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The invasion biology of tomato begomoviruses in Costa Rica reveals neutral synergism that may lead to increased disease pressure and economic loss

Minor R. Maliano [a,b], Maria R. Rojas [a], Monica A. Macedo [a,c], Natalia Barboza [d], Robert L. Gilbertson [a,*]

[a] Department of Plant Pathology, University of California, Davis, California 95616 USA
[b] Institute for Molecular Virology, University of Wisconsin–Madison, Madison, Wisconsin, 53706 USA
[c] Instituto Federal Goiano, Campus Urutaí, Urutaí, Goiás, 75790-000, Brazil
[d] Centro de Investigación en Biología Celular y Molecular (CIBCM), Escuela de Tecnología de Alimentos, Centro Nacional en Ciencia y Tecnología de Alimentos (CITA), Universidad de Costa Rica, 2060 San José, Costa Rica

A B S T R A C T

Since the late 1980s, tomato production in Costa Rica has been affected by diseases caused by whitefly-transmitted begomoviruses. The first was tomato yellow mottle virus (ToYMoV), a locally evolved New World (NW) bipartite begomovirus associated with the tomato yellow mottle disease (ToYMoD). In the late 1990s, the invasive NW bipartite tomato leaf curl Sinaloa virus (ToLCSiV) was detected in Costa Rica and has become established and associated with ToYMoD. Finally, the invasive Old World (OW) monopartite tomato yellow leaf curl virus (TYLCV) was detected in Costa Rica in 2012 and has also become established and is causing tomato yellow leaf curl disease (TYLCD). In the present study, we investigated the invasion biology of these tomato-infecting begomoviruses in Costa Rica in terms of (i) their biological and genetic properties and (ii) disease symptoms and viral DNA accumulation in tomato plants having single and mixed infections. We first generated infectious DNA-A and DNA-B clones and agroinoculation systems for ToYMoV and ToLCSiV isolates recovered from archival ToYMoD samples collected in Costa Rica in 1990 and 2002, respectively. Tomato plants agroinoculated with the infectious clones of both viruses developed ToYMoD symptoms, completing Koch's postulates for ToYMoV, and showing that ToLCSiV also causes this disease. However, pseudorecombinants formed between the DNA components of these viruses were not infectious, which is consistent with independent evolution in different lineages and limits genetic interactions. Furthermore, ToYMoV is well-adapted to tomato, has a narrow host range and is mechanically transmissible. The DNA-A component has a recombination event in the hot spot area and induced a symptomless infection in agroinoculated *Nicotiana benthamiana* and tomato plants. Tomato plants co-infected with two or all three viruses developed more severe symptoms compared with plants infected with each virus alone. Symptoms induced by the NW bipartite ToYMoV and ToLCSiV appeared earlier (~7 d post-inoculation [dpi]) than those induced by TYLCV (~10 dpi), but TYLCD symptoms became predominant in single and mixed infections by 14 dpi. Viral DNA accumulation was quantified by qPCR and generally revealed a neutral synergistic interaction in which the viruses co-existed in mixed infections. A transient reduction in accumulation of ToYMoV and ToLCSiV was detected in mixed infections at 7 dpi, whereas TYLCV accumulation was not affected in mixed infections and was uniform among treatments and time points. Together our results suggest that this neutral synergistic interaction will lead to increased begomovirus disease severity in Costa Rica. We discuss this in terms of begomovirus invasion biology and disease management.

1. Introduction

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are small plant viruses with a circular single-stranded DNA genome encapsidated in twin quasi-icosahedral virions (18 × 30 nm) (Hanley-Bowdoin et al., 2013; Rojas et al., 2005; Zerbini et al., 2017). Members of this genus are transmitted in nature by whiteflies of the *Bemisia tabaci* cryptic species complex and are prevalent in tropical and subtropical regions of the world (De Barro et al. 2011; Gilbertson et al. 2015; Zerbini et al. 2017; Navas-Castillo et al. 2011). The begomovirus genome is either monopartite, with a single genomic DNA of ~2.6–2.8 kb; or bipartite, with two DNA components of ~2.6 kb designated as DNA-A and DNA-B (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Zerbini et al. 2017; Navas-Castillo et al. 2011). Sequences of the DNA-A and DNA-B components are different, except for a shared ~200 nucleotide (nt) noncoding sequence known as the common region. Within this sequence resides *cis*-acting elements involved in replication and gene expression (i.e., the origin of replication [*ori*] and two bidirectional RNA polymerase II promoters), as well as sequences important for maintaining the bipartite genome (Hanley-Bowdoin et al., 2013).

It is well established that begomoviruses have a phylogeographic distribution, in which most bipartite species occur in the New World

* Corresponding author.
E-mail address: rlgilbertson@ucdavis.edu (R.L. Gilbertson).

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(NW), whereas most monopartite ones occur in the Old World (OW) and are often associated with satellite DNAs that are either required for disease development (betasatellites) or have minimal effect on virulence (alpha- and deltasatellites) (Briddon et al., 2010; Dry et al., 1997; Fiallo-Olivé et al., 2012; Fiallo-Olivé and Navas-Castillo, 2020; Lozano et al., 2016; Yang et al., 2019; Zhou, 2013). However, there are notable exceptions, including the identification of indigenous NW monopartite begomoviruses causing leaf curl and distortion diseases of tomato in Latin America (Gilbertson et al., 2015; Macedo et al., 2017; Márquez-Martín et al., 2011; Melgarejo et al., 2013; Romay et al., 2019).

The emergence of new begomovirus diseases has been mediated by the *B. tabaci* species Middle East Asia Minor 1 (MEAM1), which is a supervector of plant viruses (Gilbertson et al. 2015; Navas-Castillo et al. 2011). However, before the global spread of this whitefly supervector, which started in the early 1990s (Byrne and Bellows, 1991; DeBarro et al., 2011), it is important to note that begomovirus diseases were widely distributed in noncultivated plants (e.g., weeds) and in some economically important crops in the NW (e.g., common bean and tomato) and OW (e.g., cassava and tomato) and presumably were spread by indigenous whitefly species (Bock, 1983; Costa, 1976; Harrison et al., 2002). Moreover, although these locally evolved begomoviruses were divergent genetically, they tended to induce similar disease symptoms in crop plants, a phenomenon referred to as local or parallel evolution (Gilbertson et al., 1993; Zhou et al., 2008). Following the worldwide establishment of the polyphagous MEAM1, new combinations of indigenous begomoviruses were introduced into a wider range of plant species, accelerating viral evolution and the appearances of new diseases (Gilbertson et al. 2015; Navas-Castillo et al. 2011). Human activities also have facilitated the long-distance intercontinental transport of some economically important begomoviruses and, in some cases, crossing the geographic separation of OW and NW begomoviruses (Gilbertson et al. 2015; Navas-Castillo et al. 2011; Rojas et al. 2018). Notable examples include the introductions of the OW monopartite begomovirus tomato yellow leaf curl virus (TYLCV) into the NW in the early 1990s (Mabvakure et al. 2016; Lefeuvre et al. 2010; Salati et al. 2002; Nakhla et al. 1994) and the NW squash leaf curl virus (SLCuV) into the Middle East (Akad et al., 2008; Antignus et al., 2003; Lapidot et al., 2014; Ruiz et al., 2015; Varma et al., 2011).

Tomato is one of the most important vegetables in the world (Dorais et al., 2008). In Latin America and Costa Rica, tomatoes are produced locally as a fresh market crop (Blanca et al., 2012; Passam et al., 2007; Schreinemachers et al., 2018). Unfortunately, cultivated tomato is highly susceptible (permissive) to begomovirus infection, which is reflected by the >90 tomato-infecting species recognized by the International Committee on Taxonomy on Viruses (ICTV) (Zerbini et al. 2017; Rojas et al. 2018). In Costa Rica, tomato production has been negatively impacted by begomovirus diseases since the late 1980s (Hilje and Stansly, 2008; Karkashian et al., 1998; Nakhla et al., 1994; Polston and Anderson, 1997). In Costa Rica, the prevalence and importance of these diseases and the viruses involved has changed due to three emergence/invasion events. The first was the emergence of the indigenous locally evolved NW bipartite tomato yellow mottle virus (ToYMoV), previously referred to as tomato geminivirus-Costa Rica (TGV-CR) (Patel et al., 1993), in the late 1980s and in association with ToYMoD (Karkashian et al., 1998; Nakhla et al., 1994). The second was the introduction of the invasive NW bipartite tomato leaf curl Sinaloa virus (ToLCSiV) in the late 1990s (Idris et al. 1999), and the subsequent detection in ToYMoD samples collected in 2011 (Barboza et al. 2018). The third event was the introduction of the invasive OW monopartite TYLCV around 2012, which was followed by spread and establishment in many tomato producing regions of the country (Barboza et al. 2014, 2018).

In the present study, we investigated the invasion biology of these tomato begomoviruses in Costa Rica. Infectious DNA-A and DNA-B clones and an agroinoculation system were generated for an isolate of ToYMoV recovered from an archival ToYMoD sample collected in Costa Rica in 1990, and used to determine biological, genetic and molecular properties of the virus, including the fulfilling of Koch's postulates for ToYMoD. Similarly, infectious DNA-A and DNA-B clones and an agroinoculation system were generated for an isolate of the invasive ToLCSiV recovered from an archival ToYMoD sample collected in Costa Rica in 2002, and shown to also cause ToYMoD. Pseudorecombinants formed between the infectious cloned components of ToYMoV and ToLCSiV were not infectious, consistent with a long history of local evolution in different lineages. Together with an infectious clone of the genomic DNA of TYLCV, we next investigated virus-tomato interactions in single and mixed infections based on symptom development and viral DNA accumulation. This information can help (i) predict the future impact of these begomoviruses on tomato production in Costa Rica and (ii) guide the development of integrative pest management (IPM) approaches (Gilbertson et al., 2011; Rojas et al., 2018).

2. Materials and methods

2.1. Virus sources, DNA extraction, and detection of begomovirus DNA

Leaf samples with crumpling and yellow mottle/mosaic symptoms were collected from tomato plants in commercial fields in Costa Rica. Samples GR1 and GR2 were collected in the region of Grecia in 1990, whereas samples L1-L10 were collected in the region of Liberia in 2002 (Supplemental Fig. S1). These samples were applied to nylon membranes in Costa Rica, which were transported to the University of California at Davis (UC Davis). Total genomic DNA was extracted from the squashed leaf tissue on these membranes as previously described (Dellaporta et al., 1983; Kon and Gilbertson, 2012).

To detect begomovirus DNA-A and DNA-B components, polymerase chain reaction (PCR) tests were performed with the degenerate primer pairs PAL1v1978/PAR1c496 and PCRc2/PLB1v2039, which direct the amplification of ~1.1 kb and ~0.3-0.5 kb fragments, respectively (Rojas et al., 1993). PCR-amplified fragments were purified with the QIAquick gel extraction kit (Qiagen, Germantown, MD) and directly sequenced at the DNA Sequencing Facility of UC Davis.

2.2. Cloning full-length begomovirus DNA components

To obtain full-length begomovirus clones, rolling circle amplification (RCA) with Φ-29 DNA polymerase (TempliPhi; GE Healthcare, Piscataway, NJ) was performed as previously described (Inoue-Nagata et al., 2004). The resulting RCA products were digested with *Bam*HI, *Eco*RI, *Hind*III, *Sal*I and *Xba*I to generate restriction fragment length polymorphisms (RFLPs) to estimate the number and genetic diversity of DNA components in these samples, and to identify single-cutting enzymes for cloning full-length components into pGEM-11Z (+) (Promega Corp., Madison, WI).

For the bipartite begomovirus infecting the GR1 sample, full-length (~2.6 kb) DNA-A and DNA-B clones were generated with *Xba*I, whereas those of the isolate infecting the L1 sample were cloned with *Bam*HI and *Xba*I, respectively. Recombinant plasmids having the cloned DNA components were identified by restriction enzyme digestion analysis and DNA sequencing.

