Investigation of the resistance and susceptible responses of tomato (*Solanum lycopersicum*) to tomato yellow leaf curl virus, the invasion biology of tomato begomoviruses in Costa Rica and characterization of two weed-infecting begomoviruses in the Caribbean Basin

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Abstract

The research in this thesis addressed aspects of the tomato-begomovirus interaction: molecular analyses of resistance and susceptible determinants, invasion biology in Costa Rica and the role of weeds as reservoirs. In Chapter II, I used an RNA-Seq approach to identify differentially expressed genes (DEGs) during infection by tomato yellow leaf curl virus (TYLCV) of the near isogenic lines LA3473-R and LA3474-S, which have the *Ty-1* (resistant) and the *ty-1* (susceptible) alleles, respectively. Differences in gene expression in the resistance and susceptible responses were observed. VIGS analyses of selected DEGs further revealed that genes encoding WRKY transcription factors, nucleotide-binding site and leucine-rich repeat (NBS-LRR) containing proteins, and receptor-like protein kinases (RLKs) may play a role in the resistance and susceptible responses to TYLCV infection. Sequence analyses of the cloned *Ty-1* gene provided further insight into how some of the newly identified DEGs participate in the *Ty-1* defense response. In Chapter III, I examined the invasion biology of three emergent/introduced begomoviruses in Costa Rica to assess their interactions in terms of disease severity and viral accumulation and genetics. Here, I first completed the molecular and biological characterization of tomato yellow mottle virus (ToYMoV) using full-length infectious DNA-A and DNA-B clones to fulfill Koch's postulates for the ToYMoD and to show that the virus infects a subset of solanaceous species. Full-length infectious clones of an isolate of tomato leaf curl Sinaloa virus (ToLCSiV) from Costa Rica also

were generated, and were used to assess genetic interaction (pseudorecombination) with ToYMoV. The infectious clones of ToYMoV, ToLCSiV and TYLCV were then used to inoculate tomato plants with each virus and all combinations, and symptoms induced and viral DNA accumulation were determined. Tomato plants infected with combinations of two or all three viruses developed more severe symptoms than plants inoculated with each virus alone. Symptoms of ToLCSiV and ToYMoV appeared earlier (~7 d post-inoculation [dpi]) than those of TYLCV (~10 dpi). However, by 14 dpi and beyond, TYLCV symptoms had become dominant in all mixed infections. Virus accumulation was determined by qPCR and revealed that, although symptoms of TYCLV became predominant in mixed infections with the NW bipartite begomoviruses, levels of ToYMoV and ToLCSiV were similar to those in plants infected with the individual viruses. ToYMoV and ToLCSiV DNA levels were negatively impacted early in infection (7 dpi), but later in infection (14 and 21 dpi) a neutral synergistic interaction was observed. These results are discussed in terms of begomovirus invasion biology and implications for begomovirus disease development and management in Costa Rica. In Chapter IV, I describe the molecular and biological properties of two bipartite begomoviruses associated with striking golden/yellow mosaic symptoms in *Malachra* sp. and *Abutilon* sp. plants on Hispaniola, and assess their potential to cause disease in crop plants. Sequence and phylogenetic analyses, together with infectivity studies with infectious clones, established that the symptoms in *Malachra* sp. were caused by the crop-infecting bipartite begomovirus tobacco leaf curl Cuba virus (TbLCuCV), whereas those in *Abutilon* sp. were caused by a new species of weed-infecting begomovirus, for which the name Abutilon golden yellow mosaic virus (AbGYMV) is proposed. Host range experiments showed that TbLCuCV also induced moderate to severe disease symptoms in *Nicotiana benthamiana*, tobacco (*N. tabacum*) and common bean plants (*Phaseolus vulgaris*) plants. In contrast, AbGYMV induced mild or no

symptoms in these plants, indicating a high degree of adaptation to *Abutilon* sp. in the Dominican Republic, and low potential to cause crop diseases. Pseudorecombinants generated with the infectious clones of these viruses were highly infectious and induced severe symptoms in *N. benthamiana* and *Malachra* sp. Furthermore, both viruses co-infected *Malachra* sp., thereby increasing the probability of virus evolution via recombination and pseudorecombination. Together, these results suggest that TbLCuCV primarily infects *Malachra* sp. in the Caribbean Basin, and occasionally spills over to infect and cause disease in crops other than tomato, whereas AbGYMV is well-adapted to *Abutilon* sp. and has not been reported infecting crops.

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Chapter I

Introduction

Geminiviruses

Geminiviruses (family *Geminiviridae*) are a large and diverse group of plant viruses that possess a circular, single-stranded (ss) DNA genome that is encapsidated into twined or geminate virions (Hanley-Bowdoin et al. 2013; Zerbini et al. 2017). The virion, from which the family derived its name (from Latin geminus meaning "twin"), is about 18x30 nm and is composed of two incomplete icosahedra (T=1) that are joined together (Hesketh et al. 2018; Zhang et al. 2001).The family is subdivided into 9 genera: i.e., *Mastrevirus*, *Curtovirus*, *Topocuvirus*, *Begomovirus*, *Becurtovirus*, *Eragrovirus*, *Turncurtovirus*, *Capulavirus* and *Grablovirus*, based on insect vector, genome structure and organization and host range (Zerbini et al. 2017).

In nature, geminiviruses are transmitted by four different hemipterous insects: i.e., whiteflies, leafhoppers, treehoppers and aphids Begomoviruses are transmitted by a whitefly species (*Bemisia tabaci*) complex; curtoviruses, mastreviruses, becurtoviruses and turncurtoviruses are transmitted by different species of leafhoppers; grabloviruses and topocuviruses are transmitted by treehoppers, whereas capulaviruses are transmitted by aphids (DeBarro et al. 2011; Gilbertson et al. 2015; Zerbini et al. 2017; He et al. 2020; Rojas et al. 2018)

Geminiviruses are either monopartite, with a single genomic DNA of ~2.6-3.0 kb; or bipartite, with two DNA components of ~2.8 kb (Rojas et al. 2005; Hanley-Bowdoin et al. 2013). The genomic DNA (monopartite) and each of the DNA components of the bipartite genome are encapsidated separately (Briddon et al. 2010; Rojas et al. 2005; Hesketh et al. 2018). Thus, for bipartite geminiviruses, there are two types of a quasi-icosahedral virions: one having the DNA-A and the other having the DNA-B. Furthermore, both DNA components are require for infection and induction of typical disease symptoms and disease development (Zerbini et al. 2017; Hanley-Bowdoin et al. 2013).

The geminivirus genome is organized with overlapping virion (v)-sense and complementary (c)-sense genes transcribed in a bidirectional manner from a non-coding intergenic region (IR), which contains the *cis*-acting elements involved in replication and gene expression (i.e., the conserved stem-loop structure with the nonanucleotide sequence TAATATT↓AC that is part of the origin of replication [*ori*] and two bidirectional RNA polymerase II promoters) (Hanley-Bowdoin et al. 2013). Geminivirus *cis*-acting elements also include short repeated motifs (iterons) that are the recognition sites for binding of the replication-associated protein (Rep) (Argüello-Astorga et al. 2001, 1994). These Rep high affinity binding sites, which differ in sequence and organization among geminiviruses, are recognized by the iteron-related domain (IRD) located in the N-terminus region of the Rep protein (Argüello-Astorga et al. 2001).

Geminivirus proteins

Like other plant viruses, geminiviruses encode 6-8 multifunctional proteins that are involved in replication and gene expression, encapsidation, movement and suppression of host defenses (e.g., transcriptional [TGS] and posttranscriptional gene silencing [PTGS]) (Zerbini et al. 2017; Hanley-Bowdoin et al. 2013). Once viral DNA enters the plant cell nucleus, the Rep protein is expressed to initiate replication of the viral genome, which occurs through double-stranded (ds) DNA replicative intermediates by a combination of rolling circle replication (RCR) and recombination-dependent replication (Hanley-Bowdoin et al. 2013; Rojas et al. 2005). The transcriptional activator protein (TrAP; and C2 homolog proteins) regulates the expression of v-sense transcripts, and interferes with TGS and PTGS (suppressor of gene silencing) (Hanley-Bowdoin et al. 2013; Rojas et al. 2005; Raja et al. 2008; Trinks et al. 2005). The replication enhancer protein (REn; and C3 homolog proteins) enhances geminivirus DNA accumulation, whereas the C4 (or AC4 in bipartite begomoviruses) of some geminiviruses suppress PTGS

(Settlage et al. 2005; Amin et al. 2011; Rojas et al. 2005). The capsid protein (CP) forms the geminivirus virion and is the determinant of insect vector specificity (Briddon et al. 1990; Hanley-Bowdoin et al. 2013; Hesketh et al. 2018; Zhang et al. 2001; Azzam et al. 1994). The CP of monopartite begomovirus also functions as a nuclear shuttle protein (NSP) and mediates the nuclear export of the viral genome (Priyadarshini et al. 2011; Hanley-Bowdoin et al. 2013; Rojas et al. 2001). The V2 or pre-coat protein of some begomoviruses suppresses PTGS and mediates the movement of monopartite geminiviruses (Padidam et al. 1996; Priyadarshini et al. 2011; Rojas et al. 2001; Zhang et al. 2012). Finally, bipartite geminiviruses also encode movement proteins, which regulate viral movement into and out of the nucleus (NSP) and cell-to cell through plasmodesmata (movement protein, MP) (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Rojas et al. 2016).

Geminivirus satellites

Some geminiviruses (e.g., begomoviruses) can be associated with circular ssDNAs referred as satellites. Two main classes of satellite DNAs have been described in association with these viruses: alphasatellites and betasatellites (Zhou 2013; Lozano et al. 2016). The genome size of alphasatellites and betasatellites is ~1.4 kb, which is about half the size of the begomovirus genome, and these satellites share little sequence identity with the helper virus, with the exception of the conserved stem-loop structure that is required for RCR (Zhou 2013; Briddon et al. 2010; Yang et al. 2019). These satellites depend on their helper virus for replication (alphasatellites) and encapsidation, movement and insect transmission (alphasatellites and betasatellites) (Zhou 2013; Briddon et al. 2010; Yang et al. 2019). Interestingly, these satellites are promiscuous with respect to helper virus replication with many satellites trans-replicated by multiple begomovirus species (Zhou 2013; Briddon et al. 2010; Yang et al. 2019; Chen et al. 2009).

Alphasatellites replicate their genome through a Rep-like protein (alpha-Rep), which is similar to Rep proteins of nanoviruses (Zhou 2013; Briddon et al. 2010; Yang et al. 2019; Rojas et al. 2005). These satellites are strictly dependent on their helper virus for movement, encapsidation and insect vector transmission (Mansoor et al. 1999; Saunders et al. 1999). Alphasatellites are mostly associated with monopartite begomoviruses in the OW (Zhou 2013; Briddon et al. 2010; Yang et al. 2019; Rojas et al. 2005). However, these satellites have also been found in association with bipartite begomoviruses in the NW (Paprotka et al. 2010; Romay et al. 2010), and most recently, in association with a betasatellite and a mastrevirus in the OW (Kumar et al. 2014).

Betasatellites also occur mostly in the OW and are usually, but not exclusively, associated with monopartite begomoviruses (Saunders et al. 2000; Briddon et al. 2001; Zhou 2013; Rojas et al. 2005). Unlike alphasatellites, betasatellites do not encode a Rep-like protein. Thus, they depend completely on the helper virus for replication as well for encapsidation and whitefly transmission (Zhou 2013; Rojas et al. 2005). Instead, these satellites encode the βC1 protein, which is a major pathogenicity and symptom determinant that interferes with TGS and PTGS (suppressor of gene silencing) (Zhou 2013; Rojas et al. 2005; Hanley-Bowdoin et al. 2013).

In 1997, a type class of satellite DNAs was first described (Dry et al. 1997). These satellites were associated with some bipartite begomovirus in the NW and some monopartite begomoviruses in the NW and OW (Dry et al. 1997; Elvira et al. 2012; Lozano et al. 2016), and they were named deltasatellites. The genome size of deltasatellites is ~0.7 kb (approximately of quarter of the geminivirus genome), and has sequence and structural similarities with betasatellites (e.g., conserved stem-loop structure, an A-rich region and a region with a high nt identity with the satellite conserved region [SCR]) (Elvira et al. 2012). However, deltasatellites do not encodes any

known proteins and share no sequence identity with the helper virus (Dry et al. 1997; Elvira et al. 2012; Lozano et al. 2016). In general, deltasatellites do not seems to have a role in pathogenicity and symptom development, but in some cases (NW deltasatellites), these satellites can interfere with virus DNA accumulation (Fiallo-Olivé et al. 2020).

Geminivirus evolution

Geminiviruses have a long evolutionary history, likely having evolved from ancient phytoplasmal-type II extrachromosomal ssDNA (EcDNA) plasmids via acquisition of CP genes from an RNA virus (Krupovic 2012; Lefeuvre et al. 2011; Krupovic et al. 2009; Rojas et al. 2005). Several lines of evidence support this hypothesis. First, the existence of prokaryotic-like features in the genome of present-day geminiviruses, e.g., the promoter of the tomato golden mosaic virus CP is active in *Escherichia coli*, begomovirus ssDNA and replicative dsDNA forms have been found in plastids, and geminiviruses can be replicated in *Agrobacterium tumefaciens* (Petty et al. 1986; Gröning et al. 1990; Selth et al. 2002; Frischmuth et al. 1990; Rojas et al. 2005). Second, structural modeling of the geminiviral CP showed homology with that of icosahedral RNA viruses (Krupovic et al. 2009). Third, the presence of conserved amino acids (aa) motifs in the Reps of geminiviruses and ss DNA plasmids of Gram-positive bacteria (Koonin et al. 1992; Krupovic et al. 2009; Hanley-Bowdoin et al. 2000; Ilyina et al. 1992). Together, these findings provided strong evidence for the evolution of geminiviruses from ancient prokaryotic DNA plasmids following recombination with an RNA virus. However, this hypothesis involves the occurrence of a rare DNA/RNA recombination event in different cell compartments (geminiviruses replicate in the nucleus, whereas most plant RNA viruses replicate in the cytoplasm). Another hypothesis is that geminiviruses emerged following recombination between an ancient ssDNA virus encoding a RCR-like Rep and a marine virus (Saccardo et al. 2011; Rosario et al. 2009). Evidence supporting

this latter hypothesis came from: (i) sequence and phylogenetic analyses showing that the type II EcDNA encoded Rep is not of bacteria origin but has been acquired by phytoplasmas from an ancient Rep-encoding virus, and (ii) metagenomic data analyses have shown aa sequence similarities between the CP of geminiviruses and that of viruses from soil or marine environments (Krupovic et al. 2009; Saccardo et al. 2011; Rosario et al. 2009; Kim et al. 2008).

Although the precise origin of geminiviruses cannot be determined with complete certainty, the evolution of geminiviruses must surely have involved the acquisition and modification of additional genes required for host infection and insect vector transmission (i.e., through recombination with host DNA or other virus-like entities) (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Zerbini et al. 2017). In this scenario, these viruses have co-evolved with plants for millions of years, i.e., well before the occurrence of continental drift (Lefeuvre et al. 2011; Rojas et al. 2005; Rybicki 1994). The subsequent diversification and evolution of geminiviruses was mediated by viral genetic mechanisms such mutation, recombination and, in the case of the bipartite genome, pseudorecombination (Rojas et al. 2018; Lima et al. 2017; Duffy et al. 2009; Lefeuvre et al. 2015). Moreover, these events have ocurred multiple times in different geographic regions, giving rise to multiple viruses that infect different hosts (local evolution) (Zhou et al. 2008).

Geminivirus cause economically important diseases of food, feed, and fiber crops worldwide (Rojas et al. 2018, 2005; Hanley-Bowdoin et al. 2013), and these diseases have resulted in substantial losses to agriculture, especially in tropical and subtropical regions (Rojas et al. 2018; Zerbini et al. 2017). Moreover, the increase local populations and long distance spread to new regions of insect vectors, especially the polyphagous whitefly supervector, have accelerated the emergence of new geminiviruses in different geographical regions. The resulting diseases have

caused substantial losses to economically important crops, including common bean, cassava, cotton, cucurbits, pepper, tomato and ornamental plants.

Begomoviruses

Viruses in the genus *Begomovirus* infect dicotyledonous crops and cause economically important diseases in tropical and subtropical regions of the world. There are >422 begomovirus species officially recognized by the International Committee on Taxonomy on Viruses (ICTV), making this one the largest and most diverse genera of viruses (Zerbini et al. 2017). Begomoviruses infect a wide range of dicotyledonous crops and are transmitted by whiteflies of the *Bemisia tabaci* cryptic species complex (De Barro et al. 2011; Gilbertson et al. 2015; Rojas et al. 2005; Navas-Castillo et al. 2011; Rojas et al. 2018). The complex is formed by at least 34 morphologically indistinguishable species, which have different biological and molecular properties (DeBarro et al. 2011). For example, *B. tabaci* Middle East-Asia Minor 1 (MEAM1, formerly known as biotype B) and Mediterranean (MED, formerly known as biotype Q) are highly polyphagous, rapidly develop insecticide resistance, and are highly fecund. Moreover, transmission efficiency varies among species, depending on the nature of the virus-host combination (De Barro et al. 2011; Gilbertson et al. 2015; Navas-Castillo et al. 2011). In general, the mode of insect transmission of begomoviruses is circulative (persistent) and non-propagative (De Barro et al. 2011; Gilbertson et al. 2015; Navas-Castillo et al. 2011). However, results of some studies have shown that the invasive monopartite tomato yellow leaf curl virus (TYLCV) replicates in cells of the primary salivary glands of the whitefly adult, and that such replication requires the interaction of Rep with the whitefly DNA polymerase δ and proliferating cell nuclear antigen (PCNA) (Pakkianathan et al. 2015; He et al. 2020; Wang et al. 2016, 2021). Moreover, TYLCV can also be transmitted transovarially for at least two generations of whiteflies (Guo et al. 2010; Wang et al. 2021). Thus,

it seems that there is at least one species (no other begomovirus has been shown to replicate in the whitefly vector) of begomoviruses that have evolved to replicate in the whitefly vector, and that, this replication may only happen under certain conditions (e.g., specific organs).

Genome properties

The begomovirus genome is either monopartite, with a single genomic DNA of ~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). The DNA-A and DNA-B components share no sequence identity, except for an ~200 nucleotide (nt) noncoding conserved sequence known as the common region (CR), which encodes the *ori* and regulatory sequences (Hanley-Bowdoin et al. 2013). The monopartite genomic DNA is similar in sequence and genome organization to the DNA-A component of bipartite begomoviruses, with one gene (V1/AV1) on the v-sense strand encoding the CP, and four genes on the c-sense strand (C1/AC1, C2/AC2, C3/AC3 and C4/AC4) encoding Rep, TrAP, Ren and the C4/AC4 protein. The DNA-B component of the bipartite begomoviruses has two genes, BV1 and BC1, which encodes a nuclear shuttle protein and movement protein, respectively (Hanley-Bowdoin et al. 2013; Rojas et al. 2005). The sequence of the DNA-B component is typically more divergent than that of the DNA-A component (Briddon et al. 2010), and also has a hypervariable region (HVR) (Gilbertson et al. 1991). This HVR extends from the 5' end of the CR to the initiation codon of the BC1 open reading frame (ORF) and is the most variable region in the genome of bipartite begomoviruses.

Begomoviruses also show a phylogeographic distribution, with most bipartite species occurring in the New World (NW) and rarely associated with satellites DNAs, and most monopartite species occurring in the Old World (OW) and often in association with satellite DNAs that are either required for disease development (betasatellites) or have no obvious effect in

modulating disease symptoms (alphasatellites) (Rojas et al. 2005; Zhou 2013). However, there are exception to this, including the introduction of TYLCV into the NW in the early 1990s and the recent identification of indigenous NW monopartite begomoviruses infecting tomato in Peru, Ecuador, Venezuela and Northern Brazil (Lefeuvre et al. 2010; Melgarejo et al. 2013; Salati et al. 2002; Macedo et al. 2017; Nakhla et al. 1994).

Begomoviruses have a remarkable flexibility in genome evolution and a long evolutionary history. It is generally believed that the ancestor of modern-day begomoviruses was monopartite (Briddon et al. 2010) and that the bipartite genome likely evolved before the breakup of the supercontinent Pangea (continental drift) because bipartite begomoviruses occur in the NW and OW (Rojas et al. 2005). In this scenario, these viruses have co-evolved with plants for millions of years, i.e., well before the emergence of flowering plants and the domestication of crops (Lefeuvre et al. 2011; Rojas et al. 2005; Rybicki 1994). The subsequent diversification and evolution of OW and NW begomoviruses involved genetic mechanisms such as mutation and recombination, which are known as the major contributors to the genetic divergence of viruses (Rojas et al. 2018; Lima et al. 2017; Duffy et al. 2009; Lefeuvre et al. 2015; Rojas et al. 2005).

In terms of these mechanisms, recombination has played, and continues to play, an important role in the emergence of begomoviruses. Extensive analysis of begomovirus/satellite sequences have shown that several species of monopartite and bipartite begomoviruses have evolved extensively through recombination (Lefeuvre et al. 2010; Urbino et al. 2013; Hou et al. 1996; Lozano et al. 2016; Padidam et al. 1999; Lefeuvre et al. 2009; Lima et al. 2013). Furthermore, these studies also revealed that recombination hot spots exist in the begomovirus genome, especially the region spans the 5' end of the AC1 gene, the entire overlapping AC4 gene and the left side of the CR (Stanley 1995; Ndunguru et al. 2005; Fauquet et al. 2005; García-

Andrés, Tomás, et al. 2007; Rojas et al. 2005; Hou et al. 1996). Begomovirus genomes also showed high level of within-host variation (mutation) and substitution rates inferred for these viruses are equivalent to those of RNA viruses (Ge et al. 2007; Lima et al. 2017; Duffy et al. 2008, 2009). More recently, it was shown that mutation is likely the main source of genetic variation for begomovirus genomes (Lima et al. 2017).

The evolution of begomoviruses also involves pseudorecombination (or reassortment). This occurs when a viable virus is formed with DNA components from different bipartite viruses (Gilbertson et al. 1993; Hou et al. 1996; Silva et al. 2014; Andrade et al. 2006; Rojas et al. 2005, 2018). Pseudorecombinants are typically formed between isolates and strains of the same begomovirus species, but in some cases, they can also be formed with DNA components from different species (Melgarejo et al. 2014; Hou et al. 1996; Gilbertson et al. 1993; Idris et al. 2008; Garrido-Ramirez et al. 2000; Ramos et al. 2003; Hill et al. 1998; Pita et al. 2001; Faria et al. 1994; Andrade et al. 2006). Additionally, begomovirus genomes have the capacity to acquire and modify small circular ssDNA components. For example, acquisition of satellite DNAs has played a major role in evolution of OW monopartite begomoviruses, whereas acquisition and modification of the DNA-B components acquired by pseudorecombination has played a role bipartite begomoviruses evolution (Rojas et al. 2005; Gilbertson et al. 1993; Hou et al. 1996; Briddon et al. 2010; Rojas et al. 2008; Silva et al. 2014; Garrido-Ramirez et al. 2000; Pita et al. 2001). Finally, begomovirus also evolved locally through the world, resulting in the emergence of multiple begomovirus species that infect one particular host and cause similar symptoms (Rojas et al. 2018; Melgarejo et al. 2013).

The emergence and evolution of new begomovirus species has been facilitated by the polyphagous behavior of the whitefly supervector, especially the *B. tabaci* species MEAM1

(Gilbertson et al. 2015; Rojas et al. 2008; Navas-Castillo et al. 2011; Rojas et al. 2018). Moreover, human activities have led to the long-distance movement of numerous begomoviruses, blurring the geographic separation of OW and NW begomoviruses, e.g., introduction of squash leaf curl virus (SLCuV) into the Middle East from the NW, introduction of tomato leaf curl New Delhi virus (ToLCNDV) into the Western Mediterranean Basin from the subcontinent of Asia (India) (Antignus et al. 2003; Akad et al. 2008; Varma et al. 2011; Rojas et al. 2018; Ruiz et al. 2015; Gilbertson et al. 2015). However, none of these cases has been as important as the introduction of the invasive TYLCV into the NW during the early 1990s (Nakhla et al. 1994). From there, TYLCV quickly invaded the Southern US, Mexico and the rest of the world (Lefeuvre et al. 2010; Rojas et al. 2018; Scholthof et al. 2011; Mabvakure et al. 2016).

In general, individual begomoviruses tend to have relative narrow host ranges (Rojas et al. 2018). However, as a group, they can infect a wide range of food and fiber crops, ornamental plants and non-cultivated plants (weeds) in the OW and NW (Rojas et al. 2018, 2005; Gilbertson et al. 2015). Plants affected include crops such as tomato, cassava, common bean, cotton, cucurbits and pepper; ornamentals such as *Abutilon* spp. and *Lonicera* spp. (honeysuckle); and weeds such as *Malachra* spp., *Sida* spp., *Macroptilium* spp. and *Malva* spp. (Rojas et al. 2018).

Begomoviruses of tomato

In terms of production, tomato (*Solanum lycopersicum*) has become one of the most important vegetables crops in the world (Morales et al. 2001; Morales 2010; Nakhla et al. 2005). Tomato is infected by more begomovirus species (~90) than any other crop (Gilbertson et al. 2015; Zerbini et al. 2017; Rojas et al. 2018), and this can be attributed to a number of factors. First, production of tomato has grown quickly over the world (Rojas et al. 2018; Polston et al. 1997). Second, domesticated tomato is highly susceptible to begomovirus infection (Varma et al. 2003;

Polston et al. 1997; Rojas et al. 2018; Gilbertson et al. 2015). Third, local evolution has resulted in the emergence of multiple begomoviruses that infect tomato and cause similar symptoms (Gilbertson et al. 2015; Varma et al. 2003; Rojas et al. 2018). Together this has accelerated the emergence of numerous tomato-infecting begomoviruses in the NW and OW (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Rojas et al. 2018). The NW begomovirus disease of tomato are mainly caused by bipartite species, although monopartite are becoming more important (Rojas et al. 2005; Melgarejo et al. 2013; Macedo et al. 2017; Rojas et al. 2018; Gilbertson et al. 2015).

Several tomato-infecting begomoviruses have been reported from North and Central America, including tomato mottle virus, pepper golden mosaic virus (PepGMV), pepper huasteco yellow vein virus, chino del tomate virus, tomato golden mottle virus, tomato leaf curl Sinaloa virus (ToLCSiV), tomato yellow mottle virus (ToYMoV), tomato mosaic Havana virus (ToMHaV), tomato severe leaf curl virus (ToSLCV) and the invasive TYLCV (Nakhla et al. 2005; Salati et al. 2010; Ala-Poikela et al. 2005; Polston et al. 1997; Rojas et al. 2018). Furthermore, these begomoviruses are often found in mixed infections (e.g., ToSLCV and ToMHaV in Guatemala, ToLCSiV, ToLCSiV and PepGMV in Nicaragua and ToYMoV, ToLCSiV and TYLCV in Costa Rica), which allows for further virus evolution via recombination and pseudorecombination (Barboza et al. 2018; Ala-Poikela et al. 2005; Rojas et al. 2018).

In South America, tomato-infecting begomoviruses emerged in Brazil and other countries after the introduction of the highly polyphagous *B. tabaci* species MEAM1 in the 90s (Rocha et al. 2013; Gilbertson et al. 2015; Rojas et al. 2018; Polston et al. 1997). The first tomato begomovirus disease in Brazil was cause by the tomato golden mosaic virus (TGMV), which was reported in the early 1960s (Inoue-Nagata et al. 2016; Rojas et al. 2018; Polston et al. 1997; Albuquerque et al. 2012). Since then, at least 14 tomato-infecting begomoviruses have been

described and characterized (Albuquerque et al. 2012; Inoue-Nagata et al. 2016; Rocha et al. 2013). For example, tomato severe rugose virus (ToSRV) is important in central regions, whereas tomato mottle leaf curl virus (ToMoLCV) is more prevalent in north-eastern regions of Brazil (Inoue-Nagata et al. 2016; Rojas et al. 2018; Polston et al. 1997; Albuquerque et al. 2012). Additionally, since 2010, NW monopartite tomato-infecting monopartite begomoviruses continue to be discovered in BR, Ecuador, Peru and Venezuela (Rojas et al. 2018; Gilbertson et al. 2015; Melgarejo et al. 2013; Macedo et al. 2017; Márquez-Martín et al. 2011).

In the OW, tomato yellow leaf curl disease (TYLCD) has emerged as one of the most important diseases of tomato (Zaidi et al. 2017). The disease is caused by a complex of species of monopartite and bipartite begomoviruses (Abhary et al. 2007; Melgarejo et al. 2013; Rojas et al. 2018; Kon et al. 2012; Leke et al. 2011; Zhou et al. 2008; Moriones et al. 2017). Among these viruses, TYLCV and tomato yellow leaf curl Sardinia virus (TYLCSV) have cause significant crop losses in the Mediterranean Basin (Accotto et al. 2000; Rojas et al. 2018; Gilbertson et al. 2015; Sánchez-Campos et al. 2002; Díaz-Pendón et al. 2019). Furthermore, these viruses have been shown to participate in genetic exchanges (e.g., recombination) giving rise to resistance-breaking strains of TYLCV (Belabess et al. 2015, 2016).

The bipartite ToLCNDV, the causal agent of tomato leaf curl disease (ToLCD), is one of the most important tomato-infecting begomovirus in the Indian subcontinent (Moriones et al. 2017; Zaidi et al. 2017; Rojas et al. 2018). Moreover, in recent years, the virus has expanded its host range and spread into new geographical regions, e.g., North Africa and Southern Europe (Zaidi et al. 2017). Finally, several monopartite tomato-infecting begomoviruses have been described in Asia, including tomato yellow leaf curl China virus, tomato leaf curl China virus, tobacco curly shoot virus, tobacco leaf curl Yunnan virus, tomato leaf curl Guangxi virus, tomato leaf curl

Malaysia virus, tobacco leaf curl Japan virus and tomato yellow leaf curl Thailand virus (Kenyon et al. 2014; Yin et al. 2001; Zhou et al. 2003; Xu et al. 2007; Tsai et al. 2006; Zhang et al. 2010; Shimizu et al. 1999; Yang et al. 2011).

Begomoviruses of non-cultivated plants

In addition to infecting crop plants such as tomato, begomoviruses infect a diversity of non-cultivated plants, mostly weeds, in tropical and subtropical regions of the world (Rojas et al. 2018; García-Arenal et al. 2019; Prajapat et al. 2014; Morales et al. 2001; Gilbertson et al. 2015). Numerous begomoviruses have been reported infecting weed species in the families Asteraceae, Capparaceae, Convolvulaceae, Euphorbiaceae, Fabaceae, Malvaceae, Nyctaginaceae and Solanaceae (Barreto et al. 2013; Blawid et al. 2013; da Silva et al. 2011; Ferro et al. 2017; Fiallo-Olivé et al. 2014; Hernández-Zepeda et al. 2007; Mar et al. 2017; Melgarejo et al. 2019, 2014; Melo et al. 2016; Nascimento et al. 2016; Passos et al. 2017; Pinto et al. 2016; Romay et al. 2016; Stewart et al. 2014; Tavares et al. 2012).

Non-cultivated plants have long been believed to serve as a reservoir or alternative hosts for crop-infecting begomoviruses (Gilbertson et al. 1991; Bedford et al. 1998; Salati et al. 2002; Rojas et al. 2018; Barreto et al. 2013). However, most begomoviruses characterized from weeds are highly adapted to these hosts, and typically infect crop plants less efficiently or not at all (Melgarejo et al. 2014; Barreto et al. 2013; Fiallo-Olivé et al. 2012; Rocha et al. 2013; Melgarejo et al. 2019; Silva et al. 2012; Wyant et al. 2011). Although there are some exceptions such as tobacco leaf curl Cuba virus infecting *Malachra alceifolia* in Jamaica (Hall et al. 2008), and *Nicandra physaloides* infected with tomato severe rugose virus in Brazil (Barbosa et al. 2009).

Begomoviruses populations in non-cultivated plants are subject to a range of distinct selective pressures compared with those in crop species, as the genetic background of these wild

species is wider than those of crop species (Webster et al. 2007; Silva et al. 2012). As a result, begomovirus populations in non-cultivated plants tend to be divergent compared with those of crop-infecting begomoviruses (Melgarejo et al. 2014; Barreto et al. 2013; Fiallo-Olivé et al. 2012; Rocha et al. 2013). Together, this suggest that begomoviruses infecting crops and weeds have co-evolved with their hosts over a long period of time, such that the crop-infecting viruses inefficiently infect weeds. More likely, these weeds serve as sources of begomovirus diversity for the emergence of crop-infecting begomoviruses (Silva et al. 2012; Melgarejo et al. 2014). In this scenario, crop-infecting begomoviruses have originated from ancestral viruses infecting non-cultivated plants, e.g., during spillover events and subsequently evolving and adapting to crops, some of which are highly susceptible, e.g., tomato (Castillo-Urquiza et al. 2008; Fernandes et al. 2009; Melgarejo et al. 2019; Rojas et al. 2018; Melgarejo et al. 2013; García-Arenal et al. 2019). Several lines of evidence support this later hypothesis. First, crop-infecting begomoviruses can generally be associated with geographical regions, e.g., begomoviruses that infect crops in Brazil appear to have been indigenous (Inoue-Nagata et al. 2016; Silva et al. 2012; Prajapat et al. 2014). Second, characterization of some crop-infecting begomoviruses have shown the capacity to (still) infect weeds (Hall et al. 2008; Fernandes et al. 2006; Calegario et al. 2007; Ribeiro et al. 2007; Salati et al. 2002; Prajapat et al. 2014). Third, the well-established phenomenon of local evolution is consistent with crop-infecting begomoviruses having evolved from weed-infecting progenitors (Idris et al. 2019; Campbell et al. 2013; Zhou et al. 2007). Together, these findings indicate the importance of weeds in the epidemiology of crop plants and help explains the substantial divergence of weed- and crop-infecting begomoviruses.

Tomato yellow leaf curl virus

TYLCV is the most widely distributed and economically important begomovirus infecting tomato. TYLCV is an OW monopartite member of the genus *Begomovirus* (family *Geminiviridae*) (Rojas et al. 2018; Abhary et al. 2007; Navot et al. 1991). The genome organization of TYLCV is typical of OW monopartite begomoviruses, i.e., the genomic DNA is ~2.8 kb and encodes six ORFs, two in the v-sense strand (V1 and V2) encoding the CP and precoat protein, respectively, and four in the c-sense strand (C1, C2, C3, and C4) encoding the Rep, TrAP, REn and the AC4 protein, respectively (Rojas et al. 2001; Scholthof et al. 2011; Navot et al. 1991). TYLCV exists as a complex of genetically distinct strains and species, some of which have emerged through recombination (Fauquet et al. 2008; Brown et al. 2015; Navas-Castillo et al. 2000; Chen et al. 2009).

The TYLCV Israel strain (TYLCV-IL), which was described in the Middle East in the 1940s, is a recombinant virus derived from the Mild strain of TYLCV (TYLCV-Mld) and a tomato leaf curl virus-like ancestor (Antignus et al. 1994; Navot et al. 1991). Additionally, TYLCV-IL and TYLCV-Mld have recombined with TYLCSV to generate the tomato yellow leaf curl Malaga virus and the recombinant TYLCV-IS76 variant (Monci et al. 2002; Belabess et al. 2015). These recombinant viruses were found to have completely displaced the parental viruses in the Mediterranean Basin and are associated with TYLCD on resistant (*Ty-1*) cultivars (TYLCV-IS76) (Monci et al. 2002; Belabess et al. 2015; García-Andrés et al. 2009; Rojas et al. 2018; García-Andrés, Accotto, et al. 2007). Additionally, other characterized TYLCV strains such as the Gezira, Iran and Oman have been found to have emerged through independent, albeit similar, recombination events (Idris et al. 2005; Bananej et al. 2004; Khan et al. 2008; Navas-Castillo et al. 2000). Finally, of all of these strains, TYLCV-IL and TYLCV-Mld have the broader

distribution and are the only two strains that have disseminated outside the Middle East (Mabvakure et al. 2016; Lefeuvre et al. 2010; Chen et al. 2009).

Whitefly transmission

In nature, TYLCV is transmitted exclusively by the whitefly supervector *B. tabaci* in a persistent (circulative) and propagative manner (DeBarro et al. 2011; Gilbertson et al. 2015; Rojas et al. 2005; Navas-Castillo et al. 2011; Rojas et al. 2018; He et al. 2020; Pakkianathan et al. 2015). Collectively, members of the *B. tabaci* cryptic species complex feeds on more than 600 plant species worldwide (Jones 2003; Navas-Castillo et al. 2011). The *B. tabaci* MEAM1 and MED are considered the most invasive and damaging species that transmit TYLCV to tomato, and the efficiency of such transmission depends on the whitefly species in a sex dependent manner (DeBarro et al. 2011; Gilbertson et al. 2015; Rojas et al. 2005; Navas-Castillo et al. 2011; Rojas et al. 2018; He et al. 2020; Ning et al. 2015). Factors such as extreme fecundity, development of large population and dispersal ability, extreme polyphagy and capacity to alter plant defenses and develop insecticide resistant have made these two MEAM1 and MED species important supervectors (Gilbertson et al. 2015; Luan et al. 2011). In addition, TYLCV can be transovarially transmitted to the whitefly progeny, as well as being seed transmitted in tomato, although a recent report did not find TYCLV was seed transmitted (Ghanim 2014; Guo et al. 2010; Kil et al. 2016; Pérez-Padilla et al. 2020). Together with the intercontinental movement of infected plant material mediated by human activities, these factors have contributed to the global spread of TYLCV-IL and TYLCV-Mld (Gilbertson et al. 2015; Rojas et al. 2018; Seal et al. 2006).

Global distribution

TYLCV was first described from the Jordan Valley around 1940s and was known only in the OW (Cohen et al. 2007; Duffy et al. 2007; Lefeuvre et al. 2010). The global dissemination of

TYLCV began in the 1980s, after the emergence of TYLCV-IL and TYLCV-Mld (Duffy et al. 2008, 2007; Lefeuvre et al. 2010). Subsequently, these TYLCV strains spread from the Middle East into the Mediterranean Basin, Asia and Australia. In the early 1990s, TYLCV first entered the Caribbean Basin from the Eastern Mediterranean (Mabvakure et al. 2016; Lefeuvre et al. 2010; Salati et al. 2002; Van Brunschot et al. 2010; Nakhla et al. 1994). Moreover, other independent introductions of TYLCV into the NW also occurred when TYLCV-IL and TYLCV-Mld were introduced from the East Asian, Australian and Western Mediterranean regions (Bird et al. 2001; Mabvakure et al. 2016; Lefeuvre et al. 2010; McGlashan 1994; Zambrano et al. 2007; Kon et al. 2014). Nowadays, TYLCV has quickly spread to the southern United States, northern Mexico, Central America, the Caribbean Basin and Venezuela (Lefeuvre et al. 2010; Rojas et al. 2018; Scholthof et al. 2011; Mabvakure et al. 2016; Salati et al. 2010).

Disease symptoms

TYLCV is the most devastating tomato-infecting begomovirus worldwide (Scholthof et al. 2011). Under high whitefly pressure, outbreaks can result in yield losses of up to 100%, especially when plants are infected early in development (Salati et al. 2002; Gilbertson et al. 2015; Rojas et al. 2018; Cohen et al. 1994). Tomato plants infected with TYLCV are stunted, and show abnormal upright grow, and leaves developed upward curling with yellowing, interveinal chlorosis, crumpling and often a bushy appearance (Salati et al. 2002; Cohen et al. 1994). Moreover, flowers on infected plants commonly do not develop and abscise before fruit set, leading to major yield losses, as high as 100%. So far, TYLCV has been reported infecting other solanaceous crops, such as pepper and tobacco; fabaceous crops, such as common bean; and non-cultivated (weeds) plants, such as *Ageratum conyzoides*, *Chenopodium murale*, *Cuscuta europaea*, *Malva parviflora* and *Sida acuta*. However, tomato is by far the most economically important host of this virus, and most

of the weeds are symptomless hosts and have low virus titers (Salati et al. 2010; Papayiannis et al. 2011).

Management

Management of TYLCV requires an integrated pest management (IPM) program that involves measures before, during and after the growing season (Rojas et al. 2018; Gilbertson et al. 2011). Before the growing season, TYLCV can be controlled by using virus- and whitefly-free transplants and propagative stocks and there are now commercially available resistant cultivars generated through conventional breeding (Kenyon et al. 2014; Ji et al. 2009; Pereira-Carvalho et al. 2010; Tomás et al. 2011; Caro et al. 2015; Gilbertson et al. 2011; Vidavski et al. 2008; Lapidot et al. 2015, 2014; Yan et al. 2018; Rojas et al. 2018; Inoue-Nagata et al. 2016). In addition, a variety of transgenic strategies have been used to develop TYLCV-resistant varieties, although these are not currently grow commercially (Zrachya et al. 2007; Rojas et al. 2018). The use of insecticides to reduce whitefly populations is one of the most important, and commonly used components of a successful IPM approach during the growing season, but monitoring and proper timing of insecticide application is critical (Rojas et al. 2018). Insecticides such as neonicotenoids and cyazypyr have been used effectively to control the whitefly vector in open fields and greenhouses (Rojas et al. 2018; Gilbertson et al. 2011; Navas-Castillo et al. 2011). Unfortunately, continue usage of insecticides has led to the rapid emergence of insect vector population with resistance to various insecticides (Rojas et al. 2018; Gilbertson et al. 2011; Navas-Castillo et al. 2011; Nauen et al. 2005; Horowitz et al. 2005). Alternative measures during the growing season also involve roguing of virus-infected plants and row covers and reflective mulches (Rojas et al. 2018; Gilbertson et al. 2011; Stansly et al. 2004). After the growing season, sanitation, weed management and implementation of a host-free period can be used to reduce the viral inoculum

source and vector populations (Rojas et al. 2018; Gilbertson et al. 2011). Indeed, these approaches have been successfully used to managed TYLCV in the DO (Salati et al. 2002; Ucko et al. 1998; Rojas et al. 2018; Gilbertson et al. 2011).

