ANGÉLICA MARIA NOGUEIRA

BIPARTITE BEGOMOVIRUSES: REPLICATION, TRANSMISSION AND INTERACTION WITH ALPHASATELLITES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de Doctor Scientiae.

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RESUMO

NOGUEIRA, Angélica Maria, D.Sc., Universidade Federal de Viçosa, setembro de 2018. **Begomovírus bipartidos: Replicação, transmissão e interação com alfassatélites.** Orientador: Francisco Murilo Zerbini Júnior. Coorientadores: Eduardo Seiti Gomide Mizubuti e Camila Geovana Ferro.

O gênero Begomovirus é constituído por vírus com DNA de fita simples circular, transmitidos por moscas-brancas para plantas dicotiledôneas. A maioria dos begomovírus do Novo Mundo (NM) possuem dois componentes de DNA e compartilham uma região comum (RC) que contém a origem de replicação reconhecida pela proteína viral Rep para iniciar a replicação. Infecções mistas naturais com dois ou mais begomovírus são comuns em tomateiro. Estudos anteriores demonstraram que a interação entre os begomovírus Tomato rugose mosaic virus (ToRMV) e Tomato severe rugose virus (ToSRV) é complexa, com o ToRMV interferindo negativamente na infectividade e no acúmulo de ToSRV. Interessantemente, o ToRMV é de ocorrência esporádica no campo, ao contrário de ToSRV, que predomina no tomateiro no sudeste do Brasil. Além do efeito que um begomovírus pode exercer sobre o outro em um mesmo hospedeiro, begomovírus do NM podem ser encontrados também em plantas não-cultivadas em associação com alfassatélites, os quais dependem do begomovírus auxiliar para causar infecção sistêmica. O impacto de uma possível interação entre alfassatélites e begomovírus em plantas cultivadas como o tomateiro é desconhecido. A tese teve como objetivos: i) verificar a eficiência de transmissão de ToSRV e ToRMV por B. tabaci Middle East-Asia Minor 1 (BtMEAM1) e Mediterranean (BtMED) em infecções simples e mistas; ii) investigar se sítios divergentes na RC e na Rep desses begomovírus estão envolvidos na interferência negativa que o ToRMV exerce sobre a replicação do ToSRV; iii) verificar se o alfassatélite Euphorbia yellow mosaic alphasatellite (EuYMA) é capaz de interagir com dois begomovírus que infectam tomateiro, Tomato yellow spot virus (ToYSV) e ToSRV. Para o primeiro objetivo, ao qual corresponde o primeiro capítulo, plantas inoculadas por biobalística com cada vírus em infecções simples e mista foram utilizadas como fonte de inóculo. O ToSRV foi transmitido com a mesma eficiência em relação ao ToRMV por BtMEAM1 e em maiores taxas por BtMED em infecção simples. Entretanto, o ToRMV foi transmitido mais eficientemente do que o ToSRV em infecções mistas, sugerindo que a transmissão preferencial de ToRMV em relação a ToSRV pode estar relacionada à replicação. Para o segundo objetivo, correspondente ao segundo capítulo, mutantes do DNA-A do ToSRV contendo os mesmos nucleotídeos nas posições divergentes da RC e da IRD (domínio relacionado ao reconhecimento

de iterons) da Rep que ocorrem no ToRMV foram construídos. Em inoculações mistas com o ToRMV, foi observado um acúmulo elevado do DNA-A de ToSRV contendo as mutações na RC (em relação ao ToSRV selvagem), sugerindo que nucleotídeos específicos da RC servem como sítios de reconhecimento para ligação da Rep, aumentando a taxa de replicação e o acúmulo de DNA viral. Para o terceiro objetivo, correspondente ao terceiro capítulo, plantas de tomateiro e Nicotiana benthamiana foram inoculadas por biobalística com ToYSV e ToSRV na presença ou ausência do alfassatélite. A interação dos dois begomovírus com o EuYMA foi demonstrada, e apresentou a maior severidade de sintomas. A associação de EuYMA com o ToYSV foi menos eficiente em tomateiro do que em N. benthamiana, no entanto, entre ToSRV e EuYMA foi similar em ambos os hospedeiros, indicando diferenças na interação entre alfassatélites e diferentes espécies de begomovírus. Os resultados deste estudo fornecem novas informações que aumentam o entendimento das interações entre vírus, DNAs satélites, vetor e hospedeiro que contribuem para a adaptação e predominância dos begomovírus no campo.