2.3. Sequence analysis

Sequences of the cloned full-length DNA-A and DNA-B components of the GR1 isolate in the recombinant plasmids pGV-CR-GR1-A and pGV-CR-GR1-B, and the L1 isolate in the recombinant plasmids pGV-CR-L1-A and pGV-CR-L1-B were determined at the UC Davis DNA Sequencing Facility and analyzed with SnapGene Viewer (GSL Biotech). For the sequence of each component, a BLASTn search was initially performed to identify the ten sequences in GenBank with highest identities (Altschul et al., 1990; Benson et al., 2018; Muhire et al., 2014). Pairwise nt sequence alignments of the full-length components were performed with

MUSCLE within the Species Demarcation Tool (SDT) v.1.2 (Muhire et al. 2014). Comparisons also were made with individual open reading frames (ORFs) and non-translated regions (NTRs). Nucleotide and amino acid (aa) identities and similarities were calculated in the Sequence Manipulation Suite Server (Stothard, 2000). The *cis*-acting elements involved in begomovirus replication (i.e., iterons and the Rep iteron-related domains [IRDs]) were identified according to Argüello-Astorga and Ruiz-Medrano (2001).

2.4. Phylogenetic analysis

For the phylogenetic analyses, we used the complete nt sequences of the DNA-A and DNA-B components of: (i) the GR1 and L1 isolates; (ii) ToYMoV isolates from Costa Rica (both components for one isolate and the DNA-A components of two others); (iii) ToLCSiV isolates from Costa Rica and Nicaragua; (iv) the ten begomovirus sequences with highest identities to the GR1 isolate revealed by the BLASTn search; and (v) sequences of selected begomoviruses representing the Abutilon mosaic virus (AbMV), Brazil, SLCuV, bean golden yellow mosaic virus (BGYMV) and Boerhavia golden mosaic virus (BoGMV) lineages of NW begomoviruses. Multiple sequence alignments (MSA) were generated with the MAFFT algorithm implemented in the Guidance2 Server (Katoh, 2002; Sela et al., 2015). The alignment quality was analyzed and unreliable regions (poorly aligned) were removed with the GUIDANCE algorithm (Sela et al., 2015). The resulting alignments were then exported as Nexus files, and phylogenic trees were constructed with a Bayesian inference and Markov chain Monte Carlo (MCMC) simulation implemented in MrBayes V3.2 (Ronquist et al., 2012). The best fit model of nt substitution for each data set was determined with the program MrModeltest V2.2 (Darriba et al., 2012). The analyses were carried out by running 2,000,000 generations and sampling at every 100 generations, resulting in 20,000 trees. The first 10% of samples were discarded as a burn-in. Trees were visualized with Archaeopteryx tree viewer and exported in Newick format (Han and Zmasek 2009). Trees were manually edited with MEGA X (Kumar et al., 2018). The DNA-A and DNA-B phylogenetic trees were rooted with the sequences of the genomic DNA of the OW monopartite TYLCV and the DNA-B component of the OW bipartite African cassava mosaic virus (ACMV), respectively.

2.5. Recombination analysis

Preliminary datasets of 584 complete DNA-A and 240 DNA-B sequences were assembled, which included the complete nt sequences of the DNA-A and DNA-B components of: (i) the GR1 and L1 isolates; (ii) ToYMoV isolates from Costa Rica; (iii) ToLCSiV isolates from Costa Rica and Nicaragua; and (iv) all full-length begomovirus sequences available in GenBank. SDT and the Recombination Detection Program version 4.0 (RDP4) (Martin et al., 2015) were used to remove sequences that were identical to each other or had nt sequence identities <70%. Final datasets of 503 DNA-A sequences and 201 DNA-B sequences were used for the recombination analyses. MSA were generated with Muscle within MEGA X (Edgar, 2004; Kumar et al., 2018), and the alignment was manually edited and exported as FASTA files. Recombination events and breakpoints and potential parental viruses were determined with RDP4. The recombination analyses were performed with default settings and a Bonferroni-corrected *p*-value cut-off of 0.05. Only recombination events detected with three or more methods were considered.

2.6. Infectivity of the full-length monomers of the DNA-A and DNA-B components determined by particle bombardment

Full-length linear double-stranded monomers of the DNA-A and DNA-B components of the GR1 and L1 isolates, thereafter referred to as ToYMoV and ToLCSiV, respectively, based on comparisons of PCR-amplified fragments, were inoculated into leaves of *Nicotiana benthamiana* plants at the three to five leaf stage (~3 wk old) and tomato (cv. Glamour) seedlings at the one to two leaf stage (~2 wk old) by particle bombardment as described by Paplomatas et al. (1994).

The negative control was equivalent plants bombarded with gold particles alone. Inoculated plants were maintained in a controlled environment chamber (photoperiod, 16 h light/8 h dark; light intensity, 300 μEinsteins; temperature of 25°C and relative humidity of 60%). Disease symptoms were evaluated at 14 d post-bombardment (dpb), and virus infection was determined by PCR tests with the degenerate primer pairs PAL1v1978/PAR1c496 and PCRc1/PLB1v2039 and DNA sequencing as described above.

2.7. Production of multimeric clones and agroinoculation systems

To develop agroinoculation systems for infectivity studies, recombinant plasmids with multimeric copies of infectious cloned DNA-A and DNA-B components were generated as described by Paplomatas et al. (1994). For the DNA-A component of the ToYMoV GR1 isolate, an ~0.5 kb *Eco*RI-*Xba*I fragment containing the common region was cloned into pCAMBIA 1300 to generate the 0.2-mer pGV-CR-GR1-A0.2. The full-length DNA-A monomer was released from pGV-CR-GR1-A with *Xba*I and cloned into *Xba*I-digested pGV-CR-GR1-A0.2 to generate the 1.2-mer pGV-CR-GR1-A1.2. For the DNA-B component, an ~0.9 kb *Xba*I-*Sal*I fragment containing the common region was cloned into pCAMBIA 1300 to generate the 0.4-mer pGV-CR-GR1-B0.4. The full-length DNA-B monomer was released from pGV-CR-GR1-B with *Xba*I and cloned into the *Xba*I-digested pGV-CR-GR1-B0.4 to generate the 1.4-mer pGV-CR-GR1-B1.4.

For the DNA-A component of the ToLCSiV L1 isolate, an ~1.3 kb *Sal*I-*Bam*HI fragment containing the common region was cloned into pCAMBIA 1300 to generate the 0.5-mer pGV-CR-L1-A0.5. The full-length DNA-A monomer was released from pGV-CR-L1-A with *Bam*HI and cloned into *Bam*HI-digested pGV-CR-L1-A0.5 to generate the 1.5-mer pGV-CR-L1-A1.5. For the DNA-B component, an ~1.2 kb *Xba*I-*Sal*I fragment containing the common region was cloned into pCAMBIA 1300 to generate the 0.5-mer pGV-CR-L1-B0.5. The full-length DNA-B monomer was released from pGV-CR-L1-B with *Xba*I and cloned into *Xba*I-digested pGV-CR-L1-B0.5 to generate the 1.5-mer pGV-CR-L1-B1.5. Recombinant plasmids having the multimeric clones were identified by restriction enzyme digestion, and then transformed into electrocompetent *Agrobacterium tumefaciens* cells (strain C58C1) by electroporation.

2.8. Infectivity and host range experiments

The infectivity of the cloned multimeric DNA-A and DNA-B components of ToYMoV GR1 isolate and ToLCSiV L1 isolate in binary plasmids in *A. tumefaciens*, hereafter referred to as agroinoculation systems, was initially determined by agroinoculating *N. benthamiana* plants at the three-to-five true leaf-stage with mixtures of suspensions of *A. tumefaciens* cells (optical density of 600 nm = 1.0) containing binary plasmids with the cloned multimeric DNA-A and DNA-B components of each virus by needle puncture of the stem just beneath the shoot apex (Hou et al., 1998).

For the ToYMoV GR1 isolate, a partial host range was determined by agroinoculating tomato (*Solanum lycopersicum* cv. Glamour), *N. benthamiana*, tobacco (*Nicotiana tabacum* cv. Havana), *Datura stramonium*, common bean (*Phaseolus vulgaris* cv. Topcrop), cucumber (*Cucumis sativus*), cantaloupe melon (*Cucumis melo* cv. Minnesota Midget), honeydew melon (*C. melo* cv. Sweet Delight), pumpkin (*Cucurbita maxima* cv. Sugarpie) and *Chenopodium amaranticolor*. The negative control was equivalent plants agroinoculated with cell suspensions of an *A. tumefaciens* strain carrying the empty vector (pCAMBIA 1300).

Because certain common bean and pepper varieties are recalcitrant to agroinoculation (Garrido-Ramirez et al., 2000; Li et al., 2000; Zhou et al., 2008), these were inoculated with the cloned multimeric DNA-A

and DNA-B components of the ToYMoV GR1 isolates by particle bombardment as described by Paplomatas et al. (1994). Pepper plants (*Capsicum annuum* cv. Cayenne) at the two true leaf-stage were bombarded with these plasmids at 450 psi. The positive control in these experiments was equivalent pepper plants bombarded with the infectious multimeric DNA-A and DNA-B clones of the pepper-infecting begomovirus pepper huasteco yellow vein virus (PHYVV), whereas the negative control was equivalent plants bombarded with gold particles alone.

For common bean, hypocotyls of seedlings of cultivars representing the Andean (cv. Topcrop) and Middle American (cvs. Carioca, Black Turtle Soup [BTS] and Othello) gene pools were bombarded with the ToYMoV DNA-A and DNA-B multimers as previously described by Paplomatas et al. (1994). The positive control was equivalent seedlings of cv. Topcrop bombarded with multimers of the infectious DNA-A and DNA-B components of bean golden mosaic virus (BGMV), whereas the negative control was equivalent seedlings bombarded with gold particles alone.

Inoculated plants were maintained in a controlled environment chamber as described above. Symptom development was assessed visually and recorded at 14 d post-inoculation (dpi). In selected symptomatic and all symptomless plants, the presence of the inoculated DNA-A and DNA-B components in newly emerged (non-inoculated) leaves was determined by PCR tests with component-specific primers (Supplemental Table S1).

2.9. Sap Inoculation

Leaves of *N. benthamiana* and tomato (cv. Glamour) plants were rub-inoculated with sap prepared with ice-cold 0.1 M phosphate buffer (pH 7.2) as previously described (Gilbertson et al., 1991; Wang et al., 1996). Sap was prepared by grinding symptomatic leaf tissue collected from *N. benthamiana* plants that had been agroinoculated with the ToYMoV GR1 isolate. The positive control for *N. benthamiana* plants was equivalent plants inoculated with sap prepared from symptomatic leaf tissue collected from *N. benthamiana* plants infected with the sap-transmissible bipartite begomovirus bean dwarf mosaic virus (BDMV), whereas for tomato, the positive control was tomato mottle virus (ToMoV). The negative control was equivalent plants inoculated with buffer alone. Inoculated plants were maintained in a controlled environment chamber and symptom development was assessed visually and recorded at 14 dpi. In selected symptomatic and all symptomless plants, the presence of the ToYMoV DNA-A and DNA-B components in newly emerged leaves was determined by PCR tests with primer pairs specific for each component (Supplemental Table S1).

2.10. Pseudorecombination experiments

These experiments were performed by agroinoculating *N. benthamiana* and tomato (cv. Glamour) plants with mixtures of cell suspensions of *A. tumefaciens* strains carrying binary plasmids with the multimeric cloned DNA-A or DNA-B components of the ToYMoV GR1 and ToLCSiV L1 isolates. Controls were equivalent plants agroinoculated with the DNA-A and DNA-B components of each virus (positive) or with the empty vector (negative). Inoculated plants were maintained in a controlled environment chamber, and symptom development was assessed as previously described. The presence of the inoculated DNA-A and DNA-B components in newly emerged leaves was determined by PCR tests with component-specific primer pairs (Supplemental Table S1).