Genetic resistance to TYLCV

Domesticated tomato is highly susceptible to TYLCV, but resistance has been found in wild tomato species (Ji, Scott, et al. 2007). Breeding for resistance to TYLCV started in the 1970s and several resistance genes have been introgressed into commercial varieties of tomato (Scholthof et al. 2011; Rojas et al. 2018; Gilbertson et al. 2011). Resistance to TYLCV has been reported in a number of wild tomato species, including *S. arcanum*, *S. cheesmaniae*, *S. chilense*, *S. chmielewskii*, *S. corneliomulleri*, *S. galapagense*, *S. habrochaites*, *S. neorickii*, *S. pennellii*, *S. peruvianum*, and *S. pimpinellifolium* (Ji et al. 2009; Vidavski et al. 2008; Pereira-Carvalho et al. 2010; Tomás et al. 2011; Caro et al. 2015; Gilbertson et al. 2011; Kenyon et al. 2014; Lapidot et al. 2015, 2014; Yan et al. 2018). Currently, six resistance loci (*Ty-1* to *Ty-6*) have been mapped in tomato, and four of them have been characterized (*Ty-1*, *Ty-2*, *Ty-3* and *Ty-5*) (Verlaan et al. 2011, 2013; Yamaguchi et al. 2018; Yang et al. 2014; Lapidot et al. 2015; Anbinder et al. 2009; Ji et al. 2009; Gill et al. 2019). The *Ty-1* and *Ty-3* genes are allelic and were introgressed from *S. chilense* (Verlaan et al. 2011). Both genes are located on the long arm of chromosome 6 and encode an RNA-dependent RNA polymerase (Verlaan et al. 2011, 2013). The *Ty-2* gene originated from *S. habrochaites*, mapped to the long arm of chromosome 11 and encodes a nucleotide-binding domain and leucine-rich repeat (NB-LRR) containing protein (Yamaguchi et al. 2018; Yang et al. 2014). The *Ty-5* is a recessive gene that is believed to have been introgressed from *S. peruvianum* (Lapidot et al. 2015; Anbinder et al. 2009). This gene is located on chromosome 4 and encodes a loss-of-function allele of the messenger RNA surveillance factor Pelota, which is involved in

ribosome recycling during translation (Lapidot et al. 2015). Two additional resistance genes have been mapped on the tomato chromosome. The *Ty-4* gene, which has a minor effect on TYLCV resistance, was introgressed from *S. chilense* and is located on the long arm of chromosome 3 (Ji et al. 2009). The *Ty-6* gene is located on the long arm of chromosome 10 and is believed to have been introgressed from *S. chilense* (Gill et al. 2019). However, relatively little is known about the mechanism(s) underlying this resistance.

Over the last 30 years, a major focus of breeding programs was the introgressions of the *Ty-1*, *Ty-2* and *Ty-3* genes into commercial varieties of tomato (Vidavski et al. 2008; Rojas et al. 2018; Gilbertson et al. 2011; Butterbach et al. 2014). Indeed, commercial varieties with some degree of TYLCV resistance are now widely available for open field and protected culture. However, previous reports has showed that the resistance conferred by these genes can be overcome under high TYLCV pressure, especially after early infections (García-Cano et al. 2008; Pérez de Castro et al. 2013; Rojas et al. 2018). For example, breakdown of *Ty-2*-mediated resistance have been reported for TYLCSV and TYLCV-Mld (Barbieri et al. 2010; Ohnishi et al. 2016), whereas the recombinant TYLCV-IS76 has been associated with the overcoming of *Ty-1* resistant in morocco (Belabess et al. 2015, 2016).

The *Ty-1* resistance gene

Ty-1 is a partially dominant resistant gene that was introgressed from the wild tomato species *S. chilense* LA1969 (Zamir et al. 1994; Verlaan et al. 2013). High levels of resistance in *S. chilense* LA1969 was first reported in Israel in the early 1990s (Zamir et al. 1994). Resistance derived from this line is mainly attributed to this gene, although two minor genes associated with TYLCV resistance have also been identified on chromosomes 3 and 7 (Zamir et al. 1994; Verlaan et al. 2013). The *Ty-1*-mediated resistance was subsequently confirmed in numerous *Ty-1*-

introgression lines, and under different experimental conditions (Scott et al. 1991; Czosnek et al. 1993; Michelson et al. 1994; Pico et al. 1999). Tomato lines with the *Ty-1*-introgression are not immune, as TYLCV can replicate and moves systemically, although viral titters are substantially reduced and do not exceed more than 10% of those in susceptible tomato lines (Verlaan et al. 2013; Scott et al. 1991; Czosnek et al. 1993; Michelson et al. 1994; Pico et al. 1999; Rojas et al. 2018). Furthermore, this gene confers high levels of resistance to TYLCV and other begomoviruses (e.g., ToSRV and ToMoLCV), but do not provide immunity or resistance to all begomoviruses, especially bipartite ones (Rojas et al. 2018; Gilbertson et al. 2011; Butterbach et al. 2014). Furthermore, the resistance conferred by this gene can be overcome in mixed infections of TYLCV with cucumber mosaic virus (RNA virus) and with ageratum yellow vein virus betasatellite under laboratory conditions (Voorburg et al. 2020; Butterbach et al. 2014).

The *Ty-1* gene is located in the long arm of chromosome 6 (Verlaan et al. 2013; Zamir et al. 1994; Ji, Schuster, et al. 2007). The *Ty-1* locus is composed of numerous allelic variants, and *Ty-1*-like alleles are present in both resistant and susceptible lines (e.g., *Ty*-1/Ty-*3* and the susceptible *ty-1*) (Caro et al. 2015; Verlaan et al. 2013). Analysis of the *Ty-1* coding region has identified five allele-specific polymorphisms that differed between *Ty-1*- and *ty*-1-like alleles (Caro et al. 2015; Verlaan et al. 2013). In addition, a 12 bp insertion was identified in the coding sequences of the resistant *Ty*-1/Ty-*3* alleles, but this insertion is also present in some susceptible *ty-1* alleles (Caro et al. 2015; Verlaan et al. 2013). Moreover, expression analysis of these alleles have showed that the expression of the *Ty-1* allele was significantly higher compared to the *ty*-1 allele, and that these differences correlate with the resistant phenotype (Caro et al. 2015; Verlaan et al. 2013).

The *Ty-1* encodes an RNA-dependent RNA polymerase of the γ-class (RDR-γ), which possesses an atypical DFDGD aa motif in the catalytic domain located at the C-terminus (Verlaan et al. 2013). RDR-γ are encoded by plants and fungi; however, no clear function has been established for these proteins (Willmann et al. 2011). Early attempts to establish the function of the *Ty-1* gene showed that the accumulation of TYLCV in inoculated tissues and the long-distance movement of the virus were impaired in lines containing this gene, suggesting a role in suppression of cell-to-cell movement (Michelson et al. 1994). However, it was later demonstrated that in resistant *Ty-1* plants, but not in susceptible *ty-1* plants, small interfering RNA (siRNA) levels are increased, and that the presence of these siRNA correlates with cytosine methylation of the viral genome (Butterbach et al. 2014). This suggest that the *Ty-1* is likely involved in TGS. So far, little is known about the function of this gene and the current understanding of the *Ty-1* mediated defense response is incomplete.

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Chapter II

Transcriptomic- and VIGS-based approaches to identify determinants of resistance and susceptibility of tomato (*Solanum lycopersicum*) to tomato yellow leaf curl virus (TYLCV) and some properties of the *Ty-1* resistance gene

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Abstract

The whitefly-transmitted begomovirus tomato yellow leaf curl virus (TYLCV) is one of the most devastating tomato-infecting viruses worldwide, and breeding for resistance (e.g., with the *Ty-1* to *Ty-6* resistance genes) is an effective management strategy. The partially dominant *Ty-1* gene encodes for an RNA-dependent RNA polymerase (RdRp), which is involved in transcriptional gene silencing of the TYLCV genome. Here, we used an RNA-Seq approach to identify differentially expressed genes (DEGs) during TYLCV infection of the near isogenic lines LA3473-R and LA3474-S, which have the *Ty-1* (resistant) and the *ty-1* (susceptible) alleles, respectively. We observed differences in gene expression in the resistance and susceptible responses in terms of numbers of DEGs and stage of infection. A total of 679 genes (468 upregulated and 211 downregulated DEGs) and 58 long non-coding RNAs (lncRNAs) were differentially expressed during TYLCV infection. Gene ontology (GO) analyses of DEGs revealed similar types of genes in both resistance and susceptible responses, although GO terms involved in defense response and signaling transduction pathways were enriched in the resistant LA3473-R line during TYLCV

infection. VIGS analyses of selected DEGs further revealed that genes encoding WRKY transcription factors, nucleotide-binding site and leucine-rich repeat (NBS-LRR) containing proteins, and receptor-like protein kinases (RLKs) may play a role in the resistance and susceptible responses to TYLCV infection. In subcellular localization studies, Ty-1-EGFP localized to the nucleus and the cell periphery (e.g., cytoplasm). A prediction of the tertiary structure of the Ty-1 protein revealed similarities to this region of the RdRp of *Neurospora crassa*, suggesting a conserved role for these proteins. Finally, our results suggest that the signal transduction pathway that underlies the *Ty-1*-mediated defense response is a complex network of genes encoding proteins involved in virus detection (NBS-LRR), signaling cascades (RLK) and transcriptional activation (WRKY transcription factors).

Introduction

Tomato yellow leaf curl disease (TYLCD) can cause devastating losses to tomato (*Solanum lycopersicum*) production worldwide (Cohen et al. 2007; Rojas et al. 2018). The disease is widely distributed in the Old World (OW) and New World (NW), and is caused by several begomoviruses, including tomato yellow leaf curl virus (TYLCV) (Rojas et al. 2018; Abhary et al. 2007; Navot et al. 1991). Regardless of the virus involved, symptoms of TYLCD include severe stunting, distorted and upright growth and leaves with upcurling, crumpling, interveinal chlorosis, and yellowing (Salati et al. 2002; Cohen et al. 1994). Tomato is highly susceptible to TYLCV infection and, under high disease pressure (e.g., high whitefly populations), outbreaks can result in substantial yield losses, especially when plants are infected at early stages of development (Salati et al. 2002; Gilbertson et al. 2015; Rojas et al. 2018; Cohen et al. 1994).

Because the whitefly vector is difficult to control, an important strategy for disease management is planting of resistant tomato varieties that possess resistance loci introgressed from

wild species (Rojas et al. 2018; Vidavski et al. 2008; Cohen et al. 1994; Pico et al. 1999; Yan et al. 2018). Currently, six resistance loci (*Ty-1* to *Ty-6*) have been mapped in tomato (Verlaan et al. 2011, 2013; Yamaguchi et al. 2018; Yang et al. 2014; Lapidot et al. 2015; Anbinder et al. 2009; Ji et al. 2009; Gill et al. 2019). The *Ty-1* and *Ty-3* genes are allelic and were introgressed from *S. chilense* (Verlaan et al. 2011). The *Ty-2* gene originated from *S. habrochaites* and encodes a nucleotide-binding site-leucine-rich repeat (NBS-LRR) containing protein (Yamaguchi et al. 2018; Yang et al. 2014). The *Ty-4* gene is located on the long arm on chromosome 3, and was introgressed from *S. chilense* (Ji et al. 2009). The recessive *Ty-5* gene encodes the messenger RNA surveillance factor Pelota, and is believed to have been introgressed from *S. peruvianum* (Lapidot et al. 2015; Anbinder et al. 2009). Finally, the *Ty-6* gene is located on the long arm of chromosome 10, and was introgressed from *S. chilense* (Gill et al. 2019).

The *Ty-1* is a partially dominant resistant gene that was introgressed from the *S. chilense* line LA1969 (Zamir et al. 1994; Verlaan et al. 2013). The *Ty-1* locus is composed of three allelic variants, and *Ty-1*-like alleles are present in both resistant and susceptible tomato lines (e.g., *Ty*-1, *Ty-3* and the susceptible *ty-1*) (Caro et al. 2015; Verlaan et al. 2013). Expression analyses of these alleles revealed that the *Ty-1 gene expression was significantly higher compared to that of the ty-1* allele, and that these differences correlate with the resistant phenotype (Caro et al. 2015; Verlaan et al. 2013). The *Ty-1* gene encodes an RNA-dependent RNA polymerase (RdRp) of the γ-class, but with an atypical DFDGD amino acid (aa) motif in the catalytic domain located at the C-terminus (Verlaan et al. 2013). The γ-RdRp is found in plants and fungi, but no clear function has been established for these proteins (Willmann et al. 2011).

Investigation of the *Ty-1* function has shown that the accumulation of TYLCV in inoculated tissues and the long-distance movement of the virus were impaired in *Ty-1* lines,

suggesting a role in suppression of cell-to-cell movement (Michelson et al. 1994). However, it was later demonstrated that TYLCV-specific small interfering RNA (siRNA) levels were higher in resistant *Ty-1* plants compared with those in susceptible *ty-1* plants (Butterbach et al. 2014). Furthermore, the presence of these siRNA was correlated with cytosine methylation of the viral genome (Butterbach et al. 2014). This suggested that the *Ty-1* gene is involved in transcriptional gene silencing (TGS). So far, little is known about the functional properties of the Ty-1 protein, and the nature of the signal transduction pathway involved in the defense response.

Here, we generated cDNA libraries from samples of inoculated stems and newly emerged leaves of the near isogenic lines (NILs) LA3473-R and LA3474-S, respectively. We used an RNA-Seq approach to identify differentially expressed genes (DEGs) during the resistant and susceptible responses to TYLCV infection. VIGS analyses were then used to assess the role of selected DEGs in the resistant response to TYLCV infection. Finally, we cloned and sequenced the *Ty-1* gene and determine some properties of the Ty-1 protein to provided further insight into how some of the newly identified DEGs participate in the TYLCV-mediated defense response. These results will be discussed in terms of the mechanism of resistance and associated signal transduction pathways.

Materials and methods

Plant materials.

NILs of tomato with the *Ty-1* gene (resistant line, LA3473-R) or *ty-1* gene (susceptible line, LA3474-S) were used for these experiments (Michelson et al. 1994). Seeds of these NILs were obtained from the Tomato Genetics Resource Center, UC Davis. Tomato plants of both lines were grown in a greenhouse, allowed to be self-pollinated and fruits were harvested. Seeds were extracted and treated with 2.7% sodium hypochlorite for 30 min and rinsed with MilliQ water. Treated seeds were planted into sunshine mix 1 potting mix (Sunshine® Mix #1) in a controlled

environment chamber (light intensity, 300 μEinsteins; temperature, 25 C and relative humidity of approximately 60%).

Confirmation of TYLCV resistance in tomato lines LA3473-R and LA3474-S.

To confirm the resistant and susceptible phenotypes of plants produced from increased seeds, NILs LA3473-R and LA3474-S, respectively, seedlings at the three- to five-leaf-stage (3 wks old) were agroinoculated with cell suspensions (optical density of 600 nm = 1.0) of a strain of *Agrobacterium tumefaciens* containing a binary plasmid with the multimeric infectious clone of TYLCV from California (TYLCV-[US:CA:06]) (Rojas et al. 2007) by needle puncture inoculation of the stem just beneath the shoot apex (Hou et al. 1998). The positive control in these experiments were seedlings of the susceptible tomato plants cv. Glamour agroinoculated with TYLCV, whereas the negative control was seedlings agroinoculated with the empty vector (pCAMBIA 1300). Inoculated plants were maintained in a controlled environment chamber, and symptom development was assessed visually and recorded at 7, 14 and 21 d post infection (dpi) based on the severity scores described in Lapidot et al. (2002).

Absolute viral DNA accumulation was quantified at 7, 14 and 21 dpi by quantitative polymerase chain reaction (qPCR) tests according to the protocol described by Mason et al. (2008). Total genomic DNA was extracted from newly emerged leaves according to the method of Dellaporta et al. (1983). A virus-specific primer pair (PTYv372/PTYc523) for qPCR detection was designed to direct the amplification of an ~150 base pair (bp) fragment from the capsid protein (CP) gene of TYLCV (Supplemental Table S2.1). The specificity of this primer pair was predicted based on BLAST search (Altschul et al. 1990), and confirmed experimentally by conventional PCR with DNA extracts of tomato plants agroinoculated with TYLCV and non-inoculated plants. The PCR-amplified TYLCV fragment was cloned into pCR-Blunt II-TOPO (Zero Blunt® PCR

Cloning Kit; Invitrogen) to generate a standard for qPCR assays. Recombinant plasmids containing the cloned PCR-amplified TYLCV fragment were quantified with a NanoDrop1000 spectrophotometer (Thermo Scientific), and plasmid copy number (Cn) was adjusted to 10^7 copies/µl with the Avogadro's constant ($6.022140857 \times 10^{23}$). Standard curves for qPCR were prepared with tenfold serial dilutions ranging from 10^1 to 10^6 copies of plasmid DNA. These standard curves were used to estimate the viral Cn for each sample. The qPCR was conducted on a *QuantStudio™ 6 Flex Real-Time PCR System* (Thermo Fisher Scientific) with 100 ng of total genomic DNA in a 20-µl reaction mix with the SsoFast EvaGreen Supermix kit (Bio-Rad, Richmond, CA).

To detect the presence of the *Ty-1* or *ty-1* genes in the NILs LA3473-R and LA3474-S, respectively, restriction fragment length polymorphism (RFLP) analyses of the amplified fragments with a marker to *Ty-1*/*ty-1* alleles were performed. Here, leaf samples were collected from non-inoculated leaves of LA3473-R and LA3474-S plants and total genomic DNA was extracted as previously described. PCR tests were performed with the C2_At5g61510-F/C2_At5g61510-R primer pair (Prasanna et al. 2015), which direct the amplification of an ~1.0 kb fragment. PCR-amplified fragments were purified with the QIAquick gel extraction kit (Qiagen, Germantown, MD) and digested with the five-base-cutting enzyme *Hinf*I and the reaction analyzed by agarose gel electrophoresis.

Library preparation for Illumina sequencing.

For the RNA-sequencing (RNA-seq) experiments, a cell suspension (optical density of 600 nm = 1.0) of an *A. tumefaciens* (C58C1) strain containing the multimeric infectious clone of TYLCV-[US:CA:06] or the empty vector was used for agroinoculation of seedlings (1-2 true leaf

stage) of the NILs LA3473-R (resistant) and LA3474-S (susceptible) as previously described. A total of three plants per treatment were inoculated per experiment, and the experiment was repeated three times. Tissue samples of the resistant and susceptible lines were individually collected and pooled together in each treatment from inoculated stems (0, 12, 24 and 48 h post-inoculation [hpi]) and, from other inoculated plants, from newly emerged leaves (5, 7, 10 and 14 dpi) for a total of eight-time points (Supplemental Table S2.2). Samples were frozen immediately in liquid nitrogen. High-throughput RNA-seq library preparation was performed according to the method described in Kumar et al. (2012). Briefly, double-stranded complementary DNA (cDNA) was prepared with random hexamer priming, and the resulting cDNA was fragmented, end-repaired and A-tailed. DNA barcodes for multiplexing Illumina DNA-Seq libraries were added to the cDNA fragments during adapter ligation, and the adapter-ligated cDNA libraries were enriched with 13 cycles of PCR amplification followed by size selection of ~200-500 bp fragments. A total of 96 cDNA libraries we generated, which included the eight-time points each for the resistant and susceptible lines and for the three independent experiments. Finally, barcoded libraries for each independent experiment were pooled together and sequenced on two lanes on Illumina HiSeq 2000 platform at the UC Davis Genome Center.

RNA-seq data analyses.

Raw sequence data were filtered/trimmed for low-quality reads and technical sequences with Trimmomatic (Bolger et al. 2014). Trimmed RNA-Seq reads were aligned to the high quality, non-redundant database generated from the tomato genome (cv. Heinz 1706) by the International Tomato Annotation Group (ITAG) with Hisat2 (Kim et al. 2015). The latest ITAG database was obtained from Sol Genomics Network (SGN) (Mueller et al. 2005). FeatureCount was used to count the number of reads mapped to each gene (Liao et al. 2014). DEGs were identified with

Cuffdiff, a method that estimates the relative transcript abundance (Trapnell et al. 2012). Gene expression levels were normalized with fragments per kilobase of exon per million mapped reads (FPKM) values, and the false discovery rate (FDR) was used to determine the differentially expressed *p*-value threshold. In addition, an independent DEGs analysis was performed with DEseq and EdgeR methods (Anders et al. 2010; Robinson et al. 2009), and data were visualized with Galaxy (Batut et al. 2018). In the present study, genes were considered to be differentially expressed only when their absolute value of $\log_2$ fold change was >1.5 and *p*-value was <0.05. To identify the potential function of DEGs involved in the *Ty-1* resistance response to TYLCV infection, the functional classes of DEGs were identified with gene ontology enrichment analyses with PANTHER within the Gene Ontology (GO) project (Ashburner et al. 2000; Mi et al. 2019).

Validation of selected DEG by reverse transcription quantitative PCR (RT-qPCR).

Twelve DEGs (4 upregulated and 8 downregulated) were selected and validated with RT-qPCR. For these analyses, gene-specific primers were designed to direct the amplification of 100-250 bp fragments from the coding region of each target genes (Supplemental Table S2.1). The sequences of these 12 genes were obtained from the SGN database and used to designed primers for RT-qPCR. Primer specificity was evaluated by comparing sequences against the NCBI and SGN databases with BLASTn and confirmed experimentally in conventional PCR tests with total DNA (Mueller et al. 2005; Benson et al. 2018). A primer pair designed to amplify a 180 bp fragment of the *β-actin gene of tomato* was used as the standard internal control (Ghandi et al. 2016).

Tomato seedlings from lines LA3473-R and LA3474-S were agroinoculated with the multimeric infectious clone of TYLCV-[US:CA:06] or the empty vector as previously described. Samples of newly emerged leaves were taken from these infected tomato plants at 48 hpi, 7 dpi

and 14 dpi, and total RNA was isolated with the TRIzol reagent (Invitrogen) following manufacturer's instructions. Total RNA was then treated with RNase-free DNase I (Invitrogen) for 15 min at 37 C to removed genomic DNA contamination, and first strand cDNA synthesis was performed with 1 µg of DNase I-treated RNA and random hexamers according to the method described in Nagalakshmi et al. (2008). The RT-qPCR was conducted with 4 µl of a 1:20 dilution of the cDNA in a 20 µl reaction prepared with the SsoFast EvaGreen Supermix kit (Bio-Rad, Richmond, CA) following manufacturer's instructions. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

Validation of *Ty-1* and selected DEGs with virus-induced gene silencing (VIGS).

Silencing of the *Ty-1* gene and the 12 selected DEG was conducted with the tobacco rattle virus (TRV)-mediated VIGS system (Dong et al. 2007). For silencing *Ty-1*, a fragment of 267 bp was amplified from cDNA prepared from RNA extracted from leaf tissue of LA3473-R plants by PCR with the primer pair TRV-180F/TRV-180R (Verlaan et al. 2013). VIGS constructs targeting DEGs were designed from sequences deposited in the SGN database. Sequences of 148 to 806 bp were selected based on the VIGS tool within SGN (Mueller et al. 2005). Primers were designed from these sequences, and specificity evaluated by conventional PCR as previously described (Supplemental Table S2.1).

Fragments of the *Ty-1* gene and the 12 selected DEGs were next amplified from LA3473-R by PCR. PCR-amplified fragments were purified with the QIAquick gel extraction kit (Qiagen, Germantown, MD) and cloned into pCR-Blunt II-TOPO. Fragments were released from pCR-Blunt II-TOPO by restriction enzyme digestion and cloned into pYL156 digested with the appropriate enzyme. Recombinant plasmids were transformed into chemically competent *Escherichia coli* One Shot® TOP10 cells (Invitrogen), and transformants were selected based on

kanamycin resistance. Plasmid DNA was extracted from selected transformants with the ZR Plasmid Miniprep kit (Zymo Research Corp). Recombinant plasmids having fragments of the *Ty-1* gene and specific DEGs were identified by restriction analysis and DNA sequencing, and then transformed into electrocompetent *A. tumefaciens* cells (strain C58C1) by electroporation.

Silencing of the *Ty-1* gene and the 12 selected DEGs was accomplished by infiltration of cotyledons of LA3473-R (resistant) and LA3474-S (susceptible) at 7 d after germination according to the method described by Velásquez et al. (2009). The positive control was equivalent seedlings agroinfiltrated with the TRV vector containing a fragment of the tomato phytoene desaturase (pTRV1 + pTRV2-*tPDS*), whereas the negative control was seedlings agroinfiltrated with the TRV empty vector (pTRV1 + pTRV2) (Yule et al. 2002). Inoculated plants were placed in a controlled environment chamber (light intensity, 300 μEinsteins; temperature, 21°C and relative humidity of approximately 60%) for two wks. The plants were then agroinoculated with TYLCV as previously described. Plants were maintained in the controlled environment chamber for 30 dpi with TYLCV, and symptom development was assessed as previously described. Relative viral DNA accumulation in these VIGS-treated plants was quantified in newly emerged leaves by qPCR with virus-specific primers as described above. Additionally, the relative expression of target genes was determined by RT-qPCR with gene-specific primers that anneal outside the region targeted for silencing, as previously described.

Cloning the *Ty-1* from tomato.

The full-length coding sequence of the *Ty-1* was amplified from cDNA generated from leaf tissues of LA3473-R plants by PCR with the primer pair Ty-1pENTRF/Ty-1pENTR-NR and the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific) (Supplemental Table S1). Blunt-end PCR products were purified and cloned into the pCR-Blunt II-TOPO vector (Zero

Blunt® PCR Cloning Kit; Invitrogen) to generate pZeroBlunt-Ty-1. The full-length coding sequence of the *Ty-1* gene in the recombinant plasmid pZero-Blunt-Ty-1 was sequenced and analyzed with MEGA X (Kumar et al. 2018). Pairwise nt sequence alignments were performed with MUSCLE within the Species Demarcation Tool (SDT) v.1.2 (Muhire et al. 2014). Nucleotide identities for total sequences and nt and aa identities of individual exons were calculated in the Sequence Manipulation Suite Server (Stothard 2000). The 3D structure of the Ty-1 protein was predicted in the Phyre2 server by homology modeling under intensive mode (Kelley et al. 2015). Putative nuclear localization signals were identified by cNLS mapper (Kosugi et al. 2009), and *N*-terminal *myristoylation* motifs were predicted by Myristoylator within the Expasy Server (Bologna et al. 2004).

Sub-cellular localization experiments with the green fluorescence protein (GFP).

To generate N- and C- terminus EGFP fusion proteins, the *Ty*-1 full-length coding sequence (3057 nt) was amplified with or without the termination codon with the high fidelity CloneAmp™ HiFi PCR Premix (Takara) and the primer pairs Ty-1-pENTR-F/Ty-1-pENTR-NR and Ty-1-pENTR-F/Ty-1-pENTR-CR, respectively (Supplemental Table S2.1). PCR products were purified and cloned into pENTR/D-TOPO (Invitrogen) to generate the entry vectors pENTR-Ty-1N and pENTR-Ty-1C, respectively. The EGFP coding sequence was fused to the N- and C-terminal regions of the *Ty-1* gene by recombining the entry clone pENTR-Ty-1 into the destination vectors pSITE-2NA (N-terminus EGFP fusion) and pSITE-2CA (C-terminus EGFP fusion), respectively, according to the method described by Chakrabarty et al. (2007). The resulting pSITE:EGFP::Ty-1 and pSITE:Ty-1::EGFP binary vectors were transformed into electrocompetent *A. tumefaciens* cells (strain C58C1) as previously described. Agroinfiltration of leaves of *Nicotiana benthamiana* plants was performed as describe by Liu et al. (2002). The

control was equivalent leaves agroinfiltrated with cell suspensions of an *A. tumefaciens* strain carrying the empty vector pSITE-2NA, from which free EGFP is expressed. Plants were kept in a controlled environment chamber for 48 h and EGFP expression was observed by laser scanning confocal microscopy as described by Lee et al. (2005).

Results

Evaluation of TYLCV resistance in tomato lines LA3473-R and LA3474-S.

To confirm that the LA3473-R and LA3474-S plants from the increased seed of these lines possess the expected resistant and susceptible phenotypes to TYLCV infection, tomato seedlings derived from the increased seed were agroinoculated with TYLCV-[US:CA:06] (Supplementary Table S2). Agroinoculated seedlings of line LA3473-R developed no obvious symptoms in newly emerged leaves by 14 dpi (Supplemental Figs S2.1A and S2.1B), whereas those of line LA3474-S were stunted and newly emerged leaves had developed symptoms of upward leaf curling, crumpling, interveinal chlorosis and yellowing (Supplemental Figs S2.1C and S2.1D), similar to symptoms induced by TYLCV in the susceptible cv. Glamour (Supplemental Figs S2.1E and S2.1F). Moreover, symptoms became progressively more severe by the 21 dpi and up to 30 dpi, with no evidence of recovery (data not shown). To measure TYLCV DNA accumulation, samples of newly emerged leaves were taken from plants of LA3473-R, LA3474-S and cv. Glamour infected with TYLCV at 7, 14 and 21 dpi. In these experiments, TYLCD symptoms were evident at 14 dpi in the susceptible LA3474-S and cv. Glamour plants, whereas not symptoms were observed in plants of the resistant LA3473-R.

Virus Cn was determined by qPCR (Supplementary Table S2.2). At all time points, leaves of the susceptible line LA3474-S and cv. Glamour accumulated substantially more virus DNA than those of line LA3473-R, although some DNA accumulation was detected in the resistant

plants (Supplemental Fig. S2.2). Although, TYLCV accumulation was detected in leaves at 7 dpi, levels increased significantly by 14 and 21 dpi, with the higher overall Cn values for leaves of the susceptible cv. Glamour (Supplemental Fig. S2.2). Equivalent tomato plants agroinoculated with the empty vector did not develop symptoms and qPCR test of these plants were negative.

The presence of the *Ty-1* locus in the NIL LA3473-R was determined with a molecular marker linked to the gene (Prasanna et al. 2015; Pérez De Castro et al. 2007). The expected ~1.0 kb and ~0.3 kb fragments were generated from *Hinf*I-digested PCR products amplified from genomic DNA of LA3473-R plants with the C2_At5g61510-F/C2_At5g61510-R primer pair, whereas ~0.7 and ~0.5 kb fragments were generated from the PCR products amplified from total genomic DNA of LA3474-S or cv. Glamour plants (data not shown). Together, these results confirmed that the LA3473-R plants from the increased seed are resistant to TYLCV, and the resistance is associated with the *Ty-1* gene.

RNA-seq data analyses.

In order to investigate the transcriptional changes in tomato stems and leaves during TYLCV infection, we performed RNA-Seq experiments with inoculated stems (0 to 48 hpi) and systemically infected leaves (5 to 14 dpi) in the NILs LA3473-R and LA3474-S. Overall, ~1140 million read pairs were obtained for 96 libraries, with an average of ~12 million read pairs per library (Supplementary Table S2.3). The reads from these libraries were trimmed and then aligned to the tomato genome in the ITAG database.

Time course comparisons of DEGs in the NILs revealed that the largest transcriptional changes in LA3473-R occurred at early stages of infection in inoculated stem tissues. A total of 797 (363 upregulated and 434 downregulated) and 660 (201 upregulated and 459 downregulated) genes were differentially expressed at 12 and 24 hpi, respectively, and dropped to 42 DEGs (37

upregulated and 5 downregulated) at 48 hpi (Fig. 2.1A). In the systemically infected leaves, there were substantially fewer DEGs, with 0, 44 (34 upregulated and 10 downregulated) and 10 (0 upregulated and 10 downregulated) genes differentially expresses at 7, 10 and 14 dpi, respectively (Fig. 2.1A).

Most of the transcriptional changes observed in the LA3474-S plants occurred from 12 to 48 hpi in inoculated stem tissues and at 7 dpi systemically infected leaves. A total of 264 (209 upregulated and 55 downregulated), 540 (169 upregulated and 371 downregulated) and 355 (157 upregulated and 198 downregulated) genes were differentially expressed at 12, 24 and 48 hpi, respectively (Fig. 2.1A). In systemic infected leaves, 356 (216 upregulated and 140 downregulated) genes were differentially expressed at 7 dpi, whereas 99 (33 upregulated and 66 downregulated) and 2 (2 upregulated and 0 downregulated) genes were differentially expressed at 10 and 14 dpi, respectively (Fig. 2.1A). Interestingly, a similar number of DEGs were induced in both resistant and susceptible responses, with 1553 and 1616 DEG in the resistant and susceptible lines, respectively. However, the percentage of upregulated genes in the susceptible LA3474-S line (48% upregulated and 52% downregulated) was higher, whereas more genes were downregulated in the resistant LA3473-R line (59% upregulated and 41 downregulated).

In order to gain insight into the mechanism or signal transduction pathway involved in resistance to TYLCV infection, we analyzed the transcriptome of resistant vs susceptible tomato plants at the eight time points (4 in inoculated stems and 4 in systemically infected leaves) during the TYLCV infection response following agroinoculation. DEGs in the resistant vs susceptible response to TYLCV infection were identified with three different statistical algorithms, and the analyses revealed 10751 genes differentially expressed by Cuffdiff, 5051 by Deseq, and 1462 by EdgeR, respectively (Fig. 2.1B). Moreover, a total of 679 (468 upregulated and 211

downregulated) genes and 58 lncRNAs were identified as differentially expressed in LA3473-R during TYLCV infection by all three methods (Fig. 2.1C). A large number of genes were differentially expressed at 24 hpi (52 upregulated and 21 downregulated), but the largest transcriptional changes (325 upregulated and 156 downregulated) occurred at 7 dpi in systemically infected leaves, a time when TYLCV is accumulating in emerging leaves (Fig. 2.1C). In contrast, a total of 50 (21 upregulated and 10 downregulated) and 17 (10 upregulated and 7 downregulated) genes were differentially expressed at 10 and 14 dpi, respectively (Fig. 2.1C).

To further study the TYLCV-mediated defense and susceptible responses in tomato during early (local) and late (systemic) infections, DEGs in protein families involved in stress responses in plants were selected for further analyses (Table 2.2). Of the DEG identified by all three methods, 12 (4 upregulated and 8 downregulated) genes were associated with protein families involved in tolerance to abiotic stress response and plant-pathogen interactions, such as WRKY transcription factors, nucleotide binding site-leucine rich repeats (NBS-LRR) proteins, receptor-like protein kinases (RLKs), leucine-rich repeat receptor kinases (LRR-RKs) and chloroplast proteins. In comparisons between resistant and susceptible responses to TYLCV infection, the mannose-1-phosphate guanyltransferase (Solyc06g051270.3) was upregulated 3.1-fold at 48 hpi, the heavy metal transport/detoxification protein (Solyc06g036310.3) upregulated 5.4-fold at 10 dpi and the WRKY transcription 46 (Solyc08g067340.3) upregulated 5.8-fold at 14 dpi (Table 2.2). Comparisons between susceptible and the control revealed that Ycf68 chromosomal protein (Solyc09g011150.1) was upregulated 4.1-fold at 14 dpi; whereas the WRKY transcription factors 45 (Solyc08g067360.3), 46 (Solyc08g067340.3) and 55 (Solyc04g072070.3), two NBS-LRR genes (Solyc04g009150.1 and Solyc12g097000.2), two LRR-RKs genes (Solyc01g005730.3 and Solyc01g005760.3) and one RLK gene (Solyc02g081040.3) were downregulated 1.6 to 6.7-fold

at 14 dpi (Table 2.2 and Fig. 2.1D). Additionally, two lncRNAs were downregulated 2.5 to 4.0-folds at 14 dpi in the susceptible response to TYLCV infection (Table 2.2 and Fig. 2.1D).

For a better understanding of the transcriptional responses to TYLCV infection in LA3473-R plants and to reveal putative functions of DEGs, GO enrichment analyses were performed with PANTHER and the tomato reference genome sequence. In these analyses, >80% of the DEGs, respectively, were annotated. Within the biological process class, the majority of DEGs belong to the category of cellular processes, metabolic processes and biological regulation (Figs. 2.1E and 2.1F). The percentage of DEGs involved in cellular processes and response to stimuli were higher for upregulated genes, whereas the percentage of DEGs involved in biological processes, localization, signaling, multicellular organismal and developmental processes were higher for downregulated genes. Similar relatively low percentages of DEGs were associated with metabolic and rhythmic processes and interspecies interaction. Additionally, a few upregulated genes were associated with immune system processes. In the molecular function class, the majority of DEGs were associated with binding and catalytic activities and these include up and down regulated genes. Those involved in structural molecule activity were upregulated, whereas those associated with molecular function regulator and transporter activities were downregulated (Figs. 2.1E and 2.1F). Finally, DEGs involved in molecular adaptor and molecular transducer activities were specifically upregulated. Together, these results suggest that the tomato defense response to TYLCV infection involved up and downregulated genes with similar molecular functions and biological processes.

In order to validate the expression levels of these 12 selected DEGs were determined with RT-qPCR (Fig. 2.2). Nine DEG were upregulated in line LA3473-R in response to TYLCV infection (Fig. 2.2). The most upregulated genes were Solyc04g072070.3 (WRKY transcription

factor 55) (Fig. 2.2E), Solyc01g005760.3 (LRR-RLK) (Fig. 2H) and Solyc08g067340.3 (WRKY transcription factor 46) (Fig. 2.2I), with 20 to 112-fold changes at 14 dpi. In contrast, Solyc09g011150.1 (Ycf68 protein) (Fig. 2.2J) and Solyc04g009150.1 (NBS-LRR) (Fig. 2K) were not differentially expressed between LA3473-R and LA3474-S, and Solyc02g081040.3 (RLK) was downregulated in LA3473-R compared with line LA3474-S (Fig. 2.2L).

Validation of *Ty-1* and selected DEGs with virus-induced gene silencing (VIGS).

In preliminary experiments, tomato plants were silenced for the PDS gene with the TRV-based VIGS vector. Resistant LA3473-R tomato plants agroinfiltrated with mixtures of cell suspensions of *A. tumefaciens* strains containing binary plasmids with the pTRV1 + pTRV2-*tPDS* vector developed photobleaching at 14 dpi (Supplemental Fig. S2.3A). Similar results were obtained for susceptible LA3474-S tomato plants, although the extent of silencing was more pronounced in these plants compared with LA3473-R plants (Supplemental Fig. S2.3B). These results confirmed that the TRV-based VIGS vector infects tomato plants from these NILs, and that it induces an efficient systemic gene silencing response.

To confirm that the *Ty-1* locus confers resistance to TYLCV, resistant LA3473-R tomato plants were silenced for the *Ty-1* gene with the TRV-based VIGS vector containing a 267 bp fragment of the gene. Fourteen d after the TRV vector agroinfiltration, plants were agroinoculated with TYLCV as previously described. By 30 dpi, newly emerged leaves of *Ty-1*-silenced plants developed obvious TYLCV symptoms of yellowing and leaf curling (Supplemental Fig. S3C). These symptoms were milder and more irregular in distribution compared with those induced by TYCLV in susceptible plants (compare Supplementary Figs. S2.1C and S2.1D with S2.3C). As expected, non-silenced LA3473-R plants agroinoculated with TYLCV did not develop symptoms by 30 dpi (Supplemental Fig. S2.3D). Furthermore, RT-qPCR analyses confirmed that the

expression level of the *Ty-1* gene was reduced ~0.4-folds compared with non-silenced plants (Supplemental Fig. S2.3E), whereas virus DNA accumulation was ~16-folds greater than in TYLCV-infected LA3473-R plants (Supplemental Fig. S2.3F).

To investigate the role of 12 selected DEGs in TYLCV resistance, VIGS experiments on individual genes were conducted as described for the silencing of the *Ty-1* gene, and symptom development and relative gene expression and virus DNA accumulation was measured at 30 dpi by RT-qPCR and qPCR, respectively. Results of RT-qPCR analyses revealed that all tested plants (three plants per experiment) were silenced for Solyc06g051270.3, Solyc06g036310.3, Solyc08g067340.3, Solyc09g011150.1, Solyc04g009150.1 and Solyc02g081040.3; however, few plants were silenced for Solyc12g097000.2, LOC101248064 and Solyc04g072070.3, and none were silenced for Solyc01g005730.3, LOC101264688 and Solyc01g005760.3 (Fig. 2.3). In qPCR tests for TYLCV accumulation, no significant differences in virus DNA accumulation were observed for Solyc06g051270.3, Solyc06g036310.3, Solyc12g097000.2, LOC101248064, Solyc04g072070.3, Solyc01g005730.3, LOC101264688, Solyc01g005760.3 and Solyc09g011150.1 (Fig. 2.4A-2.4I), whereas an increased in ~2.7-3.7-fold in virus DNA accumulation was observed in plants silenced for Solyc08g067340.3, Solyc09g011150.1, Solyc04g009150.1 and Solyc02g081040.3 compared with the non-silenced control (Fig. 2.4J-2.4L), although none of these plants developed obvious TYLCV symptoms (data not shown). Taken together, these results suggested that Solyc08g067340.3, Solyc04g009150.1 and Solyc02g081040.3 may play a role in the resistant and susceptible responses to TYLCV in tomato, but do not necessarily ruled out a role for the other genes.

Properties of the *Ty-1* gene.

To provided further insight into the role of the *Ty-1* and how some of the newly identified genes involved in TYLCV resistance may be involved in the defense response, the *Ty-1* coding sequence was cloned from cDNA generated from LA3473-R and sequenced. The full-length coding sequence of the *Ty-1* gene is 3057 nt, and is composed of 19 exons ranging in size from 71 to 331 bp. Pairwise sequence comparisons performed with SDT revealed highest identities (99.1 to 99.7% nt and 98.9 to 99.7% aa) with those of the *Ty-1*/*Ty-3* alleles of *S. chilense* lines LA1938, LA1969, LA1971, LA1932 and LA2779; with slightly lower identities (98.2% nt and 97.7% aa) with the *ty-1* allele of susceptible cv. Glamour (Table 2.1). Exons 1 and 7 were the more variable, whereas the portion of the coding sequence that is in between exons 10 and 19 was the most conserved region of the gene (Table 2.1). Here, it is worth nothing that both *Ty-1*/*Ty-3* (resistant) and *ty-1* (susceptible) alleles encoded proteins with identical DFDGD motifs in the catalytic domain.

To investigate the function(s) of the Ty-1 protein, the aa sequence of the *Ty-1* gene was screened for cellular targeting domains. The analyses did not reveal an *N*-terminal *myristoylated motifs*. *However, the analyses did reveal* a non-canonical bipartite NLSs with cut-off scores ranging from 2 to 5.8, which predicts a protein localization to both the nucleus and cytoplasm (Kosugi et al. 2009). To test the hypothesis that the *Ty-1* targets the nucleus and the cytoplasm (but not the cell periphery based on no myristoylation domains), we determined the subcellular localization of the Ty-1 protein using EGFP tagged fusion proteins transiently expressed in *N. benthamiana* leaves. To this end, we generated transient-expression vectors expressing the Ty-1 fusion proteins having EGFP at the N- and C-terminus. In control experiments with the pSITE-2NA empty vector, EGFP fluorescence was detected in the nucleus and cytoplasm, but not in the

nucleolus (Fig. 2.5A). Similarly, the Ty-1-EGFP also localized to the nucleus and cytoplasm (Fig. 2.5B), which is consistent with the NLS analyses. Similar results were obtained for EGFP-Ty-1 (data not shown). In these experiments, the Ty-1-EGFP signal was weak and limited to few cells (<1 cell per microscope field) in the infiltrated patches compared with EGFP alone (all cells per microscope field).

The three-dimensional (3D) structure of the Ty-1 protein was predicted based on the high levels of aa homology with the RdRp of *Neurospora crassa* with the Phyre2 server. The predicted 3D structure of the Ty-1 protein was similar to the catalytic core of typical RdRp, and contains the slab, head, neck and catalytic domains (Fig. 2.5C). Moreover, the catalytic domain of the Ty-1 protein contains the three proposed aspartic acid residues (D723, D725 and D727) that interacts with Mg^{2+} ions (Fig. 2.5D).

