis on understanding the complex interaction between the virus and whiteflies from two different genera. The next critical step toward achieving this goal will be to obtain direct infection on tomato by agroinoculation, thus allowing the application of the developed system also to more practical aspects such as efficient screening of tomato and wild relatives for resistance to this emerging virus.

Materials and methods

Virus isolate

The ToCV isolate used in this study was AT80/99, which was obtained from a naturally infected tomato plant collected in 1997 from a commercial crop in Málaga province (southern Spain). It was maintained in tomato cv. Moneymaker by vegetative propagation of cuttings in a growth chamber (16 h of light at 25 °C, 8 h of dark at 18 °C and 70% relative humidity) and periodic transmission by *B. tabaci*. The complete nucleotide sequence of AT80/99 isolate was previously reported (GenBank acc. nos. DQ983480 and DQ136146; Lozano et al., 2006, 2007).

Construction of full-length ToCV RNA1 and RNA2 cDNA clones

Reverse transcription and polymerase chain reactions (RT-PCR) were used to synthesize and amplify cDNA representing full-length RNA1 and RNA2 as shown in Fig.1. Double-stranded RNA (dsRNA) from AT80/99 infected tomato tissue was purified by chromatography on CF-11 cellulose as previously described (Valverde et al., 1990) and denatured by heating at 70 °C for 5 min. For cloning under the SP6 promoter, three overlapping cDNA fragments of 2.5–3.0 kb of the RNA1 were produced from denatured dsRNA by reverse transcription with Expand RT (Roche) and amplified by PCR with Expand High Fidelity (Roche) with primers based on nucleotide sequence information of the AT80/99 isolate (Lozano et al., 2007). Amplified products were purified using High Pure PCR Product Purification kit (Roche). A pair of primers, MA544 and MA562, was used to amplify the 3′ end of RNA1 (Supplementary table 1). The amplified product was digested with the restriction endonucleases *Bam*HI and *Nsi*I (Fig.1). The 5′ end of RNA1 was amplified using the primers MA543, containing SP6 RNA polymerase promoter sequence, and MA560 (Supplementary table 1). The RT-PCR product was digested with *Sph*I and *Sac*I (Fig.1). The central cDNA fragment was amplified with primers MA557 and MA559, and the RT-PCR product was digested with *Sac*I and *Nsi*I, which recognize unique sites in ToCV RNA1. A full-length RNA1 cDNA clone was generated by one-step ligation of the three RT-PCR products into pUC18 digested with *Sph*I and *Bam*HI (Fig.1). Recombinant plasmids were transformed into *Escherichia coli* DH5α (Invitrogen) and SURE (Agilent Technologies). One clone, pToCV227, was selected for analysis in protoplasts and construction of full-length ToCV RNA1 under the CaMV 35S promoter. Two overlapping RNA2 cDNA fragments of ~4.0 kb were produced from denatured dsRNA by reverse transcription with SuperscriptIII (Invitrogen) and amplified by PCR with Expand High Fidelity (Roche) with primers based on nucleotide sequence information of AT80/99 isolate (Lozano et al., 2006). A pair of primers, MA597 and MA650, was used to amplify the 3′ end of RNA2. The 5′ end of RNA2 was amplified by using the primers MA581, containing the SP6 RNA polymerase promoter sequence, and MA649 (Supplementary table 1). The two amplified cDNA products were purified using DNA Gel Extraction Kit (Millipore), separately ligated into pGEM-T Easy (Promega), and transformed into *E. coli* DH5α (Invitrogen). For each RNA2 fragment, one clone was selected and digested with *Sph*I-*Hind*III and *Hind*III-*Xma*I, respectively, to release the insert. Full-length ToCV RNA2 cDNA clones were generated by ligating the purified inserts into *SphI-Xma*I-digested pUC18 (Fig.1). Plasmids were transformed into *E. coli* DH5α (Invitrogen). Two clones, pToCV230 and pToCV231, were selected for analysis in protoplasts and pToCV230 was used for cloning under CaMV 35S promoter.

Duplicated CaMV 35S promoter and nopaline synthase terminator (NOS) from *p*35SeNOS-B plasmid (López-Moya and García, 2000) were inserted into the multiple cloning site of *p*CAMBIA2300 plasmid (Cambia) and designated pCAM2300-35SeNOS, which was used for cloning full-length ToCV cDNAs under the 35S promoter. The full-length cDNAs corresponding to RNA1 and RNA2 were amplified by PCR using PfuTurbo DNA polymerase (Stratagene) and MA682/MA683 and MA684/MA685 primers phosphorylated at the 5′ end (Supplementary table 1) corresponding to the 5′ and 3′ ends of AT80/99 isolate (Lozano et al., 2006, 2007). Clones pToCV227 (RNA1) and pToCV230 (RNA2) described above were used as a template (Fig. 1). The PCR-amplified fragments were gel purified using QIAquick Gel Extraction Kit (QIAGEN) and cloned into pCAM2300-35SeNOS digested with *Stu*I. Recombinant plasmids corresponding to RNA1 and RNA2 (Fig. 1) were transformed into the *E. coli* strains ElectroMAX Stbl4 (Invitrogen) and DH5α (Invitrogen). Two clones corresponding to each RNA were selected to transform *A. tumefaciens* strain GV3101.