2.11. Agroinoculation of tomato plants with ToYMoV, ToLCSiV and TYLCV alone and in all combinations

In these experiments, tomato plants of the susceptible cv. Glamour were agroinoculated with each virus alone or co-agroinoculated with all combinations as described above. Plants were agroinoculated with the multimeric cloned DNA-A and DNA-B components of the ToYMoV GR1 and ToLCSiV L1 isolates and the infectious multimeric clone of an isolate of TYLCV from the Dominican Republic (Kon et al., 2009). The negative control was equivalent plants agroinoculated with the empty vector. Three plants per treatment were inoculated per experiment, and the experiment was conducted three times. Inoculated plants were maintained in a controlled environment chamber, and symptom development was assessed at 7, 14 and 21 dpi. The presence of the DNA components/genomic DNA of each virus was initially determined by conventional PCR tests with primer pairs specific for components/genomic DNA of each isolate (Supplemental Table S1).

2.12. Quantitative PCR (qPCR) assay

Viral DNA accumulation in tomato leaves infected with each virus alone or in mixed infections was quantified by qPCR according to the protocol in Mason et al. (2008). Total genomic DNA was extracted from newly emerged leaves of inoculated and control tomato plants collected at 7, 14 and 21 dpi according to Dellaporta et al. (1983). Virus-specific primer pairs for qPCR detection of ToYMoV, ToLCSiV and TYLCV were designed to direct the amplification of ~150 bp fragments from the capsid protein (CP) genes of ToYMoV (PTv324/PTc476) and TYLCV (PTYv372/PTYc523) and from the AC1 gene of ToLCSiV (PSv2226/PSc2375) (Supplemental Table S1). The specificity of these primer pairs was predicted *in silico* based on sequence alignments and confirmed experimentally by PCR tests with DNA extracts of tomato plants infected with each virus and negative control plants.

To generate standards for the qPCR tests, the PCR-amplified ToYMoV, ToLCSiV and TYLCV fragments were cloned into pCR-Blunt II-TOPO vector (Zero Blunt® PCR Cloning Kit; Invitrogen) and sequenced. Recombinant plasmids containing these fragments were quantified with a NanoDrop1000 spectrophotometer (Thermo Scientific), and plasmid copy number (Cn) was adjusted to 1.0×10^7 copies/µl with the Avogadro's constant ($6.022140857 \times 10^{23}$). Standard curves were generated for each virus with tenfold serial dilutions that ranged from 10^1 to 10^6 copies. These standard curves were used to estimate the viral Cn for each sample. The qPCR was conducted with a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific) with 100 ng of total genomic DNA in a 20-µl reaction prepared with the SsoFast EvaGreen Supermix kit (Bio-Rad, Richmond, CA).

2.13. Statistical analysis

Kruskal-Wallis tests were used to analyze the significance of differences in viral DNA levels in single and mixed infections. All data are presented as the mean ± standard error of the mean and differences between treatments are considered significant when p-value < 0.05.

3. Results

3.1. Virus source, DNA extraction, and detection of begomovirus DNA

To obtain an isolate of ToYMoV, we used archival ToYMoD samples (GR1 and GR2) collected in Grecia, Costa Rica in 1990 (Supplemental Fig. 1), relatively soon after the appearance of ToYMoD in the late 1980s. In PCR tests with total genomic DNA extracted from tissue squashes of the GR1 and GR2 samples and degenerate DNA-A and DNA-B primer pairs, the expected-size ~1.1- and ~0.3-kb fragments were amplified, respectively, consistent with infection with a bipartite begomovirus. The sequences of these PCR-amplified DNA-A and DNA-B fragments were >98% identical to each other, and had the highest identities with those of the DNA-A (>98%) and the DNA-B (>96%) components of ToYMoV isolates collected in Costa Rica in 2012 (Barboza et al., 2018). Digestion of the RCA products generated from the total genomic DNA of these samples with *Eco*RI generated three

Table 1
Nucleotide (nt) identities for total and common region and hypervariable region (HVR) sequences and nt and amino acid (aa) identities and similarities (in parenthesis) of individual open reading frames (ORFs) for the DNA-A and DNA-B components of an isolate of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) and those of begomoviruses with highest identities [a].

			DNA-A component											DNA-B component [e]				
			Total	Common region	ORFs								Total	ORFs				
					AV1		AC1		AC2		AC3		AC4		BV1		BC1	
Begomovirus isolates [b]	Year [c]	Loc [d]			nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	HVR	nt	aa	nt	aa
ToYMoV-[CR:5245-35:12]	2012	CR	98	98	98	99 (100)	98	97 (99)	99	98 (98)	99	98 (99)	98	93 (94)	97	93	98	98 (98)	98	98 (99)
ToYMoV-[CR:5249-9:12]	2012	CR	98	98	98	99 (100)	98	97 (99)	99	98 (98)	99	98 (99)	98	93 (94)	NA	NA	NA	NA	NA	NA
ToYMoV-[CR:5249-11:12]	2012	CR	98	98	98	99 (100)	98	97 (99)	99	98 (98)	99	98 (99)	98	93 (94)	NA	NA	NA	NA	NA	NA
SiCMoV-[BR:Trm531.1:10]	2010	BR	79	NA	82	90 (94)	73	74 (84)	82	72 (81)	83	78 (85)	70	44 (56)	NA	NA	NA	NA	NA	NA
TbYCV-[CU:07]	2007	CU	79	60	83	89 (93)	75	77 (86)	76	66 (75)	79	75 (82)	73	47 (60)	71	52	69	70 (82)	73	77 (87)
TYVSV-[CL:VS-B4:To:2012]	2012	CL	78	60	84	92 (95)	74	76 (84)	77	64 (75)	80	77 (86)	69	53 (59)	71	52	72	71 (82)	76	81 (90)
BleICV-[MX:Cam:11]	2011	MX	78	61	81	88 (90)	76	74 (82)	83	71 (80)	83	76 (83)	69	43 (53)	71	48	74	73 (83)	74	80 (89)
CabLCV-[EC:GuaEC54Rc:17]	2017	EC	77	56	85	90 (94)	68	65 (77)	79	66 (75)	79	76 (83)	39	10 (19)	69	48	69	67 (80)	70	76 (87)
ToMYLCV-[VE:10:03]	2003	VE	77	58	83	92 (94)	67	65 (75)	84	77 (84)	84	77 (89)	39	12 (18)	71	48	70	74 (81)	76	86 (94)
ToMoWV-[AR:Pic:398:08]	2008	AR	77	62	82	91 (95)	73	73 (83)	76	63 (73)	79	77 (86)	71	47 (58)	70	49	71	68 (82)	75	81 (90)
BCaMV-[MX:86]	1984	MX	77	63	82	91 (94)	68	66 (79)	82	72 (80)	84	78 (86)	39	15 (22)	72	51	75	77 (84)	75	83 (92)
BLCrV-[CO:HA:15]	2015	CO	77	60	82	89 (92)	67	67 (78)	82	70 (78)	84	83 (89)	37	10 (17)	72	51	72	72 (83)	78	87 (94)
EuMV-[USA:Flo:Eu4:11]	2011	US	77	49	83	93 (95)	66	65 (76)	84	76 (83)	85	79 (87)	39	12 (20)	70	45	71	70 (81)	75	82 (90)

[a] The ten begomoviruses with highest identities based on a BLASTn search.
[b] GenBank accession numbers are as follows: ToYMoV-[CR:5245-35:12]: KY064009 and KY064021; ToYMoV-[CR:5249-9:12]: KY064010; ToYMoV-[CR:5249-11:12]: KY064015; SiCMoV-[BR:Trm531.1:10]: NC038990; TbYCV-[CU:07]: FJ213931 and HQ896204; TYVSV-[CL:VS-B4:To:2012]: KC136337 and KC136338; BleICV-[MX:Cam:11]: JX827487 and JX827488; CabLCV-[EC:GuaEC54Rc:17]: MH359394 and MH359395; ToMYLCV-[VE:10:03]: AY927277 and EF547938; ToMoWV-[AR:Pic:398:08]: JQ714137 and KM243017; BCaMV-[MX:86]: AF110189 and AF110190; BLCrV-[CO:HA:15]: KX857725 and KX857726; EuMV-[USA:Flo:Eu4:11]: JQ963887 and JQ963888.
[c] Year collected.
[d] Geographic location: CR = Costa Rica, BR = Brazil, CU = Cuba, CL = Chile, MX = Mexico, EC = Ecuador, VE = Venezuela, AR = Argentina, CO = Colombia and US = United States.
[e] NA = not available.

fragments (0.9, 1.7 and 2.6 kb) that totaled ~5.2 kb, consistent with infection with a bipartite begomovirus. Together, these results indicated that the ToYMoD symptoms observed in Grecia, Costa Rica (Supplemental Fig. 1) in 1990 were caused by a isolate of ToYMoV. RCA-RFLP results indicated that both components were linearized with *Xba*I. Thus, the full-length (~2.6 kb) DNA-A and DNA-B components of the ToYMoV GR1 isolate were cloned from *Xba*I-digested RCA products to generate the recombinant plasmids pToYMoV-CR-GR1-A and pToYMoV-CR-GR1-B.

A second set of archival ToYMoD samples (L1-L10) were collected from tomato plants with ToYMoD symptoms in Liberia, Costa Rica in 2002 (Supplemental Fig. 1). These samples also were squashed onto nylon membranes in Costa Rica and then transported to UC Davis. In PCR tests with the degenerate primer pairs, the expected-size DNA-A and DNA-B fragments were amplified from all 10 samples, indicating infection with a bipartite begomovirus. The sequences of the PCR-amplified DNA-A and DNA-B fragments were >99% identical to each other and had the highest identities with the sequences of the DNA-A (>99%) and DNA-B (>97%) components of ToLCSiV isolates from Costa Rica and Nicaragua. RCA-RFLP analysis with *Sal*I generated five fragments (0.1, 0.3, 0.8, 1.3 and 2.6 kb) that totaled ~5.2 kb, indicating infection with a bipartite begomovirus. Together, these results indicated that the ToYMoD symptoms observed in Liberia, Costa Rica in 2002 were caused by a isolate of ToLCSiV. RCA-RFLP results indicated that the DNA-A and DNA-B components were linearized with *Bam*HI and *Xba*I, respectively. Thus, the full-length (~2.6 kb) DNA-A and DNA-B components of the ToLCSiV L1 isolate were cloned from RCA products digested with *BamH*I and *Xba*I, respectively, to generate the recombinant plasmids pToLCSiV-CR-L1-A and pToLCSiV-CR-L1-B).

3.2. *Genomic properties of full-length DNA-A and DNA-B components of new isolates of ToYMoV and ToLCSiV from Costa Rica*

The sequences of the cloned full-length DNA-A and DNA-B components of the GR1 isolate of ToYMoV were 2,574 (GenBank accession number: KC176780) and 2,547 nt (GenBank accession number: KC176781), respectively. Those of the L1 isolate of ToLCSiV were 2,610 (accession number MH019225) and 2,563 nt (accession number MH019226), respectively. The genome organization of both isolates is typical of NW bipartite begomoviruses, with the DNA-A components having a single gene on the virion (v)-sense (AV1) that encodes the CP, and four in the complementary (c)-sense (AC1, AC2, AC3, and AC4) encoding the Rep, the transcriptional activator protein (TrAP), the replication enhancer (REn) and the AC4 protein, respectively. Additionally, the PWRlsAgT motif in the N-terminus of the CP and the AVRFATDr motif in the C-terminus of REn, which are characteristic of NW begomoviruses, were present in both viruses (Harrison et al., 2002; Mauricio-Castillo et al., 2014). The AC4 aa sequences contain the N-terminal myristoylation domain (MGXLIS) required for membrane targeting (Rojas et al., 2001; Torres-Herrera et al., 2019). The DNA-B components have two ORFs, one in the v-sense (BV1) encoding the nuclear shuttle protein (NSP), and one in the c-sense (BC1) that encodes the movement protein (MP).