Discussion

TYLCV is one of the most widely distributed and economically important tomato-infecting viruses worldwide (Cohen et al. 2007; Rojas et al. 2018). Moreover, domesticated tomato is highly susceptible to TYLCV infection, although resistance has been identified in wild tomato species (Ji et al. 2009; Vidavski et al. 2008; Pereira-Carvalho et al. 2010; Tomás et al. 2011; Caro et al. 2015; Gilbertson et al. 2011; Kenyon et al. 2014; Lapidot et al. 2015, 2014; Yan et al. 2018). The *Ty-1* locus represents one of the major genetic resistance sources in tomato to TYLCV, and amino acid sequence analysis of the *Ty-1* gene has suggested it plays a role in TGS (Verlaan et al. 2013; Caro et al. 2015; Butterbach et al. 2014). However, little is known about the signal transduction pathway that underlies the *Ty-1* mediated-resistance and susceptibility responses of tomato plants infection to TYLCV.

Here, we expanded the current understanding of the *Ty-1*-mediated defense response by comparing the transcriptional changes occurring in the resistant and susceptible responses to TYLCV infection, and by determining some properties of the Ty-1 protein. In order to investigate the *Ty-1*-mediated defense response in tomato, the resistance response of LA3473-R and LA3474-S plants to infection by a TYLCV isolate from an outbreak in California was first evaluated. Our results showed that these NILs exhibited the expected resistant and susceptible phenotypes to TYLCV infection. This was supported by several lines of evidence. First, LA3473-R plants agroinoculated with TYLCV-[US:CA:06] developed no symptoms, whereas LA3474-S plants developed typical symptoms of TYLCV infection. This, is consistent with previous reports (Barbieri et al. 2010; Pérez de Castro et al. 2013; Lapidot et al. 1997; Fargette et al. 1996), and that this isolate of TYLCV is virulent in the susceptible LA3474-S but not in LA3473-R. Second, virus DNA accumulation was substantially higher in LA3474-S compared with LA3473-R. Interestingly, virus DNA accumulation was detected in newly emerged leaves of LA3473-R plants agroinoculated with TYLCV, indicating that these plants are not immune to the virus. These results are consistent with previous observations showing that tomatoes containing the *Ty-1* gene are not immune to TYLCV infection as the virus can efficiently replicates and moves systemically (Verlaan et al. 2013; Scott et al. 1991; Czosnek et al. 1993; Michelson et al. 1994; Pico et al. 1999; Rojas et al. 2018). Third, LA3473-R plants silenced for the *Ty-1* gene developed TYLCV symptoms, albeit in a more uneven distribution, perhaps reflecting the distribution of the TRV vector or silencing signal (e.g., siRNA). Together, these results confirmed that resistance to TYLCV-[US:CA:06] in LA3473-R is associated with the *Ty-1* gene.

Next, we investigate the transcriptional changes in tomato during TYLCV infection by conducting RNA-Seq experiments with local infection in agroinoculated stems (0 to 48 hpi), and

systemic infected newly emerged leaves (5 to 14 dpi) in the NILs LA3473-R and LA3474-S. We showed that the transcriptional responses in the local and systemically infected tissue were different in terms of numbers of DEGs and stage of infection. Similar transcriptional differences between local and systemic infection have been observed in comparisons of differential gene expression between local and systemic infection of *N. benthamiana* agroinoculated with TYLCV (Wu et al. 2019). The majority of the transcriptional changes in both resistant and susceptible lines occurred in agroinoculated stems during the first 12 to 24 hpi, which suggests that the defense response to TYLCV is triggered relatively soon after infection, probably as the virus replicates and accumulates in cells of these tissues. However, it is important to note that some of these DEGs were probably induced in response to *A. tumefaciens* interactions with cells and to the wounding. RNA-Seq analyses of the resistance versus susceptible response to TYLCV infection revealed 679 DEGs in the resistant LA3473-R, with 69% of the total number DEGs upregulated during local (59%) and systemic infection (10%) to TYLCV. Similarly, a high percentage of genes (58%) were upregulated in comparisons between resistant versus susceptible responses to TYLCV infection of systemically infected tissue of tomato plants carrying the *Ty-2* following inoculation with viruliferous whiteflies (Chen et al. 2013). This suggest that transcriptional upregulation of DEGs (e.g., defense response genes) is associated with the successful establishment of the early (inoculated stems) and later (systemically infected leaves) defense responses to TYLCV infection. Indeed, our gene ontology analyses of DEGs in the resistance versus susceptible response to virus infection revealed that upregulated genes were enriched in processes and functions involved defense response signaling transduction pathways such as response to stimulus, immune system processes and structural molecule and molecular transducer activities. Similar ontologies have

been reported during the host resistance response to TYLCV and tomato yellow leaf curl China virus and its betasatellite (Luo et al. 2019; Chen et al. 2013).

Analyses of DEGs in LA3473-R tomato plants having the TYLCV resistance response, revealed that genes encoding for proteins associated with carbohydrate metabolism and tolerance to abiotic stress response, as well as WRKY transcription factors, were upregulated. In contrast, these genes were not upregulated in the susceptible LA3474-S, whereas genes encoding for WRKY transcription factors, nucleotide binding site-leucine rich repeats (NBS-LRR) proteins, receptor-like protein kinases (RLKs), leucine-rich repeat receptor kinases (LRR-RKs) were downregulated in susceptible LA3474-S tomato plants in response to TYLCV infection. Most of these genes were not upregulated in the resistant LA3473-R plants infected with TYLCV. One exception is the WRKY transcription factor 46, which was upregulated in LA3473-R as well as downregulated in LA3474-S, suggesting it plays an important role in both the resistance and susceptible responses to TYLCV infection. Indeed, comparisons of DEGs in response to TYLCV and tomato chlorosis virus infection revealed that WRKY transcription factor 46 is upregulated in response to viral infection (Seo et al. 2018). The WRKY gene families are well known to be associated with defense response to plant viruses (Gururani et al. 2012; Ishihama et al. 2012; Marino et al. 2012; Puranik et al. 2012; Seo et al. 2004; Baker et al. 1994; Whitham et al. 1994; Dinesh-Kumar et al. 2000; Takahashi et al. 2002; Liu et al. 2004; Marathe et al. 2004). Interestingly, a total of 58 lncRNAs were also downregulated in LA3474-S compared with non-inoculated plants. A large number of lncRNAs have been identified as important regulators of plant stress responses (Khraiwesh et al. 2012; Wang et al. 2017; Nejat et al. 2018), including TYLCV infection (Wang et al. 2015).

RT-qPCR analyses validated that 3 DEGs were upregulated in LA3473-R (Solyc06g051270.3, Solyc06g036310.3 and Solyc08g067340.3), whereas 5 DEGs (Solyc12g097000.2, Solyc04g072070.3, Solyc01g005730.3, Solyc01g005760.3 and Solyc08g067340.3) and 2 lncRNA (LOC101248064 and LOC101264688) were downregulated in LA3474-S, suggesting that changes in expression of these DEGs in the NILs are associated with the establishment of the resistant and susceptible response to TYLCV in tomato. To investigate the putative role of these DEGs in the TYLCV defense response, we attempted to silence these genes in LA-3473R plants with the TRV VIGS system and then challenge with TYLCV at 14 d post silencing, with virus DNA accumulation measured at 30 dpi. As expected, and in agreement with previous studies (Chen et al. 2013; Seo et al. 2018), qPCR tests showed that virus DNA accumulation was ~2.7-3.7-folds higher in plants silenced for the Solyc08g067340.3 (WRKY transcription factor 45), Solyc04g009150.1 (NBS-LRR) and Solyc02g081040.3 (RLK) than in the non-silenced LA-3473R control, although despite the increased titer no TYLCV symptoms developed in the silenced plants. WRKY transcription factors, NBS-LRRs and RLKs are three of the largest families of signaling response regulators in plants and have been shown to participate in many plant processes including virus-mediated resistance (e.g., pathogen associated molecular patter-triggered immunity [PTI] and effector-triggered immunity [ETI]) (Pandey et al. 2009; Chen et al. 2019; Li et al. 2016, 2017; de Ronde et al. 2014; Yeam 2016; Gururani et al. 2012; Ishihama et al. 2012; Marino et al. 2012; Puranik et al. 2012; Seo et al. 2004; Baker et al. 1994; Whitham et al. 1994; Dinesh-Kumar et al. 2000; Takahashi et al. 2002; Liu et al. 2004; Marathe et al. 2004).

We were unable to establish a role in defense for the other 9 selected DEGs. In qPCR tests, TYLCV DNA accumulation in plants silenced for Solyc06g051270.3 (mannose-1-phosphate guanyltransferase), Solyc06g036310.3 (heavy metal transport/detoxification protein) and

Solyc09g011150.1 (Ycf68 protein), virus DNA accumulation did not change significantly, suggesting that these genes were not differentially expressed in response to TYLCV infection. However, their role in defense can not be completely rule out as plant stress responses are well known to be redundant (e.g., duplicate gene functions) (Zhang et al. 2019; Li et al. 2018; Anderson et al. 2011). In the case of 4 other selected DEGs and 2 lncRNAs, we were unable to achieve silencing. In VIGS experiments with Solyc12g097000.2 (TIR-NBS-LRR), Solyc04g072070.3 (WRKY transcription factor 55) and LOC101248064 (lncRNA), few plants were silenced, whereas none were silenced for Solyc01g005730.3 and Solyc01g005760.3 (LRR-RLKs) and for LOC101264688 (lncRNA). It is possible that our failure to silence these genes can be attributed to several reasons. First, the TRV VIGS system provides only transient silencing, which led to a reduction in transcript accumulation, but not a complete knock down of the target gene (Padmanabhan et al. 2009; Velásquez et al. 2009). Second, the asynchronous nature of viral infection, in which mixtures of cells are generally at different phases of infection, could yield a mosaic of silenced and non-silenced phenotype (i.e., dilution of the overall silencing effect) (Liu et al. 2008). Indeed, silencing of the *Ty-1* or the PDS control results in non-uniform patchy phenotypes in newly emerged leaves (Fig.3). This effect may be even more dramatic for cells of the phloem, which are exclusively infected by TYLCV (Rojas et al. 2001). Finally, several studies have shown that different gene fragments can yield different silencing efficiencies (Luo et al. 2004; Liu et al. 2008; Padmanabhan et al. 2009).

The *Ty-1* gene is partially dominant and confers resistance against monopartite and some bipartite begomoviruses, including TYLCV and tomato severe rugose viruses (Voorburg et al. 2020; Butterbach et al. 2014; Verlaan et al. 2013). The *Ty-1* gene encodes for an RdRp (Butterbach et al. 2014; Verlaan et al. 2013), which is consistent with the predicted structure of the protein

being similar to that of the RdRp of *Neurospora crassa* (Salgado et al. 2006). Sequence analyses of the complete coding sequence of the *Ty-1* encoded by the LA3473-R genome revealed highest identities (99.1 to 99.7% nt and 98.9 to 99.7% aa) with *Ty-1*/*Ty-3* alleles from *S*. *chilense* lines, and slightly lower identities (98.2% nt and 97.7% aa) with the *ty-1* allele of *S*. *lycopersicum* (cv. Glamour). This is consistent with previous studies showing very low levels of genetic variation between these alleles (Caro et al. 2015; Verlaan et al. 2013), and suggests that some function(s) of these genes are conserved in both resistant and susceptible tomato plants. We identified a non-canonical bipartite NLSs in the *Ty-1* gene, and did not find a N-terminal myristoylated motif, suggesting the protein can localizes to the nucleus and is nor membrane targeted. Further insight came from localization of Ty-1-GFP to the nucleus and cytoplasm, but not the cell periphery. This was consistent with the targeting associated with these motifs and a role in TGS as well as yet to be identified cellular processes. These results further suggest that the *Ty-1* gene, as well as other plant RdRps, may participate in processes in addition to TGS in the cell (Hoffer et al. 2011; Willmann et al. 2011; Luo et al. 2007; Salgado et al. 2006).

In conclusion, we used an RNA-Seq approach to identify differentially expressed transcripts during the resistant and susceptible responses to TYLCV infection. Analyses of DEGs showed that the transcriptional changes in response to TYLCV infection are different in LA3473-R and LA3474-S tomato plants, and that specific changes in the resistance response were associated with GO terms involved in defense and signaling transduction pathways. In VIGS analyses of DEGs, genes encoding for WRKY transcription factors, NBS-LRR and RLKs negatively impacted viral accumulation as revealed by VIGS assays. Our results suggest that the signal transduction pathway that underlies the *Ty-1* mediated resistance and susceptibility responses to TYLCV is a complex network including genes involved in virus detection (NBS-

LRR), signaling cascades (RLK) and transcriptional activation (WRKY transcription factors) proteins. Finally, sequence and subcellular localization analyses of the Ty-1 revealed properties that are consistent with different roles in the cell, including TGS.

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Table 2.1. Nucleotide (nt) identities for total sequences and nt and amino acid (aa) identities of individual exons of the full-length coding sequence of the *Ty-1* gene and those of the *Ty-1* alleles of other species of tomato

			Total		Exon																	
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Line [a]	Species	Allele	nt	aa	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
LA1938	*S. chilense*	*Ty-1*	99.7	99.7	99.7	99.1	100	99.7	99.7	99.0	99.9	100	100	100	100	100	100	100	99.3	100	100	100	99.1
LA1969	*S. chilense*	*Ty-1*	99.6	99.7	99.4	99.1	100	100	100	99.0	98.9	100	100	100	100	100	100	99.6	99.3	100	99.3	100	99.6
LA1971	*S. chilense*	*Ty-1*	99.2	99.1	98.2	98.2	100	100	100	98.6	97.7	100	100	100	100	100	100	99.6	100	100	99.3	99.4	97.8
LA1932	*S. chilense*	*Ty-1*	99.1	98.9	98.5	99.1	98.6	100	98.7	98.6	97.7	100	98.7	97.2	100	100	100	99.6	99.3	99.4	98.6	100	98.7
LA2779	*S. chilense*	*Ty-3*	99.1	98.8	98.8	99.1	100	100	98.7	98.6	97.7	100	94.7	97.2	100	100	100	99.6	100	98.8	98.6	100	98.7
Moneymaker	*S. lycopersicum*	*ty-1*	98.2	97.7	95.5	98.6	99.3	97.3	98.0	98.6	95.5	100	94.7	98.6	100	97.3	99.6	98.7	99.3	99.4	99.3	97.6	98.7

[a] The full-length coding sequences of the *Ty-1* alleles of *Solanum lycopersicum* cv. Moneymaker, and those of *S. chilense* LA1969 and LA2779 were obtained from Verlaan et al. (2013); and those of *S. chilense* LA1932, LA1938 and LA1971 were obtained from Caro et al. (2015).

Table 2.2. A list of putative defense related genes from the analyses of differentially expresses genes in response of tomato yellow leaf curl virus infection.

Gene	Annotation[a]	Ontology	Time point	Fold change
Resistance vs susceptible				
Solyc06g051270.3	Mannose-1-phosphate guanyltransferase	Carbohydrate metabolism	48 hpi	5.5
Solyc06g036310.3	Heavy metal transport/detoxification protein	Tolerance to abiotic stresses	7 dpi	3.1
Solyc08g067340.3	WRKY transcription factor 46	Stress response	14 dpi	5.8
Susceptible vs control				
Solyc09g011150.1	Ycf68 protein	Unknow	14 dpi	4.0
Solyc08g067360.3	WRKY transcription factor 45	Stress response	14 dpi	-4.8
Solyc08g067340.3	WRKY transcription factor 46	Stress response	14 dpi	-5.2
Solyc04g072070.3	WRKY transcription factor 55	Stress response	14 dpi	-4.3
Solyc04g009150.1	NBS-LRR	Stress response	14 dpi	-6.7
Solyc12g097000.2	TIR-NBS-LRR	Stress response	14 dpi	-1.3
Solyc02g081040.3	RLK	Stress response	14 dpi	-1.4
Solyc01g005760.3	LRR-RLK	Stress response	14 dpi	-1.5
Solyc01g005730.3	LRR-RLK	Stress response	14 dpi	-1.7
TCONS.00059979.XLOC.046416	lncRNA	LOC101264688	14 dpi	-4.0
TCONS.00016405.XLOC.012494	lncRNA	LOC101248064	14 dpi	-2.5

[a] Annotations are based in the latest International Tomato Annotation Group database in Sol genomic network. Abbreviations: NBS-LRR = nucleotide-binding site and leucine-rich repeats; TIR-NBS-LRR = toll interleukin1 receptor-nucleotide binding site-leucine-rich repeats; RLK = receptor-like protein kinase; LRR-RLK = leucine-rich repeats receptor-like protein kinase and lncRNA = long non-coding RNA.

Supplemental Table S2.1. Sequences of the oligonucleotide primers used in this study.

Primer	Nucleotide sequence	Reference
TYLCV accumulation		
PTYv372	5′-ACTTCGACAGCCCATACAG-3′	This study
PTYc523	5′-TGGGCCTTCACATCCAC-3′	This study
Ty-1 markers		
C2_At5g61510-F	5′-ATTCCTACTGCGCCGCTGCTTC-3′	Prasanna et al. 2015
C2_At5g61510-R	5′-AGCATGAACAAGTACTGTGTGCCCACG-3′	Prasanna et al. 2015
ApsF	5′-GGCAGGAGAATATGCCAAAA-3′	Pérez De Castro et al. 2007
ApsR	5′-CGTTCCATTCTCAACCCATT-3′	Pérez De Castro et al. 2007
DEG qPCR		
Q-Ty-R/SF	5′-AATTGATGTTCCTTCTTGTATAC-3′	This study
Q-Ty-R/SR	5′-TTGAGCATTTCTAATGACCC-3′	This study
Q-1270F	5′-TGTTCTCAAGAAACTGAACCAC-3′	This study
Q-1270R	5′-CACCATTAAAGAAGCCTCCC-3′	This study
Q-6310F	5′-CAACAACCTTATCCACAGTACC-3′	This study
Q-6310R	5′-AGTCCACTAACTCATCACAAATG-3′	This study
Q-7340F	5′-ACAAATGGCGTCTTCGTTGT-3′	This study
Q-7340R	5′-ACAATTTAATCCTCTCAACAAACAGT-3′	This study
Q-7000F	5′-TGCTTCTTCTTCCAAAGTCTG-3′	This study
Q-7000R	5′-TGATTGTCCTGCCTCCAACC-3′	This study
Q-1150F	5′-GCTCTCAGCCACATGGATAG-3′	This study
Q-1150R	5′-TAGAACTCGAGAGAACACCA-3′	This study
Q-in8064F	5′-ACGAGAGAACAAAGAACAGACC-3′	This study
Q-in8064R	5′-TTGCCAACACCCAAACACC-3′	This study
Q-9150F	5′-TTCCCTACACACGCTTGGAT-3′	This study
Q-9150R	5′-CCACCACAAGGTATTTGCGT-3′	This study
Q-2070F	5′-CTACAAGTGTTCAAGTGGAG-3′	This study
Q-2070R	5′-TTCCAGTAAATGATAAAGGGAC-3′	This study
Q-5730F	5′-CGTGGAGTGGGAAATTGGTC-3′	This study
Q-5730R	5′-CTGCACTTCATAAGCTGGACA-3′	This study
Q-in4688F	5′-ACTATACACCTCTCACATTTCC-3′	This study
Q-in4688R	5′-TCTCATTTTTATTCTCAGCTGC-3′	This study
Q-5760F	5′-TCACAATCATCTCGTTGGATGC-3′	This study
Q-5760R	5′-CTTGATCATCACGGCCACAA-3′	This study
Q-1040F	5′-CTGCTACAGAGGCATTGTTTTCAAC-3′	This study
Q-1040R	5′-CAAACTGCTAAAAGGGCAACCAC-3′	This study
T-β-actinF	5′-GGAAAAGCTTGCCTATGTGG-3′	Ghandi et al. 2016
T-β-actinR	5′-CCTGCAGCTTCCATACCAAT-3′	Ghandi et al. 2016

VIGS cloning		
TRV2-180F	5′-ATGAAGACAAAAACTGCTTCTTTG-3′	This study
TRV2-180R	5′-ACCATTTTCATCCCGACAAA-3′	This study
TRV2-1270F	5′-CTCGAGACAAGCTGGTAGATGATTC-3′	This study
TRV2-1270R	5′-GAGCTCTCACATCACTATTTCTGG-3′	This study
TRV2-6310F	5′-ATGATGAAAGTAGTGCTGAAGTTGGAG-3′	This study
TRV2-6310R	5′-CTAGCAGATCACACAAGAGTTAGAGTC-3′	This study
TRV2-7340F	5′-ACAACATCAATAGAAGTCCGATACCGCGTG-3′	This study
TRV2-7340R	5′-GATGTCTTTCATGAATTTGTTCAAAAATGTTGGATCTG-3′	This study
TRV2-7000F	ATCTATCTAGACTTTCAAAGACGATGAACG	This study
TRV2-7000R	GATGAGAGCTCCATCTTTGAACCTTCTCC	This study
TRV2-1150F	ATCTATCTAGATGATAGGCACCAGGGCGG	This study
TRV2-1150R	GATGAGAGCTCGGCCACGGGTCATAACGC	This study
TRV2-in8064F	ATCTATCTAGATATGTAGTAAAGAATGGAG	This study
TRV2-in8064R	GATGAGAGCTCTAACAGATGGAGATTTAAAAAAAG	This study
TRV2-9150F	ATCTATCTAGACTCTTTGGAACTCAGCATTG	This study
TRV2-9150R	GATGAGAGCTCACTTCATATGAGATATATG	This study
TRV2-2070F	ATCTATCTAGACAACACAAACCACATATATC	This study
TRV2-2070R	GATGAGAGCTCGTTTGTGTAATTGGAATAA	This study
TRV2-5730F	ATCTATCTAGAACTTGTTTACGCGTCTTC	This study
TRV2-5730R	GATGAGAGCTCAAATTCAACGTACGAAGTC	This study
TRV2-in4688F	ATCTATCTAGAGGGAATTTATGGATGTGTTAAC	This study
TRV2-in4688R	GATGAGAGCTCGAAGACAAGATACAAACATG	This study
TRV2-5760F	ATCTAGGATCCAGTGAACTTCAAGTTTTG	This study
TRV2-5760R	GATGAGAGCTCGACTCGAATTTTAGCAAAC	This study
TRV2-1040F	ATCTATCTAGAATGATTTTTTTTCAAGAAAATAG	This study
TRV2-1040R	GATGAGGATCCATCAAACTGATGTCCCATG	This study
Ty-1 cloning		
Ty-1-pENTR-F	5′-CACCATGGGTGATCCGTTGATTGAAGAAATTG-3′	This study
Ty-1-pENTR-NR	5′-CTAGAGTATTTCCTGCAAAACCGATGGC-3′	This study
Ty-1-pENTR-CR	5′-GAGTATTTCCTGCAAAACCGATGGC-3′	This study

Supplemental Table S2.2. Disease rate and viral copy number (Cn) of resistant and susceptible tomato lines infected with tomato yellow leaf curl virus (TYLCV) at 7 to 21 days post-infection (dpi).

Line	Time point	Infectivity [a]	Symptoms [b]	Disease rate [c]	Ct value [d]	Cn
LA-3473-R	7 dpi	9/9 (100)	NS-i	0	21.2	6
	14 dpi	9/9 (100)	Y	1	17.6	32
	21 dpi	9/9 (100)	Y	1	18.3	22
LA-3474-S	7 dpi	9/9 (100)	Y	1	15.1	267
	14 dpi	9/9 (100)	D, S, U, Y	3	12.9	1025
	21 dpi	9/9 (100)	D, F, S, U, Y	4	13.2	819
cv. Glamour	7 dpi	9/9 (100)	Y	1	13.6	650
	14 dpi	9/9 (100)	D, S, U, Y	3	12.3	1524
	21 dpi	9/9 (100)	D, F, S, U, Y	4	12.5	1405

[a] Infectivity (number of infected plants/number inoculated) was determined at 7 to 21 dpi based on symptom development and detection of TYLCV genomic DNA by qPCR with specific primers. Percentage of infection is indicated in parentheses; data represents a total of three independent experiments.

[b] Symptom abbreviations: D = distorter growth; F = flower abortion; NS-i = no symptoms infected; S = stunting; U = upward leaf curling and Y = yellowing.

[c] Disease severity ratings were as follows: 0 = no symptoms; 1 = very slight yellowing of leaflet margins on apical leaf (very mild symptom); 2 = some yellowing and minor curling of leaves (mild symptom); 3 = stunting and leaves with distorted growth and yellowing and upward leaf curling (severe symptom); and 4 = severe stunting and flower abortion, distorted growth and leaves with yellowing and upward leaf curling (very severe symptom).

[d] TYLCV DNA was detected in all symptomatic and non-symptomatic tomato plants at 7 dpi. Ct = cycle threshold.

Supplementary Table S2.3. Number of reads used for RNA-Seq analyses [a]

Line	Treatment [b]	Number of raw reads [c]			
		Experiment 1	Experiment 2	Experiment 3	Total
LA-3473-R	TYLCV-0 hpi	14080252	12782852	11366003	38229107
	TYLCV-12 hpi	12646492	11772152	12147601	36566245
	TYLCV-24 hpi	10788503	9652588	10645930	31087021
	TYLCV-48 hpi	13456716	10547000	14398155	38401871
	TYLCV-5 dpi	14934676	10036689	9413118	34384483
	TYLCV-7 dpi	10839242	12619450	9855122	33313814
	TYLCV-10 dpi	10992769	13032018	9963254	33988041
	TYLCV-14 dpi	12101800	9239902	11399973	32741675
	Control-0 hpi	13260323	12352884	11639114	37252321
	Control-12 hpi	9947124	12021596	13010559	34979279
	Control-24 hpi	10850270	10840957	13063016	34754243
	Control-48 hpi	10514907	11339650	13305135	35159692
	Control-5 dpi	12225088	10938717	11903089	35066894
	Control-7 dpi	10903826	12202798	9268431	32375055
	Control-10 dpi	11603086	12905860	12302754	36811700
	Control-14 dpi	10750768	10736435	9547412	31034615
LA-3474-S	TYLCV-0 hpi	12010707	10874571	13617197	36502475
	TYLCV-12 hpi	8913342	10480115	13240398	32633855
	TYLCV-24 hpi	12112034	24765678	15118741	51996453
	TYLCV-48 hpi	11718915	10976430	14101644	36796989
	TYLCV-5 dpi	9213040	12227539	8340289	29780868
	TYLCV-7 dpi	8888479	12035103	10810151	31733733
	TYLCV-10 dpi	11874224	26585682	9581968	48041874
	TYLCV-14 dpi	10256351	9530146	9334475	29120972
	Control-0 hpi	14918843	12801018	3410	27723271
	Control-12 hpi	10387572	10958049	26843175	48188796
	Control-24 hpi	11065879	11976508	13029843	36072230
	Control-48 hpi	11462008	13433894	12087705	36983607
	Control-5 dpi	14049742	13116750	8076247	35242739
	Control-7 dpi	10924840	9710224	10262586	30897650
	Control-10 dpi	13076822	12236964	10677951	35991737
	Control-14 dpi	11303524	15720782	9485375	36509681
	Total	372072164	400451001	367839821	1140362986

[a] A total of 96 libraries were prepared from the near isogenic lines (NILs) LA3473-R (resistant) and LA3474-S (susceptible).

[b] *Agrobacterium tumefaciens* (C58C1) containing a multimeric clone of the TYLCV-[US:CA:06] or the empty vector control (pCAMBIA 1300) was used for agroinoculation of the NILs LA3473-R (resistant) and LA3474-S (susceptible) seedlings at the 1-2 true-leaf stage (25 days post germination). Tissue samples of the resistant and susceptible lines were collected from inoculated stems (0, 12, 24 and 48 hours post inoculation [hpi]) and newly emerged leaves (5, 7, 10 and 14 days post inoculation [dpi]) for a total of eight time-points.

[c] Each library pool was run on 2 lanes of Illumina HiSeq 2000 at the Genome Center, UC Davis.

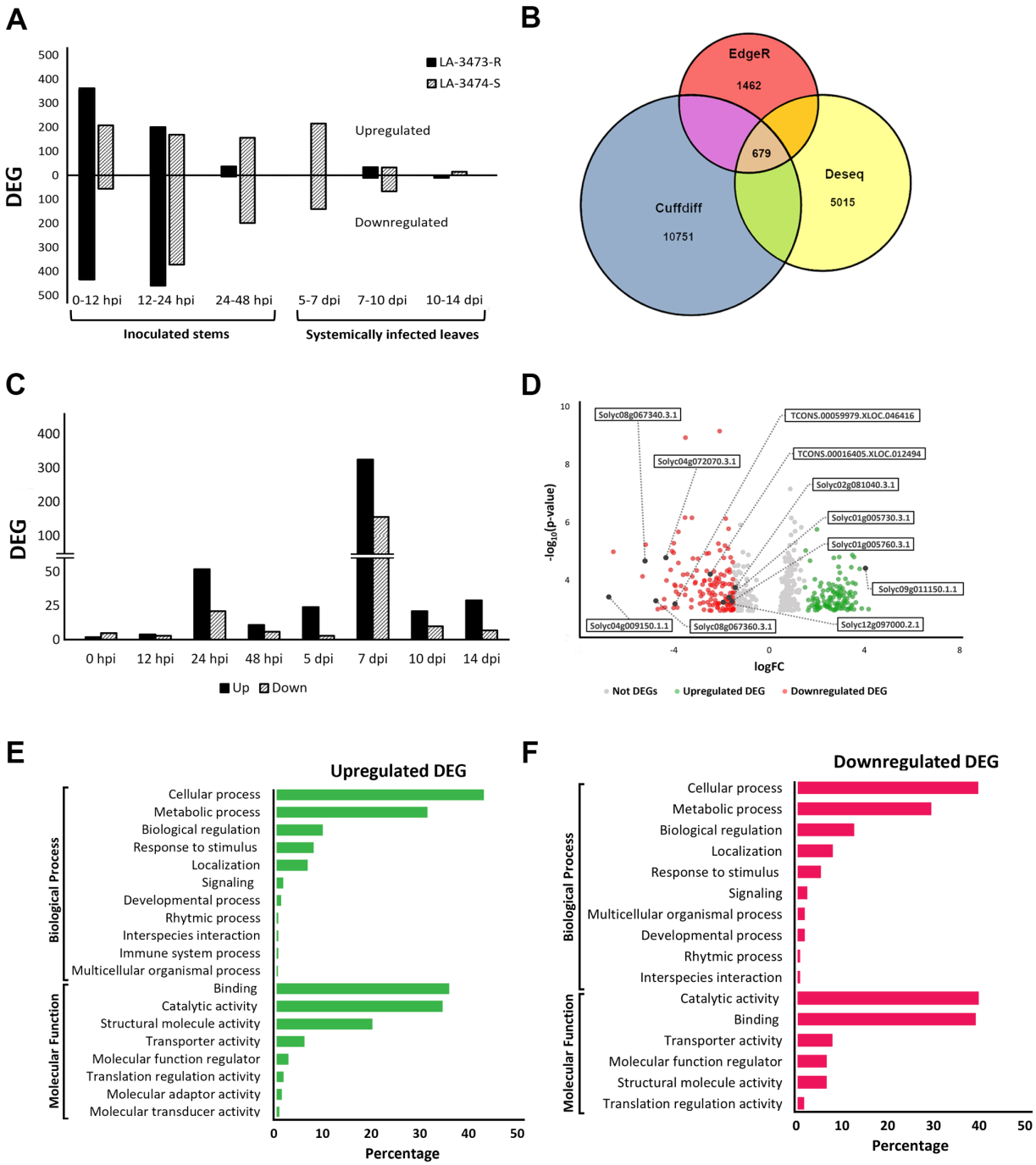


Fig. 2.1. Differentially expressed genes (DEGs) in the near isogenic lines (NILs) LA3473-R (resistant) and LA3474-S (susceptible) infected with the multimeric infectious clone of TYLCV from California (TYLCV-[US:CA:06]) from 0 hours post infection (hpi) to 14 days post infection (dpi). (A) Time course comparisons of DEGs during local infection of stems (0 to 48 hpi) or

systemic infection of leaves (5 to 14 dpi) in the NILs LA3473-R and LA3474-S agroinoculated with TYLCV. (B) Venn diagram showing the number of DEGs identified in comparisons between the resistance vs susceptible responses to TYLCV infection with the Cuffdiff, DEseq and EdgeR methods. (C) Time course comparisons of DEGs and showing the number of upregulated and downregulated genes during the resistance vs susceptible responses to TYLCV infection. (D) A volcano plot showing statistically significant DEGs in LA3474-S tomato plants infected with TYLCV at 14 dpi compared with noninfected control plants. (E) Gene ontology (GO) enrichment analyses of upregulated DEGs identified in comparisons between the resistance vs susceptible responses to TYLCV infection. (F) Gene ontology (GO) enrichment analyses of downregulated DEGs identified in comparisons between the resistance vs susceptible responses to TYLCV infection.

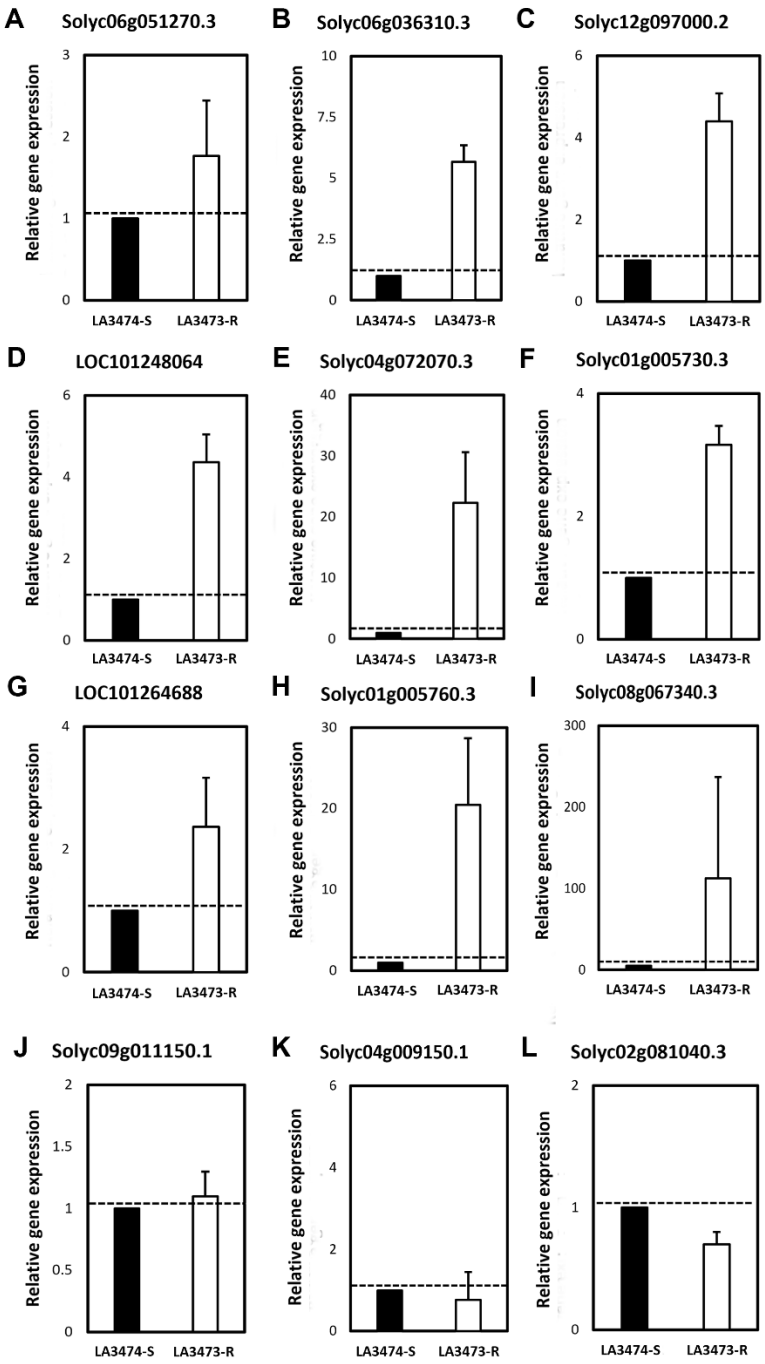


Fig. 2.2. Validation of differentially expressed genes (DEGs) during resistance and susceptible responses to TYLCV infection by reverse transcription quantitative PCR (RT-qPCR). Changes in expression levels of 12 selected DEGs were measured by RT-qPCR and the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001). The 12 selected DEGs were annotated as (A) mannose-1-phosphate guanyltransferase; (B) heavy metal transport/detoxification protein; (C) toll interleukin 1 receptor-nucleotide binding

site-leucine-rich repeat (TIR-NBS-LRR) class disease resistance protein; (D) long non-coding RNA (lncRNA); (E) WRKY transcription factor 55; (F) leucine-rich repeat receptor-like protein kinase (LRR-RLK); (G) lncRNA; (H) LRR-RLK; (I) WRKY transcription factor 46; (J) Ycf68 protein; (K) NBS-LRR class disease resistance protein and (L) receptor-like protein kinase. Relative gene expression was expressed as fold changes of transcript levels in the resistant LA3473-R line compared with those in the susceptible LA3474-S line. Relative gene expression was normalized based on the expression levels of the β-actin gene of tomato (*Solanum lycopersicum*). Error bars represent the standard deviation of three biological replicas.

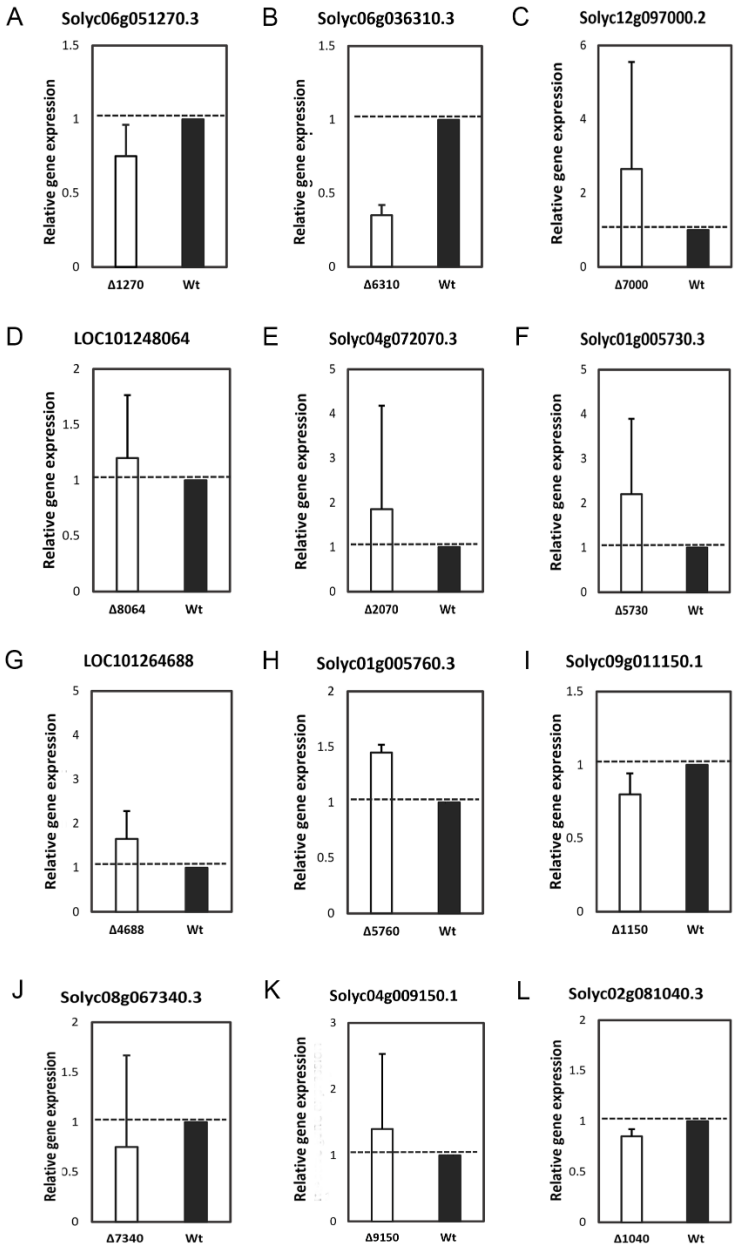


Fig. 2.3. Relative gene expression of 12 selected differentially expressed genes (DEGs) in virus-induced gene silencing (VIGS)-treated resistant tomato plants from line LA3473-R inoculated with TYLCV at 30 after agroinoculation. The 12 selected DEGs were annotated as (A) mannose-1-phosphate guanyltransferase; (B) heavy metal transport/detoxification protein; (C) toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) class disease resistance protein; (D) long non-coding RNA (lncRNA); (E) WRKY transcription factor 55; (F) leucine-rich

repeat receptor-like protein kinase (LRR-RLK); (G) lncRNA; (H) LRR-RLK; (I) WRKY transcription factor 46; (J) Ycf68 protein; (K) NBS-LRR class disease resistance protein and (L) receptor-like protein kinase. Changes in expression levels of 12 selected DEGs were measured by RT-qPCR and the $2^{-\Delta\Delta CT}$ method. Relative gene expression was expressed as fold changes of transcript levels in VIGS-treated plants compared with those in the non-silenced control. Relative gene expression was normalized based on the expression levels of the β-actin gene of tomato (*Solanum lycopersicum*). Error bars represent the standard deviation of three independent experiments.