In vitro transcription and transfection of Nicotiana benthamiana and tomato mesophyll protoplasts

Capped (Ribo m7G Cap Analog [Promega]) transcripts were synthesized from *Bam*HI-linearized pToCV227 (RNA1) and *Xma*I-linearized pToCV230 and pToCV231 (RNA2) with SP6 RNA polymerase (Roche). Following transcription, RNAs were analyzed on 1% non-denaturing agarose gels in TAE and transcripts were stored at −70 °C until use.

The method used for *N. benthamiana* protoplast isolation and inoculation were as described by Navas-Castillo et al. (1997), except that 0.5% Onozuka Cellulase R10 and 0.25% Macerase pectinase in enzyme solution were used, and inoculations were performed using 5–10 μg of *in vitro* transcripts of linearized plasmid per 10^6 protoplasts with 30% polyethyleneglycol (PEG) 8000 in 3 mM $CaCl_2$ and 400 mM D-mannitol. For RNA extraction, 10^6 protoplasts were disrupted in 500 μl of 50 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, pH 9.0, plus 2% SDS and analyzed by northern blot hybridization.

Protoplasts from tomato plants (cultivar Moneymaker) were prepared and inoculated essentially by procedures described by Tan et al. (1987), but without using sucrose. Inoculations were performed with PEG 4000 using 5 μg of *in vitro* transcripts of linearized pToCV227 clone, as described above.

Agrobacterium-mediated transmission

Agrobacterium tumefaciens GV3101 carrying RNA1 and RNA2 clones corresponding to the full-length ToCV genome components

were grown at 28 °C for 48 h either on Luria-Bertani (LB) broth containing rifampicin (25 μg/mL), gentamicin (20 μg/mL), tetracycline (2.5 μg/mL) and kanamycin (50 μg/mL), 10 mM morpholineethanesulfonic acid (MES) and 20 μM acetosyringone in vigorous shaking or LB agar medium with the above mentioned antibiotics. The bacterial cells were harvested either by centrifugation (for bacteria grown in liquid medium) and resuspended into induction buffer (10 mM MgCl2, 10 mM MES pH 5.6, 150 μM acetosyringone) to a final OD600 of 1.0 (for both RNA1 and RNA2 clones) and then kept at room temperature for 2 h without shaking, or by scraping the bacteria from agar medium (for bacteria grown on solid medium).

Agroinoculation of *N. benthamiana*, tomato cv. Moneymaker and *A. thaliana* Columbia was carried out with bacterial suspensions corresponding to both RNA components mixed in a 1:1 ratio. Agroinoculation of *N. benthamiana* plants (3–5 leaf stage) was performed by infiltration on the underside of leaves with a 1 mL syringe without a needle. *A. thaliana* plants were agroinoculated in the same way in the stage of 6–8 leaves or by apex inoculation using an entomological needle number 0 dipped into the *A. tumefaciens* inoculum culture (agar medium) inserted 6–8 times in the shoot apex of the rosette. Tomato plants were agroinfiltrated at the cotyledon and/or 2–4 leaf stages as described for *N. benthamiana* and also stems were injected with a cell suspension with a 1 mL syringe with a 25 gauge needle. Furthermore, other forms of inoculation were assayed for tomato plants: (i) *Agrodrench* of 10 plants using 5 mL of *Agrobacterium* suspension as previously described (Ryu et al., 2004) and (ii) maintaining 10 plants with the exposed roots immersed in 5 mL of *Agrobacterium* suspension for 4 days.

Both RNAs clones were also co-inoculated with clones transiently expressing viral silencing suppressors, ToCV *p*22 (Cañizares et al., 2008) and *Potato virus Y* (PVY) HC- Pro (Hamilton et al., 2002). These suppressor proteins were cloned into the binary vectors pBin19 and pBin61, respectively. Agroinoculation assays are summarized in Table 1. Plants agroinoculated with *A. tumefaciens* strain GV3101 transformed with the vector pCAM2300-35SeNOS were used as negative control. Agroinoculated plants were kept in a growth chamber with 16 h of light at 25 °C, 8 h dark at 18 °C and 70% relative humidity. The inoculated plants were analyzed by molecular hybridization of leaf petiole cross-sections (tissue printing) and/or northern blots of infiltrated area and/or apical leaves (~100 mg).