Pairwise sequence comparisons performed with SDT and full-length DNA-A and DNA-B component sequences of the ToYMoV GR1 isolate revealed the highest nt identities with those of the DNA-A (98%) and DNA-B (98%) components of ToYMoV isolates collected in Costa Rica in 2012. This confirmed that the bipartite begomovirus infecting the GR1 sample is a isolate of ToYMoV and is named tomato yellow mottle virus-[Costa Rica:Grecia:GR1:1990] (ToYMoV-[CR:Gre:GR1:90]). Consistent with these results, the ORFs of both components all had very high nt and aa identities, i.e., ≥97%, except for the AC4 aa sequence and the hypervariable region (HVR) of the DNA-B component, which were slightly more divergent (93%) (Table 1). The next highest identities for the DNA-A sequence were with NW bipartite begomoviruses from Latin America, including Sida chlorotic mottle virus from Brazil (79%), tobacco yellow

crinkle virus from Cuba (79%) and tomato yellow vein streak virus from Chile (78%). For the DNA-B sequence, the next highest identities were with bean calico mosaic virus (BCaMV) from Mexico (72%) and bean leaf crumple virus (BLCrV) from Colombia (72%).

The SDT analyses performed with the full-length DNA-A and DNA-B component sequences of the ToLCSiV L1 isolate revealed the highest identities with those of the DNA-A (99%) and DNA-B (98%) components of ToLCSiV isolates from Costa Rica and Nicaragua (Supplemental Table 2), whereas identities were lower (93–94%) with partial sequences of isolates from Mexico. Similar results were obtained in comparisons with nt and aa sequences of the ORFs of both components (98–99% identities), whereas identities for the HVR sequences of the DNA-B component were slightly lower (95–98%) (Supplemental Table 2). This confirmed that the bipartite begomovirus infecting the L1 sample is a isolate of ToLCSiV and is named tomato leaf curl Sinaloa virus-[Costa Rica:Liberia:L1:2002] (ToLCSiV-[CR:Lib:L1:02]). The viruses with the next highest sequence identities were NW bipartite begomoviruses mostly from Mexico, including Sida interveinal bright yellow virus (SiIBYV) (87% for DNA-A and 80% for DNA-B), Sida yellow vein virus (86% for DNA-A and 80% for DNA-B) and chino del tomate virus (CdTV) (85% for DNA-A and 80% for DNA-B).

3.3. Analyses of the common region sequences of ToYMoV and ToLCSiV

The DNA-A and DNA-B components of ToYMoV-[CR:Gre:GR1:90] share an identical common region sequence of 151 nt, consistent with being cognate components of a bipartite begomovirus. Comparisons performed with the common region sequence of ToYMoV-[CR:Gre:GR1:90] in SDT revealed highest identities (98%) with those of ToYMoV isolates collected in Costa Rica in 2012 (Table 1), and next highest identities with BCaMV from Mexico (63%) and tomato mottle wrinkle virus from Argentina (62%). The ToYMoV common region contains *cis*-regulatory elements implicated in replication and gene expression, including the conserved geminivirus stem-loop structure with the nonanucleotide sequence TAATATT↓AC, the Rep high-affinity binding site (iterons), the AC1 TATA box and the G-box (Eagle and Hanley-Bowdoin, 1997; Fontes et al., 1994; Hanley-Bowdoin et al., 2013). The Rep high-affinity binding site is composed of a direct repeat of the GGTGT iteron, which is adjacent to the AC1 TATAbox, and an upstream inverted repeat of this iteron, ACACC (Fig. 1). The Rep iteron-related domain

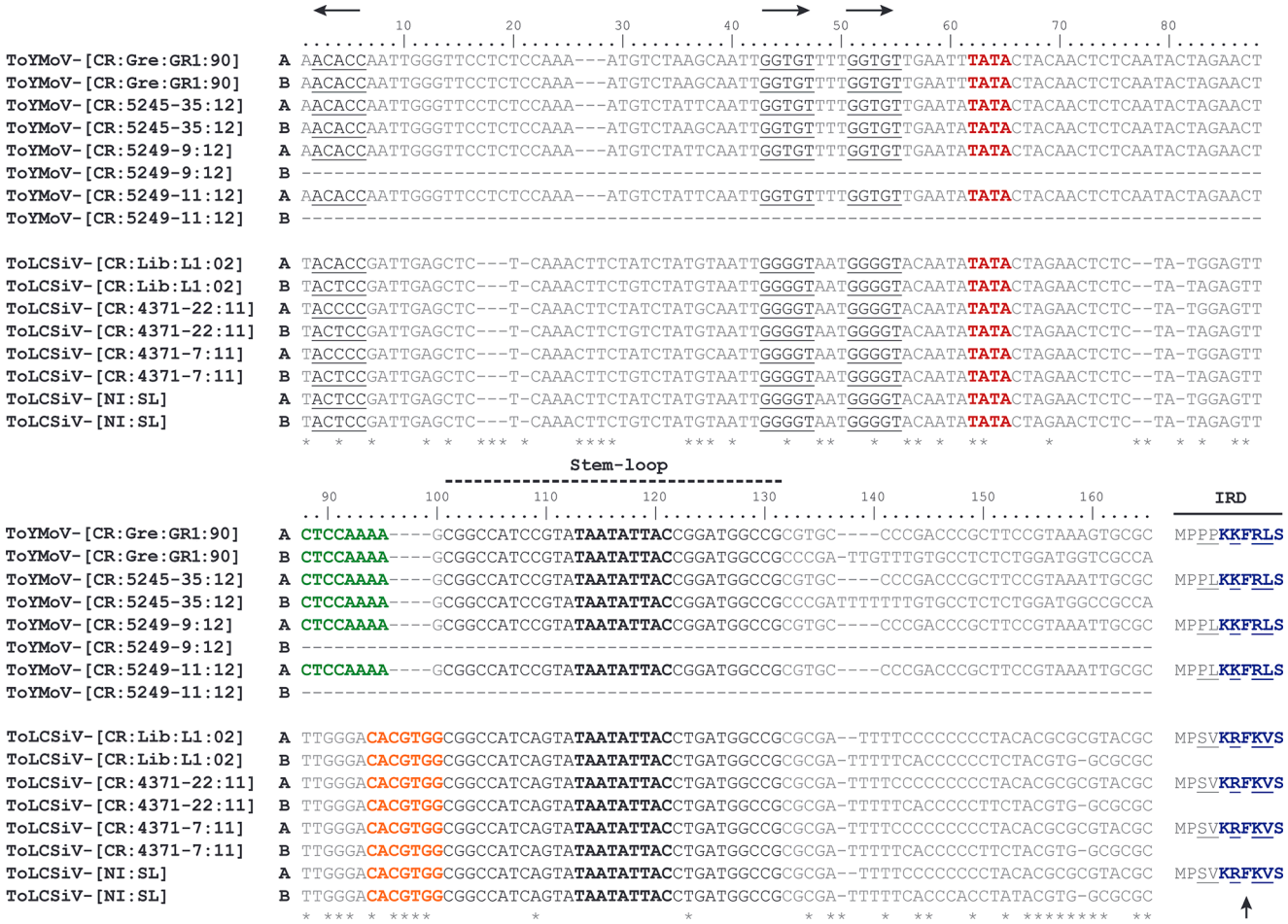

Fig. 1. Alignment of a portion of the common region sequences of isolates of tomato yellow mottle virus (ToYMoV) and tomato leaf curl Sinaloa virus (ToLCSiV) from Costa Rica and Nicaragua. The replication-associated protein (Rep) high affinity binding site (two direct repeats and one upstream inverted repeat of the core iteron) are shown in black letters and underlined, and orientation is shown with arrows at the top of the alignment. The TATA box of the AC1 (Rep) gene is indicated in red bold letters, the G-box with orange bold letters, and the GYA box with green bold letters. The characteristic geminivirus stem-loop structure is highlighted in black under the broken line, and the conserved geminivirus nonanucleotide sequence is shown in black bold letters. The Rep iteron-related domain (IRD) located in the N-terminus of Rep is shown to the lower right of the alignment, with the six amino acids involved in iteron recognition indicated in blue bold letters, the highly conserved F residue indicated with an arrow and variable positions are underlined. Numbers on top of the alignment indicate nt positions in respect to the inverted repeat, and nt differences among virus isolates are indicated with an asterisk at the bottom of the alignments. Abbreviations and GenBank accession numbers are as follows: ToYMoV-[CR:Gre:GR1:90]: KC176780 and KC176781; ToYMoV-[CR:5245-35:12]: KY064009 and KY064021; ToYMoV-[CR:5249-9:12]: KY064010; ToYMoV-[CR:5249-11:12]: KY064015; ToLCSiV-[CR:Lib:L1:02]: MH019225 and MH019226; ToLCSiV-[CR:4371-22:11]: KY064013 and KY064025; ToLCSiV-[CR:4371-7:11]: KY064014 and KY064020 and ToLCSiV-[NI:SL]: AJ608286 and AJ508783.

(IRD) is MPPPK**KFRLS** (key aa are shown in bold and the highly conserved F residue is underlined), and is predicted to recognize the GGTGT iteron (Fig. 1) (Argüello-Astorga and Ruiz-Medrano, 2001).

The DNA-A and DNA-B components of ToLCSiV-[CR:Lib:L1:02] share a common region sequence that is 174 nt and 96% identical, indicating these are cognate components of a bipartite begomovirus. Pairwise sequence comparisons performed with the common region sequence of ToLCSiV-[CR:Lib:L1:02] in SDT revealed highest identities (93–97%) with those of ToLCSiV isolates from Costa Rica and Nicaragua (Supplemental Table 2). The next highest identities were with SiIBYV (84%) and CdTV (79%) from Mexico, whereas identities with the common region sequence of ToYMoV-[CR:Gre:GR1:90] were substantially lower (~60%). The ToLCVSiV Rep high affinity binding site consists of a direct repeat of the GGGGT iteron, which is adjacent to the AC1 TATAbox, and an upstream imperfect inverted repeat, ACTCC (Fig. 1). The Rep IRD is MPSV**KRFKVS,** which is predicted to recognize the GGGGT iteron (Argüello-Astorga and Ruiz-Medrano, 2001).

3.4. Phylogenetic analyses

In the phylogenetic tree generated with DNA-A/genomic DNA sequences, the ToYMoV isolates from Costa Rica (four sequences including ToYMoV-[CR:Gre:GR1:90]) were placed in a strongly supported clade (Fig. 2), consistent with the low level of sequence divergence among these isolates (Table 1). This ToYMoV clade was a sister to the strongly supported SLCuV lineage (Fig. 2), which is composed mostly of NW bipartite begomoviruses that infect cucurbits in the Southern US, Mexico and Central America (Rojas et al., 2018). However, in the phylogenetic tree generated with the DNA-B component sequences, ToYMoV isolates (only two sequences) were placed in a strongly supported clade that was included with BLCrV from Colombia in a highly supported clade that was placed within the strongly supported SLCuV lineage (Supplemental Fig. S2).