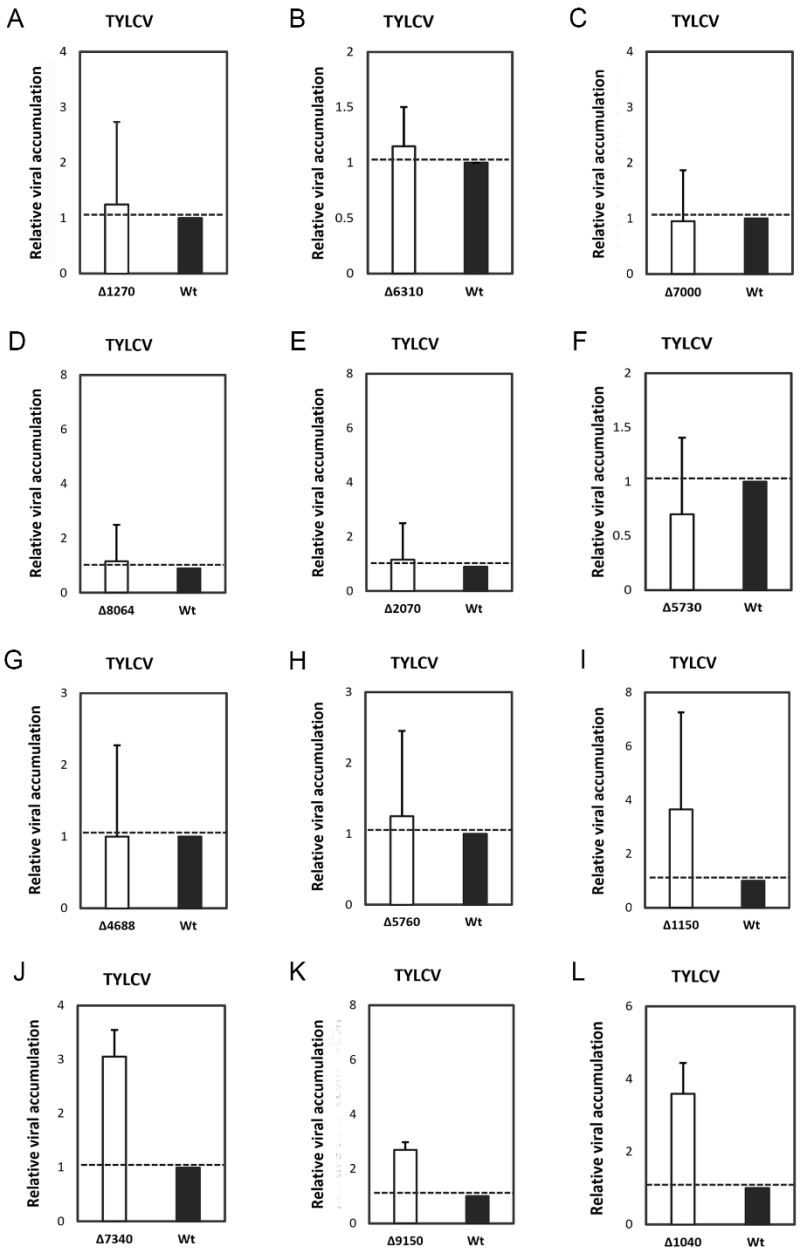


Fig. 2.4. Virus DNA accumulation in virus-induced gene silencing (VIGS)-treated resistant tomato plants from line LA3473-R inoculated with TYLCV at 30 after agroinoculation. The 12 selected DEGs were annotated as (A) mannose-1-phosphate guanyltransferase; (B) heavy metal transport/detoxification protein; (C) toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) class disease resistance protein; (D) long non-coding RNA (lncRNA); (E) WRKY transcription factor 55; (F) leucine-rich repeat receptor-like protein kinase (LRR-RLK);

(G) lncRNA; (H) LRR-RLK; (I) WRKY transcription factor 46; (J) Ycf68 protein; (K) NBS-LRR class disease resistance protein and (L) receptor-like protein kinase. Changes in expression levels of 12 selected DEGs were measured by RT-qPCR and the $2^{-\Delta\Delta CT}$ method. Relative gene expression was expressed as fold changes of transcript levels in the resistant LA3473-R line compared with those in the susceptible LA3474-S line. Relative gene expression was expressed as fold changes of transcript levels in VIGS-treated plants compared with those in the non-silenced control. Relative gene expression was normalized based on the expression levels of the β-actin gene of tomato (*Solanum lycopersicum*). Error bars represent the standard deviation of three independent experiments.

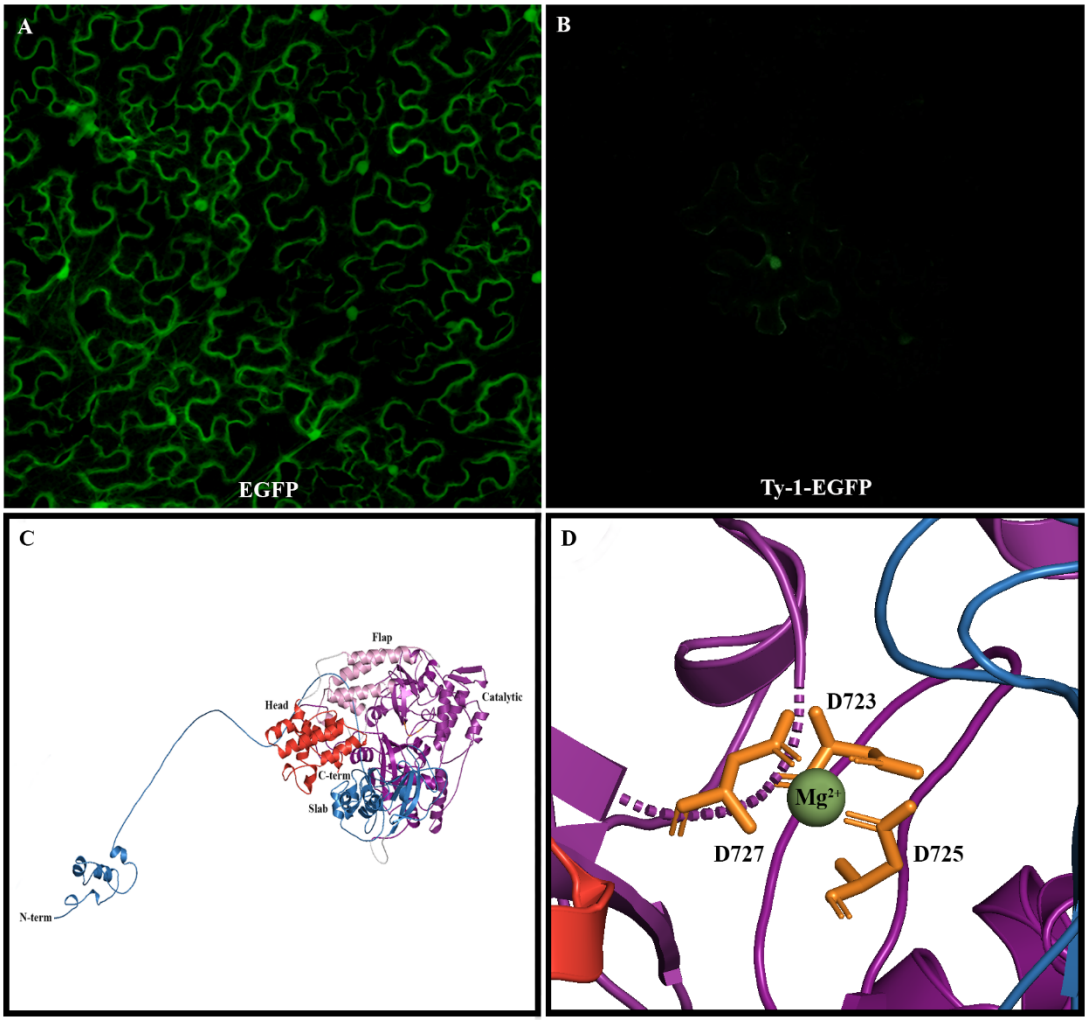
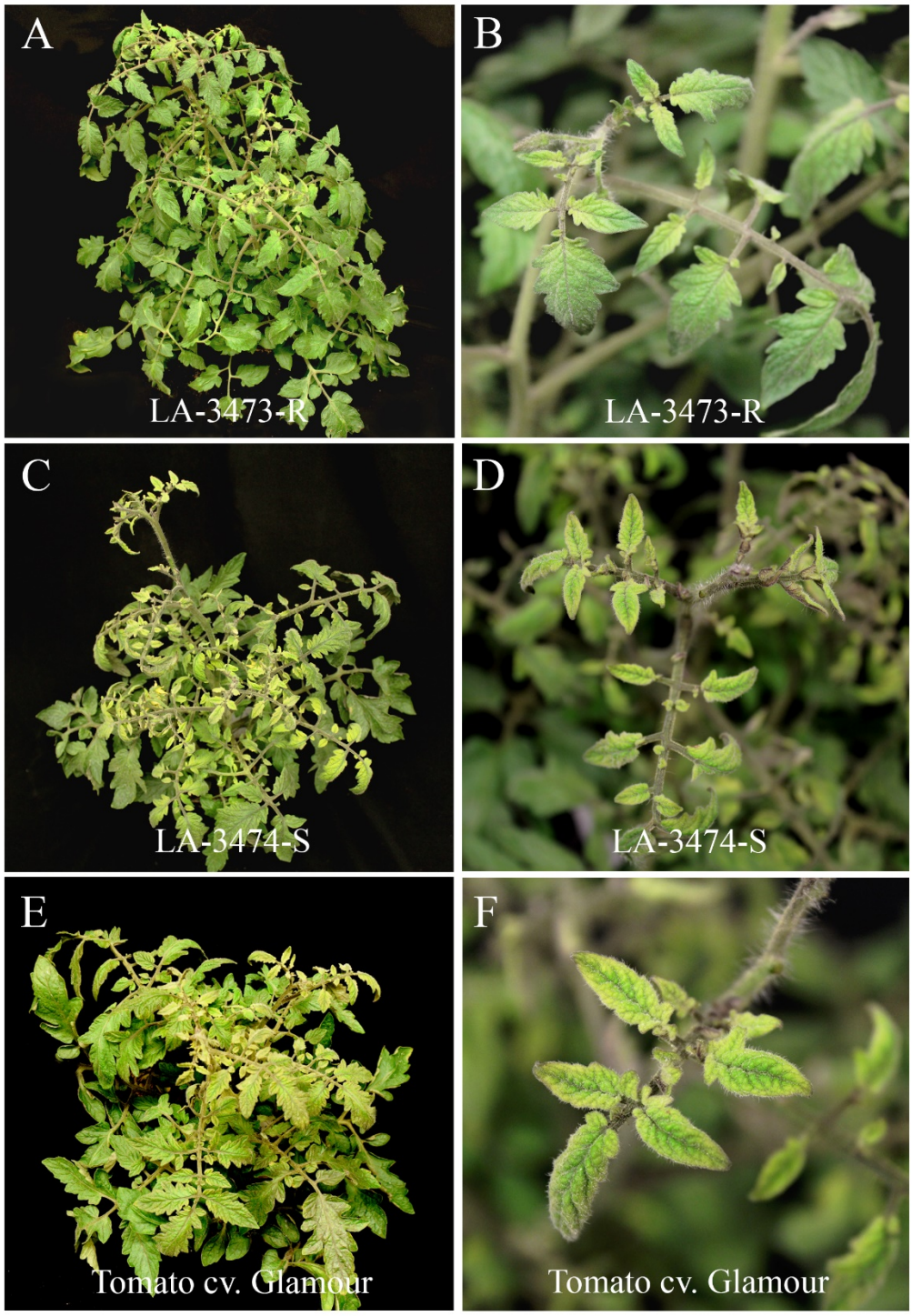
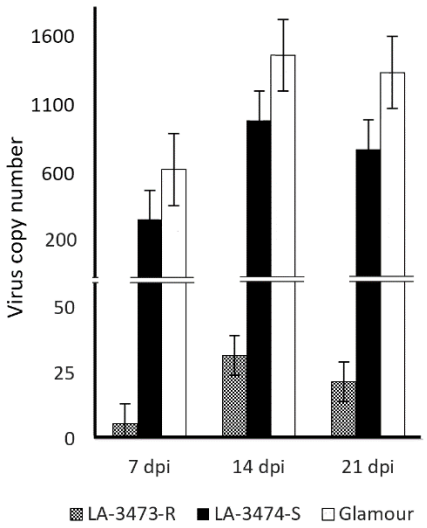


Fig. 2.5. Subcellular localization and predicted structure of the Ty-1 protein. Confocal microscopy analyses showing the subcellular localization of: (A) the green fluorescent protein (EGFP) and (B) the Ty-1-EGFP fusion protein in agroinfiltrated leaves of *Nicotiana benthamiana* plants. (C) Predicted structure of the Ty-1 protein colored according to domains: slab, blue; catalytic, purple; flap, light purple and head, orange. (D) Zoom of the active region of the Ty-1 protein containing three proposed aspartic acid residues (D723, D725 and D727) that interacts with Mg^{2+} ions.

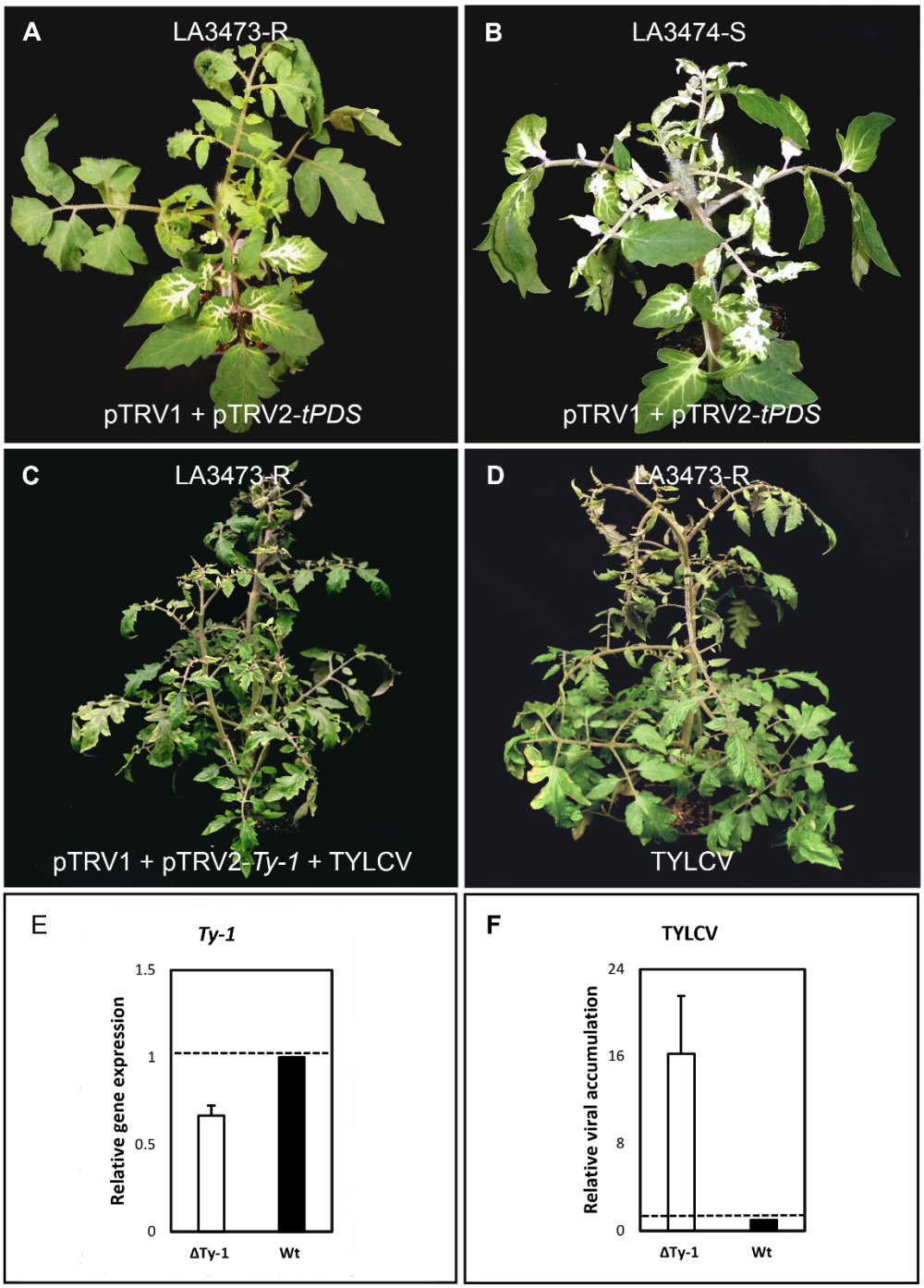


Supplemental Fig. S2.1. Disease symptoms induced following agroinoculation with the infectious multimeric clone of an isolate of tomato yellow leaf curl virus from California (TYLCV-[US:CA:06]) in (A) a resistant tomato plant of LA3473-R, (B) a susceptible tomato plant of

LA3474-S, and (C) a susceptible tomato plant cv. Glamour. Plants were photograph on 21 days after agroinoculation.



Supplemental Fig. S2.2. Time course analyses of absolute viral DNA accumulation in the near isogenic lines LA3473-R (resistant) and LA3474-S (susceptible) and in the susceptible tomato cv. Glamour inoculated with the multimeric infectious clone of an isolate of tomato yellow leaf curl virus from California (TYLCV-[US:CA:06]). Total DNA was extracted from newly emerged leaves at 7, 14 and 21 days post inoculation and used in qPCR assays with specific primers. Error bars represent the standard deviation of three independent experiments.



Supplemental Fig. S2.3. Validation of VIGS assays in the near isogenic lines LA3473-R (resistant) and LA3474-S (susceptible). A-B, photobleaching symptoms induced by the pTRV1 + pTRV2-*tPDS* vector in a resistant tomato plant LA3473-R and a susceptible tomato plant LA3474-S, respectively. Plants were photographed 14 days after agroinfiltration of cotyledonary leaves at

7 days after germination. C-D, disease symptoms induced by tomato yellow leaf curl virus (TYLCV) in a resistant tomato plant LA3473-R inoculated with the pTRV1 + pTRV2-*Ty-1* vector; and those induced by the pTRV1 + pTRV2 vector in a resistant tomato plant LA3473-R, respectively. Plants were photographed 30 days after agroinoculation. E-F, expression levels of the *Ty-1* and virus DNA accumulation, respectively, was measured with qPCR in VIGS-treated resistant tomato plants LA3473-R inoculated with TYLCV at 30 after agroinoculation. Relative gene expression was expressed as fold changes of transcript levels in LA3473-R plants compared with those in the non-silenced control. Relative gene expression was normalized based in the expression levels of the β-actin gene of tomato (*Solanum lycopersicum*). Error bars represent the standard deviation of three biological replicas.

Chapter III

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

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Abstract

Tomato production in Costa Rica (CR) is affected by diseases caused by whitefly-transmitted begomoviruses. Since the late 1980s, the main virus involved has been tomato yellow mottle virus (ToYMoV), a New World (NW) bipartite begomovirus associated with stunting and yellow mosaic/mottle of leaves. In addition, the exotic NW bipartite begomovirus tomato leaf curl Sinaloa virus (ToLCSiV) and the Old World (OW) monopartite tomato yellow leaf curl virus (TYLCV) were introduced in 1998 and in 2012, respectively. In the present study, we examined the invasion biology of these viruses, i.e., their interactions with each other and the susceptible tomato host. We first generated full-length cloned DNA-A and DNA-B components of ToYMoV and ToLCSiV isolates from samples collected in CR in 1990 and 2002, respectively. These clones induced yellow mosaic/mottle symptoms in tomato, and this fulfilled Koch's postulates for ToYMoV and ToLCSiV and these diseases. Pseudorecombination experiments revealed no capacity to form infectious reassortants between these NW bipartite begomoviruses. The interaction among these NW and OW begomoviruses was next investigated by agroinoculating tomato plants with the

infectious clones of ToYMoV, ToLCSiV and TYLCV alone or in all combinations. Tomato plants infected with combinations of two or all three viruses developed more severe symptoms than plants inoculated with each virus alone. Symptoms of ToLCSiV and ToYMoV appeared earlier (~7 d post-inoculation [dpi]) than those of TYLCV (~10 dpi). However, by 14 dpi and beyond, TYLCV symptoms had become dominant in all mixed infections. Virus DNA accumulation in single and mixed infections were then determined by qPCR. Although symptoms of TYCLV became predominant in mixed infections with the NW bipartite begomoviruses, levels of ToLCSiV and ToYMoV were similar to those in single infections. Furthermore, ToYMoV and ToLCSiV DNA levels were negatively impacted early in infection (7 dpi). However, later in infection (14 and 21 dpi), this negative effect was no longer observed and was replaced by a neutral synergistic interaction. These results are discussed in terms of begomovirus invasion biology and implications for begomovirus disease development and management in CR.

Introduction

Begomoviruses (genus *Begomovirus*) are small plant viruses with a circular, single-stranded (ss) DNA genome encapsidated in twin quasi-icosahedral virions (18 x 30 nm) (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Zerbini et al. 2017). Members of this genus are transmitted in nature by whiteflies of the *Bemisia tabaci* cryptic species complex (DeBarro et al. 2011; Gilbertson et al. 2015; Zerbini et al. 2017). 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; Gilbertson et al. 2015; Navas-Castillo et al. 2011). The DNA-A and DNA-B components share no sequence identity, except for an ~200 nucleotide (nt) noncoding conserved sequence known as the common region. Within this sequence are the *cis*-acting elements involved in

replication and gene expression (i.e., the origin of replication [*ori*] and two bidirectional RNA polymerase II promoters) (Hanley-Bowdoin et al. 2013). These sequences are also critical for maintaining the bipartite genome.

Worldwide begomoviruses show a phylogeographic distribution, with most bipartite ones (~85%) occurring in the New World (NW), and most monopartite ones occurring in the Old World (OW), and often in association with satellites DNAs that are either required for disease development (e.g., betasatellites) or have no obvious effect on virulence (e.g., alphasatellites) (Zhou 2013; Briddon et al. 2010; Yang et al. 2019). However, there are notable exception to this distribution, including the identification of indigenous NW monopartite begomoviruses infecting tomato in Peru, Ecuador and Northern Brazil (Melgarejo et al. 2013; Macedo et al. 2017; Márquez-Martín et al. 2011). The long distance spread and emergence of new begomoviruses has been mediated by the whitefly *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; Rojas et al. 2018). Furthermore, before the global spread of the whitefly supervector, begomovirus diseases were widely distributed in noncultivated plants (e.g., weeds) in the NW and OW, with these viruses presumably spread by indigenous species of whiteflies (Gilbertson et al. 2015; Navas-Castillo et al. 2011). However, after the global spread of the polyphagous *B. tabaci* MEAM1 in the 1990s, indigenous begomoviruses were introduced into new cultivated and non-cultivated plant species (Gilbertson et al. 2015; Navas-Castillo et al. 2011). This has resulted in the emergence of similar diseases of crops and some weeds (e.g., *Sida* spp.) in different geographical regions. For example, the NW bipartite begomoviruses bean golden mosaic virus (BGMV) and bean golden yellow mosaic virus (BGYMV) are different species that have independently evolved to cause bean

golden mosaic disease of common bean in South America and *North* and *Central America and the Caribbean Basin, respectively* (Garrido-Ramirez et al. 2000; Gilbertson et al. 1993).

Human activities have led to the long-distance intercontinental movement of numerous begomoviruses, which has further blurred the geographic separation of OW and NW begomoviruses, and accelerated the worldwide spread of NW bipartite economically important begomovirus diseases, e.g., introduction of NW bipartite squash leaf curl virus (SLCuV) into the Middle East from the NW, and appearance of tomato leaf curl New Delhi virus (ToLCNDV) in the Western Mediterranean Basin from the subcontinent of Asia (India) (Varma et al. 2011; Rojas et al. 2018; Ruiz et al. 2015; Gilbertson et al. 2015). However, the most well documented and economically important example is the worldwide dissemination of the invasive OW monopartite begomovirus tomato yellow leaf curl virus (TYLCV) (Mabvakure et al. 2016; Lefeuvre et al. 2010; Salati et al. 2002; Nakhla et al. 1994). TYLCV was first introduced into the Dominican Republic during the early 1990s (Mabvakure et al. 2016; Lefeuvre et al. 2010; Salati et al. 2002; Nakhla et al. 1994; Gilbertson et al. 2007), and it has now invaded the Southern US, Mexico (MX) and the rest of the world, following the spread of the whitefly supervector (Mabvakure et al. 2016; Lefeuvre et al. 2010). Unfortunately, the tomato crop is highly permissive host for begomovirus infection, with ~90 tomato-infecting begomovirus species recognized by the International Committee on Taxonomy on Viruses (ICTV) (Gilbertson et al. 2015; Zerbini et al. 2017; Rojas et al. 2018).

Tomato is one of the most consumed vegetables in the world (Dorais et al. 2008). In Central America, tomato has become one of the most important crops in terms of area of cultivation and production (Polston et al. 1997; Rojas et al. 2018; Schreinemachers et al. 2018; Passam et al. 2007; Blanca et al. 2012). In Costa Rica (CR), tomato is one of the most important vegetable crops, and

is locally produced and sold as a fresh market crop often by small holder farmers. However, since the late 1980s, tomato production in CR has been impacted by different begomoviruses representing three examples of emergence and invasion: (i) indigenous locally evolved, (ii) invasive from the region and (iii) exotic from a different continent. The indigenous tomato-infecting begomovirus in CR is tomato yellow mottle virus (ToYMoV), previously referred to as tomato geminivirus-Costa Rica (TGV-CR). ToYMoV is a NW bipartite begomovirus associated with stunting and yellow mosaic/mottle of leaves (Nakhla et al. 1994; Polston et al. 1997; Karkashian et al. 1998; Barboza et al. 2018; Hilje et al. 2008). Growers first observed the tomato yellow mottle disease (ToYMoD) in the late 1980s (Nahkla et al. 1994; D. P. Maxwell, personal communication), and ToYMoV was the predominant tomato-infecting begomovirus in CR until the introduction of the NW bipartite begomovirus tomato leaf curl Sinaloa virus (ToLCSiV) in the late 1990s and the OW monopartite TYLCV in 2012 (Idris et al. 1999; Karkashian et al. 1998; Barboza et al. 2014, 2018). Therefore, we consider ToYMoV to represent an indigenous, locally evolved begomovirus, ToLCSiV as an introduced virus from the region and TYLCV as an introduced virus from outside the region.

Here, we utilized the tomato begomovirus situation in CR to examine their invasion biology, i.e., their interactions in terms of disease development, viral accumulation and viral genetics. To do this, we first completed the molecular and biological characterization of ToYMoV using full-length infectious DNA-A and DNA-B clones to fulfill fulfilling Koch's postulates for the ToYMoD and to show that the virus primarily infected solanaceous species. Phylogenetic and sequence analyses indicated that ToYMoV may comprise a distinct lineage that is closely related to the squash leaf curl virus (SLCuV) lineage of NW begomoviruses. This is consistent with the long period of local evolution in the region. Full-length infectious clones of an isolate of ToLCSiV

from CR were generated and used to assess genetic interaction (pseudorecombination) with ToYMoV. The infectious clones of ToYMoV, ToLCSiV and TYLCV were then used to inoculate tomato plants with each virus clone and all combinations, and symptoms induced and viral DNA accumulation were determined. These findings are discussed in terms of the nature of the invasion biology of these viruses and to predict the future impact of these begomoviruses on tomato product in CR.

Materials and methods

Virus source, 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 CR (Supplemental Fig. S3.1). Samples GR1 and GR2 were collected from plants in Grecia in 1990, whereas samples L1-L10 were collected from plants in Liberia in 2002. These samples were transported to the University of California at Davis (UC Davis), and total genomic DNA was extracted from tissue according to the method of Dellaporta et al. (1983). 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 sequenced at the DNA Sequencing Facility of UC Davis.

Cloning of 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 used to amplify circular DNA genomes (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 polymorphism (RFLP) to estimate the number and genetic diversity of DNA components in the samples, and to identify single cutting enzymes for cloning full-length components into pGEM-11Z (+) (Promega Corp., Madison, WI).

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

Sequence analysis.

Sequences of the cloned full-length DNA-A and DNA-B components in the recombinant plasmids pGV-GR1-A, pGV-GR1-B, pGV-L1-A and pGV-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 sequences in GenBank with highest identities (Benson et al. 2018). Pairwise nt sequence alignments were performed with MUSCLE within the Species Demarcation Tool (SDT) v.1.2, and with full-length DNA-A component sequences of the ten begomoviruses with the highest identities revealed by BLASTn (Muhire et al. 2014; Altschul et al. 1990). The same analysis was also performed with full-length sequences of DNA-B components. Comparisons also were made with individual open reading frames (ORFs) and non-translated regions (NTRs) of both components. Nucleotide identities 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).

Phylogenetic analysis.

For the phylogenetic analyses, we used the complete nt sequences of the DNA-A and DNA-B components of: (i) the bipartite begomoviruses from the GR1 and L1 samples; (ii) ToYMoV isolates from CR (both components for one isolate and the DNA-A components of two others); (iii) ToLCSiV isolates from CR and Nicaragua (NI); (iv) the ten begomoviruses 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, 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 (Sela et al. 2015; Katoh 2002). 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 then 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 begomoviruses TYLCV and the DNA-B component of the OW bipartite African cassava mosaic virus (ACMV), respectively.

Recombination analysis.

Preliminary datasets of 584 complete DNA-A sequences and 240 complete DNA-B sequences were assembled, which included the complete nt sequences of the DNA-A and DNA-B components of: (i) the bipartite begomoviruses from the GR1 and L1 samples; (ii) ToYMoV isolates from CR; (iii) ToLCSiV isolates from CR and NI; and (iii) sequences of selected viruses retrieved from 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. Detection of recombination events and breakpoints and identification of potential parental viruses were assessed with RDP4. The recombination analysis was 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 *bona fide* events.

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

Infectivity of the full-length DNA-A and DNA-B clones was initially determined by particle bombardment inoculation of excised linear double-strand (ds) monomers 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) as described by Paplomatas et al. (1994). The negative control in these experiments was equivalent plants bombarded with gold particles alone. Inoculated plants were maintained in a controlled environment chamber (light intensity, 300 µEinsteins; temperature, 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.

Production of multimeric clones and agroinoculation systems.

To develop an agroinoculation system, 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 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-GR1-A0.2. The full-length DNA-A monomer was released from pGV-GR1-A with *Xba*I and cloned into *Xba*I-digested pGV-GR1-A0.2 to generate the 1.2-mer pGV-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-GR1-B0.4. The full-length DNA-B monomer was released from pGV-GR1-B with *Xba*I and cloned into the *Xba*I-digested pGV-GR1-B0.4 to generate the 1.4-mer pGV-GR1-B1.4.

For the DNA-A component of the 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-L1-A0.5. The full-length DNA-A monomer was released from pGV-L1-A with *Bam*HI and cloned into *Bam*HI-digested pGV-L1-A0.5 to generate the 1.5-mer pGV-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-L1-B0.5. The full-length DNA-B monomer was released from pGV-L1-B with *Xba*I and cloned into *Xba*I-digested pGV-L1-B0.5 to generate 1.5-mer pGV-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.

Infectivity and host range experiments.

The infectivity of the cloned multimeric DNA-A and DNA-B components was initially assessed by agroinoculation of *N. benthamiana* plants at the three to five leaf-stage (3 wk old). Plants were agroinoculated with mixtures of *A. tumefaciens* cell suspensions (optical density of 600 nm = 1.0) of strains containing binary plasmids with each of the multimeric DNA-A and DNA-B clones of the GR1 and L1 isolates by needle puncture inoculation of the stem just beneath the shoot apex (Hou et al. 1998).

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

Because of recalcitrance to agroinoculation (Gilbertson et al. 1991), some species were inoculated with the multimeric DNA-A and DNA-B components of the GR1 isolate by particle bombardment as previously described. Pepper (*Capsicum annuum* cv. Cayenne) plants were bombarded with the multimers and the positive control in these experiments were equivalent pepper seedlings bombarded with the multimeric DNA-A and DNA-B clones of the pepper-infecting begomovirus pepper huasteco yellow vein virus (PHYVV), and the negative control was equivalent seedlings bombarded with gold particles alone. Seedlings of common bean cultivars

representing the Andean (cv. Topcrop) and Middle American (cvs. Carioca, BTS and Othello) gene pools, also were inoculated with the GR1 multimers by particle bombardment. The positive control in these experiments was equivalent seedlings inoculated with the multimeric DNA-A and DNA-B clones of the bean-infecting begomovirus 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 by Melgarejo et al. (2014). Symptom development was assessed visually and recorded at 14 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 S3.1).

Sap Inoculation.

Leaves of *N. benthamiana* and tomato (cv. Glamour) seedlings were rub-inoculated with sap as described by Gilbertson et al. (1991). Sap was prepared by grinding symptomatic leaf tissue from *N. benthamiana* plants (4-6 leaf stage) that had been agroinoculated with the multimeric DNA-A and DNA-B clones of the GR1 isolate as previously described. The positive control was equivalent plants inoculated with sap prepared from symptomatic leaf tissue from *N. benthamiana* plants infected with the sap-transmissible bipartite begomoviruses bean dwarf mosaic virus (BDMV) and tomato mottle virus (ToMoV), respectively, whereas the negative control was equivalent plants inoculated with 0.1 M phosphate buffer (pH 7.2) 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 inoculated DNA-A and DNA-B components in newly emerged (non-inoculated) leaves was

determined by PCR tests with primers specific for components of each isolate (Supplemental Table S3.1).

Pseudorecombination experiments with cloned DNA components of ToYMoV and ToLCSiV.

Pseudorecombination 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 ToYMoV and ToLCSiV. Controls were equivalent plants agroinoculated with the DNA-A and DNA-B components of ToYMoV or ToLCSiV (positive control), or with the empty vector alone (negative control). Inoculated plants were maintained in a controlled environment chamber, and symptom development was assessed as previously described. The presence of DNA-A and DNA-B components in newly emerged (non-inoculated) leaves was determined by PCR tests with component specific primers for of each virus (Supplemental Table S3.1).

Inoculation of tomato plants with ToYMoV, ToLCSiV and TYLCV.

Here, tomato (cv. Glamour) seedlings were co-agroinoculated with each virus alone or all combinations. Plants were agroinoculated with cell suspensions of *A. tumefaciens* strains carrying binary plasmids with the multimeric cloned DNA-A and DNA-B components of ToYMoV and ToLCSiV (DNA-A and DNA-B) and the multimeric infectious clone of the genomic DNA of an isolate of TYLCV from the Dominican Republic (TYLCV-[DO]) (Salati et al. 2002). Plants were agroinoculated with individual viruses or mixtures of all combinations, whereas the negative control was plants agroinoculated with the empty vector. 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 of each virus was determined by PCR tests with primers specific for components of each isolate (Supplemental Table S3.1).

Quantitative PCR (qPCR) assay.

Viral DNA accumulation in plants infected with individual viruses or mixtures of viruses was quantified at 7, 14 and 21 dpi by qPCR according to the protocol described by Mason et al. (2008). Total genomic DNA was extracted from newly emerged leaves as described above. 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 S3.1). 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 agroinoculated with each virus and non-inoculated 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 on a *QuantStudio™ 6 Flex Real-Time PCR System* (Thermo Fisher Scientific) with 100 ng of total genomic DNA in a 20-µl reaction mix using the SsoFast EvaGreen Supermix kit (Bio-Rad, Richmond, CA).

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 mean

and differences between treatments are considered significant when p-value < 0.05. Variability scores were determined by calculation of standard deviation. Statistical significance of this variation was analyzed for each time point with 3-way analysis of variance (ANOVA) using the R statistical analysis software.

Results

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

To investigate the invasion biology of tomato begomoviruses in CR, we needed to complete the characterization of ToYMoV and fulfill Koch's postulates for ToYMoD. To this end, we went back to samples collected in Grecia, CR in 1990, prior to the introduction of ToLCSiV and TYLCV. In PCR tests with total genomic DNA extracted from the GR1 and GR2 samples and primers degenerate for the begomovirus DNA-A and DNA-B components, the expected-sized ~1.1- and ~0.3-kb fragments, were amplified, respectively, indicating infection with a bipartite begomovirus. Furthermore, restriction enzyme digestion of the RCA products generated from these samples with *Eco*RI generated three fragments (~0.9, 1.7 and 2.6 kb) that added up to ~5.2 kb, consistent with infection with a single bipartite begomovirus (data not shown). Based upon BLASTn analyses, the sequences of the PCR-amplified DNA-A and DNA-B fragments from the GR1 and GR2 samples were >98% identical to each other and had highest identities with sequences of isolates of ToYMoV from CR (>98% for DNA-A and >96% for DNA-B sequences). These results indicated that the yellow mosaic/mottle disease of tomato observed in two commercial fields in Grecia, CR in 1990 was caused by ToYMoV.

The same analyses were performed with the L1-L10 samples and all were determined to be infected with a bipartite begomovirus (data not shown). Moreover, restriction enzyme digestion of the RCA products generated from these samples with *Sal*I generated four fragments,

respectively, that added up to ~5.2 kb, consistent with infection with a single bipartite begomovirus (data not shown). In contrast to the GR1 and GR2 samples, the sequences of the PCR-amplified DNA-A and DNA-B fragments from the L1-L10 samples were >96% identical to each other and had highest identities (>99% for DNA-A and >97% for DNA-B sequences) with sequences of isolates of ToLCSiV from CR and NI. These results indicated that the yellow mosaic/mottle disease of tomato observed in three commercial fields in Liberia, CR in 2002 was caused by ToLCSiV. The GR1 isolate of ToYMoV from Grecia and the L1 isolate of ToLCSiV from Liberia were selected for further characterization. For the GR1 isolate, putative full-length (~2.6 kb) linear ds DNA-A and DNA-B components were generated with RCA products with *Xba*I for both components, and for isolate L1, with *Bam*HI and *Xba*I, respectively.

Genomic properties of full-length clones of new isolates of ToYMoV and ToLCSiV.

The complete sequences of the cloned full-length DNA-A and DNA-B components of the GR1 isolate of ToYMoV (in recombinant plasmids pGV-GR1-A and pGV-GR1-B) collected in Grecia in 1990 and the L1 isolate of ToLCSiV (in recombinant plasmids pGV-L1-A and pGV-L1-B) collected in Liberia in 2002 were 2,574 nt (GenBank accession number: KC176780) and 2,547 nt (GenBank accession number: KC176781), respectively, and 2,610 nt (accession number MH019225) and 2,563 nt (accession number MH019226), respectively. The genome organization of these isolates is typical of NW bipartite begomoviruses, i.e., that is, a single gene on the virion (v)-sense strand (AV1) that encodes the CP, and four in the complementary (c)-sense strand (AC1, AC2, AC3, and AC4) encoding the Rep, the transcriptional activator protein (TrAP), the replication enhancer (REn) and the AC4 protein, respectively. Additionally, the CP and REn aa sequences of these isolates possess the N- and C-terminal motifs PWRlsAgT and AVRFATDr (lowercase indicates variable aa residues), respectively, which are characteristic of NW

begomoviruses (Harrison et al. 2002; Melgarejo et al. 2014; Mauricio-Castillo et al. 2014). The AC4 aa sequence contains the N-terminal myristoylation domain (MGXLIS) required for membrane targeting (Torres-Herrera et al. 2019; Rojas et al. 2001). The DNA-B components of these isolates have two ORFs, one in the v-sense strand (BV1) encoding the nuclear shuttle protein (NSP), and one on the c-strand strand (BC1) that encodes the movement protein (MP).

Pairwise sequence comparisons performed with SDT and the sequences of full-length DNA-A and DNA-B components of the GR1 isolate from Grecia revealed the highest identities with those of the DNA-A (~98%) and DNA-B (~98%) components of isolates of ToYMoV from CR (Table 3.1). Consistent with these results, the ORFs of these components all had very high identities, i.e., all nt and aa sequence identities were ≥97%, with the exception of the AC4 aa sequence (~93%). Similar results were obtained for NTRs, including the common region (~98%) and the hypervariable region (HVR) of the DNA-B component (~93%) (Table 3.1). The next highest identities for the DNA-A component sequence were with NW bipartite begomoviruses from Latin America, including Sida chlorotic mottle virus from Brazil (~79%), tomato yellow leaf distortion virus from Cuba (~79%) and tomato yellow vein streak virus from Chile (~78%). These results confirmed that the begomovirus infecting tomato plants with yellow mosaic/mottle symptoms in Grecia in 1990 was a variant of ToYMoV, which was named tomato yellow mottle virus-[CR:Grecia:1990] (ToYMoV-[CR:Gre:90]). The SDT analysis performed with the sequences of the complete DNA-A and DNA-B components of the L1 isolate from Liberia revealed the highest identities with those of the DNA-A (~99%) and DNA-B (~98%) components of isolates of ToLCSiV from CR and NI (Supplemental Table S3.2), whereas identities were lower (~93-94%) with available partial sequences of isolates from MX. Similar results were obtained in comparisons made with nt and aa sequences of the ORFs (~98-99%), whereas common region

identities ranged from ≥93 to 97% and identities for HVR of the DNA-B component ranged from ≥95 to 98% (Supplemental Table S3.2). The next highest identities for the sequences of the DNA-A component were with NW bipartite begomoviruses from Latin America, including Sida interveinal bright yellow virus from MX (~87%), Sida yellow vein virus from Honduras (~86%) and chino del tomate virus from MX (~85%). These results confirmed that the begomovirus infecting the tomato plants with yellow mosaic/mottle symptoms in Liberia in 2002 was a variant of ToLCSiV, and is named tomato leaf curl Sinaloa virus-[CR: Liberia: 2002] (ToLCSiV-[CR:Lib:02]). Thus, this isolate represents the first invasion event: a new bipartite begomovirus indigenous to the regions (MX and Central America).

Analyses of the common region sequences of ToYMoV and ToLCSiV.

The DNA-A and DNA-B components of ToYMoV-[CR:Gre:90] share a common region of 151 nt, which is 100% identical, indicating these are cognate components of a bipartite begomovirus species. The ToYMoV common region sequence contains all the *cis*-regulatory elements implicated in virus replication and gene expression, e.g., the conserved geminivirus stem-loop structure with the nonanucleotide sequence TAATATT↓AC, the Rep high-affinity binding site (iterons) and the canonical AC1 TATA box and G-box (Eagle et al. 1997; Fontes et al. 1994; Hanley-Bowdoin et al. 2013). The Rep high-affinity binding site is composed of two direct repeats of GGTGT adjacent to the AC1 TATA-box, and an upstream inverted repeat ACACC (Fig. 3.1). The Rep iteron-related domain (IRD) is MPPPK**K<u>F</u>RLS** (key aa are shown in bold and the highly conserved F residue is underlined), which is predicted to interact with the core iteron sequence GGTGT (Fig. 1) (Argüello-Astorga et al. 2001). Here, it is worth nothing that it has been proposed that ToYMoV may possess a unique 8 nt iteron sequence (TTGGTGTT) that is found in all

members of the SLCuV lineage (Fig. 3.1) (Argüello-Astorga et al. 1994, 2001; Torres-Herrera et al. 2019).

The DNA-A and DNA-B components of ToLCSiV share a common region of 174 nt, which is 96% identical, indicating these are cognate components. The Rep high affinity binding site of ToLCSiV consists of two direct repeats of the GGGGT adjacent to the AC1 TATA-box, and one inverted ACTCC motif (Fig. 3.1). The Rep IRD is MPSV**KRFKVS** (key aa are shown in bold and the highly conserved F residue is underlined)**,** which is predicted to recognize the GGGGT iteron (Argüello-Astorga et al. 2001).

Phylogenetic analysis.

In the phylogenetic tree generated with the sequences of the complete DNA-A components, the ToYMoV isolates from CR were placed together in a strongly supported clade (Fig. 3.2), consistent with the low level of sequence divergence among these isolates collected ~22 years apart (1990, present study and 2012, Barboza et al. 2018). This clade was erected as a sister group of the SLCuV lineage, which is composed primarily of cucurbit-infecting begomoviruses from the Southern US, MX and Central America. In the phylogenetic analysis performed with the complete DNA-B sequences, ToYMoV was also placed as a sister clade of the SLCuV lineage (Supplemental Fig. S3.2). Interestingly, in this DNA-B tree, the ToYMoV isolates were most closely related with bean leaf crumple virus (BLCrV) from Colombia (Supplemental Fig. S3.2).

The ToLCSiV isolates from CR (2002, present study and 2012, Barboza et al. 2018) form a strongly supported clade with the isolate from NI (Fig. 2). This clade was part of the AbMV lineage, which includes crop- and weed-infecting begomoviruses from North and Central America and the Caribbean Basin, such as chino del tomate virus (CdTV) from MX and ToMoV from Florida (Fig. 3.2). In the tree generated with the complete DNA-B sequences, the ToLCSiV

isolates from CR and NI were also placed together in a strongly supported clade in the AbMV lineage (Supplemental Fig. S3.2).