ABSTRACT

NOGUEIRA, Angélica Maria, D.Sc., Universidade Federal de Viçosa, September, 2018. **Bipartite begomoviruses: Replication, transmission and interaction with alphasatellites.** Adviser: Francisco Murilo Zerbini Júnior. Co-advisers: Eduardo Seiti Gomide Mizubuti and Camila Geovana Ferro.

The genus Begomovirus includes viruses with circular single-stranded DNA, transmitted by whiteflies to dicotyledonous plants. Most New World (NW) begomoviruses have two genomic components that share a common region (CR), which contains the origin of replication recognized by the viral protein Rep to initiate replication. Natural mixed infections with two or more begomoviruses in tomato are common in the field. Previous work demonstrated that the interaction between two begomoviruses, Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV), is complex, with ToRMV negatively interfering in the infectivity and accumulation of ToSRV. Interestingly, ToRMV is not widely disseminated in the field, unlike ToSRV, which predominates in tomato fields in southeastern Brazil. In addition to the effect that one begomovirus can exert on the other in the same host, NW begomoviruses have also been found in non-cultivated plants in association with alphasatellites, which depend on the helper begomovirus to cause systemic infection. The impact of a possible interaction between alphasatellites with begomoviruses in cultivated hosts such as tomato is unknown. The objectives of this thesis included: i) to verify the transmission efficiency of ToSRV and ToRMV by B. tabaci Middle East-Asia Minor 1 (BtMEAM1) and Mediterranean (BtMED) in single and mixed infections; ii) to investigate if divergent sites in the CR and Rep of these begomoviruses are involved in the negative interference that ToRMV exerts on the replication of ToSRV; iii) to verify if the alphasatellite Euphorbia yellow mosaic alphasatellite (EuYMA) is capable of interacting with two tomato-infecting begomoviruses, Tomato yellow spot virus (ToYSV) and ToSRV. For the first objective, corresponding to the first chapter, plants that were biolistically inoculated with each virus in single and mixed infections were used as inoculum sources. ToSRV was transmitted with the same efficiency in relation to ToRMV by BtMEAM1 and at higher rates by BtMED in single infections. However, ToRMV was transmitted more efficiently than ToSRV in mixed infections, suggesting that this preferential transmission of ToRMV over ToSRV may be related to replication. For the second objective, corresponding to the second chapter, ToSRV DNA-A mutants containing the same nucleotides of ToRMV DNA-A at the divergent positions in the CR and within the IRD (iteron-related domain) of the Rep gene were constructed. In mixed inoculation with ToRMV, high accumulation of ToSRV DNA-A

containing mutations in the CR was observed (as compared to wild-type ToSRV), suggesting that the mutated CR sites serve as specific recognition sites for Rep binding, increasing viral replication and viral DNA accumulation. For the third objective, corresponding to the third chapter, tomato and Nicotiana benthamiana plants were biolistically inoculated with ToYSV and ToSRV in the presence or absence of the alphasatellite. Interaction of the two begomoviruses with EuYMA was demonstrated, and shown to be associated the increased symptom severity. The association of EuYMA with ToYSV was less efficient in tomato than in N. benthamiana, while that between ToSRV and EuYMA was similar in both hosts, indicating differences in the interaction between the alphasatellite and different tomato-infecting begomoviruses., The results of this study provide new information that increases the understanding of virus, DNA satellite, vector and host interactions that contribute to the adaptation and predominance of begomoviruses in the field.