The DNA-A sequences of the ToLCSiV isolates from Costa Rica and Nicaragua (three sequences including ToLCSiV-[CR:Lib:L1:02]) were placed in a strongly supported clade within the AbMV lineage (Fig. 2). This lineage includes crop- and weed-infecting bipartite begomoviruses from the Southern US, the Caribbean Basin and South America, but not

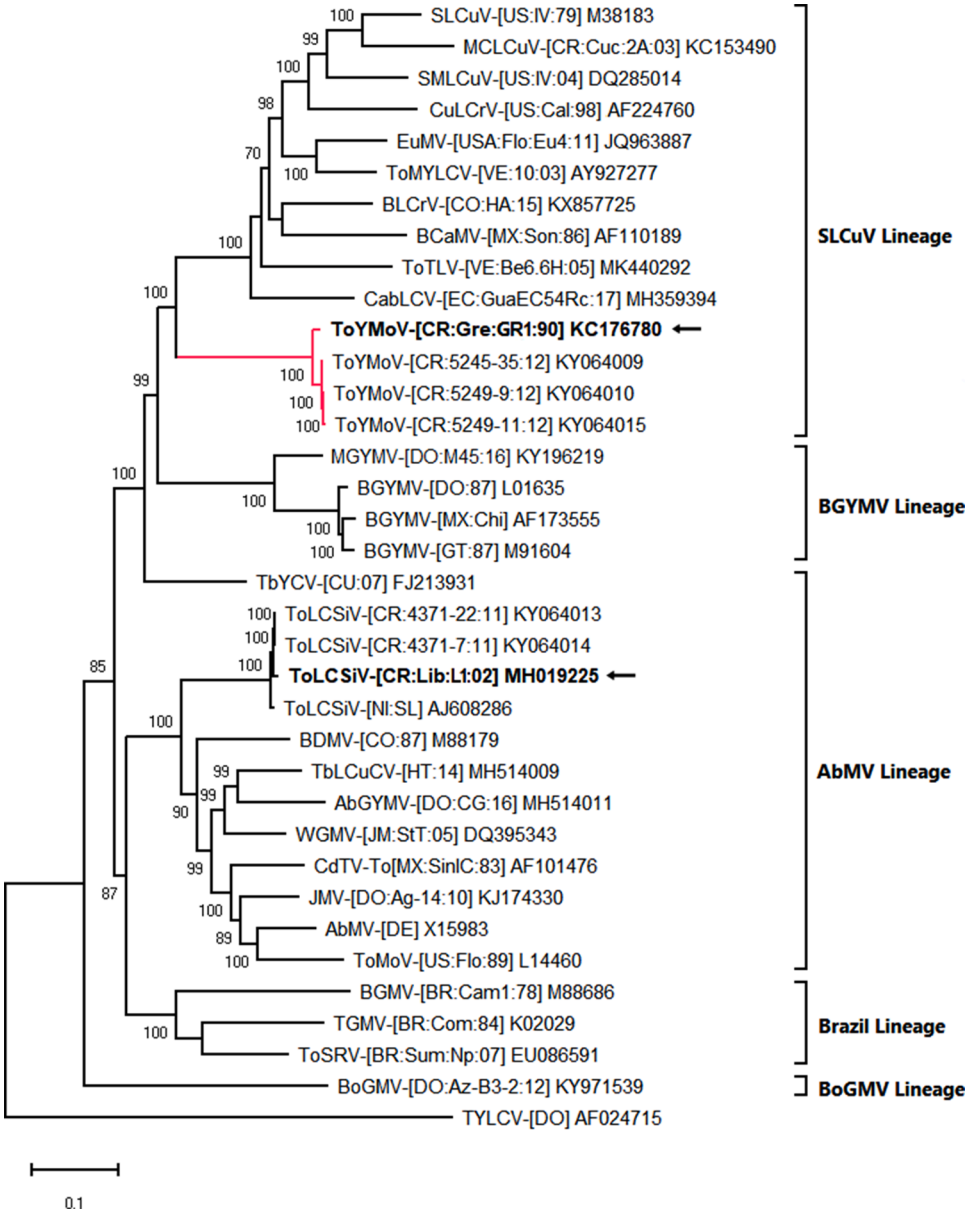

Fig. 2. Bayesian phylogenetic consensus tree based on an alignment of complete nucleotide sequences of DNA-A components and showing the relationship of the new isolates of tomato yellow mottle virus (ToYMoV-[CR:Gre:GR1:90]) and tomato leaf curl Sinaloa virus (ToLCSiV-[CR:Lib:L1:02]) from Costa Rica (shown in bold and with arrows) with: (i) previously characterized isolates of ToYMoV and ToLCSiV from Costa Rica and an isolate of ToLCSiV from Nicaragua; (ii) the ten begomoviruses with highest identities to ToYMoV-[CR:Gre:GR1:90] based on a BLASTn search; and (iii) selected begomoviruses representing the Abutilon mosaic virus (AbMV), Brazil, bean golden yellow mosaic virus (BGYMV), squash leaf curl virus (SLCuV) and Boerhavia golden mosaic virus (BoGMV) lineages of New World begomoviruses (lineages are indicated with brackets). The ToYMoV lineage is highlighted in red. Sequences were obtained from GenBank, and virus abbreviations are as described in Brown et al. (2015). Branch strengths were evaluated by Bayesian posterior probabilities. The phylogenetic consensus tree was rooted with the sequence of the genomic DNA of the Old World monopartite begomovirus tomato yellow leaf curl virus from the Dominican Republic (TYLCV-[DO]). The length of horizontal branches indicates the rate of substitution per nucleotide.

from Central America (Fig. 2). The DNA-B sequences of the ToLCSiV isolates from Costa Rica and Nicaragua were also placed in a strongly supported clade in the AbMV lineage, but in a strongly supported clade with CdTV from Mexico (Supplemental Fig. S2).

3.5. Recombination analyses

The RDP4 analyses revealed a recombination event in the DNA-A component of all four ToYMoV isolates, whereas no recombination was detected in the ToYMoV DNA-B component or in the DNA-A and DNA-B components of the ToLCSiV isolates. This event was identified by the RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq methods, with *p*-values of 5.846×10^{-15}, 1.049×10^{-08}, 1.294×10^{-04}, 8.048×10^{-04}, 1.006×10^{-04}, 2.345×10^{-08} and 6.492×10^{-06}, respectively. This recombination event is 424 nt and spans nts 2056 to 2479, which is in the well-known recombination hot-spot in the DNA-A component/genomic DNA (Hou and Gilbertson, 1996; Lefeuvre et al., 2009; Padidam et al., 1999) and includes the 5' end of the AC1 ORF, most of the AC4 ORF and 43 nts of the 5' end of the common region that included the inverted repeat but not the direct repeat (Supplemental Fig. S3). Thus, this recombinant DNA-A component possesses the IRD of the minor parent and the iteron direct repeat of the Rep high affinity binding site from the major parent. Furthermore, this IRD is predicted to recognize the iteron of the major parent (Argüello-Astorga and Ruiz--Medrano, 2001). These results suggest that the parents of ToYMoV were closely related, e.g., from the same lineage. Further evidence comes from the high identity (98%) of the highly variable AC4 nt sequence (Table 1).

The RDP further predicted that the recombinant sequence in the ToYMoV DNA-A component was derived from an uncharacterized minor parent, whereas the major parent was most identical to tomato chlorotic leaf distortion virus (TCLDV), a NW bipartite begomovirus from Venezuela (GenBank accession number JN241632) that is a member of the AbMV lineage (Zambrano et al., 2011). However, the TCLDV DNA-A sequence is highly divergent (25%) from that of the ToYMoV DNA-A, suggesting that the true major parent of ToYMoV has not been characterized.

3.6. Infectivity of the cloned full-length DNA-A and DNA-B components of ToYMoV-[CR:Gre:GR1:90] and ToLCSiV-[CR:Lib:L1:02]

All *N. benthamiana* and tomato plants bombarded with the full-length DNA-A and DNA-B monomers of ToYMoV-[CR:Gre:GR1:90] were stunted and newly emerged leaves showed epinasty, crumpling, yellow mosaic/mottle and vein yellowing by 14 dpb (Figs. 3A and 3B). By 21 dpb, symptoms in *N. benthamiana* and tomato plants had become relatively milder. PCR tests with component-specific primer pairs revealed both ToYMoV components in leaves of symptomatic plants. Notably, the symptoms in ToYMoV-infected tomato plants were similar to those of ToYMoD, thereby fulfilling Kochś postulates.

The full-length DNA-A and DNA-B monomers of ToLCSiV-[CR:Lib:L1:02] also induced stunting and epinasty, crumpling and a mild yellow mosaic/mottle in newly emerged leaves of bombarded *N. benthamiana* and tomato plants by 14 dpb (Figs. 3C and 3D). By 21 dpb, symptoms became relatively milder. Here, it is important to note that the symptoms induced by ToLCSiV in tomato were similar to those induced by ToYMoV (compared Fig. 3B and 3D). This is consistent with the ToYMoD symptoms observed in Liberia, Costa Rica in 2002 being

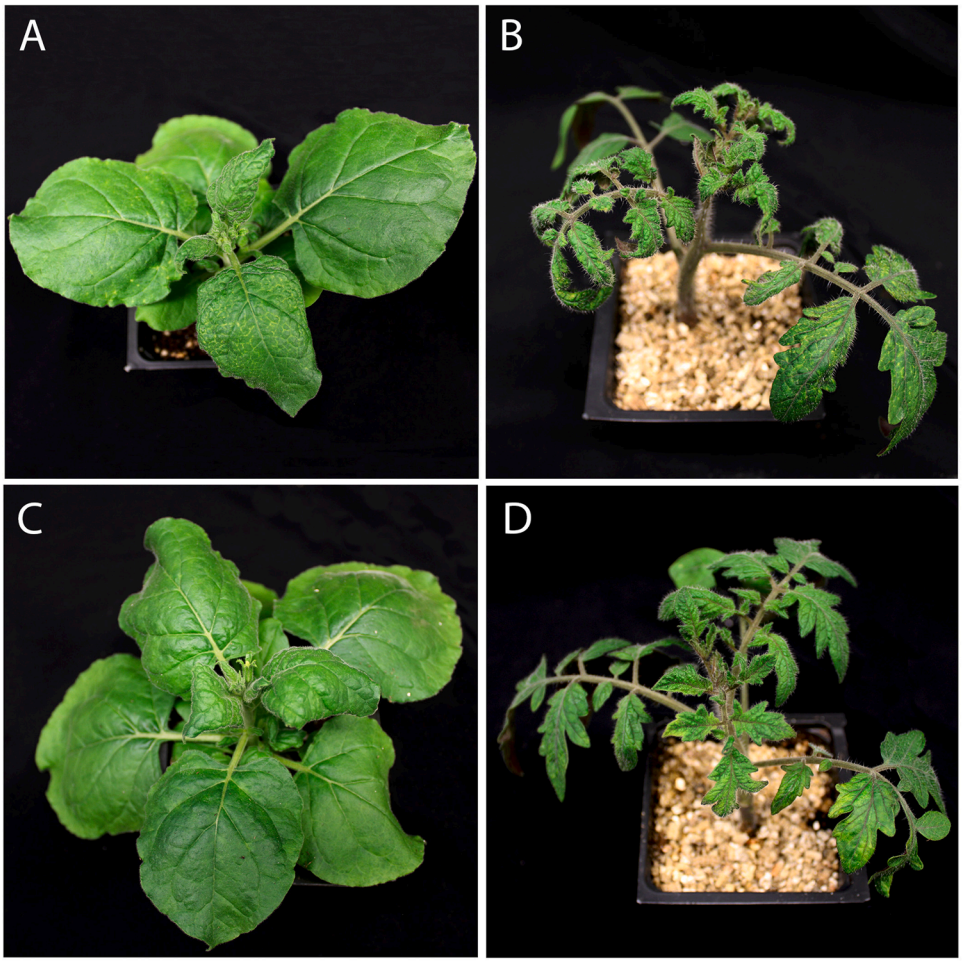

Fig 3. Disease symptoms induced by the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) in (A) *Nicotiana benthamiana* and (B) tomato (*Solanum lycopersicum* cv. Glamour) plants, and those induced by the infectious cloned DNA-A and DNA-B components of an isolate of tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:L1:02]) in (C) *N. benthamiana* and (D) tomato (*S. lycopersicum* cv. Glamour) plants. Plants were inoculated with excised linear double strand monomers by particle bombardment and photographed 14 d later.

caused by ToLCSiV.

3.7. Host range experiments

A partial host range of ToYMoV was next determined by agroinoculation and particle bombardment inoculation of a range of plant species (Table 2) (note that a partial host range for ToLCSiV was previously reported by Idris et al. [1998]). In addition to inducing symptoms in agroinoculated *N. benthamiana* and tomato plants, ToYMoV induced stunting and severe epinasty, crumpling, mosaic/mottle and yellowing of leaves of agroinoculated common bean plants (cv. Topcrop) by 14 dpi (Supplemental Fig. S4 and Table 2) and PCR tests confirmed the presence of the ToYMoV DNA-A and DNA-B components in leaves of symptomatic plants. A symptomless DNA-A infection was detected in 38% of pumpkin plants agroinoculated with the ToYMoV DNA-A and DNA-B components, whereas ToYMoV did not infect *N. tabacum*, pepper, *D. stramonium*, cucumber, cantaloupe and honeydew melons and *C. amaranticolor* plants (Table 2). Plants agroinoculated with the empty vector did not develop symptoms and tested negative for ToYMoV infection in PCR tests.