Recombination analysis.

RDP4 analysis reveal a single recombination event in the DNA-A component of all four ToYMoV isolates (Supplemental Fig. S3.3), whereas no recombination was detected in the DNA-B component nor in the DNA-A and DNA-B components of ToLCSiV. The recombination event in the ToYMoV DNA-A was 424 nt and spans nts 2056 to 2479 and includes the 5' end of the AC1 ORF, the entire AC4 ORF and the 5´ sequence of the common region. Thus, this event was in the well-known begomovirus recombination hot-spot region (Lefeuvre et al. 2009; Padidam et al. 1999; Hou et al. 1996). This recombination event was strongly supported by six methods implemented in RDP4 (*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} for the RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq recombination methods, respectively). The RDP4 analysis further indicated that the recombinant region was derived from an uncharacterized minor parent, whereas the major parent was most similar to tomato chlorotic leaf distortion virus (TCLDV) from Venezuela (GenBank accession number JN241632).

Infectivity and host range experiments.

The infectivity and pathogenicity of the full-length cloned DNA-A and DNA-B components (monomers) of ToYMoV-[CR:Gre:90] were established by particle bombardment inoculation of *N. benthamiana* and tomato seedlings (Table 3.2). By 14 dpb, all of the bombarded *N. benthamiana* plants were stunted and newly emerged leaves showed epinasty, crumpling, yellow mosaic/mottle and vein yellowing (Fig. 3.3A); whereas tomato seedlings were stunted and newly emerged leaves had developed epinasty, crumpling and mild yellow mosaic/mottle by 14

dpb (Fig. 3.3B). In *N. benthamiana* and tomato plants, symptoms in ToYMoV-infected plants became progressively milder by 21 dpb (data not shown). Notably, symptoms in tomato plants following bombardment of the infectious ToYMoV clones were similar to those of the ToYMoD of tomato in the field in CR, thereby fulfilling Koch´s postulates for this disease. The partial host range of ToYMoV was next investigated by agroinoculation and particle bombardment inoculation of a range of plant species with the infectious ToYMoV clones (Table 3.2). In addition to *N. benthamiana* and tomato, symptomless DNA-A infections were detected in ~38% of agroinoculated pumpkin plants, whereas ToYMoV did not infect the solanaceous species *N. tabacum* and *D. stramonium*; cucumber, cantaloupe and honeydew melons; and *C. amaranticolor* plants (Table 3.2).

PCR tests with component-specific primers confirmed that all symptomatic plants tested were infected with the ToYMoV DNA-A and DNA-B components and revealed the symptomless infections of pumpkin plants. Plants agroinoculated with the empty vector control or bombarded with gold particles alone did not develop symptoms and were negative in PCR tests for ToYMoV/ToLCSiV DNA-A and DNA-B components.

The infectivity and pathogenicity of the full-length cloned DNA-A and DNA-B components (monomers) of ToLCSiV-[CR:Lib:02] were also assessed by particle bombardment inoculation of *N. benthamiana* and tomato seedlings. All bombarded were stunted and newly emerged leaves developed epinasty, crumpling and mild yellow mosaic/mottle by 14 dpb (Fig. 3.3C and 3.3D). By 21 dpb, symptoms became progressively milder (data not shown). These results were consistent with those reported by Idris et al. (1998) and established that the crumpling and yellow mottle/mosaic symptoms of tomato leaves observed in Liberia in 2002 was caused by ToLCSiV (note that the partial host range of ToLCSiV has been done by Idris et al. 1998).

Sap inoculation.

Sap transmission is a property of some bipartite begomoviruses and can reveal insight into tissue tropism, i.e., phloem-limitation (Yarwood 1957; Walkey et al. 1991). To determine if ToYMoV is mechanically transmissible, sap prepared from symptomatic *N. benthamiana* leaves from plants infected with ToYMoV via agroinoculation was rub-inoculated onto leaves of *N. benthamiana* and tomato seedlings. In these experiments, ToYMoV was sap-transmitted to ~56% of *N. benthamiana* plants, but not to tomato plants (total of three independent experiments) (Table 3.2). Disease symptoms in *N. benthamiana* plants infected followed sap transmission were indistinguishable from those infected after agroinoculation (data not shown). In contrast, a 100% of *N. benthamiana* plants rub-inoculated with the sap-transmissible BDMV developed stunting, distortion and severe leaf epinasty by 14 dpi, and 100% of tomato plants rub-inoculated with ToMoV developed stunting, epinasty and yellow mottling by 14 dpi. In all these experiments, the presence of the ToYMoV DNA-A and DNA-B components was confirmed in newly emerged leaves of representative symptomatic plants by PCR tests with component-specific primers (Supplemental Table S3.1). The expected-size PCR fragments were amplified from symptomatic *N. benthamina* and tomato plants infected with BDMV and ToMoV (positive controls), respectively, whereas no DNA fragments were amplified from an extract prepared from plants inoculated with buffer alone (data not shown).

Pseudorecombination experiments with cloned DNA components of ToYMoV and ToLCSiV.

To further investigate the relationship between ToYMoV and ToLCSiV, pseudorecombination experiments were conducted in which *N. benthamiana* and tomato plants were agroinoculated with the DNA-A and DNA-B components of the wild-type viruses and the pseudorecombinants (PRs). *N. benthamiana* plants agroinoculated with the ToYMoV DNA-A

(TA) and ToLCSiV DNA-B (SB) PR did not developed symptoms, although ToYMoV DNA-A (TA) only infections were detected in all inoculated plants (Table 3.3). Plants agroinoculated with the ToLCSiV DNA-A and ToYMoV DNA-B PR also did not develop symptoms, but in contrast to the TA + SB PR, ToLCSiV DNA-A only infections were not detected. Similar results were obtained in equivalent experiments conducted with tomato plants, although rates for ToYMoV DNA only infections were lower (~30%) (Table 3.3). Together, these results established that the DNA components of these viruses cannot form infectious PRs, which is consistent with ToYMoV and ToLCSiV belonging to different lineages and having different iterons and IRDs.

Invasion biology experiments with ToYMoV, ToLCSiV and TYLCV.

To understand the potential impact of these two invasion events on begomovirus disease of tomato in CR, we next compared single and mixed infections of these viruses in terms of symptom development and severity and viral DNA accumulation. Consistent with agroinoculation results, ToYMoV and ToLCSiV each induced stunting and epinasty, crumpling and yellow mosaic/mottle symptoms in newly emerged leaves of all agroinoculated tomato plants by 21 dpi (Fig. 3.4A-B and 3.5A-B). Here it should be note that the ToYMoD symptoms induced in cv. Glamour by these two viruses were indistinguishable. Equivalent tomato plants agroinoculated with the infectious clone of TYLCV were stunted and developed the characteristic symptoms of tomato yellow leaf curl disease (TYLCD), including upward leaf curling, crumpling and interveinal and marginal yellowing in newly emerged leaves by 21 dpi (Fig. 3.4C and 3.5C). Tomato plants co-inoculated with ToYMoV and ToLCSiV were not more stunted than plants infected with each virus alone, but co-infected plants developed more severe epinasty, crumpling and yellow mosaic/mottle by 21 dpi (Fig. 3.4A-B and 3.5E). By 21 dpi, tomato plants co-infected

with ToYMoV or ToLCSiV and TYLCV were more stunted and showed mostly TYLCD symptoms (Fig. 3.4A-C).

The symptoms of ToLCSiV and ToYMoV (epinasty, crumpling and yellow mosaic/mottle) appeared earlier (~7 dpi) that those of TYLCV (~10 dpi). However, by 21 dpi, TYLCD symptoms had become dominant, regardless of the combination (Fig. 3.4A-C and 3.5A-D). Furthermore, plants having mixed infections with TYLCV showed a marked symptoms transition in leaves, in which the crumpling and yellow mosaic/mottle associated with bipartite begomoviruses initially appeared in the lower leaves, whereas those of TYLCV appeared later and became dominant by 21 dpi (Fig 3.5E-G). Finally, plants infected with all three viruses were severely stunted and foliar symptoms appeared at ~7 dpi, eventually developed unusually severe TYLCD symptoms (Fig. 3.4A-C and 3.5H). These results revealed either a synergistic interaction or an additive effect in plants co-inoculated with these viruses.

In order to measure virus accumulation over time, samples of newly emerged leaves were collected from tomato plants agroinoculated with each virus alone and in all combinations at 7, 14 and 21 dpi, and viral DNA accumulation was quantified by qPCR. In single infections, all viruses were detected in newly emerged leaves and had accumulated to similar levels in newly emerged leaves by 7 dpi, even though symptoms had not appeared in plants infected with TYLCV (Supplemental Fig. S3.4A and S3.4B). Levels of TYLCV and ToLCSiV increased by 14 and 21 dpi, whereas those of ToYMoV remained the same or decreased slightly (Supplemental Fig. S3.4A and S3.4B). In mixed infections, ToYMoV DNA levels were significantly reduced at 7 dpi, and similar trend was observed for ToLCSiV; however, levels of both viruses increased by 14 and 21 dpi and were not significantly different from those of plants infected with these viruses alone (Fig. 3.6A-B). TYLCV DNA levels were unaffected by the co-infecting bipartite begomoviruses. Thus,

other than this transient negative effect, there was no evidence of any virus having levels consistently reduced in the presence of a co-infecting virus. Variability was high or increased for the bipartite in mixed infections, especially with TYLCV (Supplemental Fig. S3.5A-B). In contrast, the viral DNA levels of TYLCV were not affected in mixed infection with either or both of the bipartite begomoviruses (Fig. 3.6C). Levels of variability for TYLCV were higher at 7 dpi for single and mixed infections, but then became much lower at 14 and 21 dpi (Supplemental Fig. S3.5C). Interestingly, overall TYLCV variability was greater in single infected compared with plants having mixed infections. Together, our results suggest that these viruses can co-infect tomato plants without negatively impacting replication of co-infecting viruses.

Discussion

In the present study, we further analyzed the three emergence/introduction events for tomato-infecting begomoviruses in CR to investigate the invasion biology of these viruses, i.e., the investigation of interactions among these viruses and the susceptible host and how this will impact begomovirus disease of the tomato crop in CR. The three emergence/introduction events were: (i) local evolution of ToYMoV, (ii) introduction of an invasive NW bipartite begomovirus from elsewhere in the region (ToLCSiV) and (iii) introduction of the OW monopartite TYLCV. However, because our approach required infectious clones of each virus, we first generate infectious clones and agroinoculation systems for isolates of ToYMoV and ToLCSiV from CR. In the case of ToYMoV, this allowed us to complete the characterization of the virus, including fulfilling Koch's postulates for the ToYMoD. Host range studies performed via agroinoculation showed that ToYMoV has a relatively narrow host range, infecting and causing symptoms in tomato and *N. benthamiana*, but not in all solanaceous species (e.g., pepper). This is consistent with previous reports in which ToYMoV was never found in association with begomovirus

diseases on pepper in CR (Barboza et al. 2018). Cucurbits do not appear to be a host of ToYMoV, with the exception of a small number of symptomless DNA-A only infections of pumpkin. The sap transmissibility of ToYMoV, albeit only to *N. benthamiana* plants, indicates some capacity for cell-to-cell movement and egress from the phloem. Taken together with previous results, ToYMoV is a locally evolved virus that has been associated with ToYMoD in CR for over a 30 yrs period, with relatively little divergence (2%) over ~20 years (Barboza et al. 2018; Hilje et al. 2008). Thus, these results suggests that ToYMoV has reached stabilizing selection and that it is well-adapted to tomato. Furthermore, reports of ToYMoV from NI and Panama indicated capacity for regional spread in Central America. The placement of ToYMoV in the SLCuV lineage was expected, based on a previous study (Barboza et al. 2018), but our phylogenetic analyses further indicated that the four ToYMoV isolates comprise a strongly supported clade that is distinct from the rest of the viruses in the SLCuV lineage. Thus, this may represent a new lineage that is closely related (sister lineages) to the SLCuV lineage. This hypothesis is supported by several lines of evidence. First, although the closest relatives of ToYMoV are members of the SLCuV lineage, the relatively low identities (<79%), indicate a long period of diversification via random mutation (Lima et al. 2017; Duffy et al. 2008). Second, the ToYMoV common region (iterons and corresponding Rep IRD) are different from those of members of the SLCuV lineage, such that they would be predicted to not form infectious PRs. Third, the ToYMoV AC4 differs from those of members of the SLCuV lineage in size (~85 aa compared with ~126 aa) and in possessing an N-terminal myristoylation motif. Finally, there has been substantial host adaptation of ToYMoV to infect tomato, whereas most of the members of the SLCuV lineage are adapted to and cause disease in cucurbits. These two lineages probably arose from an ancestral begomovirus infecting a non-cultivated plants species in the region, and then underwent local evolution to adapt to infect tomato or cucurbits.

The long branch that separates ToYMoV and the other member of the SLCuV lineage also was indicative of recombination playing a role in viral evolution (Lima et al. 2017; Martin et al. 2011). Indeed, a recombination event was detected in the well-known hot-spot region of the begomovirus genomic DNA/DNA-A component, and includes the Rep binding site, thereby changing replication specificity (Martin et al. 2011; Hou et al. 1996; Lefeuvre et al. 2009; Padidam et al. 1999). The overall biological significance of recombination in this region is still not clear, but it has been suggested to be involved in host adaptation, including a role for AC4 (Maliano et al. 2021; Chen et al. 2019; Jupin et al. 1994; Melgarejo et al. 2013; Rojas et al. 2001). Thus, it is notable that acquire a very different AC4 via the recombination even. Thus, ToYMoV is an indigenous virus that locally evolved to infect tomato and that this was likely mediated by indigenous whiteflies (Gilbertson et al. 2015; Navas-Castillo et al. 2011). The detection of ToLCSiV, first described in MX in 1989 (Brown et al. 1993; Idris et al. 1999; Nakhla et al. 2005), in Costa Rica in 1998 was the first invasion event: the introduction of an invasive NW bipartite begomovirus from the region. Furthermore, the detection of ToYMoV and ToLCSiV in samples with stunting and yellow mosaic/mottle of leaves collected from Liberia in 2002 (present study) and from Grecia in 2012 (Barboza et al. 2018), is consistent with both viruses causing ToYMoD and with ToLCSiV having spread and become established in CR. The invasiveness of ToLCSiV has resulted in the overlapping of the geographic ranges of these viruses (Barboza et al. 2018). Mixed infections of ToYMoV and ToLCSiV will allow for genetic interactions between these viruses. However, the finding that ToYMoV and ToLCSiV did not form infectious PRs is consistent with these viruses coming from distinct lineages, and having divergent regulatory sequences. Thus, a replication incompatibility must likely underlie the lack of infectivity of the PRs. Finally, although these results seem to rule out this genetic mechanism as a means of

variability, the viruses can clearly co-infect tomato plants and exist in mixed infections, thereby allow for recombination to occur. An interesting observation from the PRs experiments was the different capacities of the DNA-A components of ToYMoV and ToLCSiV to systemically infect *N. benthamiana* and tomato plants. DNA-A alone infections have been previously reported for NW and OW bipartite begomoviruses in agroinoculated plants (Garrido-Ramirez et al. 2000; Melgarejo et al. 2019; Buragohain et al. 1994; Evans et al. 1993; Hou et al. 1998; Frischmuth et al. 1993; Galvão et al. 2003; Klinkenberg et al. 1990). As in these previous reports, the ToYMoV DNA-A infections were symptomless, consistent with the need of the DNA-B component for symptom development of bipartite begomoviruses (Rojas et al. 2005; Hanley-Bowdoin et al. 2000; Jeffrey et al. 1996; Levy et al. 2003; Sudarshana et al. 1998). The mechanism underlying the virus-specific DNA-A alone infections is not known, but it may be involved the AC4 (Rojas et al. 2001; Melgarejo et al. 2013). Furthermore, the of *N. benthamiana* and tomato by the ToYMoV DNA-A reflects the susceptibility of these species to begomovirus infection, and maybe reveal how NW monopartite begomoviruses may have emerged (Márquez-Martín et al. 2011; Macedo et al. 2017; Melgarejo et al. 2013; Romay et al. 2019; Gilbertson et al. 2015).

The introduction of TYLCV into CR in 2012 (Barboza et al. 2014) was the second invasion event: a highly invasive OW monopartite begomovirus that can cause devastating losses to tomato production worldwide (Scholthof et al. 2011; Gilbertson et al. 2015; Rojas et al. 2018; Cohen et al. 1994). Indeed, TYLCV has added a new challenge for tomato production in CR, as growers have reported increased losses due to begomoviruses disease since 2012 (Barboza et al. 2014, 2018). However, what was less clear was how TYLCV interacts with the already existing bipartite begomoviruses ToYMoV and ToLCSiV. In Florida, the introduction of TYLCV led to a reduced incidence of the indigenous bipartite begomovirus ToMoV (Polston et al. 1999). Therefore, this

provides an opportunity to investigate the invasion biology of these very different begomoviruses with the long-term objective of making predictions and management suggestions.

In the infectivity experiments, interactions were revealed based on symptom severity and viral DNA accumulation. A general synergistic interaction was observed in which mixed infections resulted in more severe symptoms, with plants infected by all three viruses showing the most severe symptoms. A similar situations has been observed for the interaction of three indigenous begomoviruses in Brazil, as measured based on symptom severity (Macedo et al. 2017). Furthermore, the interaction among begomoviruses detected in the present study is a type of neutral synergism, which has also been previously described, including for begomoviruses (Zhang et al. 2001; Mascia et al. 2016; González-Jara et al. 2004; Morilla et al. 2004; Alves-Júnior et al. 2009). For example, *N. benthamiana* and tomato plants co-inoculated with TYLCV and tomato yellow leaf curl Sardinia virus (TYLCSV) developed more severe symptoms than plants inoculated with either virus alone, and accumulation of each virus in co-infected plants was similar to that in single infections (Morilla et al. 2004). Interestingly, we did detect an initial antagonistic or negative interference effect on ToYMoV and ToLCSiV accumulation at 7 dpi in all mixed infections, indicating it was not virus specific. These results are consistent with a previous study showing a transient negative effect on viral accumulation early in mixed infections of tomato rugose mosaic virus (ToRMV) and tomato yellow spot virus (ToYSV) in *N. benthamiana* and tomato (Syller 2012; Alves-Júnior et al. 2009). The overall mechanism of antagonism in mixed infections remains to be elucidated, but may involve competition for host factors or stimulation of a more efficient defense response of the host (Syller et al. 2016; Torchetti et al. 2016). For example, potato spindle tuber viroid can interfere with TYLCSV accumulation in tomato by activation of the host DNA methylation pathways (Torchetti et al. 2016). The fact that TYLCV accumulation was not affected

during mixed infections with NW bipartite begomoviruses in the present study could be due to the combine activities of multiple viral suppressor of gene silencing, e.g., C2, C4 and V2 (Luna et al. 2012). Taken together, our results with these three begomoviruses revealed the existence of temporary antagonism (7 dpi) followed by a more sustained neutral synergism.

We further showed that in mixed infections with TYLCV, TYLCD became dominant at 14 dpi and beyond, even in the presence of the two co-infecting NW tomato bipartite begomoviruses. Indeed, this dominant TYLCD phenotype has been observed in the tomato fields in Costa Rica since the introduction of TYLCV (M. R. Rojas, personal communication). Therefore, in terms of the invasion biology of these viruses, it appears they can effectively co-exist in tomato plants, which leads to more severe disease and the persistence of all three viruses in tomato production in CR. Another observation in mixed infections with TYLCV was that, although TYLCD symptoms eventually became dominant, symptoms induced by co-infecting bipartite begomoviruses appeared earlier (~7 dpi), with TYLCD symptoms appearing at ~10 dpi. This observation can be explained in terms of the tissue tropism of these viruses. The more rapid appearance of mosaic/mottle symptoms in leaves may reflect the capacity to infect cells outside of the phloem, and more rapidly colonize and accumulate in plants. The sap transmission of ToYMoV is evidence this virus is not phloem limited.

In summary, we used the tomato begomovirus situation in CR to examine the invasion biology of three viruses, two of which were introduced. We first confirmed ToYMoV caused ToYMoD in CR and is a locally evolved NW bipartite begomovirus. We then used infectious clones to investigate interactions in mixed infections. We found that these viruses exhibited a neutral synergism, in which the viruses co-exist and induced more severe symptoms. In mixed infections with TYLCV, TYLCD became predominant. These results indicate that all three viruses

are likely persisting in CR and causing more severe symptoms and losses, particularly in the presence of TYLCV. Thus, an effective management of these complexes will require an integrate approach, including the identification of varieties with resistance to all three viruses.

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Table 3.1. 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:90]) and the most closely related begomoviruses [a]

			DNA-A component												DNA-B component [e]					
					ORFs												ORFs			
					AV1		AC1		AC2		AC3		AC4				BV1		BC1	
Begomovirus isolates [b]	Year [c]	Loc [d]	Total	Common region	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	Total	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] Based on a BLASTn analysis, the ten most closely related begomoviruses were various New World bipartite begomoviruses.

[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.

Table 3.2. Infectivity, symptomatology and host range of the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:90]).

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
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	NT	NT	NT	TBD
Phaseolus vulgaris cv. Carioca	NT	TBD	NT	TBD
Phaseolus vulgaris cv. BTS	NT	TBD	NT	TBD
Phaseolus vulgaris cv. Pinto Othello	NT	TBD	NT	TBD

[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. TBD = to be determined.

[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 and Vy = vein yellowing.

Table 3.3. Infectivity and symptomatology of pseudorecombinants (PRs) formed with the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:90]) and an isolate of tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:02]) following agroinoculation of *Nicotiana benthamiana* and tomato plants.

Plant species/ PRs [a]	Infectivity [b]				Symptoms [c]
	ToYMoV		ToLCSiV		
	DNA-A (TA)	DNA-B (TB)	DNA-A (SA)	DNA-B (SB)	
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
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 a total 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.

Supplemental Table S3.1. Sequences of the oligonucleotide primers used in this study

Primer[a]	Nucleotide sequence
PATv2469	5'-ATTGGGTTCCTCTCCAAAATGTC-3′
PATc577	5'-GTCTCCGGTCTCTAACCAGCC-3′
PASv2325	5'-TCGTGGAGCTCTCTGCAGATCTTGATG-3′
PASc102	5'-AGACGCAATATGATTGGACGACAG-3′
PBTv1263	5'- GCTAATTAAACATTGACAAGAGGC-3′
PBTc1643	5'- TAGTCCGTTCCGCATCGTCAC-3′
PBSv2224	5'- GACGACTGTTTTGTGGGTCC-3′
PBSc238	5'-CGACGGAGAGGATAAGTGGTC-3′
PTv324	5'- AGGCCCTGATGTGCC-3′
PTc476	5'- ACGCAAAAACGTTTACCTAC-3′
PSv2226	5'-GCTGACCTGGTTGGG-3′
PSc2375	5'-AACAATTCCGGTTAACAAGAAATTC-3′
PTYv372	5'- ACTTCGACAGCCCATACAG -3′
PTYc523	5'- TGGGCCTTCACATCCAC -3′

[a]Primer nomenclature is coded as follows: P = primer; A = DNA-A component; B = DNA-B component; T = ToYMoV; S = ToLCSiV; TY = TYLCV; v = viral-sense primer; c = complementary-sense primer; number = annealing position for the 5' nucleotide end of the primer.

Supplemental Table S3.2. 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 leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:02]) and the most closely related begomoviruses [a]

Begomovirus isolates [b]	Year [c]	Loc [d]	DNA-A component												DNA-B component [e]					
			Total	Common region	ORFs										Total	HVR	ORFs			
					AV1		AC1		AC2		AC3		AC4				BV1		BC1	
					nt	aa	nt	aa	nt	aa	nt	aa	nt	aa			nt	aa	nt	aa
ToLCSiV-[CR:4371-22:11]	2011	CR	99	97	99	99 (100)	99	99 (99)	99	98 (98)	99	99 (99)	99	99 (99)	98	98	99	99 (100)	99	98 (98)
ToLCSiV-[CR:4371-7:11]	2011	CR	99	94	99	99 (100)	99	99 (99)	99	98 (98)	99	99 (99)	99	99 (99)	98	98	99	99 (100)	99	98 (98)
ToLCSiV-[NI:SL]	NA	NI	99	93	99	98 (99)	99	99 (99)	99	98 (98)	99	99 (99)	99	98 (98)	98	95	98	98 (99)	99	98 (98)
SiIBYV-[MX:Que:Con1:18]	2018	MX	87	84	83	91 (96)	88	89 (93)	89	81 (85)	89	86 (92)	92	81 (85)	80	55	82	86 (90)	85	89 (94)
SiYVV-[HN]	NA	HN	86	69	85	88 (94)	85	85 (91)	87	81 (86)	86	77 (86)	90	74 (80)	80	60	83	88 (89)	86	92 (96)
CdTV-To[MX:SinIC:83]	1983	MX	85	79	83	89 (95)	86	83 (90)	87	78 (86)	89	87 (92)	91	77 (83)	80	65	79	82 (89)	84	91 (96)
SiYMYuV-[MX:Yuc:05]	2005	MX	85	78	83	89 (95)	85	84 (91)	87	81 (88)	89	84 (89)	88	72 (79)	76	48	74	77 (84)	84	92 (94)
MaBYMV-[US:Ma8S:14]	2014	US	85	63	83	91 (96)	84	81 (89)	88	80 (88)	90	85 (89)	90	76 (81)	78	53	78	83 (89)	83	89 (95)
SiYMoV-[CU:SSp159:09]	2009	CU	84	56	83	88 (95)	84	85 (91)	86	78 (88)	88	87 (91)	89	71 (80)	75	49	75	78 (87)	83	91 (96)
SiGMBuV-[JM:SE1:04]	2004	JM	84	69	80	85 (91)	86	87 (92)	85	79 (85)	84	75 (86)	90	73 (78)	76	52	73	76 (85)	82	91 (95)
OYMV-[MX:Maz3:04]	2004	MX	84	67	81	88 (96)	84	86 (91)	87	81 (88)	88	87 (91)	89	76 (83)	76	64	73	75 (84)	85	90 (95)
BWCMV-[VE:Rub932:07]	2007	VE	84	71	82	87 (94)	85	83 (89)	82	72 (83)	83	80 (89)	91	79 (84)	79	59	78	83 (88)	85	94 (96)
SiMSiV-[MX:Gua:06]	2006	MX	83	68	83	87 (94)	83	80 (88)	86	78 (86)	86	78 (90)	88	72 (78)	77	61	79	82 (88)	83	91 (94)

[a] Based on a BLASTn analysis, the ten most closely related begomoviruses were various New World bipartite begomoviruses.

[b] GenBank accession numbers are as follows: ToLCSiV-[CR:4371-22:11]: KY064013 and KY064025; ToLCSiV-[CR:4371-7:11]: KY064014 and KY064020; ToLCSiV-[NI:SL]: AJ608286 and AJ508783; SiIBYV-[MX:Que:Con1:18]: MN419242 and MN419243; SiYVV-[HN]: Y11097 and Y11098; CdTV-To[MX:SinIC:83]: AF101476 and AF101478; SiYMYuV-[MX:Yuc:05]: DQ875872 and

DQ875873; MaBYMV-[US:Ma8S:14]: KU058856 and KU058860; SiYMoV-[CU:SSp159:09]: HQ822123 and HQ822124; SiGMBuV-[JM:SE1:04]: HQ008338 and HQ009518; OYMV-[MX:Maz3:04]: DQ022611 and GU972604; BWCMV-[VE:Rub932:07]: NC022005 and NC022003; SiMSiV-[MX:Gua:06]: DQ520944 and DQ356428. NA, not available.

[c] Year collected.

[c] Geographic location: CR = Costa Rica, NI = Nicaragua, MX = Mexico, HN = Honduras, US = United States, CU = Cuba, JM = Jamaica and VE = Venezuela.

[e] NA = not available.

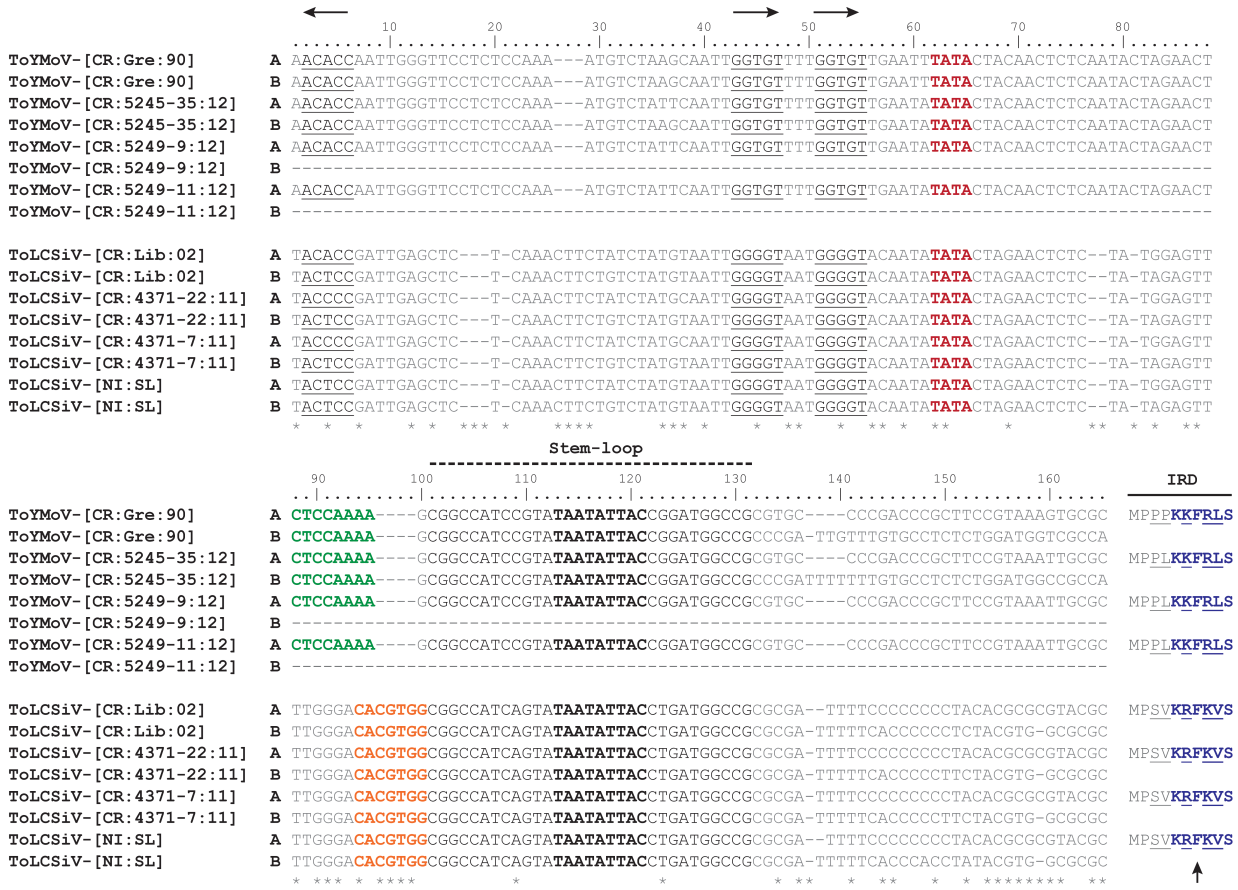


Fig. 3.1. Alignment of a portion of the common region sequences of isolates of tomato yellow mottle virus (ToYMoV) from Costa Rica (CR) and isolates of tomato leaf curl Sinaloa virus (ToLCSiV) from CR and Nicaragua (Ni). The replication-associated protein (Rep) high affinity binding site (two direct repeats and one inverted repeat of the core iteron) are shown in black letters and underlined, and their 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 is shown in orange bold letters, and the GYA box is represented in green bold letters. The characteristic geminivirus stem-loop 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 right of the alignment, with the putative six amino acids involved in iteron recognition indicated in blue bold letters; the highly conserved F residue in the IRD is

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 are indicated with an asterisk at the bottom of the alignments. Abbreviations and GenBank accession numbers are as follows: ToYMoV-[CR:Gre: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: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.

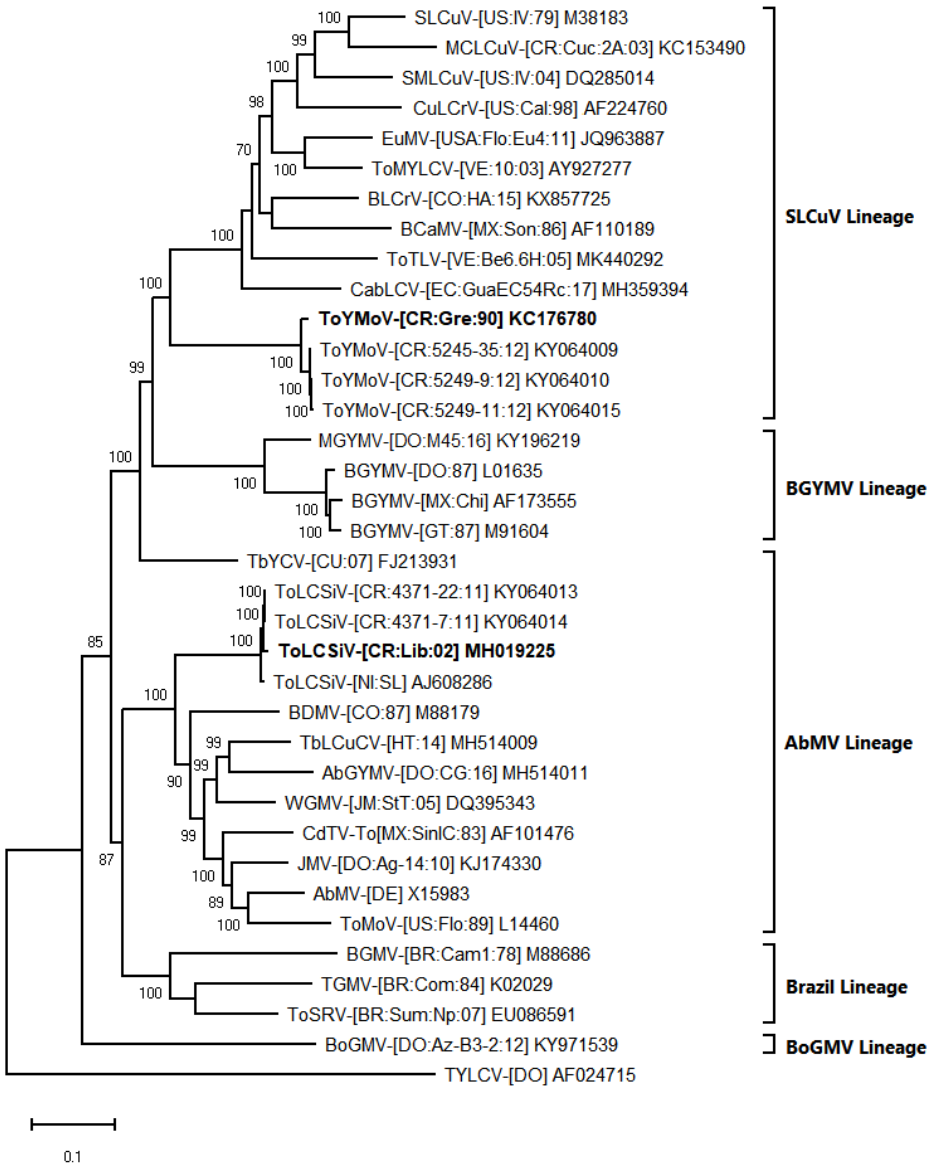


Fig. 3.2. Bayesian phylogenetic consensus tree based on an alignment of complete nucleotide sequences of DNA-A components and showing the relationship of new isolates of tomato yellow leaf curl virus (ToYMoV-[CR:Gre:90]) and tomato leaf curl Sinaloa virus (ToLCSiV-[CR:Lib:02]) from Costa Rica (shown in bold) with: (i) previously characterized isolates of ToYMoV and ToLCSiV from CR and an isolate of ToLCSiV from Nicaragua; (ii) the ten most closely related begomoviruses based on a BLAST search; and (iii) selected begomoviruses from Latin America 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 (NW) begomoviruses (lineages are indicated with brackets). 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.

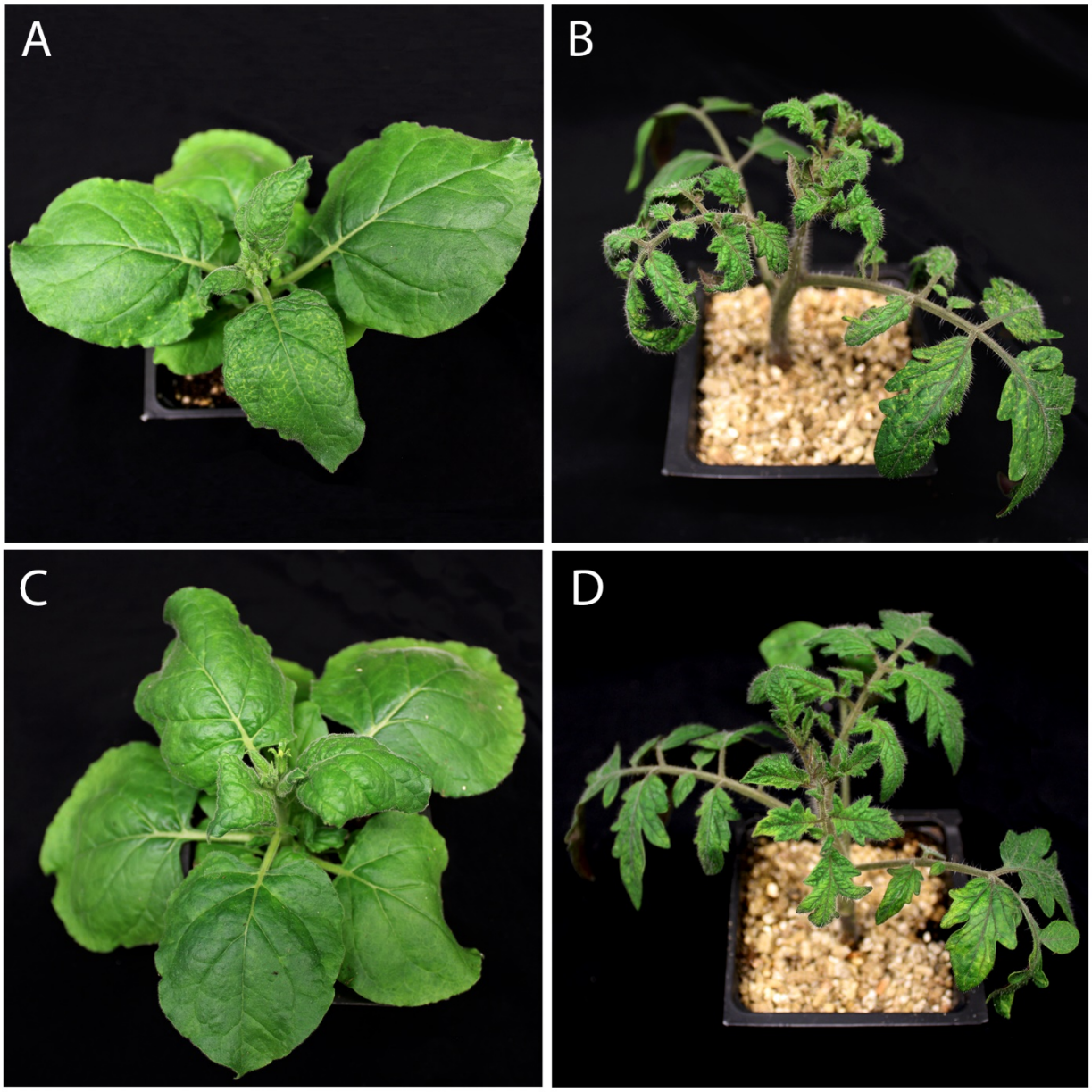


Fig. 3.3. Disease symptoms induced by the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow leaf curl virus from Costa Rica (ToYMoV-[CR:Gre:90]) in (A) *Nicotiana benthamiana* and (B) tomato (*Solanum lycopersicum* cv. Glamour); and those induced by the infectious cloned DNA-A and DNA-B components of an isolate of tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:02]) in (C) *N. benthamiana* and (D) tomato (*S.*

lycopersicum cv. Glamour). Plants were inoculated with excised linear monomers by particle bombardment and photographed 14 days later.

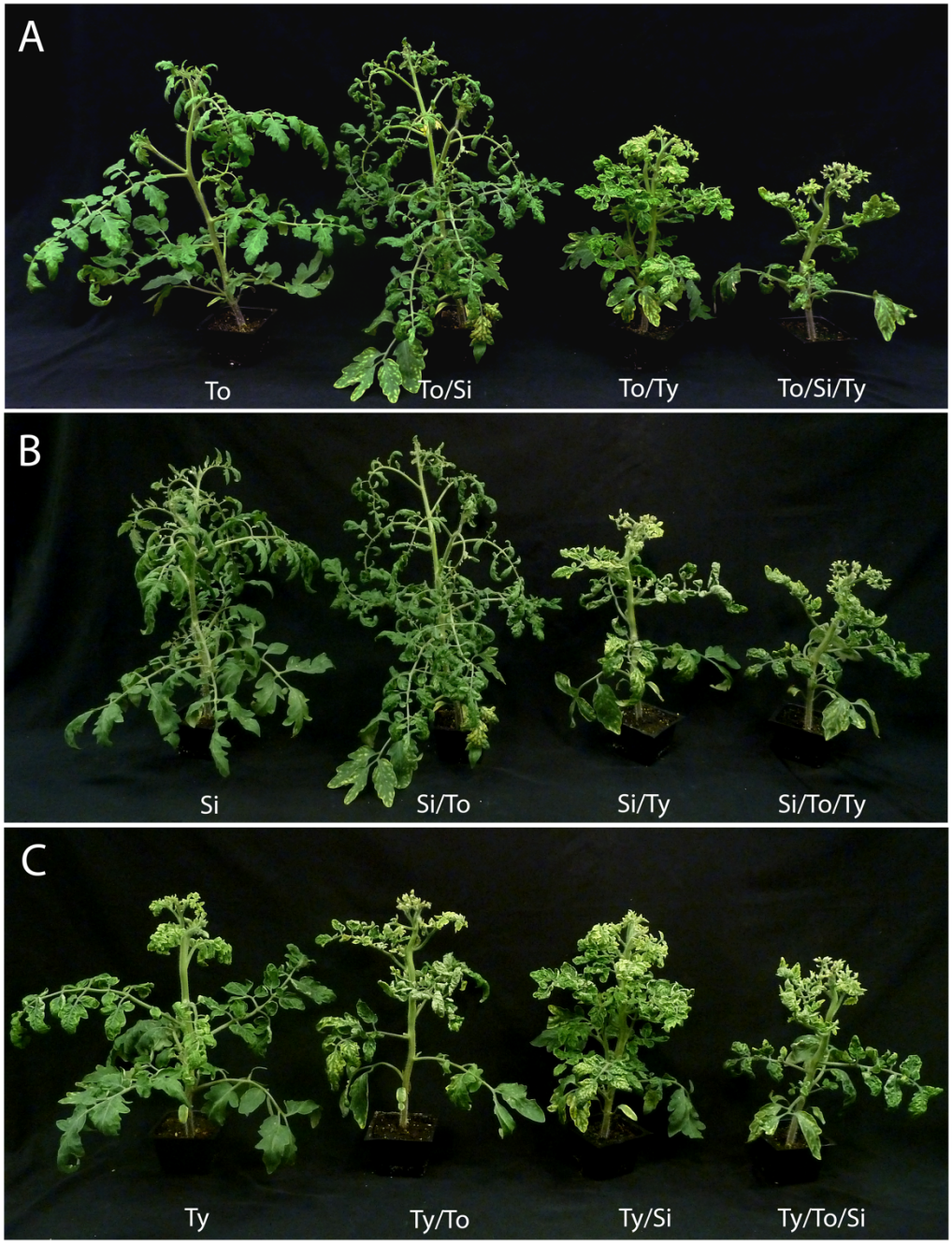


Fig. 3.4. Disease symptoms induced by the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow leaf curl virus from Costa Rica (ToYMoV-[CR:Gre:90]), an isolate of tomato leaf curl Sinaloa virus from Costa Rica (ToLCSiV-[CR:Lib:02]) and the multimeric infectious clone of tomato yellow leaf curl virus from the Dominican Republic (TYLCV-[DO]) alone or in mixed infections in tomato (*Solanum lycopersicum* cv. Glamour). (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 days later.