Grafting transmission

Healthy tomato plants cv. Moneymaker at 7 leaf stage were used as rootstock and ToCV-agroinfected *N. benthamiana* plants (35 days post infiltration) as the scions in graft inoculation experiments. Scions consisting of flowering stems were used in V-shape of side-grafting on the stem of tomato plants. Plants, covered with plastic bags to avoid dehydration for one week, were kept in a growth chamber at 25 °C during the day and 18 °C overnight with a 16 h photoperiod and 70% relative humidity. The presence of ToCV in the apical leaves of tomato plants was analyzed 15 days after grafting by tissue-printing molecular hybridization.

Whitefly transmission

Adults of *B. tabaci* Mediterranean (MED) (formerly Q biotype) and Middle East-Asia Minor 1 (MEAM1) (formerly B biotype) species (following nomenclature of De Barro et al., 2011) were used for transmission assays. Whitefly populations were reared on melon (Cucumis melon cv. ANC 42, La Mayora-CSIC seed bank) in insect-proof cages. Tomato plants infected with ToCV by grafting were used as the virus source. Whitefly-mediated transmission assays were conducted using 50 *B. tabaci* adults per clip-cage per plant, 48 h acquisition access period and 48 h inoculation access period, as described before (Fortes et al., 2012). The presence of ToCV in inoculated plants was analyzed by tissue printing molecular hybridization at 15 and 30 days post-inoculation (dpi). Inoculated plants were observed periodically to evaluate the presence of symptoms and were kept in a greenhouse (day at ~25 °C and night at ~18 °C) until 150 dpi.

RNA analysis

Total RNA was extracted from leaf tissue with TRIzol (Invitrogen) or TRIsure (Bioline) reagents following the manufacturer's instructions. Detection of ToCV RNA in inoculated plants was carried out by molecular hybridization of tissue printing, northern blotting or RT-PCR. For tissue printing, leaf petiole cross-sections were blotted on positively-charged nylon membranes (Roche Diagnostics), fixed by ultraviolet light (*Crosslinker* RPN 2500, Amersham Life Science) and hybridized with a digoxigenin-labeled negative sense RNA probe specific for the coat protein gene, as described previously (Fortes et al., 2012; Trenado et al., 2007), or for the p22 gene and 3′ end of RNA2 as described below. For northern blot analysis, total RNA (5 μg) or dsRNA (0.5 μg) was separated out by 1% formaldehyde agarose gel electrophoresis, transferred to a positively-charged nylon membrane (Roche) by applying a vacuum (VacuGene XL, HealthCare) and fixed by ultraviolet light. The membrane was hybridized with digoxigenin-labeled specific RNA probes for the RNA1 and RNA2 ends of ToCV (RNA1: 582 nt within p22 gene [7662–8243 nt of AT80/99 isolate]; RNA2: 632 nt including p7 and partial p27 genes [7613–8244 nt of AT80/99 isolate]). Digoxigenin-labeled positive and negative sense ssRNA probes were prepared by *in vitro* transcription from the appropriate linearized plasmid with SP6 or T7 RNA polymerases (Roche) and DIG RNA labeling Mix (Roche), according to the manufacturer's recommendations. The hybridization conditions used were those recommended by the manufacturer (DIG Application Manual for Filter Hybridization, Roche). Membranes were exposed to X-ray film (X-Omat AR, Kodak) and developed following a conventional photographic process. RT-PCR was performed using the primers MA380 (5′–GTGAGACCCCGATGACAGAT-3′) and MA381 (5′- TACAGTTCCTTGCCCTCGTT-3′) designed to amplify a 436 bp DNA fragment of the coat protein gene, based on the sequence of the Spanish AT80/99 ToCV isolate (Lozano et al., 2006), as previously described (Fortes et al., 2012).

Immunoelectron microscopy

Leaf samples of healthy and ToCV-infected (following graft inoculation) tomato plants were used to prepare extracts for visualization by immunoelectron microscopy. Leaves were finely chopped in the presence of sodium citrate 0.1 M (pH 6.8), 10% sucrose and 25 mM dithiothreitol. Formvar/carbon grids, previously incubated with an anti-CP ToCV antiserum (supplied by Stephan Winter, DSMZ), were allowed to float on drops of infected plant extracts and stained with 1% uranyl acetate. Grids were visualized with a JEOL JEM 1400 transmission electron microscope.

Immunoblot analysis

Serological detection of ToCV in *N. benthamiana* and tomato infected plants was carry out on cross-sections of petioles and stems onto hydrophobic polyvinylidenedifluoride (PVDF) membranes (Hybond-P, Amersham) using a polyclonal anti-CP ToCV antiserum (supplied by Stephan Winter, DSMZ) conjugated to alkaline phosphatase at a 1:500 dilution. Colorimetric detection with a NBT/BCIP system (Roche) was used following the manufacturer's instructions.

For western blot analysis, anti-CP ToCV antiserum was used and proteins were denatured by boiling in 2x protein dissociation buffer (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, 2.5% 2-mercaptoethanol) and separated in a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.032.

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