To further investigate the pathogenicity of ToYMoV in common bean, seedlings of selected cultivars of the Andean and Middle American gene pools were bombarded with the full-length DNA-A and DNA-B monomers of ToYMoV-[CR:Gre:GR1:90]. Consistent with the agroinoculation results, ToYMoV infected and caused severe symptoms in the Andean cv. Topcrop. In contrast, equivalent seedlings of the Middle American cvs. Carioca, BTS and Othello failed to develop symptoms, and viral DNA was not detected in newly emerged leaves of these plants (Table 2). Equivalent seedlings bombarded with gold particles alone did not develop symptoms and tested negative for ToYMoV infection in PCR tests. These results indicate that these Middle American cultivars are resistant to infection by ToYMoV.

3.8. Mechanical transmission

Plant viruses that are mechanically (sap) transmissible are typically not phloem-limited (Walkey and Walkey, 1991; Yarwood, 1957), including some bipartite begomoviruses such as BDMV (Wang et al., 1996; Sudarshana et al., 1998). In mechanical transmission experiments with ToYMoV, 56% of inoculated *N. benthamiana* plants developed ToYMoD symptoms by 14 dpi, and the ToYMoV DNA-A and DNA-B components were detected in symptomatic leaves. In contrast, no symptoms developed in mechanically inoculated tomato plants and ToYMoV infection was not detected in leaves of these plants (Table 2). The positive controls BDMV and ToMoV infected and caused typical symptoms by 14 dpi in all mechanically inoculated *N. benthamiana* and tomato plants, respectively, showing that this method worked in both species. Finally, no symptoms were observed in negative controls inoculated with sap prepared from uninfected plants and ToYMoV infection was not detected in these plants. These results demonstrated that ToYMoV is mechanically transmissible in a host-dependent manner.

3.9. Pseudorecombination experiments with the cloned infectious ToYMoV and ToLCSiV DNA components

To further investigate the genetic relationship between ToYMoV and ToLCSiV, pseudorecombination experiments were conducted in which *N. benthamiana* and tomato plants were agroinoculated with all combinations of the infectious DNA-A and DNA-B components. Consistent with the infectivity and host range experiments, all *N. benthamiana* and tomato plants agroinoculated with ToYMoV and ToLCSiV developed ToYMoD disease symptoms in newly emerged leaves by 14 dpi. In contrast, none of the *N. benthamiana* and tomato plants agroinoculated with the ToYMoV DNA-A and ToLCSiV DNA-B or the ToLCSiV DNA-A and ToYMoV DNA-B pseudorecombinants developed disease symptoms by 14 dpi (Table 3). Furthermore, PCR tests revealed symptomless DNA-A alone infections in all *N. benthamiana* plants agroinoculated with ToYMoV DNA-A and ToLCSiV DNA-B, but not in plants agroinoculated with ToLCSiV DNA-A and ToYMoV DNA-B (Table 3). Similar results were obtained with tomato plants, i.e., the pseudorecombinants did not induce disease symptoms in agroinoculated plants and DNA-A alone infections were detected in plants agroinoculated with the ToYMoV DNA-A and ToLCSiV DNA-B pseudorecombinant (Table 3). Notably, the incidence of ToYMoV DNA-A alone infection was substantially lower in tomato (30%) compared with the permissive host *N. benthamiana* (100%). Together, these experiments established that ToYMoV and ToLCSiV do not form infectious pseudorecombinants and also revealed a difference in biology of the DNA-A components. Moreover, these results are consistent with the placement in different lineages (Fig. 2) and having highly divergent common region sequences with incompatible iteron and IRD sequences (Fig. 1).

3.10. Virus and host interactions in single and mixed infections of a susceptible tomato cultivar

To understand the potential impact of these three emergence/

Table 2
Infectivity and symptomatology of the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) in selected plant species.

Plant species	Infectivity[a]			Symptoms[b]
	Agroinoculation	Bombardment	Sap inoculation	
Nicotiana benthamiana	9/9 (100)	9/9 (100)	5/9 (56)	Cr, E, M, S, Vy
Nicotiana tabacum cv. Havana	0/9 (0)	NT	NT	NS-ni
Solanum lycopersicum cv. Glamour	9/9 (100)	9/9 (100)	0/9 (0)	Cr, E, M, S, Vy
Capsicum annuum cv. Cayenne	NT	0/9 (0)	NT	NS-ni
Datura stramonium	0/9 (0)	NT	NT	NS-ni
Chenopodium amaranticolor	0/9 (0)	NT	NT	NS-ni
Cucurbita maxima cv. Sugarpie	6/16 (38)	NT	NT	NS-i
Cucumis sativus cv. Poinsett 76	0/9 (0)	NT	NT	NS-ni
Cucumis melo cv. Minnesota Midget	0/9 (0)	NT	NT	NS-ni
Cucumis melo cv. Sweet Delight	0/9 (0)	NT	NT	NS-ni
Phaseolus vulgaris cv. Topcrop	13/15 (87)	9/9 (100)	NT	Cr, E, M, S, Y
Phaseolus vulgaris cv. Carioca	NT	0/9 (0)	NT	NS-ni
Phaseolus vulgaris cv. BTS	NT	0/9 (0)	NT	NS-ni
Phaseolus vulgaris cv. Othello	NT	0/9 (0)	NT	NS-ni

[a] Infectivity (number of infected plants/number inoculated, with percentages in parentheses) was determined at 14 d post-inoculation based on symptom development and detection of viral DNA components in newly emerged leaves by PCR tests with component-specific primer pairs. Data represents a total of three independent experiments.

[b] Abbreviations: Cr, crumpling; E, epinasty; Ld, leaf deformation; M, mosaic/mottle; NS-I, no symptoms-infected; NS-ni, no symptoms-not infected; NT, not tested; S, stunting; Vy, vein yellowing and Y, yellowing.

Table 3
Infectivity and symptomatology of pseudorecombinants (PRs) formed with the infectious cloned DNA-A and DNA-B components of isolates of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) and tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:L1:02]) delivered via agro-inoculation of *Nicotiana benthamiana* and tomato plants.

Plant species/ PRs [a]	Infectivity [b] ToYMoV DNA-A (TA)	DNA-B (TB)	ToLCSiV DNA-A (SA)	DNA-B (SB)	Symptoms [c]
N. benthamiana					
TA + SB	9/9 (100)	0/9 (0)	0/9 (0)	0/9 (0)	NS-i
SA + TB	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)	NS-ni
TA + TB	9/9 (100)	9/9 (100)	0/9 (0)	0/9 (0)	Cr, E, M, S, Vy
SA + SB	0/9 (0)	0/9 (0)	9/9 (100)	9/9 (100)	E, I, S
Tomato					
TA + SB	5/17 (30)	0/17 (0)	0/17 (0)	0/17 (0)	NS-i
SA + TB	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)	NS-i
TA + TB	9/9 (100)	9/9 (100)	0/9 (0)	0/9 (0)	Cr, E, M, S, Vy
SA + SB	0/9 (0)	0/9 (0)	9/9 (100)	9/9 (100)	E, I, S

[a] PRs were formed by exchanging the infectious cloned DNA-A and DNA-B components of ToYMoV and ToLCSiV and agroinoculating *N. benthamiana* and tomato (cv. Glamour) plants. Controls were equivalent plants agroinoculated with the DNA-A and DNA-B components of ToYMoV and ToLCSiV (positive controls) and the empty vector (negative control).

[b] Infectivity (number of infected plants/number inoculated, with percentages in parentheses) was determined at 14 d post-inoculation based on symptom development and detection of viral DNA components by PCR tests with component-specific primer pairs. Data represents totals of three independent experiments.

[c] Symptom abbreviations: Cr, crumpling; E, epinasty; I, interveinal chlorosis; M, mosaic/mottle; NS-i, no symptoms-infected; NS-ni, no symptoms-not infected; S, stunting and Vy, vein yellowing.

invasion events on begomovirus disease of tomato in Costa Rica, we next compared single versus mixed infections of these viruses in the susceptible tomato cv. Glamour in terms of (i) development of disease symptoms and (ii) viral DNA accumulation. In plants agroinoculated with individual viruses, the symptoms induced by the NW bipartite ToYMoV and ToLCSiV appeared in newly emerged leaves ~7 dpi and, by 14 and 21 dpi, ToYMoD symptoms had developed in newly emerged leaves of all agroinoculated plants (Figs. 4A [To] and 4B [Si]). Thus, the symptoms induced by ToYMoV and ToLCSiV in this cultivar were indistinguishable. Symptoms of TYLCV infection in agroinoculated plants appeared later (~10 dpi); however, by 14 and 21 dpi, all plants had developed the characteristic symptoms of tomato yellow leaf curl disease (TYLCD), including stunting and upright growth and leaves with upward curling, crumpling, and striking interveinal and marginal yellowing (Fig. 4C [Ty]). Tomato plants co-infected with ToYMoV and ToLCSiV were not more stunted than plants infected with either virus alone, but developed more severe epinasty, crumpling and yellow mosaic/mottle symptoms by 21 dpi (compare Figs. 4A [To vs. To/Si] and 4B [Si vs. Si/To] and 5A, 5B and 5E). In contrast, tomato plants co-infected with ToYMoV or ToLCSiV and TYLCV were substantially more stunted compared with plants infected with the individual viruses, and symptoms of TYLCD became predominant by 14 and 21 dpi (compare Figs. 4A [To and To/Si vs. To/Ty], 4B [Si and Si/To vs. Si/Ty] and 4C [Ty vs. Ty/To and Ty/Si]). Plants co-infected with all three viruses were most severely stunted and foliar symptoms had the appearance of unusually severe TYLCD (compare Figs. 4A to 4C [plants on far right with other treatments] and 5H with other treatments). Another notable observation was that plants co-infected with ToYMoV or ToLCSiV and TYLCV showed a striking symptom transition in which the vein clearing, crumpling and yellow mosaic/mottle phenotype induced by bipartite begomovirus infection appeared in the first and second newly emerged leaves, whereas those induced by TYLCV became dominant in subsequent emerging leaves (compare Figs 5F to 5H). These results revealed increased symptoms in tomato plants with mixed infections, consistent with a synergistic interaction or additive effect.

To further investigate the interactions among these viruses in infected tomato plants, samples of newly emerged leaves were collected from plants infected with each virus alone and in all combinations at 7, 14 and 21 dpi, and viral DNA accumulation was determined by qPCR. In plants infected with each virus alone, viral DNA was detected in newly emerged leaves at 7 dpi and viral DNA levels were not significantly different (Supplemental Fig. S5A), even though symptoms had not appeared in plants infected with TYLCV. ToYMoV accumulation decreased over the course of the experiment, but not significantly. For ToLCSiV, accumulation decreased at 14 dpi and increased by 21 dpi; however, these differences were not significant (Supplemental Fig. S5B). In contrast, accumulation of the OW monopartite TYLCV had increased significantly by 14 and 21 dpi (Supplemental Fig. S5B).

In mixed infections, accumulation of ToYMoV was significantly reduced at 7 dpi compared with plants infected with ToYMoV alone (Fig. 6A), and a similar trend was observed for ToLCSiV (Fig. 6B). However, by 14 and 21 dpi accumulation of both viruses in mixed infections had increased and was not significantly different from that in plants infected with the viruses alone (Fig. 6A and 6B). Furthermore, although ToYMoV DNA levels decreased over the course of the experiment, they were consistently higher than those in mixed infections. This may indicate that ToYMoV does not compete as well in mixed infections. In contrast, accumulation of TYLCV was not reduced in mixed infections, and increased slightly (Fig. 6C). Overall, TYLCV DNA levels were highest at 14 dpi and remained high at 21 dpi. Thus, TYLCV DNA accumulation was not impacted in mixed infections and was remarkably uniform among treatments and time points (Fig. 6C).

Therefore, other than the transient reduction in accumulation of the bipartite begomoviruses in mixed infections at 7 dpi, co-infection did not significantly impact DNA accumulation of any virus. Thus, our results suggest that these viruses can co-infect tomato plants without negatively impacting replication and movement of each other, thereby resulting in more severe disease symptoms.