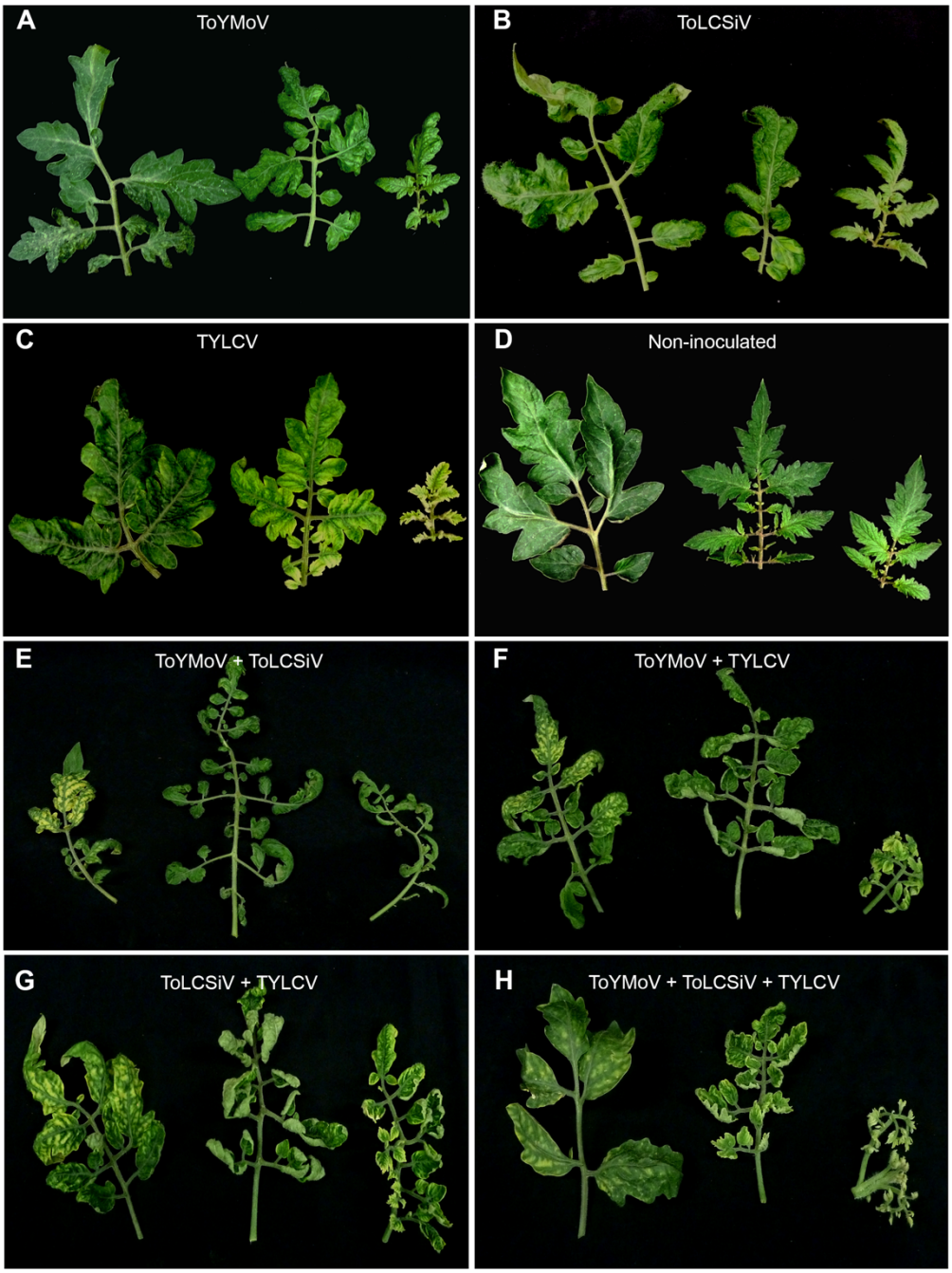


Fig. 3.5. Symptom transition in the bottom, middle and upper parts of tomato plants infected with the infectious cloned DNA-A and DNA-B components of an isolate of tomato yellow leaf curl virus from Costa Rica (ToYMoV-[CR:Gre:90]), an isolate of tomato leaf curl Sinaloa virus (ToLCSiV-[CR:Lib:02]) from CR and the multimeric infectious clone of tomato yellow leaf curl virus (TYLCV) from the Dominican Republic (TYLCV-[DO]) individually or in all combinations

following agroinoculation. (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 days after agroinoculation.

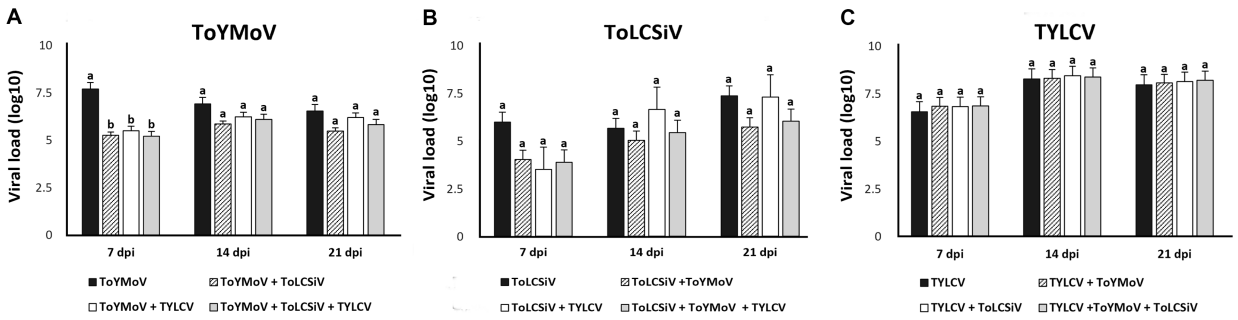
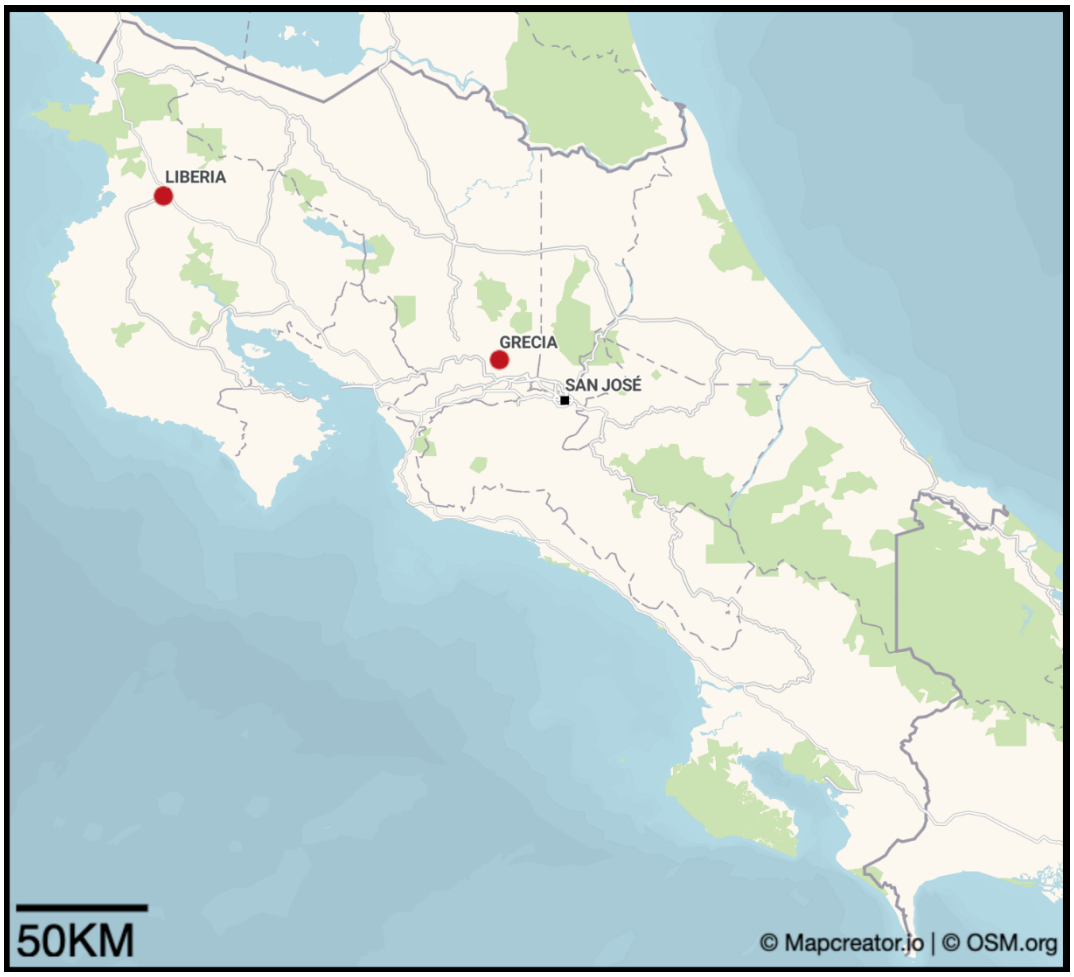
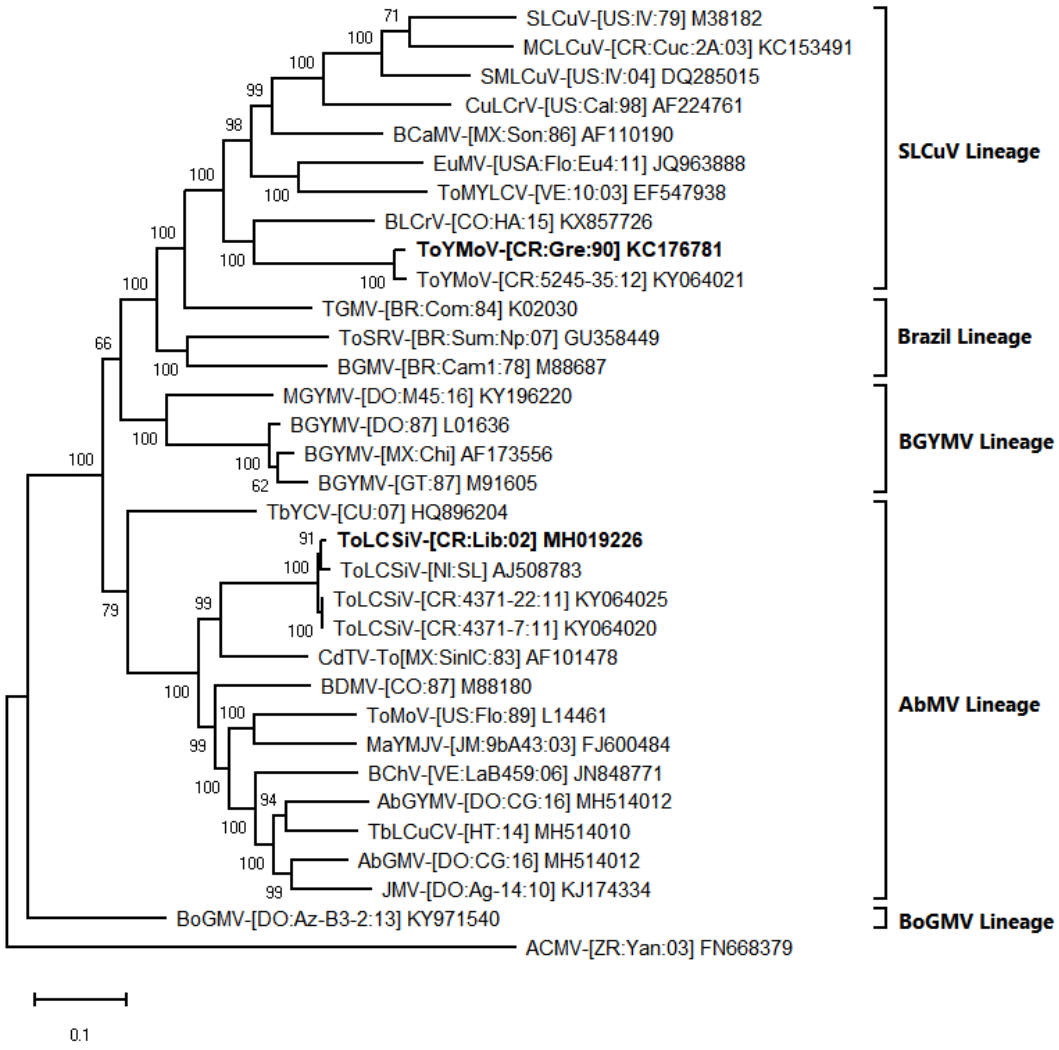


Fig. 3.6. Viral DNA accumulation in tomato (*Solanum lycopersicum* cv. Glamour) plants infected with the infectious cloned DNA-A and DNA-B components of an isolate of (A) tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:90]), (B) an isolate of tomato leaf curl Sinaloa virus from CR (ToLCSiV-[CR:Lib:02]) and (C) the multimeric infectious clone of tomato yellow leaf curl virus (TYLCV) from the Dominican Republic (TYLCV-[DO]) alone or in all combinations. Total DNA was extracted from newly emerged non-inoculated leaves at 7, 14 and 21 days after agroinoculation and used in qPCR tests with specific primers for each virus. Bars with the same letter correspond DNA amounts that do not differ statistically according to the Kruskal-Wallis test ($p < 0.05$).

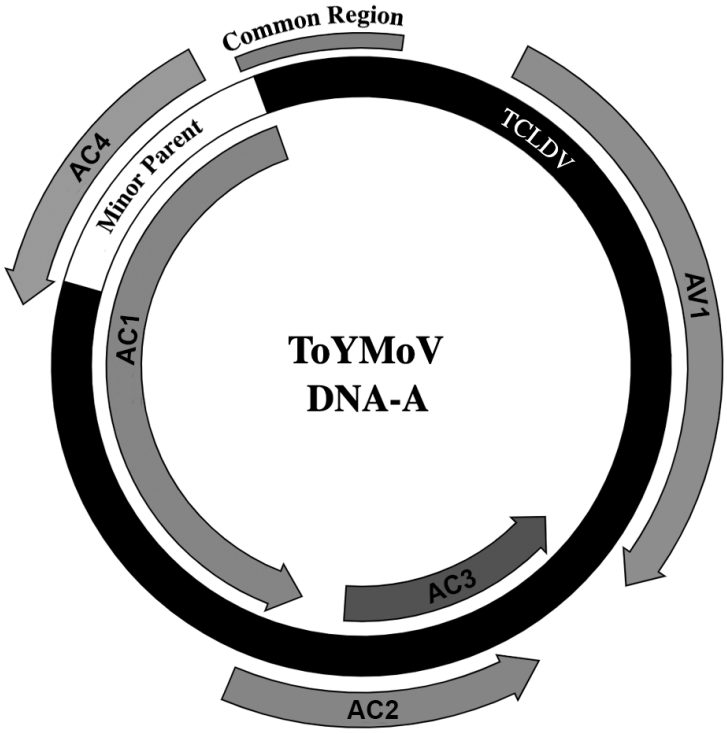


Supplemental Fig. S3.1. Map of Costa Rica showing the geographic locations where tomato plants with stunting, crumpling and yellow mosaic/mottle symptoms were collected from Grecia in 1990 and from Liberia in 2002.

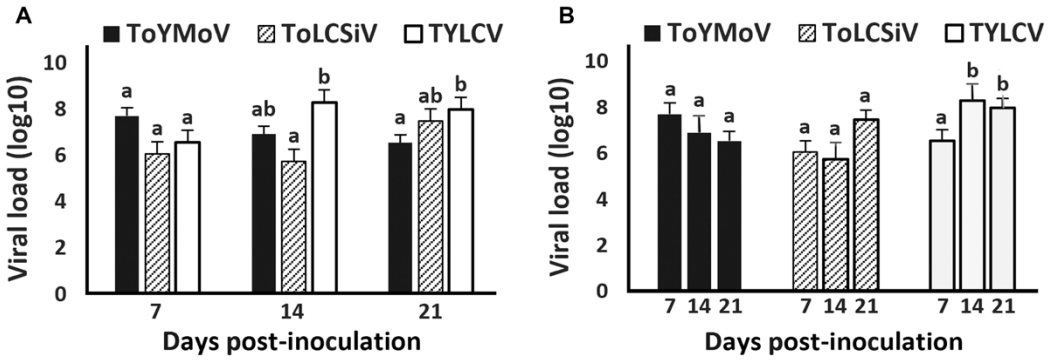


Supplemental Fig. S3.2. Bayesian phylogenetic consensus tree based on an alignment of complete nucleotide sequences of DNA-B components and showing the relationship of new isolates of tomato yellow leaf curl virus (ToYMoV-[CR:Gre:90]) and tomato leaf curl Sinaloa virus (ToLCSiV-[CR:Lib:02]) from Costa Rica (shown in bold) with: (i) previously characterized isolates of ToYMoV and ToLCSiV from CR and an isolate of ToLCSiV from Nicaragua; (ii) the ten most closely related begomoviruses based on a BLAST search; and (iii) selected begomoviruses from Latin America 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 (NW) begomoviruses (lineages are indicated with

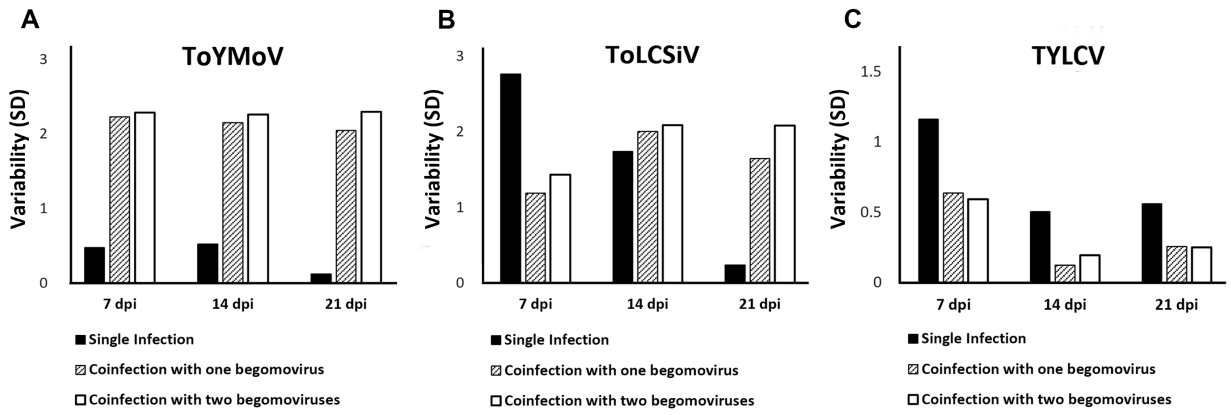
brackets). Sequences were obtained from GenBank, and virus abbreviations are as described in Brown et al. (2015). Branch strengths were evaluated by Bayesian posterior probabilities. The phylogenetic consensus tree was rooted with the sequence of the DNA-B component of the Old World bipartite begomovirus African cassava mosaic virus (ACMV). The length of horizontal branches indicates the rate of substitution per nucleotide.



Supplemental Fig. S3.3. Schematic representation of the recombination event identified in the DNA-A component of tomato yellow mottle virus (ToYMoV) from Costa Rica with the recombination detection program version 4.0 (RDP4). This strongly supported recombination event was 424 nucleotides (nts), spans nts 2056 to 2479 and includes the 5' end of the AC1 open reading frame (ORF), the entire overlapping AC4 ORF and the left side of the common region. This event is in the well-known recombination hot-spot in the begomovirus genomic DNA/DNA-A component. Furthermore, RDP4 indicated that the recombinant region came from an unidentified minor parent, and that the major parent was most similar to tomato chlorotic leaf distortion virus (TCLDV) from Venezuela (GenBank accession number JN241632). The CR is represented by a grey curved box and viral ORFs are represented by arrows.



Supplemental Fig. S3.4. Viral DNA accumulation in tomato (*Solanum lycopersicum* cv. Glamour) plants agroinoculated with the infectious cloned DNA-A and DNA-B components of tomato yellow mottle virus (ToYMoV), tomato leaf curl Sinaloa virus (ToLCSiV) and tomato yellow leaf curl virus (TYLCV) in single infections at 7, 14 and 21 days after agroinoculation. Comparisons were made as a function of virus (A) or as a function of time (B). Total DNA was extracted from newly emerged leaves (non-inoculated) at 7, 14 and 21 days after agroinoculation and used in qPCR assays with specific primers for each virus. Bars with the same letter correspond DNA amounts which do not differ statistically according to the Kruskal-Wallis test ($p < 0.05$).



Supplemental Fig. S3.5. Viral DNA variability in tomato (*Solanum lycopersicum* cv. Glamour) plants infected with the infectious cloned DNA-A and DNA-B components of an isolate of (A) tomato yellow mottle virus from Costa Rica (ToYMoV-[CR:Gre:90]), (B) an isolate of tomato leaf curl Sinaloa virus from CR (ToLCSiV-[CR:Lib:02]) and (C) the multimeric infectious clone of tomato yellow leaf curl virus (TYLCV) from the Dominican Republic (TYLCV-[DO]) alone or in all combinations. Total DNA was extracted from newly emerged non-inoculated leaves at 7, 14 and 21 days after agroinoculation and used in qPCR tests with specific primers for each virus.

Chapter IV

Weed-infecting viruses in a tropical agroecosystem pose different threats to crops and evolutionary histories

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Abstract

In the Caribbean Basin, malvaceous weeds commonly show striking golden/yellow mosaic symptoms. Leaf samples from *Malachra* sp. and *Abutilon* sp. plants with these symptoms were collected in Hispaniola from 2014 to 2020. PCR tests with degenerate primers revealed that all samples were infected with a bipartite begomovirus, and sequence analyses showed that *Malachra* sp. plants were infected with tobacco leaf curl Cuba virus (TbLCuCV), whereas the *Abutilon* sp. plants were infected with a new bipartite begomovirus, tentatively named Abutilon golden yellow mosaic virus (AbGYMV). Phylogenetic analyses showed that TbLCuCV and AbGYMV are distinct but closely related species, which are most closely related to bipartite begomoviruses infecting weeds in the Caribbean Basin. Infectious cloned DNA-A and DNA-B components were used to fulfilled Koch's postulates for these diseases of *Malachra* sp. and *Abutilon* sp. In host range studies, TbLCuCV also induced severe symptoms in *Nicotiana benthamiana*, tobacco and common bean plants; whereas AbGYMV induced few or no symptoms in plants of these species. Pseudorecombinants generated with the infectious clones of these viruses were highly infectious and induced severe symptoms in *N. benthamiana* and *Malachra* sp., and both viruses coinfected *Malachra* sp., thereby facilitating virus evolution via recombination and pseudorecombination. Together, our results suggest that TbLCuCV primarily infects *Malachra* sp. in the Caribbean

Basin, and occasionally spills over to infect and cause disease in crops; whereas AbGYMV is well-adapted to an *Abutilon* sp. in the Dominican Republic and has not been reported infecting crops.

Introduction

The genus *Begomovirus* (family *Geminiviridae*) is comprised of a large and diverse group of plant viruses that possess a circular, single-stranded (ss) DNA genome encapsidated into twin quasi-icosahedral virions (18 x 30 nm) (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Zerbini et al. 2017). These viruses infect dicotyledonous plants and cause numerous economically important diseases of fiber, fruit, ornamental and vegetable crops, mostly in tropical and subtropical regions of the world (Rojas et al. 2018). Begomoviruses are transmitted, plant-to-plant, by whiteflies of the *Bemisia tabaci* cryptic species complex (De Barro et al. 2011; Gilbertson et al. 2015; Zerbini et al. 2017; He et al. 2020).

The genome of begomoviruses is composed of either a single genomic DNA of ~ 2.8 kb (monopartite) or two ~2.6 kb DNA components (bipartite), designated as DNA-A and DNA-B (Rojas et al. 2005; Hanley-Bowdoin et al. 2013; Zerbini et al. 2017). The genomic DNA of monopartite begomoviruses is homologous to the DNA-A component of bipartite begomoviruses, and both are organized with overlapping virion (v)-sense and complementary (c)-sense genes transcribed in a bidirectional manner from an intergenic region (IR), which contains the *cis*-acting elements involved in replication and gene expression (e.g., replication-associated protein [Rep] high affinity binding sites [iterons], the origin of replication [*ori*] and two bidirectional RNA polymerase II promoters) (Hanley-Bowdoin et al. 2013). In bipartite begomoviruses, an ~200 nucleotide (nt) noncoding sequence is shared between cognate DNA-A and DNA-B components, and this common region (CR) maintains the specificity of replication for these components. Otherwise, the sequences of the DNA-A and DNA-B components are different, and both

components are needed for induction of typical disease symptoms (Hanley-Bowdoin et al. 2013; Briddon et al. 2010; Rojas et al. 2005).

In terms of begomovirus evolution, continental drift is believed to have separated ancestral monopartite and bipartite begomoviruses, resulting in the predominance of monopartite begomoviruses in the Old World (OW) and bipartite ones in the New World (NW). The subsequent independent diversification and evolution of OW and NW begomoviruses involved different combinations of mutation, recombination and acquisition and modification of foreign DNAs (Rojas et al. 2018; Lima et al. 2017; Duffy et al. 2009; Lefeuvre et al. 2015; Rojas et al. 2005). For OW monopartite begomoviruses, acquisition of satellite DNAs has played a major role in evolution, whereas acquisition and modification of the DNA-B component was essential for bipartite begomoviruses, and allowed for pseudorecombination to act as an additional mechanism of evolution (Rojas et al. 2005; Gilbertson et al. 1993; Hou et al. 1996; Briddon et al. 2010; Rojas et al. 2008; Silva et al. 2014; Garrido-Ramirez et al. 2000; Pita et al. 2001). Furthermore, the emergence of new begomoviruses has been facilitated by the global spread of the highly polyphagous *B. tabaci* species MEAM1, which can introduce mixtures of viral components/genomic DNAs into a diversity of plant species (Gilbertson et al. 2015; Rojas et al. 2008; Navas-Castillo et al. 2011; Rojas et al. 2018). Finally, human activities have led to the long-distance intercontinental movement of numerous begomoviruses, blurring the geographic separation of OW and NW begomoviruses (Gilbertson et al. 2015).

The remarkable diversification of begomoviruses has been reflected in the appearance of diseases of crop and non-cultivated plants in tropical and subtropical regions worldwide. In these agroecosystems, it is common to observe non-cultivated plants (mostly weeds) showing striking golden/yellow mosaic symptoms, which are commonly associated with begomovirus infection. In

the Caribbean Basin and other parts of Latin America, non-cultivated plants with these symptoms have been reported from species in the families Asteraceae, Capparaceae, Convolvulaceae, Euphorbiaceae, Fabaceae, Malvaceae, Nyctaginaceae and Solanaceae (Barreto et al. 2013; Blawid et al. 2013; da Silva et al. 2011; Ferro et al. 2017; Fiallo-Olivé et al. 2014; Hernández-Zepeda et al. 2007; Mar et al. 2017; Melgarejo et al. 2019, 2014; Melo et al. 2016; Nascimento et al. 2016; Passos et al. 2017; Pinto et al. 2016; Romay et al. 2016; Stewart et al. 2014; Tavares et al. 2012; Macedo et al. 2020). Importantly, characterization of begomoviruses associated with these diseases has revealed substantial genetic divergence from viruses that cause economically important crop diseases, although there are some exceptions such as the golden/yellow mosaic symptoms of *Malachra alceifolia* associated with tobacco leaf curl Cuba virus (TbLCuCV) infection in Jamaica (JM) (Hall et al. 2008), and mosaic and crumpling symptoms of *Nicandra physaloides* infected with tomato severe rugose virus in Brazil (Barbosa et al. 2009). This suggests that begomoviruses infecting crops and weeds have co-evolved independently with their hosts, with the practical implication that most of these symptomatic weeds are not major sources of inoculum for crop-infecting begomoviruses. However, these begomovirus-infected weeds can serve as a mixing vessels for evolution of viruses with the potential to infect crops (Silva et al. 2012; Melgarejo et al. 2014).

The family Malvaceae, commonly referred to as mallows, is comprised of >4225 species of annual and perennial plants (Christenhusz et al. 2016; Kubitzki 2014). Members of this family are distributed worldwide, and occur in temperate, tropical and subtropical regions (Fryxell 1997; Kubitzki 2014). Some species are important crops, such as cotton and okra (Fryxell 1997); others are grown as ornamentals or for medicinal purposes; and others are considered invasive weeds, e.g., *Abutilon* spp., *Sida* spp. and *Malachra* spp. (Fryxell 1997). Moreover, these malvaceous

weeds are commonly infected by begomoviruses and develop striking golden/yellow mosaic symptoms (Jeske et al. 1980; Torres-Herrera et al. 2019; Wyant et al. 2011; Paprotka et al. 2010; Roy et al. 2018; Ortega-Acosta et al. 2018; Hall et al. 2008; Melgarejo et al. 2019, 2014; Stewart et al. 2014).

As part of a long-term study to characterize begomoviruses causing golden/yellow mosaic symptoms in weeds and assesses the potential of these viruses to cause diseases of crop plants in the Dominican Republic (DO), we describe here the molecular and biological properties of two bipartite begomoviruses associated with these symptoms in *Malachra* sp. and *Abutilon* sp. plants on Hispaniola. Sequence and phylogenetic analyses together with infectivity studies with infectious clones were used to establish that the symptoms in *Malachra* sp. were caused by the crop-infecting bipartite begomovirus TbLCuCV, whereas those in *Abutilon* sp. were caused by a new species of weed-infecting begomovirus for which the name Abutilon golden yellow mosaic virus (AbGYMV) is proposed. Host range experiments showed that TbLCuCV also induced moderate to severe disease symptoms in *Nicotiana benthamiana*, tobacco (*N. tabacum*) and common bean plants (*Phaseolus vulgaris*) plants. In contrast, AbGYMV induced mild or no symptoms in these plants, indicating a high degree of adaptation to *Abutilon* sp. from the DO and low potential to cause crop diseases. TbLCuCV and AbGYMV are closely related species in the Abutilon mosaic virus (AbMV) lineage of NW begomoviruses and we present evidence that recombination and pseudorecombination play a role in the evolution of these viruses.

Materials and methods

Sample collection and DNA extraction

Leaf samples were collected from malvaceous weeds with golden/yellow mosaic symptoms (Supplemental Fig. S4.1) in disturbed areas around agricultural fields at five locations

on the island of Hispaniola from 2014 to 2020 (Supplemental Fig. S4.2). Sample M1 was collected in Port-au-Prince, Haiti (HT) in 2014; samples M2-M4 were collected from three locations in the DO in 2016: Juan Gomez (sample M2), Manzanillo (sample M3) and Cerro Gordo (sample M4); and samples M5-M10 were collected from two locations in the DO in 2020: Cerro Gordo (samples M5-M8) and Monte Cristi (samples M9-M10) (Supplemental Fig. S4.2). Note that Monte Cristi is ~1 to 2 km from the border with HT, in the northern part of Hispaniola.

Leaf pieces (~2 cm^2) of the M1 sample were ground in buffer and sap was applied to Agdia absorption strips (Agdia, Elkhart, IN, USA) as described by Melgarejo et al. (2014). The strips were dried overnight at room temperature and transported to the University of California at Davis (UC Davis). For samples M2 to M10, leaf tissue was squashed onto FTA Elute Micro Cards (Whatman). The FTA cards were kept at room temperature and transported to UC Davis. Total genomic DNA was extracted from the dried plant sap on the absorption strips or FTA cards as described by Dellaporta et al. (1983).

DNA barcode identification of malvaceous weeds

To determine the identity of the malvaceous weeds from which the samples were collected, the internal transcribed spacer (ITS) of the nuclear ribosomal DNA was amplified by the polymerase chain reaction (PCR) with the primer pair ITS-p5/ITS-u4 (Cheng et al. 2016). PCR-amplified fragments (~800 bp) were purified with the QIAquick gel extraction kit (Qiagen, Germantown, MD) and directly sequenced with the ITS-p5 and ITS-u4 primers at the UC Davis DNA Sequencing Facility.

Detection, characterization and cloning of begomovirus DNA components

To detect begomovirus DNA-A and DNA-B components, PCR tests were performed with the degenerate primer pairs PAL1v1978/PAR1c496 and PCRc1/PLB1v2040, respectively (Rojas

et al. 1993). PCR-amplified fragments were purified with the QIAquick gel extraction kit and directly sequenced with the PAL1v1978/PAR1c496 and PCRc1/PLB1v2040 primers.

To estimate the number and genetic diversity of begomovirus DNAs present in the samples and to identify single-cutting restriction enzymes for obtaining full-length clones, restriction fragment length polymorphism (RFLP) analyses of circular DNAs generated by rolling circle amplification (RCA) with Φ-29 DNA polymerase (TempliPhi; GE Healthcare, Piscataway, NJ) were performed (Inoue-Nagata et al. 2004). The RCA products were first digested with the four-base-cutting enzyme *Msp*I to generate RFLPs for estimating the number of begomovirus DNA components infecting the samples. Next, RCA products were digested with selected six-base-cutting enzymes to identify sites in each DNA component for obtaining full-length clones. The linearized DNA components were ligated into pGEM11Z (+) (Promega Corp., Madison, WI) or pSL1180 (Amersham Biosciences) digested with the appropriate enzyme. Recombinant plasmids having the full-length DNA-A and DNA-B components were identified by restriction enzyme digestion and DNA sequence analyses. Based upon sequencing and RCA results, the begomovirus isolates from the M1 and M4 samples were selected for further studies. Thus, full-length DNA-A and DNA-B clones were obtained from sample M1 (in plasmids pM-GV1-HT-A and pM-GV1-HT-B), sample M2 (in plasmids pM-GV2-JG-A and pM-GV2-JG-B), sample M3 (in plasmids pM-GV3-M-A and pM-GV3-M-B) and sample M4 (in plasmids pAb-GV4-CG-A and pAb-GV4-CG-B).

Sequence analyses

The complete sequences of the cloned full-length DNA-A and DNA-B components of the bipartite begomoviruses from samples M1-M4 were determined and analyzed with Vector NTI advance software (Invitrogen, Carlsbad, CA). A BLASTn search was initially performed to

identify sequences in GenBank with highest identities (Benson et al. 2018). Pairwise nt sequence alignments were performed with MUSCLE within the Species Demarcation Tool (SDT) v.1.2, and with full-length DNA-A and DNA-B sequences of the ten begomoviruses with the highest identities revealed by the BLASTn search (Muhire et al. 2014; Altschul et al. 1990). The Vector NTI advance software was used to make more extensive comparisons, including individual open reading frames (ORFs) and non-translated regions (NTRs) from both components. The *cis*-acting elements involved in begomovirus replication (i.e., iterons and the Rep iteron-related domains [IRDs]) were identified as described in Argüello-Astorga and Ruiz-Medrano (2001).

Phylogenetic analyses

For the phylogenetic analyses, we used the complete nt sequences of the DNA-A and DNA-B components of: (i) the bipartite begomoviruses from the M1-M4 samples; (ii) TbLCuCV isolates from CU (the DNA-A and DNA-B of one isolate and the DNA-A of two other isolates); (iii) the ten most identical viruses revealed by the BLASTn search; and (iv) selected viruses representing the AbMV, Brazil, squash leaf curl virus (SLCuV), bean golden yellow mosaic virus (BGYMV) and Boerhavia golden mosaic virus (BoGMV) lineages of NW begomoviruses. Multiple sequence alignments (MSA) for the DNA-A and DNA-B component sequences were generated with the MAFFT algorithm implemented in the Guidance2 Server (Sela et al. 2015; Katoh 2002). 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. Phylogenetic 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 begomovirus tomato yellow leaf curl virus (TYLCV) and the DNA-B component of the OW bipartite begomovirus African cassava mosaic virus (ACMV), respectively.

Recombination analysis

Preliminary datasets of complete sequences of 584 DNA-A and 240 DNA-B components were assembled. This included the complete nt sequences of the DNA-A and DNA-B components of: (i) the bipartite begomoviruses from the M1-M4 samples; (ii) the TbLCuCV isolates from CU; and (iii) sequences of selected viruses retrieved from GenBank. SDT and the Recombination Detection Program version 4.0 (RDP4) (Martin et al. 2015) were used to remove sequences that were identical or having nt sequence identities <70%. Final datasets of complete sequences of 488 DNA-A and 201 DNA-B components were used for recombination analyses. MSA were generated with MUSCLE within MEGA X (Edgar 2004; Kumar et al. 2018), and the alignments were manually edited and exported as FASTA files. Detection of recombination breakpoints and identification of potential parental viruses were performed with RDP4. The recombination analysis was performed with default settings and a Bonferroni-corrected *p*-value cut-off of 0.05. Only recombination events detected with three or more methods coupled with significant phylogenetic support were considered *bona fide* events.

Production of multimeric clones and agroinoculation systems

Based upon sequencing and RCA-RFLP results, the begomovirus isolates from the M1 and M4 samples were selected for infectivity studies. Here, multimeric clones of the DNA-A and DNA-B components were generated in the pCAMBIA 1300 binary vector (Hajdukiewicz et al. 1994). For the DNA-A component of the M1 isolate, an ~1.6 kb *Sal*I-*Hin*dIII fragment containing the CR was cloned into pCAMBIA 1300 to generate the 0.6-mer pM-GV1-HT-A0.6. The full-length DNA-A monomer was released with *Hin*dIII from pM-GV1-HT-A and cloned into the *Hin*dIII-digested pM-GV1-HT-A0.6 to generate pM-GV1-HT-A1.6. For the DNA-B component, an ~1.5 kb *Sac*I-*Xba*I fragment containing the CR was cloned into pCAMBIA 1300 to generate the 0.6-mer pM-GV1-HT-B0.6. The full-length DNA-B monomer was released with *Xba*I from pM-GV1-HT-B and cloned into the *Xba*I-digested pM-GV1-HT-B0.6 to generate pM-GV1-HT-B1.6.

For the DNA-A component of the M4 isolate, an ~2.1 kb *Eco*RI fragment containing the CR was cloned into pCAMBIA 1300 to generate the 0.8-mer pAb-GV4-CG-A0.8. The full-length DNA-A monomer was released with *Bgl*II from pAb-GV4-CG-A and cloned into the *Bgl*II-digested pAb-GV4-CG-A0.8 to generate pAb-GV4-CG-A1.8. For the DNA-B component, an ~1.2 kb *Bam*HI fragment containing the CR was cloned into pCAMBIA 1300 to generate the 0.5-mer pAb-GV4-CG-B0.5. The full-length DNA-B monomer was released with *Bam*HI from pAb-GV4-CG-B and cloned into the *Bam*HI-digested pAb-GV4-CG-B0.5 to generate pAb-GV4-CG-B1.5. Recombinant plasmids having the multimeric clones were identified for each component by restriction enzyme digestion. Selected plasmids with the confirmed multimeric clones were then transformed into electrocompetent *Agrobacterium tumefaciens* cells (strain C58C1) by electroporation.

Infectivity and host range experiments

The infectivity of the cloned DNA components was initially assessed by agroinoculation of *N. benthamiana* plants at the three- to five-leaf-stage (3 weeks old). Plants were agroinoculated with mixtures of *A. tumefaciens* cell suspensions (optical density of 600 nm = 1.0) of strains containing binary plasmids with the multimeric DNA-A and DNA-B clones of the M1 and M4 isolates by needle puncture inoculation of the stem just beneath the shoot apex (Hou et al. 1998). A host range experiment was conducted by agroinoculation of *Malachra* sp. and *Abutilon indicum* and *Abutilon* sp. plants (from seeds collected from the DO), *N. benthamiana*, *N. tabacum* cvs. Havana and Turkish, *N. glutinosa, Solanum lycopersicum* cv. Glamour, *Datura stramonium, Chenopodium amaranticolor, Cucurbita maxima* cv. Sugarpie and *P. vulgaris* cv. Topcrop. The negative control was equivalent plants of these species agroinoculated with cell suspensions of an *A. tumefaciens* strain containing the empty vector (pCAMBIA 1300). Infectivity in pepper (*Capsicum annuum* cv. Cayenne) was determined by particle bombardment inoculation with the multimeric DNA-A and DNA-B clones of each isolate, as described in Paplomatas et al. (1994). The positive control was equivalent plants bombarded with the multimeric DNA-A and DNA-B clones of the pepper-infecting bipartite begomovirus pepper huasteco yellow vein virus (PHYVV), whereas the negative control was plants bombarded with gold particles alone.

Inoculated plants were maintained in a controlled environment chamber as described by Melgarejo et al. (2014). Symptom development was assessed visually and recorded at 14 days post inoculation (dpi). In selected symptomatic and all non-symptomatic 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 for each virus (Supplemental Table S4.1).

Pseudorecombination experiments with cloned DNA components of TbLCuCV and AbGYMV

These experiments were performed by agroinoculating *N. benthamiana* and *Malachra* sp. plants with all combinations of the multimeric DNA-A or DNA-B clones of the M1 and M4 isolates. The negative control was equivalent plants agroinoculated with the empty vector. 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 primers for each virus (Supplemental Table S4.1).

Co-infection studies with TbLCuCV and AbGYMV

For these experiments, *Malachra* sp. seedlings were co-agroinoculated with a mixture of cell suspensions of *A. tumefaciens* strains containing binary plasmids with the multimeric DNA-A or DNA-B clones of the M1 and M4 isolates. Control plants were agroinoculated with the DNA-A and DNA-B components of the M1 or M4 isolates or with the empty vector. Inoculated plants were maintained in a controlled environment chamber, and symptom development was assessed as previously described. The presence of the inoculated DNA components in newly emerged leaves was determined by PCR tests with component-specific primers for each virus (Supplemental Table S4.1).

Results

DNA barcode identification of malvaceous weeds

Samples of leaves with golden/yellow mosaic symptoms typical of begomovirus infection were collected from two types of malvaceous weeds: a large plant (height of 1 to 2 m) with malva-like leaves (samples M1 from HT and M2 and M3 from the DO, Supplemental Fig. S4.1A), and a

smaller shrub-like plant (height of 0.5 to 1 m) with heart-shaped leaves (samples M4 to M10 from the DO, Supplemental Fig. S4.1B). In PCR tests with the plant-specific ITS primer pairs, the expected-size ~0.8 kb fragment was amplified from samples M1-M10. BLASTn analyses revealed that the ITS sequences from samples M1-M3 (large-size plant) had highest identities (94%) with those of *Malachra* spp., whereas those of samples M4-M10 (smaller-size plant) had highest identities (94%) with those of *Abutilon* spp. Thus, these results indicated that the large malva-like weed was *Malachra* sp., whereas the smaller shrub-like malvaceous weed was *Abutilon* sp.

Detection, characterization and cloning of begomovirus DNA components

In PCR tests with degenerate DNA-A and DNA-B primer pairs, the expected-sized ~1.1- and ~0.5-kb DNA fragments, respectively, were amplified from samples M1-M10, indicating infection with a bipartite begomovirus. Based upon BLASTn analyses, the sequences of the PCR-amplified DNA-A and DNA-B fragments from the M1-M3 samples were >95% identical to each other and had highest identities (>96% for DNA-A and >94% for DNA-B sequences) with sequences of isolates of TbLCuCV from CU and JM. The sequences of the PCR-amplified DNA-A and DNA-B fragments from the M4-M10 samples were >95% identical to each other and had highest identities (~86% for DNA-A and ~85% for DNA-B sequences) with sequences of the TbLCuCV isolates and various weed-infecting begomoviruses from the Caribbean Basin (data not shown). Furthermore, *Msp*I digestion of the RCA products generated from these samples yielded fragments that totaled ~5.2 kb, indicating infection with a single bipartite begomovirus (data not shown). Taken together, these results suggest that the *Malachra* sp. plants with golden/yellow mosaic symptoms were infected with variants of TbLCuCV, whereas the *Abutilon* sp. plants with these symptoms were infected with variants of a putative new begomovirus species.

The M1-M3 isolates from *Malachra* sp. and the M4 isolate from *Abutilon* sp. were selected for further characterization. For the M1 sample, full-length (~2.7 kb) linear ds DNA-A and DNA-B components were generated with RCA products with *Hin*dIII and *Xba*I, respectively, and cloned; for samples M2 (Juan Gomez) and M3 (Manzanillo) with *Hin*dIII and *Sal*I, respectively; and for sample M4 (Cerro Gordo) with *Bgl*II for both components.

Genomic properties of full-length begomovirus DNA components

The complete sequences of the cloned full-length DNA-A and DNA-B components of the M1-M4 isolates were determined. The DNA-A and DNA-B components of the M1 isolate from HT are 2,611 (GenBank accession number MH514009) and 2,567 nt (GenBank accession number MH514010), respectively; those of the M2 and M3 isolates from Juan Gomez (DO) and Manzanillo (DO) are 2,610 (GenBank accession number MK059404) and 2,565 nt (GenBank accession number MK059405) and 2,609 (GenBank accession number MK059402) and 2,565 nt (GenBank accession number MK059403), respectively; and those of the M4 isolate from Cerro Gordo (DO) are 2,638 (GenBank accession number MH514011) and 2,585 nt (GenBank accession number MH514012), respectively.

The genome organization of the DNA-A and DNA-B components of the begomovirus isolates of the M1-M4 samples is typical of NW bipartite begomoviruses, i.e., the DNA-A is ~2.6 kb and encodes five ORFs, one in the v-sense strand (AV1) encoding the capsid protein (CP), and four in the c-sense strand (AC1, AC2, AC3, and AC4) encoding the Rep, the transcriptional activator protein (TrAP), the replication enhancer (REn) and the AC4 protein, respectively. Additionally, the CP and REn amino acid (aa) sequences of all four isolates possess the N- and C-terminal motifs PWRpMAGT and AVRFATdR (lowercase indicates variable aa residues), respectively, which are characteristic of NW begomoviruses (Harrison et al. 2002; Melgarejo et

al. 2014; Mauricio-Castillo et al. 2014). The DNA-B components has two ORFs, one in the v-sense strand (BV1) that encodes the nuclear shuttle protein (NSP), and one on the c-sense strand (BC1) encoding the movement protein (MP).

SDT analysis of the sequences of the complete DNA-A component of the M1-M3 isolates from *Malachra* sp. revealed highest identities (>96%) with isolates of TbLCuCV from CU (Supplemental Table S4.2). Similar results were obtained for the complete DNA-B component sequences, i.e., identities of >94% with an isolate of TbLCuCV from CU. Comparisons made with the nt and aa sequences of individual ORFs revealed similar identities (>94%) across all ORFs, whereas CR identities range from ≥92 to 98% and identities for the hypervariable region (HVR) of the DNA-B component range from ≥91 to 93% (Supplemental Table S4.2). The next highest identities for the DNA-A component sequence were with NW bipartite begomoviruses from Latin America, including tobacco mottle leaf curl virus (87%), Sida yellow mottle virus (SiYMoV) (87%) and Wissadula golden mosaic virus (WGMV) (87%) (Supplemental Table S2). In the case of the DNA-B component sequence, the next highest identities were with the M4 isolate from *Abutilon* sp. (82%) and WGMV (81%) (Supplemental Table S4.2). These results confirmed that *Malachra* sp. plants with golden/yellow mosaic symptoms from which the M1-M3 samples were collected were infected with variants of TbLCuCV. These new isolates are named tobacco leaf curl Cuba virus-[Haiti:2014] (TbLCuCV-[HT:14]), tobacco leaf curl Cuba virus-[Dominican Republic:Juan Gomez:2016] (TbLCuCV-[DO:JG:16]) and tobacco leaf curl Cuba virus-[Dominican Republic:Manzanillo:2016] (TbLCuCV-[DO:M:16]).

The SDT analysis performed with the complete DNA-A component sequence of the M4 isolate from *Abutilon* sp. revealed highest identities (85 to 86%) with those of the isolates of TbLCuCV from Hispaniola (present study) and CU and slightly lower identities with NW weed-

infecting bipartite begomoviruses from Latin America, including Jatropha mosaic virus (85%), WGMV (85%) and Sida golden mosaic virus (84%) (Supplemental Table S3). Given that the current species demarcation value for new begomovirus species is <91% nt sequence identity for the complete DNA-A sequence (Brown et al. 2015), the M4 isolate represents a new bipartite begomovirus species, for which the name Abutilon golden yellow mosaic virus-[Dominican Republic:Cerro Gordo:2016] (AbGYMV-[DO:CG:16]) is proposed. For the DNA-B component sequence, the highest identities also were with those of the TbLCuCV isolates from CU (82%) and with weed-infecting viruses from the Caribbean Basin, including WGMV (81%) and SiYMoV (81%). The CR sequences had the highest identities (85-88%) with those of TbLCuCV isolates from Hispaniola and CU and with JMV (86%). The HVR of the DNA-B component was the most divergent region of the genome, having highest identities of 54 to 67% with tomato yellow leaf distortion virus and Corchorus yellow spot virus (Supplemental Table S4.3). Comparisons of the nt and aa sequences of individual ORFs of both components revealed a wide range of identities, some of which were lower than those expected for closely related species. For example, whereas the AC1, AC2, AC3 and BV1 ORFs had similar levels of identity for the nt and aa sequences, nt identities for the AV1 and BC1 ORFs were considerably lower (84 to 90%) than those for aa sequences (88 to 97%). In contrast, nt identities for the AC4 ORF were higher (78 to 86%) than those for aa sequences (54 to 68%) (Supplemental Table S3).

Analyses of the CR sequences of TbLCuCV and AbGYMV

The DNA-A and DNA-B components of the three new isolates of TbLCuCV and the isolate of AbGYMV share a CR of ~200 nt, with identities ranging from 90-98% as expected for cognate components. These CR sequences contain the *cis*-acting elements implicated in virus replication and gene expression, e.g., the conserved stem-loop structure with the nonanucleotide sequence

TAATATT↓AC and the canonical AC1 TATA box and G-box, which interact with the TATA-binding protein and the G-box transcription factor, respectively (Fig. 4.1) (Eagle et al. 1997; Fontes et al. 1994; Hanley-Bowdoin et al. 2013). The Rep high affinity-binding site (iterons) in the CR is typically organized with a direct repeat and a single upstream inverted repeat of a 5 nt core sequence motif GGN1N2N3 (Argüello-Astorga et al. 2001), which are specifically recognized by the iteron-related domain (IRD) located in the N-terminus region of the Rep protein, which has a canonical $X_{-n}...X_{-2}\ X_{-1}\ F\ X_1\ X_2\ X_3$ motif where X_{-n} is the first residue of the motif and F is a highly conserved phenylalanine residue (Argüello-Astorga et al. 2001).

The CR sequences of the TbLCuCV isolates from CU and Hispaniola have nearly identical Rep binding sites, which are organized with two direct repeats of the GGGGG core and an upstream inverted repeat CCCCC (Fig. 4.1). The N1 position of the first iteron (GGN1GG) was variable (N1 = G, A or T), whereas the second iteron (GGGGG) was invariant among TbLCuCV isolates. The IRD of these TbLCuCV isolates is MPRK**GSSIAN** (key amino acids shown in bold), which is atypical in (i) lacking the highly conserved F residue, instead having a serine (S) residue (underlined) (Fig. 4.1), and (ii) not predicted to recognize the GGGGG core sequence (Argüello-Astorga et al. 2001).

The Rep high affinity binding site in the CR of AbGYMV also consist of two direct repeats of the GGGGG core and an upstream inverted repeat CCCCC (Fig. 4.1). The Rep IRD aa sequence is MPRK**GSFSIK**, which possesses the conserved F residue and is predicted to recognize the GGGGG core sequence (Argüello-Astorga et al. 2001). Finally, it is worth noting that the portion of the TbLCuCV and AbGYMV CR sequences that lies between the inverted repeat and G-box (104 nt) was highly divergent (41%).

Phylogenetic analyses

In the phylogenetic tree generated with the complete DNA-A sequences, the TbLCuCV isolates from Hispaniola formed a strongly supported clade with the isolates from CU. Within this clade there was evidence of genetic divergence between isolates from CU and Hispaniola, consistent with geographical separation (note that complete DNA-A sequences for TbLCuCV isolates from JM are not available) (Fig. 4.2). In this tree, AbGYMV was placed on a distinct branch (sister clades), which was included in a larger strongly supported clade with the TbLCuCV isolates. This clade was part of the larger C1 clade of the AbMV lineage, which includes mostly weed-infecting begomoviruses from the Caribbean Basin (Fig. 2), whereas the other large clade (C2) included crop- and weed-infecting begomoviruses from many countries of Latin America (Fig. 4.2).

The phylogenetic tree generated with the complete DNA-B sequences revealed a similar overall topology, but with some notable differences. The TbLCuCV isolates from Hispaniola and CU were placed in a strongly supported clade in the AbMV lineage (Supplemental Fig. S4.3). In contrast to the DNA-A tree, AbGYMV did not form a sister clade with the TbLCuCV isolates, but was placed together with the TbLCuCV isolates and other weed-infecting bipartite begomoviruses from the Caribbean Basin in the strongly supported C1 clade of the AbMV lineage (Supplemental Fig. S4.3).

In the DNA-B tree, the C2 clade included viruses from North and Central America and the Caribbean Basin, whereas more distantly related viruses from South America were placed in a paraphyletic group (C3 clade) (Supplemental Fig. S4.3). Finally, whereas the DNA-A tree clearly separates the BGYMV, Brazil, SLCuV and BoGMV lineages, these clades clustered together in a larger clade in the DNA-B tree (compare Fig. 4.2 and Supplemental Fig. S4.3). Taken together

with the SDT analysis and sequence comparisons, the results of the phylogenetic analyses are consistent with TbLCuCV and AbGYMV representing distinct but closely related species, which are most closely related to NW bipartite begomovirus species infecting weeds in the Caribbean Basin.

Recombination analysis

RDP4 analysis did not reveal evidence of recombination in the DNA-A and DNA-B components of the TbLCuCV isolates from Hispaniola and CU. For AbGYMV, a single recombination event of 625 nt was detected in the DNA-A component, whereas no recombination was not detected in the DNA-B component. The recombination event in the AbGYMV DNA-A component was strongly supported by six methods implemented in RDP4 (*p*-values of 9.178×10^{-15}, 3.563×10^{-17}, 1.059×10^{-09}, 3.883×10^{-10}, 1.462×10^{-19} and 9.246×10^{-05} for the RDP, GENECONV, MaxChi, Chimaera, SiScan and 3Seq recombination methods, respectively). This region spans nts 1994 to 2618, which includes the 5' end of the AC1 ORF, the entire AC4 ORF and the left side of the CR (Supplemental Fig. S4.4). Thus, this event is in the well-known recombination hot-spot in the begomovirus genomic DNA/DNA-A component (Hou et al. 1996; Lefeuvre et al. 2009; Padidam et al. 1999), and explains the higher levels of genetic divergence detected in this region (Supplemental Table S4.3). The RDP4 analysis further indicated that the recombinant region was derived from an uncharacterized minor parent, whereas the major parent was tomato yellow leaf distortion virus (GenBank accession number FJ174698).

Infectivity and host range experiments

In a preliminary experiment, *N. benthamiana* plants agroinoculated with the multimeric cloned DNA-A and DNA-B components of TbLCuCV-[HT:14] were stunted and newly emerged leaves showed epinasty, crumpling, deformation, mosaic and vein yellowing by 14 dpi (Fig. 4.3A).

In the host range experiment, the infectious cloned DNA-A and DNA-B components of TbLCuCV induced stunting and golden/yellow mosaic in newly emerged leaves of all agroinoculated *Malachra* sp. plants by 14 dpi (Fig. 4.3B). These symptoms were similar to those observed in *Malachra* sp. plants in the field in Hispaniola (Supplemental Fig. S4.1A), thereby fulfilling Koch's postulates for the golden/yellow mosaic disease of *Malachra* sp. TbLCuCV also induced stunting and epinasty and crumpling of newly emerged leaves of agroinoculated *N. tabacum* (cvs. Havana and Turkish) and *N. glutinosa* plants, and stunting and epinasty, deformation, chlorosis and mosaic of newly emerged leaves of agroinoculated common bean (cv. Topcrop) plants by 14 dpi (Fig. 4.3C). *D. stramonium* plants agroinoculated with TbLCuCV developed chlorotic spots in newly emerged leaves, whereas symptomless DNA-A and DNA-B infections were detected in some (<50%) agroinoculated tomato plants by 14 dpi (Table 4.1). TbLCuCV did not infect Cayenne long pepper (inoculated by particle bombardment), pumpkin and *C. amaranticolor* plants.

N. benthamiana plants agroinoculated with the multimeric cloned DNA-A and DNA-B components of AbGYMV were stunted and developed mild symptoms of epinasty and crumpling in newly emerged leaves and no obvious mosaic or vein yellowing by 14 dpi (Table 4.1, Fig. 4.3D). These symptoms became progressively milder by 21 dpi (data not shown). In the host range experiment all *Abutilon* sp. plants (derived from seeds collected in the DO) agroinoculated with the infectious DNA-A and DNA-B components of AbGYMV were stunted and developed epinasty and striking golden/yellow mosaic of newly emerged leaves by 14 dpi (Fig. 4.3E). Moreover, these symptoms were similar to those observed in *Abutilon* sp. plants in the DO (Supplemental Fig. S4.1B), thereby fulfilling Koch's postulates for the golden/yellow mosaic disease of *Abutilon* sp. in the DO. In contrast, agroinoculated *Malachra* sp. plants developed no symptoms and only a small number of plants had DNA-A only infections (Table 4.1). AbGYMV induced mild upward

leaf curling symptoms in *N. glutinosa*, and very mild symptoms of leaf epinasty in common bean by 14 dpi (Table 4.1 and Fig. 4.3F). Symptomless DNA-A and DNA-B infections were detected in agroinoculated *N. tabacum* (cvs. Havana and Turkish) and *D. stramonium* plants, whereas symptomless DNA-A only infections were detected in some tomato by 14 dpi (Table 4.1). AbGYMV did not infect Cayenne long pepper, pumpkin, *C. amaranticolor* and *A. indicum* plants.

In all these experiments, the presence of the inoculated DNA-A and DNA-B components was confirmed in newly emerged leaves of representative symptomatic and in all non-symptomatic plants by PCR tests with component-specific primers (Supplemental Table S4.1). Plants agroinoculated with the empty vector or bombarded with gold particles alone did not develop symptoms and were negative for the TbLCuCV/AbGYMV DNA-A and DNA-B components.

Pseudorecombination experiments with cloned DNA components of TbLCuCV and AbGYMV

To further investigate the relationship between TbLCuCV and AbGYMV, pseudorecombination experiments were conducted in *N. benthamiana* and *Malachra* sp. plants (note that *Abutilon* sp. seeds from the DO were not available for these experiments) (Table 4.2). In *N. benthamiana*, pseudorecombinants (PRs) formed with the TbLCuCV DNA-A (TA) and AbGYMV DNA-B (AbB) or AbGYMV DNA-A (AbA) and TbLCuCV DNA-B (TB) were highly infectious (100%) and induced severe symptoms by 14 dpi. The TA + AbB PR induced epinasty, crumpling, deformation, mosaic and vein yellowing symptoms, which were more similar to those induced by the TbLCuCV parent (compared Fig. 4.4A with Fig. 4.4C). In contrast, the AbA + TB PR induced mostly epinasty and crumpling symptoms, which were more similar to those induced by the AbGYMV parent (compared Fig. 4.4B with Fig. 4.4D). Thus, the symptoms induced by these PRs were associated with the source of the DNA-A component. Furthermore, the symptoms

induced by both PRs were more severe than those induced by the AbGYMV parent (compared Fig. 4.4B with Fig. 4.4C and 4.4D). Taken together, these results suggest an important role for the DNA-A component in symptom development in this host.

In equivalent experiments conducted in *Malachra* sp., both PRs were infectious, but at lower rates than in *N. benthamiana*. Furthermore, the PRs differed markedly in infectivity, with the TA + AbB PR having an infection rate of 80%, whereas that of the AbA + TB was only 22%. The symptoms induced by these PRs were different compared with those induced by the parental viruses. Thus, both PRs induced more severe symptoms than those induced by the AbGYMV parent (compared Fig. 4.4G and 4.4H with Fig 4.4F). Furthermore, the TA + AbB PR induced epinasty, crumpling and deformation, but little yellow mosaic (Fig 4.4G); whereas the AbA + TB PR induced epinasty, crumpling, deformation as well as yellow mosaic by 14 dpi (Fig. 4.4H). These results suggest an important role for the DNA-A component in infectivity and a role for the DNA-B component in symptom development in *Malachra* sp.

In PCR tests with component-specific primers, the inoculated DNA components were detected in newly emerged leaves of all symptomatic plants. Together, these results established that the components of these viruses are interchangeable, consistent with the conservation of critical CR sequences (e.g., iterons) and their close phylogenetic relationship (Fig. 4.1 and 4.2). Moreover, infectivity and symptoms were host-dependent, involved both components and revealed evidence of differential adaptation of these viruses.

Co-infection studies with TbLCuCV and AbGYMV

Malachra sp. plants were co-agroinoculated with the infectious cloned DNA-A and DNA-B components of TbLCuCV and AbGYMV (four component inoculation) to determine if these viruses can co-infect this species. Here, 80% of *Malachra* sp. plants co-agroinoculated with these

components were stunted and newly emerged leaves showed epinasty, crumpling, deformation and golden/yellow mosaic by 14 dpi (Table 4.3). These symptoms were indistinguishable from those induced by the TbLCuCV DNA-A and DNA-B components in *Malachra* sp. (Fig. 4.3B). PCR tests with component-specific primers revealed that all of the symptomatic plants were infected with the TbLCuCV DNA-A and DNA-B components, whereas the AbGYMV DNA-A and DNA-B components were detected in 22% and 33% of these plants, respectively. These results indicate that TbLCuCV and AbGYMV can co-infect *Malachra* sp. and that TbLCuCV may enhance infectivity of AbGYMV in *Malachra* sp., but there was no evidence of synergism in terms of disease symptoms.

Discussion

In the present study, we determined the etiology of golden/yellow mosaic diseases of two malvaceous weed species in Hispaniola, as part of a long-term project to characterize begomoviruses infecting weeds and determine their potential to cause diseases of crop plants in the DO. We used DNA barcoding to identify the larger malva-like weed as *Malachra* sp. and the smaller bushy plant as *Abutilon* sp. *Malachra* sp. is an invasive weed found in association with irrigation ditches and disturbed areas around agricultural fields in Hispaniola and other countries of the Caribbean Basin, whereas *Abutilon* sp. also occurs in this environment, but was less common based in our surveys.

Infection of *Malachra* sp. by TbLCuCV in HT and the DO is the first report of this virus in these countries, and extends on a previous report of TbLCuCV infecting *M. alceifolia* in JM (Hall et al. 2008). Together with reports of TbLCuCV infecting tobacco and common bean in CU Morán et al. 2006; Leyva et al. 2016), it appears that this virus is widely distributed in the Caribbean Basin, where it infects crops and weeds. Infectivity and host range experiments with

infectious clones confirmed that TbLCuCV induces golden/yellow mosaic symptoms in *Malachra* sp. and stunting and epinasty, crumpling and mosaic/mottling in tobacco and common bean, thereby fulfilling Koch's postulates for these diseases. This raises the question of what is the prevalent host of this virus in the Caribbean Basin, and several lines of evidence suggest it may be *Malachra* sp. First, TbLCuCV has now been detected in *Malachra* spp. in multiple countries in the Caribbean Basin, whereas disease outbreaks in crops (tobacco and common bean) have only been reported from CU and seem to be sporadic (Leyva et al. 2016; Morán et al. 2006). Second, TbLCuCV is most closely related to other weed-infecting bipartite begomoviruses from the Caribbean Basin (Fig. 2), consistent with evolution of these viruses from a common ancestor and adapted to non-cultivated (weed) species. Third, numerous weed-infecting begomoviruses have been reported to also infect and cause disease symptoms in crop plants under laboratory conditions, but such diseases are rarely observed in the field (Domínguez et al. 2009; Morán et al. 2006; Melgarejo et al. 2014; Barbosa et al. 2011; Blawid et al. 2013; Pramesh et al. 2013; Tahir et al. 2015). In this scenario, *Malachra* sp. is the prevalent host of TbLCuCV and, under certain conditions, e.g., high whitefly populations, the virus can spill over into crops and causes disease outbreaks. Thus, although TbLCuCV can infect crop species, it is not a *bona fide* crop-infecting begomovirus such as BGYMV, which is highly adapted to common bean and is rarely detected in weeds (Gilbertson 1991). Finally, we hypothesize that the golden/yellow mosaic disease of *Malachra* sp. caused by TbLCuCV also occurs in CU and may be the source of inoculum for outbreak in crops.

A different disease etiology and biology was determined for the golden/yellow mosaic disease of *Abutilon* sp. in the DO. Here, we identified a new bipartite begomovirus, AbGYMV, in samples of plants with these symptoms collected in two location in the DO. We further showed

that the infectious clones of this virus DNA components of this virus induced striking golden/yellow mosaic symptoms in plants of *Abutilon* sp. from the DO, thereby fulfilling Koch's postulates for this disease. Furthermore, host range experiments showed that AbGYMV is highly adapted to this species of *Abutilon* sp. from the DO, as it induced mild or no symptoms in other species tested, e.g., *N. benthamiana*, common bean, tobacco, *Malachra* sp., and *A. indicum* from India. These results may indicate a long period of virus-host co-evolution, with the interaction having reached an equilibrium. Evidence for this comes from the nature of the disease symptoms, which were mostly striking golden/yellow mosaic with little or no distortion, crumpling or curling of leaves. A similar situation has been described for the striking golden/yellow mosaic symptoms induced by AbMV in *A. hybridum* (Jeske et al. 1980; Valverde et al. 2012). AbMV is the NW bipartite begomovirus for which the AbMV lineage was named and it originated in the Caribbean Basin (West Indies). Furthermore, years of graft-transmission (>150 years) has resulted in the selection of abutilon plants showing only the striking golden/yellow mosaic disease (i.e., virus-host equilibrium). Indeed, these symptoms are so aesthetically pleasing that these plants are sold commercially as a variegated variety of flowering maple (Jeske et al. 1980; Valverde et al. 2012).

In the phylogenetic analyses, TbLCuCV and AbGYMV were placed together as sister groups in a strongly supported clade, consistent with evolution from a common ancestor followed by diversification driven through host adaptation. The long branch separating AbGYMV from the clade ancestor further suggests a relatively long period of evolution, which often indicates recombination (Lima et al. 2017; Martin et al. 2011). Indeed, a recombination event was detected in the well-known hot-spot region of the AbGYMV DNA-A component (Martin et al. 2011; Hou et al. 1996; Lefeuvre et al. 2009; Padidam et al. 1999). Furthermore, the high degree of sequence divergence across the genomes of TbLCuCV and AbGYMV, including in the CR and HVR, are

consistent with evolution via mutation, another major mechanism of begomovirus diversification (Lima et al. 2017; Duffy et al. 2008). The common ancestor of TbLCuCV and AbGYMV was probably a bipartite begomovirus infecting non-cultivated plants on Hispaniola. Subsequent local evolution and host adaptation, presumably driven by indigenous whiteflies, led to the emergence of viruses adapted to non-cultivated weed species, such as *Malachra* sp. and *Abutilon* sp., respectively. This is supported by two lines of evidence. First, TbLCuCV and AbGYMV are most closely related to each other and to the C1 clade of the AbMV lineage, which contains bipartite weed-infecting begomoviruses from the Caribbean Basin (e.g., JMV and WGMV) (Fig. 2 and Supplemental Fig. 2). Second, the results of the infectivity studies showed that TbLCuCV and AbGYMV are well-adapted to *Malachra* sp. and *Abutilon* sp., respectively. It is also worth noting that it is not clear weather *Malachra* sp. and *Abutilon* sp. are native or introduced species on Hispaniola.

The capacity of these bipartite begomoviruses to form infectious PRs can provide insight into relatedness and gene function (Melgarejo et al. 2014; Hou et al. 1996; Gilbertson et al. 1993; Idris et al. 2008; Garrido-Ramirez et al. 2000; Ramos et al. 2003; Hill et al. 1998; Von-Arnim et al. 1992; Hou et al. 2000; Hussain et al. 2005; Briddon et al. 2010). The highly infectious nature of the PRs formed between the DNA-A and DNA-B components of TbLCuCV and AbGYMV likely reflects the importance of the conserved CR iterons, because there was substantial divergence in the CR sequence outside of these elements (Fig. 1). Furthermore, the Rep IRDs of these viruses are different, with the TbLCuCV IRD lacking the highly conserved F residue and not predicted to recognize the "GGGGG" core iteron (Argüello-Astorga et al. 2001). In the case of the IRD, the substitution of the aromatic non-polar F residue with a polar S residue suggests a non-essential role for the aromatic side chain and some shared biochemical properties between these

aa that allow IRD function. Thus, this is another example of unexpected genetic flexibility in the IRD-iteron interactions revealed by the infectivity of PRs formed between viruses from different lineages (Garrido-Ramirez et al. 2000). Moreover, an efficient interaction between components of these viruses was revealed by the highly infectious and virulent TbLCuCV/AbGYMV PRs in *N. benthamiana* and *Malachra* sp., despite the CR/IRD differences. In fact, both PRs were more virulent than the AbGYMV parent, which is atypical for PRs. This may be explained in terms of the host adaptation and co-evolution of AbGYMV with the local species of *Abutilon* sp. In this regard, AbGYMV may have become so well-adapted to this *Abutilon* sp. that it is not well suited to infect and induce symptoms in *N. benthamiana* and *Malachra sp*. However, this equilibrium in the virus-host interaction can be disrupted when PRs are formed with the TbLCuCV DNA components. Thus, the higher virulence of both PRs may be due to the uncoupling of aspects of the host adaptation of the AbGYMV DNA-A and DNA-B components when combined with the components of TbLCuCV.

The disease phenotypes of the PRs also revealed that infectivity and pathogenicity determinants mapped to both DNA components and in a host-dependent manner. In *N. benthamiana*, the symptom phenotypes were associated with the source of the DNA-A component, whereas in *Malachra* sp. the DNA-A component played an important role in infectivity and the DNA-B component was associated with the symptom phenotype (Table 2). These results are in agreement with previous studies showing that host specificity and symptom development are complex phenomena that involves interaction among multiple virus- and host-encoded factors as well as non-translated regions (Hou et al. 1996; Petty et al. 2000; Zhou et al. 2007; Lazarowitz 1991; Ingham et al. 1993; Qin et al. 1998). Thus, there are numerous examples of DNA-A sequences/gene products being symptoms determinants (Hou et al. 1996; Petty et al. 2000; Zhou

et al. 2007; Qin et al. 1998), possibly involving virus-host interactions associated with replication, gene expression or suppression of host defenses. The very low rate of infectivity of AbA + TB in *Malachra* sp. may be related to early establishment of infection in this host, e.g., capacity for movement or defense suppression, rather than a deficiency of replication, because some AbA + TB plants developed severe symptoms and had wild-type DNA levels based on semi-quantitative PCR test results (data not shown). This low rate of infectivity also cannot be explained by incompatibility of the DNA-B-encoded proteins, as the PRs induced symptoms as severe or more so than the parental viruses. Thus, the low infectivity in *Malachra* sp. may reflect differences in interactions with host factors, such as those involved in gene expression or silencing mediated by the AbA-encoded proteins, including expression of the TB-encoded NSP and MP. In *Malachra* sp., the role of the DNA-B component in symptom development is in agreement with previous studies that showed both DNA-B encoded proteins are symptom determinants (Von-Arnim et al. 1992; Hou et al. 2000; Hussain et al. 2005; Briddon et al. 2010; Rojas et al. 2005; Gilbertson et al. 1993; Hou et al. 1996; Garrido-Ramirez et al. 2000; Zhou et al. 2007). Indeed, these aspects of the TbLCuCV/AbGYMV interaction may make this system useful for further mapping the host-specific role of the DNA-B encoded protein in symptom development.

In conclusion, we showed that the etiology of the golden/yellow mosaic diseases of two malvaceous species in Hispaniola are caused by TbLCuCV and AbGYMV, respectively. TbLCuCV also infects common bean or tobacco, consistent with causing occasional disease outbreaks, whereas AbGYMV induces few or no symptoms in crop plants. TbLCuCV and AbGYMV are closely related viruses in the AbMV lineage of NW begomoviruses, and can form infectious PRs and coinfect *Malachra* sp. plants, which allows for further virus evolution. Finally, our results indicated that TbLCuCV and AbGYMV do not appear to pose a threat to crop

production in the DO, although TbLCuCV-infected *Malachra* sp. could serve as sources of inoculum for sporadic spillover outbreaks in crops, such as in tobacco grown in the Cibao Valley in the northern DO.

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Table 4.1. Infectivity, host range and symptomatology of the infectious cloned DNA-A and DNA-B components of an isolate of tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) and Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]).

Host plant/Begomovirus species	Infectivity[a]		Symptoms[b]
	Agroinoculation	Bombardment	
Nicotiana benthamiana			
TbLCuCV	10/10 (100)	NT	Cr, E, Ld, M, S, Vy
AbGYMV	9/9 (100)	NT	Cr, E, S
Nicotiana tabacum cv. Havana			
TbLCuCV	9/9 (100)	NT	Cr, E, S
AbGYMV	9/9 (100)	NT	NS-i
Nicotiana tabacum cv. Turkish			
TbLCuCV	9/9 (100)	NT	Cr, E, S
AbGYMV	9/9 (100)	NT	NS-i
Nicotiana glutinosa			
TbLCuCV	9/9 (100)	NT	Cr, E, M, S
AbGYMV	9/9 (100)	NT	U
Solanum lycopersicum cv. Glamour			
TbLCuCV	4/9 (44)	NT	NS-i
AbGYMV	1/9 (10)	NT	NS-i
Capsicum annuum cv. Cayenne			
TbLCuCV	NT	0/9 (0)	NS-ni
AbGYMV	NT	0/9 (0)	NS-ni
Datura stramonium			
TbLCuCV	4/9 (44)	NT	Cs
AbGYMV	6/9 (66)	NT	NS-i
Chenopodium amaranticolor			
TbLCuCV	0/9 (0)	NT	NS-ni
AbGYMV	0/9 (0)	NT	NS-ni
Cucurbita maxima cv. Sugarpie			
TbLCuCV	0/9 (0)	NT	NS-ni
AbGYMV	0/9 (0)	NT	NS-ni
Phaseolus vulgaris cv. Topcrop			
TbLCuCV	10/10 (100)	NT	Ch, E, Ld, M, S
AbGYMV	17/17 (100)	NT	E
Malachra sp.			
TbLCuCV	12/12 (100)	NT	Cr, E, Ld, M, S
AbGYMV	1/12 (8)	NT	NS-i
Abutilon indicum			
TbLCuCV	0/9 (0)	NT	NS-ni
AbGYMV	0/9 (0)	NT	NS-ni
Abutilon sp.[c]			
AbGYMV	5/5 (100)	NT	Cr, E, M, S

[a] Infectivity (number of infected plants/number inoculated) was determined at 14 days after inoculation based on symptom development and detection of viral DNA components in newly emerged leaves by PCR tests with component-specific primer pairs. Percentage of plants infected

is indicated in parentheses; NT = not tested. Data represents a total of three independent experiments, except for *Abutilon* sp., for which a single experiment was performed.

[b] Abbreviations: Ch, chlorosis; Cr, crumpling; Cs, chlorotic spots; E, epinasty; Ld, leaf deformation; M, mosaic/mottle; NS-i, no symptoms-infected; NS-ni, no symptoms-not infected; S, stunting; U, upward leaf curling and Vy, vein yellowing.

[c] *Abutilon* sp. plants were derived from seeds collected from plants in the Dominican Republic.

Table 4.2. Infectivity and symptomatology of pseudorecombinants (PRs) formed with the infectious cloned DNA-A and DNA-B components of an isolate of tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) and Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]) following agroinoculation of *Nicotiana benthamiana* and *Malachra* sp. plants.

Plant species/ PRs[a]	Infectivity[b]				Symptoms[c]
	TbLCuCV		AbGYMV		
	DNA-A	DNA-B	DNA-A	DNA-B	
N. benthamiana					
TA + AbB	9/9 (100)	0/9 (0)	0/9 (0)	9/9 (100)	Cr, E, Ld, M, S, Vy
AbA + TB	0/9 (0)	9/9 (100)	9/9 (100)	0/9 (0)	E, M, Ld, S
TA + TB	9/9 (100)	9/9 (100)	0/9 (0)	0/9 (0)	Cr, E, Ld, M, S, Vy
AbA + AbB	0/9 (0)	0/9 (0)	9/9 (100)	9/9 (100)	E, S
***Malachra* sp.**					
TA + AbB	7/9 (80)	0/9 (0)	0/9 (0)	7/9 (80)	Cr, E, Ld, S
AbA + TB	0/9 (0)	2/9 (22)	2/9 (22)	0/9 (0)	Cr, E, Ld, M, S
TA + TB	9/9 (100)	9/9 (100)	0/9 (0)	0/9 (0)	Cr, E, Ld, M, S
AbA + AbB	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)	NS-ni

[a] Virus abbreviation: TA, TbLCuCV DNA-A; TB, TbLCuCV DNA-B; AbA, AbGYMV DNA-A and AbB, AbGYMV DNA-B. PRs were formed by exchanging the infectious cloned DNA-A and DNA-B components of TbLCuCV and AbGYMV via agroinoculation of *N. benthamiana* and *Malachra* sp. plants. Controls were equivalent plants agroinoculated with the DNA-A and DNA-B components of TbLCuCV or AbGYMV (positive controls) and the empty vector alone (negative control).

[b] Infectivity (number of infected plants/number inoculated) was determined at 14 days post inoculation based on symptom development and detection of viral DNA components by PCR tests with component-specific primer pairs; Percentages of infection is indicated in parentheses. Data represents a total of three independent experiments.

[d] Symptom abbreviations: Cr, crumpling; E, epinasty; Ld, leaf deformation; M, mosaic/mottle; NS-ni, no symptoms-not infected; S, stunting; and Vy, vein yellowing.

Table 4.3. Infectivity and symptomatology in *Malachra* sp. plants co-agroinoculated with the infectious cloned DNA-A and DNA-B components of an isolate of tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) and Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]).

Plant species / Combination[a]	Infectivity[b]				Symptoms[c]
	TbLCuCV		AbGYMV		
	DNA-A	DNA-B	DNA-A	DNA-B	
TA + TB + AbA + AbB	7/9 (80)	7/9 (80)	2/9 (22)	3/9 (33)	Cr, E, Ld, M, S
TA + TB	5/5 (100)	5/5 (100)	0/9 (0)	0/9 (0)	Cr, E, Ld, M, S
AbA + AbB	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)	Ni-ni
Empty vector	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)	Ni-ni

[a] Abbreviation: TA, TbLCuCV DNA-A; TB, TbLCuCV DNA-B; AbA, AbGYMV DNA-A; AbB, AbGYMV DNA-B. Experiments were performed by agroinoculation of the infectious cloned DNA-A and DNA-B components of TbLCuCV and AbGYMV in *Malachra* sp. plants. Controls were equivalent plants agroinoculated with the DNA-A and DNA-B components of TbLCuCV or AbGYMV (positive controls) and the empty vector alone (negative control).

[b] Infectivity (number of infected plants/number inoculated) was determined at 14 days post inoculation based on symptom development and detection of viral DNA components by PCR tests with component-specific primers; Percentages of infection is indicated in parentheses. Data represents a total of three independent experiments.

[c] Symptom abbreviations: Cr, crumpling; E, epinasty; Ld, leaf deformation; M, mosaic/mottle; NS-ni, no symptoms-not infected and S, stunting.

Supplemental Table S4.1. Sequences of the oligonucleotide primers used in this study

Primer[a]	Nucleotide sequence
PACUv2353	5'-CGGAGTATTTACTTTCTGTAATTGGG-3′
PACUv1889	5'-GGAACGTTAGTGAAAGAGGAGAGTTG-3′
PACUc174	5'-GTCCATACAGGCCCAAACAGCTTAAA-3′
PBCUv2171	5'-ATTCGGGAGAAGAAGAGAGGAATAG-3′
PBCUv1779	5'-GATCTATGTTGCATCTGATCGG-3′
PBCUc159	5'-CAAATTGTCAGGCAGAAATCGG-3′
PADRv1980	5'-AATCCTATCGAGATTTGCCCTTAG-3′
PADRc205	5'-TTAAAGAAAAGGGACCAATCAGCTTTTC-3′
PADRv1918	5'-GGCACGTTAGTAAAAGAGGAGAGTTG-3′
PADRc2402	5'-TTACTTAAACTCCAAACCCCATCCAAG-3′
PBDRv2241	5'-GCGTTGGATAAGATGGAAGAAGG-3′
PBDRc167	5'-GTAAAACAATTCGCGCGACAGAAG-3′

[a] Primer nomenclature is as follows: P, primer; A, DNA-A component; B, DNA-B component; CU, TbLCCuV; DR, AbGYMV; v, viral sense primer; c, complementary sense primer; and number of the annealing position for the 5' end of the primer.

Supplemental Table S4.2. Nucleotide (nt) identities for total (Tot) and common region (CR) 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 tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) with TbLCuCV isolates from the Dominican Republic and Cuba and the most closely related begomoviruses[a]

		DNA-A												DNA-B[d]					
				ORFs												ORFs			
				AV1		AC1		AC2		AC3		AC4				BV1		BC1	
Species[b]	Loc[c]	Tot	CR	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	Tot	HVR	nt	aa	nt	aa
TbLCuCV-[DO:JG:16]	DO	97	98	97	99 (100)	98	97 (98)	98	98 (99)	96	97 (96)	99	99 (98)	97	92	98	98 (99)	97	99 (99)
TbLCuCV-[DO:M:16]	DO	97	92	97	99 (100)	98	98 (99)	99	98 (99)	97	98 (98)	99	99 (100)	97	93	98	99 (99)	98	99 (99)
TbLCuCV-[CU:VC-CU2015:14]	CU	96	94	96	98 (99)	96	97 (98)	99	98 (99)	98	98 (98)	98	95 (94)	NA	NA	NA	NA	NA	NA
TbLCuCV-[CU:frijol-8:14]	CU	96	92	96	99 (100)	97	96 (98)	99	98 (99)	98	98 (99)	98	96 (95)	94	91	96	96 (98)	95	98 (99)
TbLCuCV-[CU:Tag:05][e]	CU	96	93	96	98 (99)	96	97 (97)	98	98 (99)	98	98 (98)	*	*	NA	NA	NA	NA	NA	NA
TbMoLCV -[CU:SSp:03]	CU	87	93	87	92 (95)	87	83 (86)	91	76 (80)	91	89 (92)	92	86 (86)	NA	NA	NA	NA	NA	NA
SiYMoV-[CU:SSp159:09]	CU	87	85	88	96 (98)	88	87 (93)	91	88 (90)	91	91 (93)	91	82 (81)	78	59	77	75 (87)	88	95 (97)
WGMV-[JM:Alb:08]	JM	87	80	89	90 (94)	86	86 (90)	90	91 (94)	93	92 (93)	88	82 (81)	81	71	81	83 (88)	90	95 (97)
JMV-[JM:ST1:04]	JM	87	89	89	92 (96)	86	84 (91)	90	86 (90)	91	89 (92)	91	79 (80)	79	68	81	79 (86)	86	94 (96)
SiGYVV-[US:Sa19:12]	US	86	81	86	91 (95)	87	87 (91)	91	90 (92)	90	87 (89)	90	80 (81)	76	59	75	73 (83)	84	90 (95)
AbGYMV-[DO:CG:16]	DO	86	85	88	94 (98)	84	84 (90)	93	89 (92)	91	90 (94)	86	66 (74)	82	58	85	86 (91)	89	96 (98)
ToYLDV-[CU:5E17:07]	CU	86	87	84	88 (95)	85	83 (89)	93	89 (94)	93	90 (92)	88	76 (78)	80	59	82	80 (88)	87	92 (96)
OYMV-[MX:Maz3:04]	MX	86	76	88	94 (98)	86	88 (92)	90	84 (87)	90	90 (94)	87	69 (77)	74	60	72	70 (82)	84	92 (96)
MaYMJV-[JM:9bA43:03]	JM	85	82	85	92 (96)	86	85 (90)	90	84 (86)	90	90 (90)	87	74 (76)	74	58	77	75 (83)	82	90 (96)
RhRGMV-[CU:Cam:171:09]	CU	85	87	87	95 (97)	83	82 (89)	91	88 (90)	90	90 (93)	88	79 (81)	70	57	73	68 (82)	76	86 (89)

[a] Based on a BLASTn analysis, the most closely related begomoviruses were various New World bipartite begomoviruses.