4. Discussion

In the present study, we investigated the relationships and interactions among three tomato-infecting begomoviruses that emerged or were introduced over different periods of time in Costa Rica. Because ToLCSiV and TYLCV represent NW and OW invasive species in Costa Rica, respectively, this situation provided an opportunity to investigate the invasion biology of these viruses. To do this, we generated infectious clones of the DNA-A and DNA-B components and agroinoculation systems for isolates of ToYMoV and ToLCSiV recovered from archival ToYMoD samples collected in Costa Rica in 1990 and 2002, respectively. The time of collection of these samples facilitated the recovery of these viruses, as 1990 was soon after the appearance of ToYMoV, and 2002 was soon after the introduction of ToLCSiV (Idris et al., 1999).

The infectivity studies with the cloned DNA-A and DNA-B components of ToYMoV allowed us to fulfill Koch's postulates for ToYMoD. The finding that sequence comparisons of ToYMoV-[CR:Gre:GR1:90] and isolates collected more than 20 years later (2012) showed a low level of sequence divergence (~2%) suggests that the virus is under stabilizing selection (Barboza et al., 2018). The host range study showed that ToYMoV has a narrow host range mostly within the Solanaceae and is well adapted to tomato. The only non-solanaceous species infected was the common bean cv. Topcrop, which is susceptible to infection by many NW bipartite begomoviruses, including many in the SLCuV lineage. However, like many of these viruses, ToYMoV did not infect plants of the Middle American gene pool, which possess more resistance to

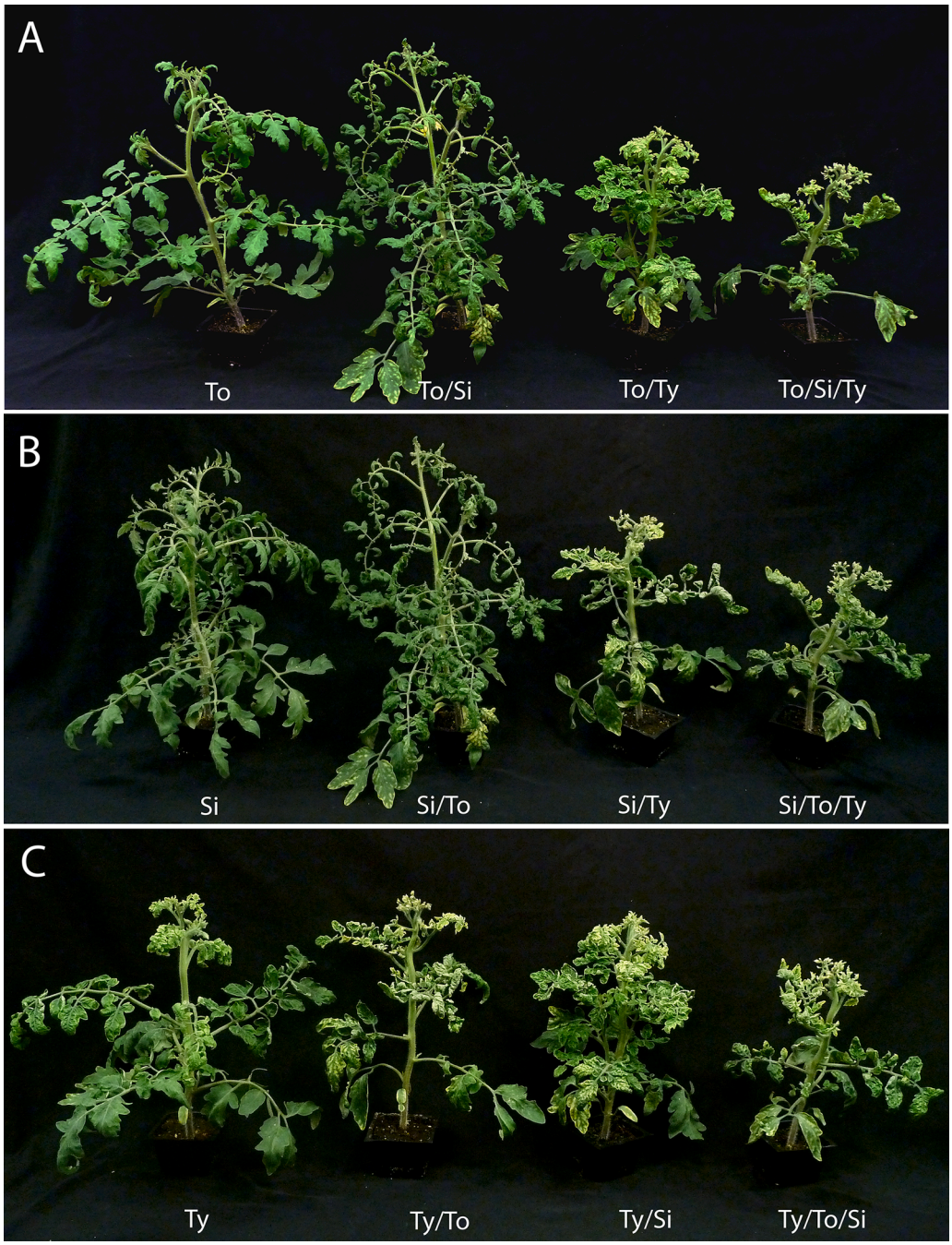

Fig 4. Disease symptoms induced by the infectious cloned DNA-A and DNA-B components of isolates of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]), tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:L1:02]) and the infectious clone of the genomic DNA of tomato yellow leaf curl virus from the Dominican Republic (TYLCV-[DO]) individually or in all combinations in agroinoculated tomato (*Solanum lycopersicum* cv. Glamour) plants. (A) ToYMoV (To), ToYMoV and ToLCSiV (To/Si), ToYMoV and TYLCV (To/Ty) and ToYMoV, ToLCSiV and TYLCV (To/Si/Ty); (B) ToLCSiV (Si), ToLCSiV and ToYMoV (Si/To), ToLCSiV and TYLCV (Si/Ty) and ToLCSiV, ToYMoV and TYLCV (Si/To/Ty); and (C) TYLCV (Ty), TYLCV and ToYMoV (Ty/To), TYLCV and ToLCSiV (Ty/Si), and TYLCV, ToYMoV and ToLCSiV (Ty/To/Si). Plants were agroinoculated and photographed 21 d later.

begomovirus infection (Hagen et al., 2008; Seo et al., 2004). Furthermore, because Middle American types are mostly grown in Costa Rica, ToYMoV should not pose a threat to common bean production. This is consistent with the failure to detect ToYMoV in samples of common bean with begomovirus symptoms collected in Costa Rica (Nakhla et al., 2005). Cucurbits are also not hosts of ToYMoV and the small number of symptomless ToYMoV DNA-A alone infections detected in pumpkin plants were mediated by agroinoculation, as discussed below. These results indicate that the primary crop host of ToYMoV is tomato, something also known for ToLCSiV and TYLCV (Cohen and Antignus, 1994; Idris and Brown, 1998; Salati et al., 2002). Thus, a tomato-free period of 2-3 months is a management strategy for all three viruses.

It was previously shown that ToYMoV is most closely related to members of the SLCuV lineage of NW begomoviruses (Barboza et al., 2018). In the phylogenetic analysis performed with the ToYMoV-[CR:Gre:GR1:90] DNA-A sequence, the four ToYMoV isolates were placed in a strongly supported subclade within the SLCuV lineage (Fig. 2). This 'ToYMoV subclade' may actually be a sister lineage that has undergone a long period of evolution and host adaptation, independent of the other members of the lineage. This notion is supported by numerous distinct properties of ToYMoV including (i) a high level of nt sequence divergence (~20%), (ii) different common region iteron and Rep IRD sequences, (iii) different AC4 properties (the ToYMoV AC4 is 85 aa and possessing a N-terminal myristoylation motif, whereas those of the other members are 126 aa and do not possess the myristoylation domain) and (iv) infecting tomato rather than cucurbits.

The recombination event detected in all the ToYMoV DNA-A components was in the well-known recombination hot-spot (Hou and Gilbertson, 1996), and involved two relatively closely related parental viruses. Based on the breakpoints predicted by RDP, the minor parent

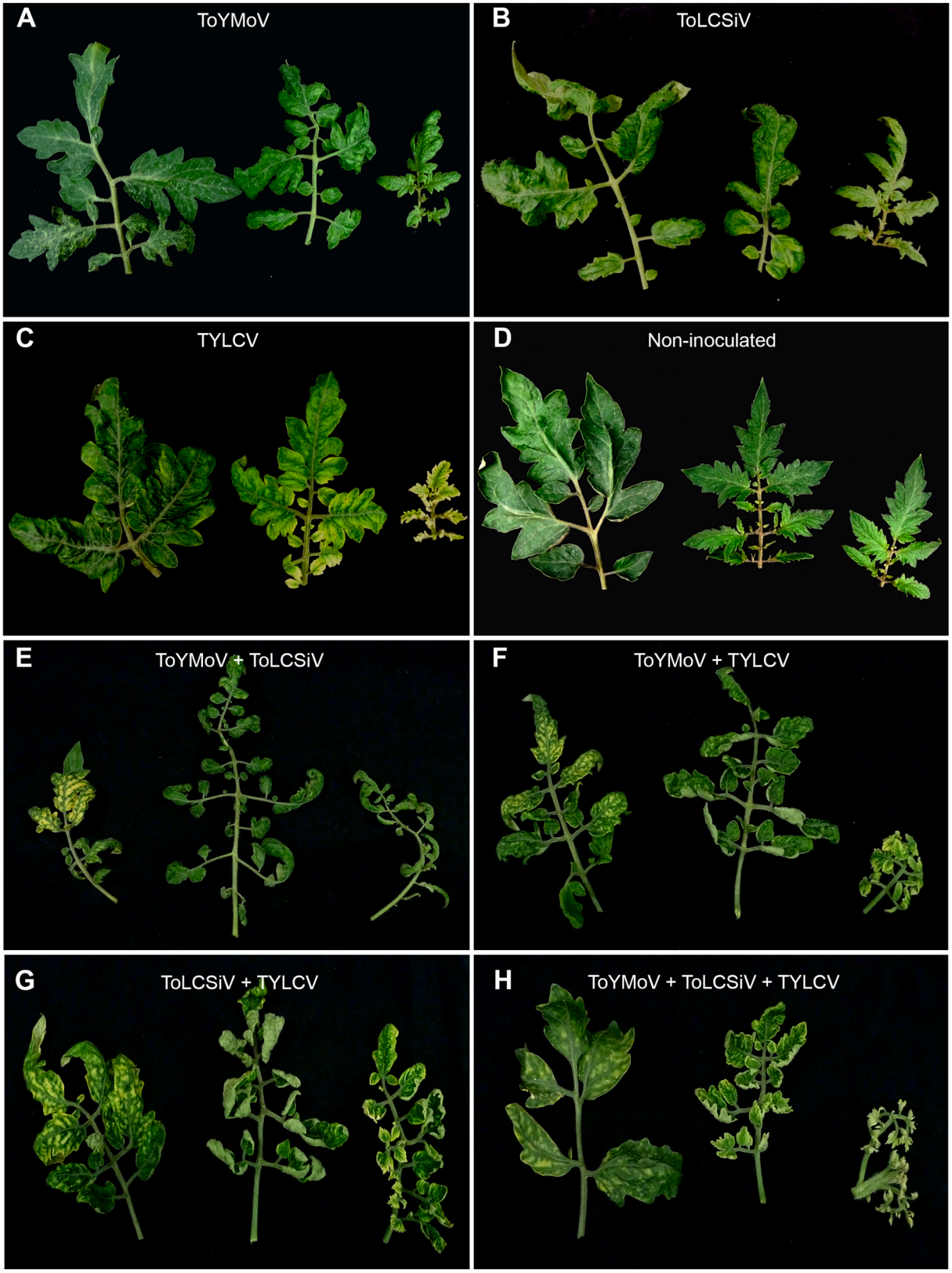

Fig 5. Symptom transition in the lower, middle and upper leaves of tomato plants agroinoculated with the infectious cloned DNA-A and DNA-B components of isolates of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]) and tomato leaf curl Sinaloa virus (ToLCSiV-[CR:Lib:L1:02]) from Costa Rica and the infectious clone of the genomic DNA of tomato yellow leaf curl virus (TYLCV) from the Dominican Republic (TYLCV-[DO]) individually or in all combinations. (A) ToYMoV; (B) ToLCSiV; (C) TYLCV; (D) non-inoculated tomato plant; (E) ToYMoV and ToLCSiV; (F) ToYMoV and TYLCV; (G) ToLCSiV and TYLCV; (H) ToYMoV, ToLCSiV and TYLCV. Plants were photographed 21 d after agroinoculation.