[b] GenBank accession numbers are as follows: TbLCuCV-[HT:14]: MH514009 and MH514010; TbLCuCV-[DO:JG:16]: MK059404 and MK059405; TbLCuCV-[DO:M:16]: MK059402 and MK059403; TbLCuCV-[CU:VC-CU2015:14]: KU562963; TbLCuCV-[CU:frijol-8:14]: KX011471 and KX011472; TbLCuCV-[CU:Tag:05]: AM050143; TbMoLCV -[CU:SSp:03]: NC038893; SiYMoV-

[CU:SSp159:09]: HQ822123 and HQ822124; WGMV-[JM:Alb:08]: GQ355488 and GQ355487; JMV-[JM:ST1:04]: KF723258 and KF723261; SiGYVV-[US: Sa19:12]: KT879816 and KT879818; AbGYMV-[DO:CG:16]: MH514011 and MH514012; ToYLDV-[CU:5E17:07]: FJ174698 and NC017913; OYMV-[MX:Maz3:04]: DQ022611 and GU972604; MaYMJV-[JM:9bA43:03]: FJ600482 and FJ600484 and RhRGMV-[CU:Cam:171:09]: NC038805 and NC038804.

[c] Geographic location: DO = Dominican Republic, CU = Cuba, JM = Jamaica, US = United States and MX= Mexico.

[d] NA = not available.

[e] *ORF AC4 is truncated.

Supplemental Table S4.3. Nucleotide (nt) identities for total (Tot) and common region (CR) 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 Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]) and the most closely related begomoviruses[a]

		DNA-A												DNA-B[d]					
				ORFs												ORFs			
				AV1		AC1		AC2		AC3		AC4				BV1		BC1	
Species[b]	Loc[c]	Tot	CR	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	Tot	HVR	nt	aa	nt	aa
TbLCuCV-[HT:14]	HT	86	85	88	94 (98)	84	84 (90)	93	89 (92)	91	90 (94)	86	66 (74)	82	59	85	86 (91)	89	96 (98)
TbLCuCV-[CU:frijol-8:14]	CU	86	88	89	95 (98)	79	78 (86)	92	89 (93)	91	90 (95)	84	62 (71)	82	56	85	86 (91)	89	97 (98)
TbLCuCV-[DO:JG:16]	DO	86	87	88	95 (98)	84	83 (90)	92	88 (92)	90	89 (93)	85	65 (73)	82	60	84	85 (90)	90	97 (98)
TbLCuCV-[CU:VC-CU2015:14]	CU	86	87	88	94 (97)	83	83 (90)	92	88 (92)	91	90 (96)	84	61 (70)	NA	NA	NA	NA	NA	NA
TbLCuCV-[DO:M:16]	DO	86	86	88	94 (98)	84	84 (90)	92	88 (92)	90	90 (94)	85	66 (74)	82	59	85	86 (91)	89	96 (98)
TbLCuCV-[CU:Tag:05][e]	CU	85	88	88	94 (97)	83	83 (89)	91	88 (92)	91	89 (94)	*	*	NA	NA	NA	NA	NA	NA
JMV-[JM:ST1:04]	JM	85	86	86	93 (96)	85	85 (94)	90	84 (88)	89	87 (92)	84	64 (70)	80	61	83	83 (90)	89	96 (97)
WGMV-[JM:Alb:08]	JM	85	78	86	89 (94)	82	82 (89)	90	94 (96)	91	89 (94)	84	68 (73)	81	59	85	89 (93)	90	96 (97)
SiGMV-[US:Flo]	US	84	77	88	93 (98)	82	81 (89)	91	89 (92)	89	87 (93)	84	62 (70)	76	61	80	81 (87)	87	96 (97)
CoYSV-[MX:Yuc:05]	MX	84	76	87	94 (97)	83	82 (90)	89	84 (87)	87	85 (91)	84	64 (69)	80	67	78	80 (85)	87	95 (97)
ToYLDV-[CU:5E17:07]	CU	84	84	84	88 (95)	83	82 (90)	92	90 (95)	90	89 (93)	85	66 (71)	80	54	86	87 (91)	88	94 (97)
SiYMoV-[CU:SSp159:09]	CU	84	84	87	95 (98)	81	80 (87)	90	86 (90)	90	88 (93)	86	67 (73)	81	66	79	78 (89)	89	96 (98)
RhRGMV-[CU:Cam:171:09]	CU	84	83	86	94 (97)	80	81 (89)	92	90 (92)	90	89 (93)	86	67 (72)	70	56	72	74 (84)	77	88 (90)
MaYMJV-[JM:9bA43:03]	JM	84	79	86	92 (96)	81	81 (87)	90	84 (88)	88	86 (89)	81	61 (69)	74	57	77	77 (85)	84	92 (96)
MaYMHeV-[JM:Ma179A5:05]	JM	83	74	85	91 (96)	82	81 (89)	92	87 (92)	88	86 (89)	78	54 (64)	NA	NA	NA	NA	NA	NA

[a] Based on a BLASTn analysis, the most closely related begomoviruses were various New World bipartite begomoviruses.

[b] GenBank accession numbers are as follows: AbGYMV-[DO:CG:16]: MH514011 and MH514012; TbLCuCV-[HT:14]: MH514009 and MH514010; TbLCuCV-[CU:frijol-8:14]: KX011471 and KX011472; TbLCuCV-[DO:JG:16]: MK059404 and MK059405; TbLCuCV-[CU:VC-CU2015:14]: KU562963; TbLCuCV-[DO:M:16]: MK059402 and MK059403; TbLCuCV-[CU:Tag:05]:

AM050143; JMV-[JM:ST1:04]: KF723258 and KF723261; WGMV-[JM:Alb:08]: GQ355488 and GQ355487; SiGMV-[US:Flo]: AF049336 and AF039841; CoYSV-[MX:Yuc:05]: DQ875868 and DQ875869; ToYLDV-[CU:5E17:07]: FJ174698 and NC017913; SiYMoV-[CU:SSp159:09]: HQ822123 and HQ822124; RhRGMV-[CU:Cam:171:09]: NC038805 and NC038804; MaYMJV-[JM:9bA43:03]: FJ600482 and FJ600484 and MaYMHeV-[JM:Ma179A5:05]: NC038452.

[c] Geographic location: HT = Haiti, CU = Cuba, DO = Dominican Republic, JM = Jamaica, US = United States and MX= Mexico.

[d] NA = not available.

[e] *ORF AC4 is truncated.

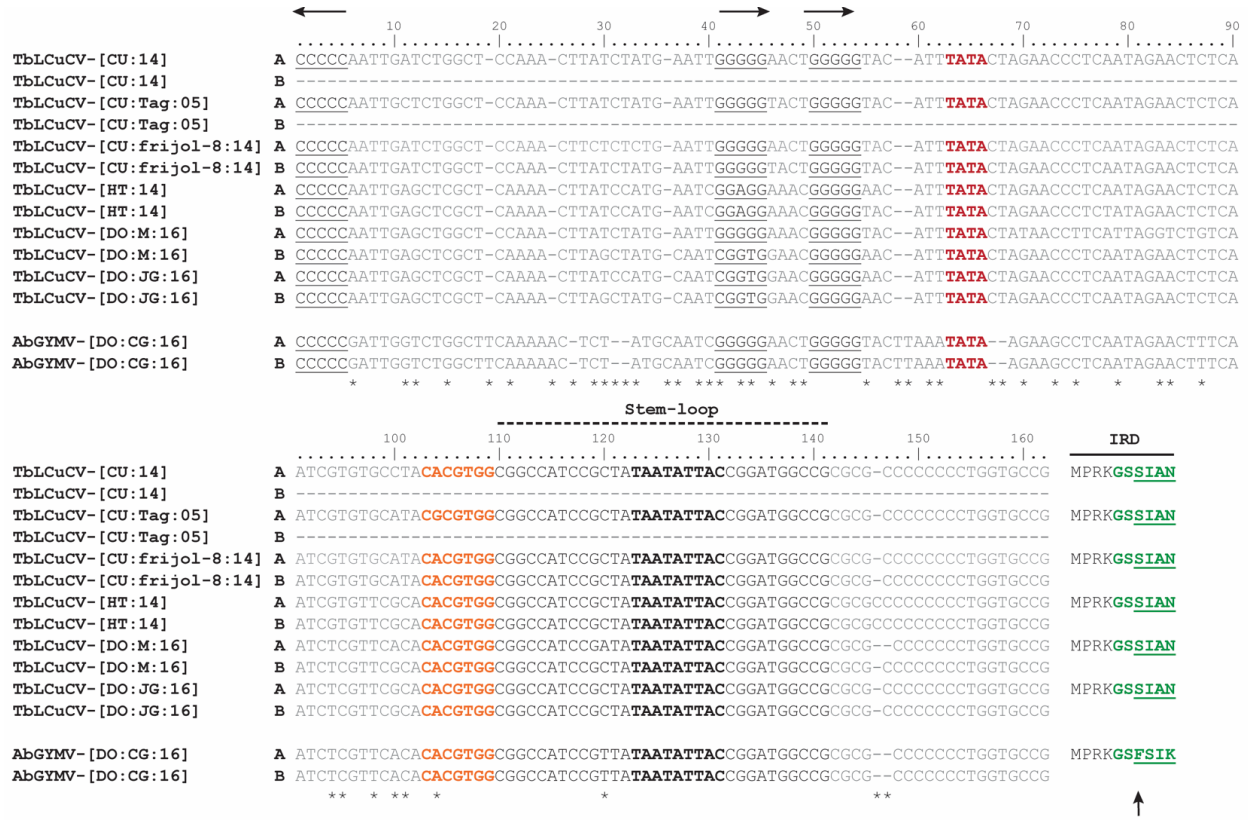


Fig 4.1. Alignment of a portion of the common region (CR) sequences of isolates of tobacco leaf curl Cuba virus (TbLCuCV) from Cuba, Haiti and the Dominican Republic (DO) and the putative new bipartite begomovirus Abutilon golden yellow mosaic virus (AbGYMV) from the DO. The replication-associated protein (Rep) high affinity binding site (two direct repeats and one inverted repeat of the core iteron) are shown in black letters and underlined, and their orientation is shown with arrows at the top of the alignment. The motif with the TATA box of the AC1 (Rep) gene is indicated in red bold red letters, whereas the G-box is represented in orange bold letters. The characteristic geminivirus stem-loop forming sequence is highlighted in black under the broken line, with the conserved geminivirus nonanucleotide sequence shown in black bold letters. The Rep iteron-related domain (IRD) located in the N-terminus of Rep, is shown to the right of the alignment, with the putative six amino acids involved in iteron recognition indicated in green bold letters and variable positions are underlined. The highly conserved F residue in the IRD is indicated

with an arrow. The numbers on top of the alignment indicate nt positions in respect to the inverted repeat, and nt differences are indicated with an asterisk. Abbreviations and GenBank accession numbers are as follows: TbLCuCV-[CU:14]: KU562963; TbLCuCV-[CU:Tag:05]: AM050143; TbLCuCV-[CU:frijol-8:14]: KX011471 and KX011472; TbLCuCV-[HT:14]: MH514009 and MH514010; TbLCuCV-[DO:M:16]: MK059402 and MK059403; TbLCuCV-[DO:JG:16]: MK059404 and MK059405 and AbGYMV-[DO:CG:16]: MH514011 and MH514012.

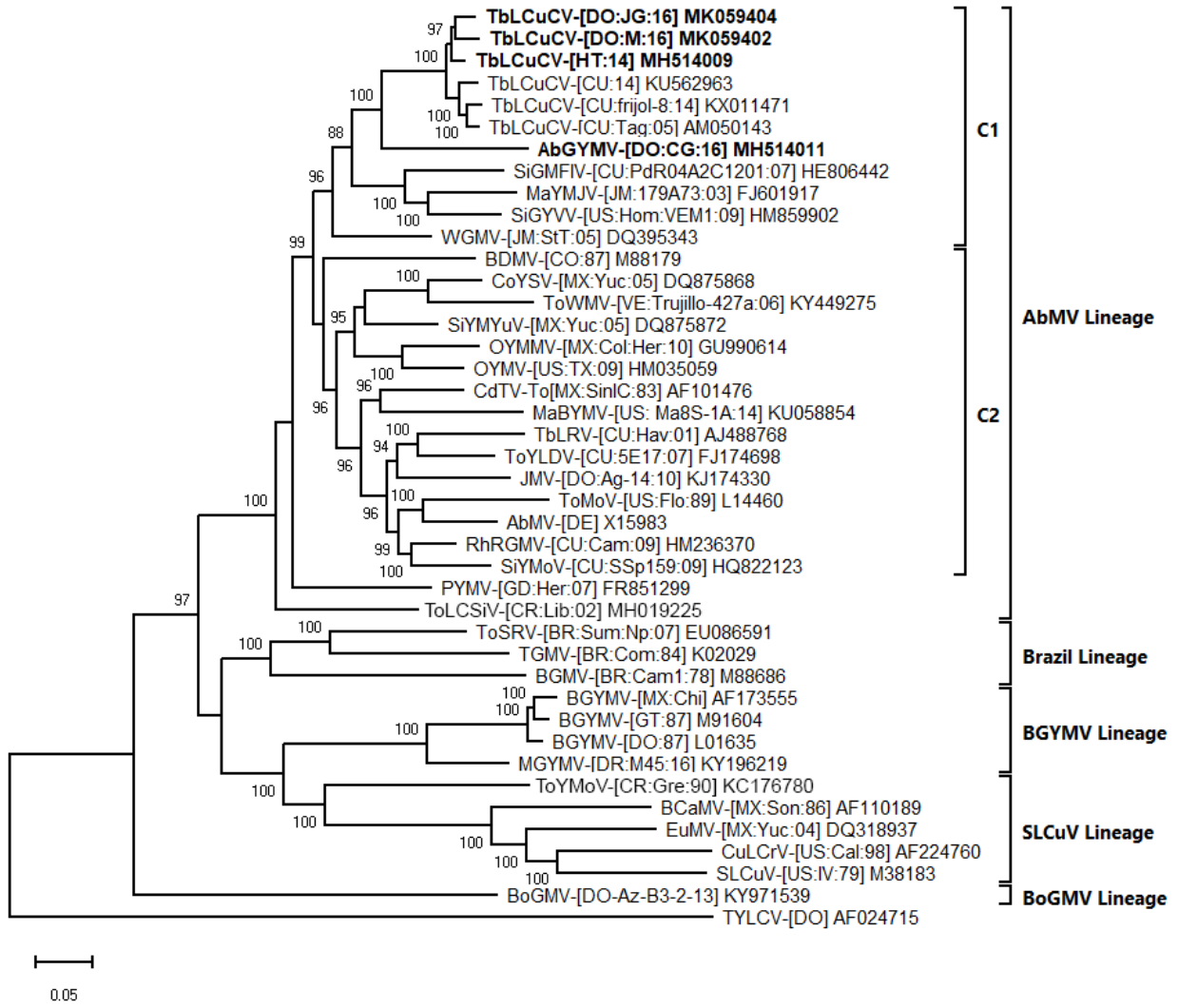


Fig. 4.2. Bayesian phylogenetic consensus tree constructed with complete nucleotide sequences of the DNA-A components and showing the relationship of three isolates of tobacco leaf curl Cuba virus (TbLCuCV) from Hispaniola and the putative new bipartite begomovirus species Abutilon golden yellow mosaic virus (AbGYMV) from the Dominican Republic (shown in bold) with: (i) the most closely related begomoviruses identified based on a BLASTn search and (ii) selected begomoviruses representing the Abutilon mosaic virus (AbMV), Brazil, squash leaf curl virus (SLCuV), bean golden yellow mosaic virus (BGYMV) and Boerhavia golden mosaic virus (BoGMV) lineages of New World (NW) bipartite begomoviruses (lineages are indicated with brackets). The two major clades of the AbMV lineage, C1 and C2, are shown with inner brackets.

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 complete sequence of the genomic DNA of the Old World monopartite begomovirus tomato yellow leaf curl virus (TYLCV). The length of horizontal branches indicates the rate of substitution per nucleotide.

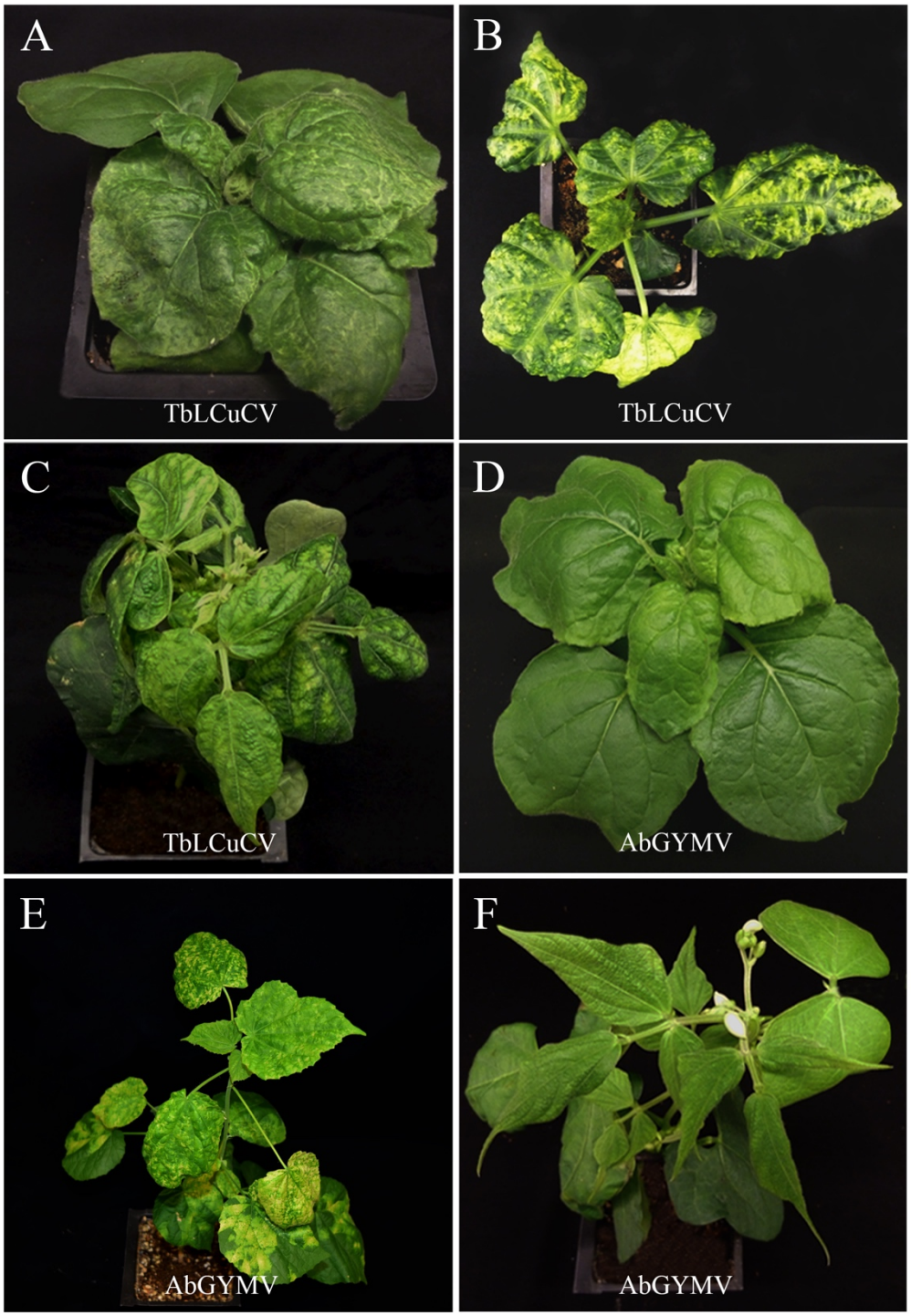


Fig. 4.3. Disease symptoms induced by the infectious cloned DNA-A and DNA-B components of an isolate of tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) in: (A) *Nicotiana benthamiana*, (B) *Malachra* sp. and (C) common bean (*Phaseolus vulgaris* cv. Topcrop); and by

those induced by the infectious cloned DNA-A and DNA-B components of the putative new bipartite begomovirus species Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]) in (D) *N. benthamiana*, (E) *Abutilon* sp. from the DO and (F) common bean (*P. vulgaris* cv. Topcrop). Plants were photographed 14 days after agroinoculation.

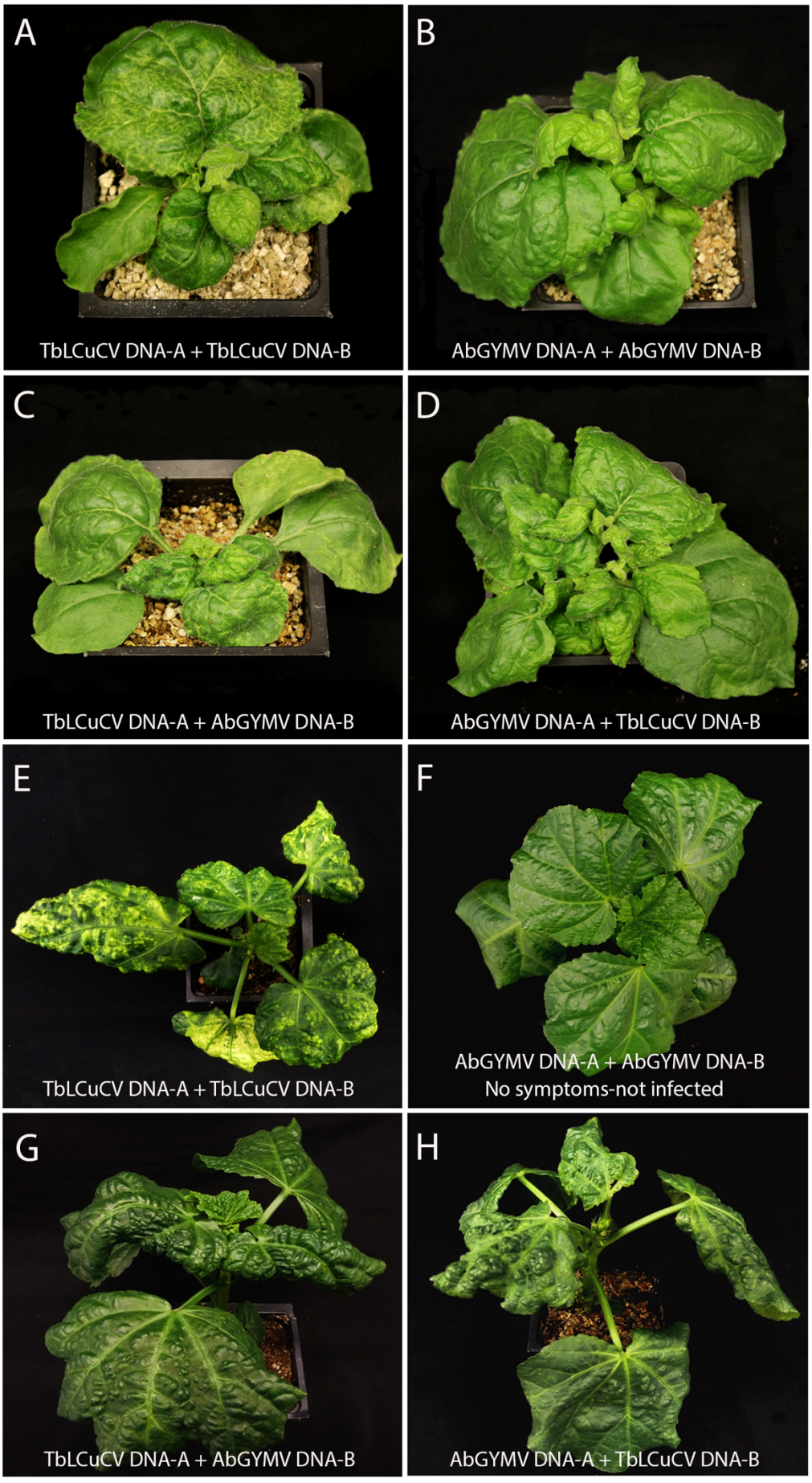
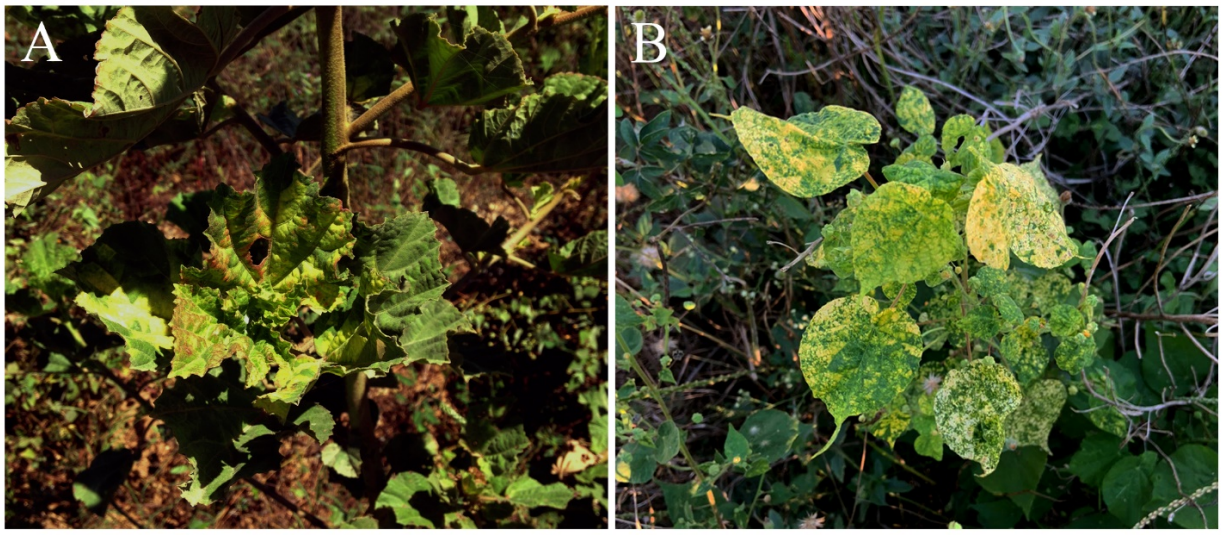
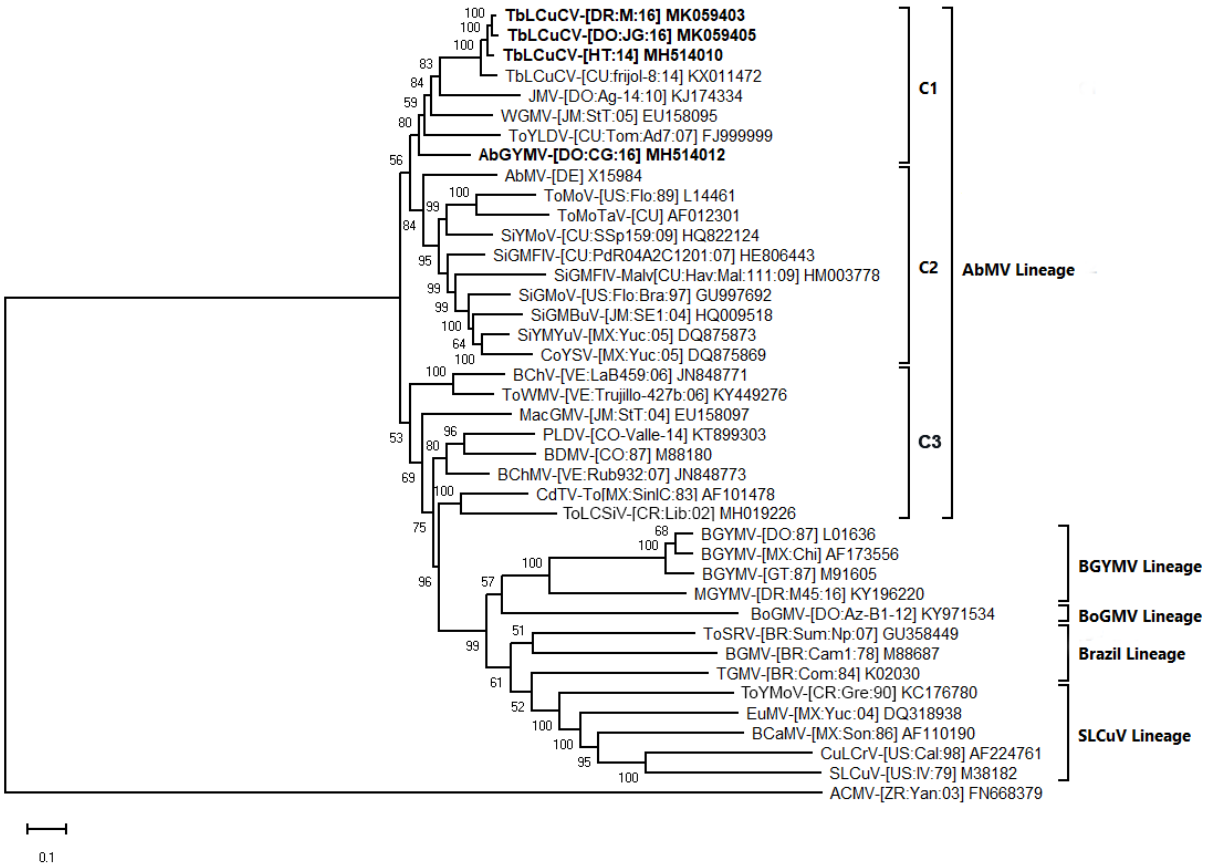

A
TbLCuCV DNA-A + TbLCuCV DNA-B
B
AbGYMV DNA-A + AbGYMV DNA-B
C
TbLCuCV DNA-A + AbGYMV DNA-B
D
AbGYMV DNA-A + TbLCuCV DNA-B
E
TbLCuCV DNA-A + TbLCuCV DNA-B
F
AbGYMV DNA-A + AbGYMV DNA-B
No symptoms-not infected
G
TbLCuCV DNA-A + AbGYMV DNA-B
H
AbGYMV DNA-A + TbLCuCV DNA-B

Fig. 4.4. Disease symptoms induced by the pseudorecombinants (PRs) formed between the infectious cloned DNA-A and DNA-B components of an isolate of tobacco leaf curl Cuba virus from Haiti (TbLCuCV-[HT:14]) and the putative new bipartite begomovirus species Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]) in: *Nicotiana benthamiana* (panels A-D) and *Malachra* sp. (panel E-H). Symptoms induced by TbLCuCV DNA-A and DNA-B are shown in panels A and E, whereas those induced by AbGYMV DNA-A and DNA-B are shown in panels B and F. Note that because AbGYMV did not infect *Malachra* sp. plants in these experiments, panel F shows an inoculated plant that did not become infected. Symptoms induced by TbLCuCV DNA-A (TA) and AbGYMV DNA-B (AbB) are shown in panels C and G, whereas those induced by AbGYMV DNA-A (AbA) and TbLCuCV DNA-B (TB) are shown in panels D and H. Plants were photographed 14 days after agroinoculation.



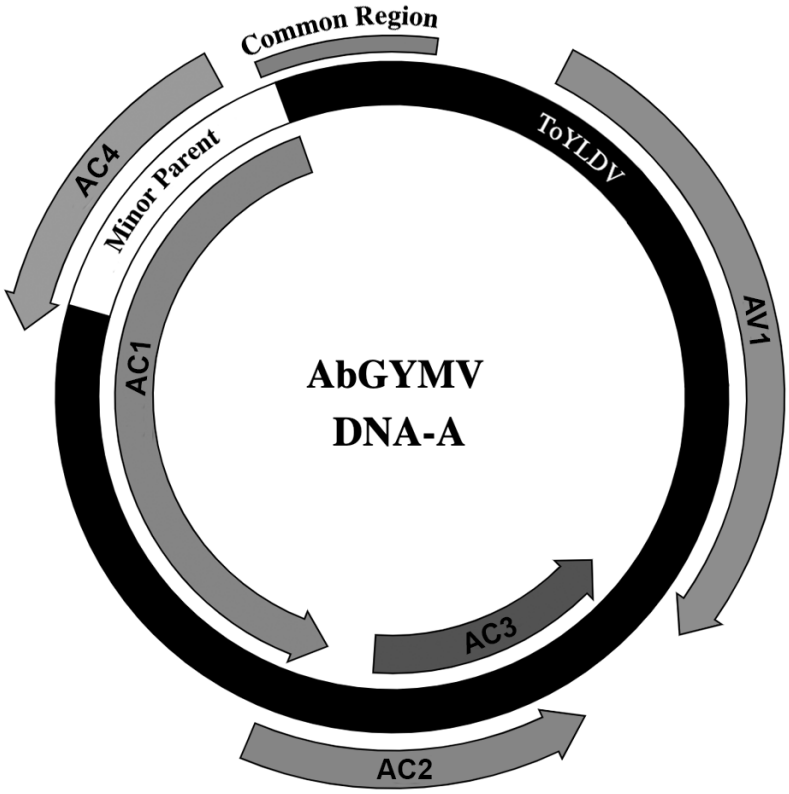
Supplemental Fig. S4.1. (A) *Malachra* sp. plant with leaf crumpling and yellow vein and mosaic/mottle symptoms associated with infection by the New World bipartite begomovirus, tobacco leaf curl Cuba virus (TbLCuCV) in the Dominican Republic (DO) in 2016. (B) *Abutilon* sp. plant with yellow mosaic/mottle symptoms associated with infection by Abutilon golden yellow mosaic virus (AbGYMV), a putative new bipartite begomovirus species, in Cerro Gordo, DO in 2020.

Supplemental Fig. S4.2. Map of the island of Hispaniola showing the geographic locations where malvaceous weeds with golden/yellow mosaic symptoms were collected from Port-Au-Prince, Haiti in 2014; from three locations in the Dominican Republic (DO) in 2016: Juan Gomez, Manzanillo and Cerro Gordo; and from two locations in the DO in 2020: Cerro Gordo and Monte Cristi.



Supplemental Fig. S4.3. Bayesian phylogenetic consensus tree constructed with complete nucleotide sequences of the DNA-B components and showing the relationship of three isolates of tobacco leaf curl Cuba virus (TbLCuCV) from Hispaniola and the putative new bipartite begomovirus species Abutilon golden yellow mosaic virus (AbGYMV) from the Dominican Republic (shown in bold) with: (i) the most closely related begomoviruses identified based on a BLASTn search and (ii) selected begomoviruses representing the Abutilon mosaic virus (AbMV), Brazil, squash leaf curl virus (SLCuV), bean golden yellow mosaic virus (BGYMV) and Boerhavia golden mosaic virus (BoGMV) lineages of New World (NW) bipartite begomoviruses (lineages are indicated with brackets). The two major clades of the AbMV lineage, C1 and C2, as well as the paraphyletic group C3 are shown with inner brackets. 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 complete sequence of the DNA-B component of the Old World bipartite begomovirus African cassava mosaic virus (ACMV). The length of horizontal branches indicates the rate of substitution per nucleotide.



Supplemental Fig. S4.4. Schematic representation of the recombination event identified in the DNA-A component of Abutilon golden yellow mosaic virus from the Dominican Republic (AbGYMV-[DO:CG:16]) with the recombination detection program version 4.0 (RDP4). This strongly supported recombination event was 625 nucleotides (nts), spans nts 1994 to 2618 and includes the 5' end of the AC1 open reading frame (ORF), the entire overlapping AC4 ORF and the left side of the common region. This event is in the well-known recombination hot-spot in the begomovirus genomic DNA/DNA-A component. Furthermore, RDP4 indicated that the recombinant region came from an unidentified minor parent, and that the major parent was tomato yellow leaf distortion virus (ToYLDV) (GenBank accession number FJ174698). The CR is represented by a grey curved box and viral ORFs are represented by arrows.

Chapter V

Future directions

Further investigation of gene function(s) of host factors associated with the *Ty-1* mediated resistance to tomato yellow leaf curl virus (TYLCV) in tomato

In Chapter II, we identified putative differentially expressed genes (DEGs) in response to TYLCV infection in the resistant LA3473-R, including WRKY transcription factors, nucleotide-binding site and leucine-rich repeat (NBS-LRR) containing proteins and receptor-like protein kinases (RLKs). The next step is to identify the functions of these proteins and determine if they play a role in the signal transduction pathway that underlines the *Ty-1* mediated resistance and susceptibility to TYLCV in tomato.

The functions of transcription factors can be assessed by identifying the promoter and genes that are regulated (Birkenbihl et al. 2017). Thus, to investigate the functions of WRKY transcription factors 45, 46 and 55, a combination of bioinformatic and mutational analyses and transactivation experiments with synthetic promoters can be used to determine the cis-acting response elements associated with these transcription factors. Alternatively, genome-wide chromatin immunoprecipitation (ChIP) experiments can be used for further identification of binding sites of WRKY transcription factors (e.g., at promoter regions) and annotation of potential target genes. In the case of NBS-LRR and RLKs proteins, protein-protein interaction assays such as yeast two-hybrid and co-immunoprecipitation (Co-IP) can be used to identified putative interacting proteins (e.g., host factors and viral proteins).

It has been previously established that the *Ty-1*-mediated resistance to TYLCV is associated with enhanced cytosine methylation of the viral genome and transcriptional gene silencing (TGS) (Butterbach et al. 2014). In this regard, these proteins can be overexpressed in the susceptible LA3474-S (e.g., in transient assays or in transgenic plants), and the effect on viral DNA methylation assessed with bisulfite genomic sequencing analyses (Li and Tollefsbol 2011)

to test the hypothesis that there will be enhanced methylation, and possibly a resistant phenotype. Additionally, these DEGs can be mutated or alter (e.g., by CRISPR) and their role in resistance evaluated by viral replication and methylation as well as the levels of TYLCV-derived small-interfering RNAs (siRNAs) with Northern blot or small RNA sequencing (Small RNA-Seq) assays (T'Hoen et al. 2013).

Further characterization of the *Ty-1* gene

In Chapter II, we also characterized some molecular and biological properties of the *Ty-1* gene. In agreement with Verlaan et al. (2013), we established that both resistant *Ty-1/Ty-3* and susceptible *ty-1* alleles have high levels of nucleotide (nt) and amino acid (aa) sequence identity (>98.2% nt and >97.7% aa). In this regard, it will be of great interest to determine the functional differences of Ty-1 and ty-1 proteins. An *in vitro* cell-free translation system can be used to express the Ty-1 and ty-1 proteins and used them to perform RNA-dependent RNA polymerase (RdRp) assays. Thus, properties such as primer dependent initiation, template specificity, nucleic acid binding assays, selectivity of nucleotide incorporation and divalent cations dependence experiments can be used to establish whether the catalytic domains of these two proteins are functionally different (Kim et al. 2007; Fullerton et al. 2007; Tan et al. 1991).

Additionally, *in situ* PCR and immunolocalization experiments can be used to establish subcellular localization of genes and proteins. For this purpose, the Ty-1 protein can be expressed with *in vivo* systems (e.g., *Escherichia coli* and *Pichia pastoris*) to generated antibodies against the protein. These antibodies can also be used in co-immunoprecipitation-coupled mass spectrometry (IP-MS) assays to identify putative Ty-1 interacting host factors. Finally, the Ty-1 protein can be crystallized and its tertiary structure determined with X-ray crystallography.

Characterization of viral synergism of tomato-infecting begomoviruses

In Chapter III, we examined the invasion biology of three tomato-infecting begomoviruses from Costa Rica. We established that the interaction between these viruses and the susceptible tomato host was shown to be a neutral synergism, i.e., an increase in disease symptom severity with no substantial negative or positive impact on virus DNA accumulation. However, in mixed infections there was transient negative effect on accumulation of the bipartite begomoviruses tomato yellow mottle virus (ToYMoV) and tomato leaf curl Sinaloa virus (ToLCSiV). It would be of interest to investigate this phenomenon by examining more and earlier time points and assessing interactions.

The next step is to investigate the molecular mechanisms of these effects on tomato plants inoculated with these different combinations of viruses. For this purpose, small RNA-Seq assays can be used to compare the profile of virus-derived small-interfering RNAs (vsiRNAs) on tomato plants inoculated in single and mixed infections, thereby revealing inside into gene silencing targets. This will help answer the question if this neutral synergistic interaction is somehow associated with suppression of plant defense responses (e.g., suppression of gene silencing). Additionally, bioinformatic analyses of vsiRNAs can be used to establish if putative defense-related plant transcripts are targeted (Zhou et al. 2017). Finally, virus mutants can be generated to study potential viral genes involved in these interactions

Our results also showed that in mixed infections, symptoms of ToLCSiV and ToYMoV appeared earlier (~7 dpi) that those of TYLCV (~10 dpi), but later in infection (~14 dpi and beyond), tomato yellow leaf curl disease (TYLCD) symptoms become predominant. In this regard, it will be very interesting to determine if the movement functions of the viruses are affected by the presence of co-inoculating viruses. For example, *in situ* hybridization and immunolocalization

experiments can be done to established if the movement proteins of ToLCSiV and ToYMoV can facilitate TYLCV invasion into non-phloem tissues.

In our sequence and phylogenetic analyses, we showed that ToYMoV isolates may comprise a distinct lineage but closely related to the SLCuV lineage of New World begomoviruses (sister lineages). One very interesting difference is the AC4 proteins, with that of ToYMoV predicted to be smaller (85 aa compared with 126 aa) and possessing an N-terminal myristoylation motif. Here, ToYMoV AC4 can be used for further study the differences of the functions of AC4 proteins encoded by members of the SLCuV lineage. Finally, as part of an integrated pest management (IPM) approach for growers, varieties of tomato resistant to all three viruses can be identified and used as part of an IPM program for tomato growers in Costa Rica (Rojas et al. 2018).

Surveys of tobacco leaf curl Cuba virus (TbLCuCV) and Abutilon golden yellow mosaic virus (AbGYMV) in the Caribbean Basin and study of the pathogenicity determinant properties of these weed-infecting begomoviruses

In Chapter IV, we established that TbLCuCV is widely distributed in the Caribbean Basin, where it infects crops (tobacco and common bean) and weeds (*Malachra* sp.) (Morán et al. 2006; Leyva et al. 2016), whereas AbGYMV is well-adapted to an *Abutilon* sp. in the Dominican Republic and has not been reported infecting crops. This raises the question of what is the most prevalent host of TbLCuCV and AbGYMV in the Caribbean Basin. In this regard, it will be a great interest to survey other countries in the Caribbean Basin for the presence of TbLCuCV and AbGYMV infecting *Malachra* sp. and *Abutilon* sp. plants as well as in crop plants, e.g., tobacco and common bean.

The capacity of these bipartite begomoviruses to form infectious PRs can provide insight into relatedness and gene function of bipartite begomoviruses (Melgarejo et al. 2014; Hou and Gilbertson 1996; Gilbertson et al. 1993; Idris et al. 2008; Garrido-Ramirez et al. 2000; Ramos et

al. 2003; Hill et al. 1998; Von Arnim and Stanley 1992; Hou et al. 2000; Hussain et al. 2005; Briddon et al. 2010). Our pseudorecombination experiments showed that infectivity and pathogenicity determinants of these two viruses mapped to both DNA components and in a host-dependent manner. However, these experiments still need to be conducted in *Abutilon* sp. plants from the Dominican Republic. It will also be of interest to test the susceptibility of *Abutilon* spp. from elsewhere in the Caribbean Basin to assess the degree of adaptation of AbGYMV to this host plant. Thus, the protein from these viruses and the infectious clones can be used to investigate host adaptation (e.g., genome exchange and microRNAs profiles). Finally, whitefly transmission experiments with local and invasive whiteflies (*Bemisia tabaci* Middle East Asia Minor 1 [MEAM1]) should be conducted to assess the invasion biology of these viruses.

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