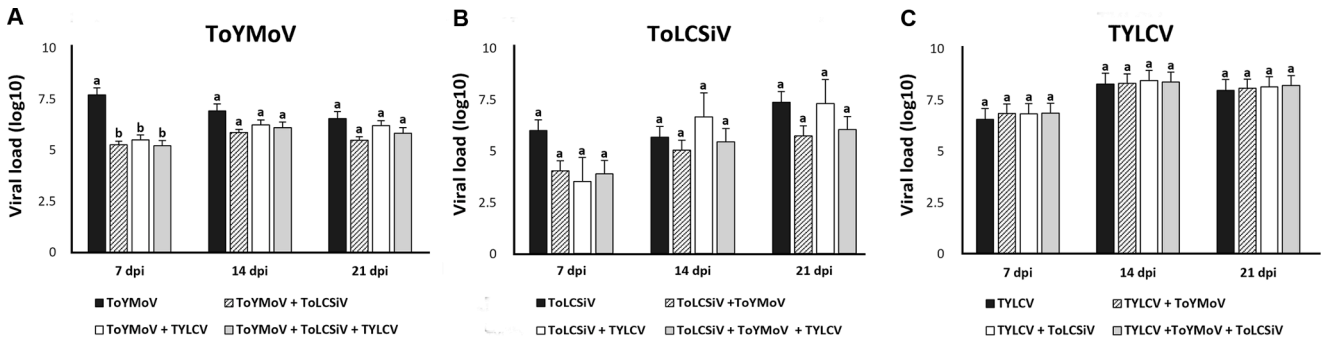

Fig 6. Viral DNA accumulation in tomato plants (*Solanum lycopersicum* cv. Glamour) infected with the DNA-A and DNA-B components of isolates of (A) tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:GR1:90]), (B) tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:L1:02]) or (C) the genomic DNA of tomato yellow leaf curl virus from the Dominican Republic (TYLCV-[DO]) alone or in all combinations. Total DNA was extracted from newly emerged leaves at 7, 14 and 21 d after agroinoculation and used in qPCR tests with specific primer pairs for the DNA-A/genomic DNA of each virus. Bars with the same letter correspond to DNA levels that do not differ statistically according to the Kruskal-Wallis test ($p > 0.05$).

contributed the Rep IRD, but the iteron direct repeat of the Rep high affinity binding site came from the major parent (Supplemental Fig. S3); however, because this IRD is predicted to recognize the iteron of the major parent, the recombinant Rep is predicted to mediate replication of the recombinant DNA-A component as well as the DNA-B component of the major parent. The selective advantage(s) provided by this recombination event may come from functional differences of the ToYMoV AC4 protein, possibly related to host adaptation or movement (Maliano et al., 2021; Torres-Herrera et al., 2019). The begomovirus C4/AC4 gene is highly variable and evolves rapidly as it is under strong positive selection (Deom et al., 2021; Medina-Puche et al., 2021). As a result, this protein can exhibit a range of different subcellular localizations, functional properties and host interactions (Deom et al., 2021; Medina-Puche et al., 2021). Taken together, our results suggest that ToYMoV evolved locally via mutation and recombination, possibly from indigenous progenitor viruses infecting solanaceous weeds and that were spread and co-inoculated into plants by indigenous whitefly vectors (Gilbertson et al., 2015; Navas-Castillo et al., 2011).

The first invasion event in Costa Rica was the introduction of the NW bipartite ToLCSiV in the late 1990s (Idris et al., 1999). This virus was first detected in Mexico in 1989, where it may have locally evolved to infect tomato (Brown et al., 1993). The sequences of the DNA-A and DNA-B clones of ToLCSiV-[CR:Lib:L1:02] were nearly identical (99%) to those of isolates from ToYMoD samples collected in Grecia in 2011 and an isolate from Nicaragua in 2000, which suggested extensive local spread and establishment (Barboza et al. 2018). The detection of mixed infections of ToYMoV and ToLCSiV in ToYMoD samples collected in Grecia in 2012 showed the geographic ranges of these viruses overlaps and that both viruses are associated with this disease in Costa Rica.

Mixed infections of bipartite begomoviruses can accelerate viral evolution via pseudorecombination (reassortment) and recombination (Hou and Gilbertson, 1996). However, our results showing that pseudorecombinants formed between the infectious DNA components of ToYMoV and ToLCSiV were not infectious revealed genetic incompatibility between components of these viruses. This is fully consistent with these viruses having highly divergent common region sequences and incompatible IRD/iteron combinations and placement in different lineages (Fig. 2). This suggest a long period of independent local evolution and that genetic interaction between these viruses may be limited.

An interesting result from the pseudorecombination experiments was the specific capacity of the ToYMoV DNA-A component to induce a symptomless systemic infection in agroinoculated *N. benthamiana* and tomato plants. This phenomenon has been reported for DNA-A components of NW and OW bipartite begomoviruses when delivered by agroinoculation (Garrido-Ramirez et al., 2000; Hou et al., 1998). Moreover, these infections do not require co-inoculation with a DNA-B component, but highlight the requirement of a DNA-B component for typical disease development (Hanley-Bowdoin et al., 2000; Rojas et al., 2005; Sudarshana et al., 1998). The finding that the ToLCSiV DNA-A component did not induce these infections, even in the permissive *N. benthamiana*, revealed a biological difference between these DNA-A components. The mechanism underlying DNA-A alone infections is not known, but it may also involve the rapidly evolving and multifunctional AC4, which can be targeted to the cell periphery and plays a role in movement (Petty et al., 2000; Rojas et al., 2001). Thus, this type of AC4 protein may also mediate DNA-A alone infections in a virus-specific manner (Medina-Puche et al., 2021). In this regard, it is worth noting that the AC4 nt and aa sequences of ToYMoV and ToLCSiV are relatively divergent with 72% and 42% identity, respectively, although both are 85 aa and possess the myristylation domain. The role of AC4 and other genes or sequences need to be determined experimentally.

The detection of the invasive OW monopartite TYLCV in 2012 revealed the second invasion event in Costa Rica (Barboza et al., 2014). This event was not unexpected given the extensive spread of the virus in the Caribbean Basin, Mexico and Central America since it was introduced into the Dominican Republic in the early 1990s (Rojas et al., 2018; Salati et al., 2002). Moreover, as TYLCV becomes established in Costa Rica (Barboza et al., 2018), a major question was the impact of TYLCV on the bipartite ToYMoV and ToLCSiV and disease symptoms in Costa Rica. In Florida, USA, introduction of TYLCV led to a greatly reduced incidence of the indigenous bipartite ToMoV (Polston et al., 1999). However, two NW monopartite begomoviruses (tomato leaf curl purple vein virus and tomato mottle leaf curl virus) and the bipartite tomato severe rugose virus from Brazil co-existed and caused more severe symptoms in mixed infections (Alves-Júnior et al., 2009; Macedo et al., 2017). A similar synergistic interaction was observed in mixed infections of pepper golden mosaic virus and pepper huasteco yellow vein virus in pepper plants (Méndez-Lozano et al., 2003; Rentería-Canett et al., 2011), and in mixed infections of the OW monopartite TYLCV and tomato yellow leaf curl Sardinia virus (TYLCSV) in *N. benthamiana* and tomato plants (Morilla et al., 2004).

In the present study, the interaction of these three tomato-infecting begomoviruses in tomato plants was assessed based on symptom development and viral DNA accumulation in single and mixed infections. Mixed infections were associated with more severe symptoms, with the most severe symptoms observed in plants co-infected with all three viruses. Thus, these results revealed an additive synergistic interaction, similar to that described in other studies with tomato begomoviruses (Alves-Júnior et al., 2009; Macedo et al., 2017). Furthermore, notable aspects of symptom development were associated with biological properties of the viruses used in the present study. First, symptoms appeared earlier in plants infected with the bipartite begomoviruses, alone or in mixed infection, which may reflect a capacity for cell-to-cell

movement and infection of cells outside of the phloem, allowing for more rapid colonization and symptom development. The mechanical transmission of ToYMoV provided some evidence for this hypothesis. Second, although development of TYLCD symptoms was delayed by ~3 d, the predominance of these symptoms in single and mixed infections by 14 dpi revealed the efficient colonization and associated perturbation of phloem function by this invasive virus, which also encodes multiple silencing suppressors (Rojas et al., 2001). Indeed, in mixed infections of the bipartite viruses and TYLCV, there was a striking transition from symptoms of ToYMoD to those of TYLCD.

The viral DNA accumulation results provided evidence of viral co-existence, i.e., over the course of the experiment, none of the viruses significantly impacted accumulation of the other viruses. Thus, the interactions among these viruses is a type of neutral synergism, which has been previously described in plants with mixed begomovirus infections (Alves-Júnior et al., 2009). Although DNA accumulation of the bipartite ToYMoV and ToLCSiV was significantly lower in mixed infections at 7 dpi, this was a transient effect that was not observed at 14 and 21 dpi (Fig. 6). A transient negative effect on viral accumulation was also observed early in mixed infection of the NW bipartite tomato rugose mosaic virus and tomato yellow spot virus in *N. benthamiana* and tomato plants (Syller 2012, Alves-Júnior et al. 2009). This transient antagonism may involve competition for host factors or the triggering of a more robust anti-viral defense response in mixed infections (Syller and Grupa, 2016), e.g., TYLCV accumulation was reduced in tomatoes co-infected with potato spindle tuber viroid due to upregulation of methylation pathways (Torchetti et al., 2016).

In summary, we investigated the invasion biology of three tomato-adapted begomoviruses in Costa Rica: the locally evolved NW bipartite ToYMoV and the invasive NW bipartite ToLCSiV and OW monopartite TYLCV. Infectious DNA-A and DNA-B clones and an agroinoculation system were generated for an isolate of ToYMoV recovered from an archival ToYMoD samples collected in Costa Rica in 1990 and used to fulfill Koch's postulates and show that the virus has a narrow host range and is mechanically transmissible. Infectious DNA-A and DNA-B clones and an agroinoculation system were also generated for an isolate of ToLCSiV from an archival ToYMoD sample collected in Costa Rica in 2002, and were shown to induce ToYMoD and not form infectious pseudorecombinants with ToYMoV, consistent with a long period of independent evolution in different lineages. Examination of symptoms and DNA accumulation in single and mixed infections revealed a neutral synergistic interaction, in which viruses co-existed and induced more severe symptoms in mixed infections, especially those including TYLCV. A transient and significant reduction in DNA accumulation of the bipartite viruses was detected in mixed infections at 7 dpi, whereas TYLCV accumulation was unaffected in mixed infections and TYLCD symptoms became predominant by 14 dpi. Thus, our results suggested increased begomovirus disease severity as mixed infections become more common, and this is supported by anecdotal reports of increased losses to tomato production due to these diseases in Costa Rica, especially TYLCD. Finally, this information helps in the formulation of an IPM approach for these viruses, including planting varieties with resistance to all three viruses (especially TYLCV), implementing tomato-free periods and improving sanitation in tomato fields following harvest. It will also be important to conduct more extensive surveys for tomato begomovirus disease incidence and severity in Costa Rica and document the spread and prevalence of these viruses, particularly as different management approaches are implemented.

CRediT authorship contribution statement

Minor R. Maliano: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Maria R. Rojas:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Visualization. **Monica A. Macedo:** Methodology, Investigation, Writing – review & editing. **Natalia Barboza:** Methodology, Investigation, Writing – review & editing. **Robert L. Gilbertson:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198793.

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