INTRODUÇÃO GERAL

A família Geminiviridae é constituída por vírus com genoma composto por um ou dois componentes de DNA de fita simples circular encapsidados em partículas icosaédricas geminadas. A família é constituída pelos gêneros Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus e Turncurtovirus, definidos com base no tipo de inseto vetor, gama de hospedeiros, organização genômica e relacionamento filogenético (Brown et al., 2012; Varsani et al., 2014; Varsani et al., 2017; Zerbini et al., 2017). O gênero Begomovirus inclui vírus transmitidos por moscas-brancas (Bemisia tabaci) a plantas dicotiledôneas (Brown et al., 2015). Membros desse gênero incluem um grande número de patógenos de relevância econômica em culturas de grande importância econômica, como o algodão, o feijoeiro, a mandioca e o tomateiro (Rojas et al., 2018).

Com base em estudos filogenéticos e características do genoma, os begomovírus podem ser divididos em dois grupos: Velho Mundo (VM; Europa, África e Ásia) e Novo Mundo (NM; Américas) (Rybicki, 1994; Padidam et al., 1999; Paximadis et al., 1999). A maioria dos begomovírus do Novo Mundo (Américas) possuem genoma constituído por dois componentes de DNA, denominados DNA-A e DNA-B. O DNA-A contém cinco genes: Rep, que codifica a única proteína essencial à replicação, iniciadora do mecanismo de replicação por círculo rolante (Fontes et al., 1992; Orozco et al., 1997); Trap, que codifica um fator transcricional dos genes Cp e Nsp, e que também atua como supressora de respostas de defesa da planta (Voinnet et al., 1999; Wang et al., 2005); Ren, que codifica um fator acessório (não essencial) à replicação viral (Sunter et al., 1990; Pedersen e Hanley-Bowdoin, 1994); Ac4, que codifica uma proteína também envolvida na supressão de silenciamento gênico (Vanitharani et al., 2004); e Cp, que codifica a proteína capsidial, essencial também para a transmissão do vírus pelo inseto vetor (Briddon et al., 1990; Hofer et al., 1997). O DNA-B contém dois genes: Nsp, que codifica a

proteína responsável pelo transporte do DNA através do envelope nuclear (Noueiry et al., 1994; Sanderfoot e Lazarowitz, 1996) e também possui atividade de supressão de mecanismos de defesa (Zorzatto et al., 2015), e Mp, que codifica a proteína envolvida no movimento célula-a-célula do vírus por meio do aumento do limite de exclusão dos plasmodesmas (Noueiry et al., 1994). Os dois componentes compartilham baixa identidade de sequência, com exceção de uma região de aproximadamente 200 nucleotídeos denominada região comum (RC), que apresenta mais de 85% de identidade (Briddon et al., 2010).

A RC contém diversos elementos de sequência conservados, incluindo a origem de replicação do DNA viral e sequências repetidas diretas e invertidas denominadas iterons, que são reconhecidas pelo domínio relacionado aos iterons ("iteron-related domain", IRD) da proteína viral Rep para iniciar a replicação (Arguello-Astorga et al., 1994; Fontes et al., 1994; Gutierrez, 1999). A sequência de nucleotídeos dos iterons pode variar entre os begomovírus (Arguello-Astorga et al., 1994; Arguello-Astorga e Ruiz-Medrano, 2001). Os aminoácidos específicos da IRD (e também adjacentes ao motivo II de Rep) importantes para o reconhecimento dos iterons são denominados determinantes de especificidade ("specificity determinants", SPDs) (Arguello-Astorga e Ruiz-Medrano, 2001; Ramos et al., 2003; Londono et al., 2010). O sucesso na formação de pseudo-recombinantes viáveis em infecções mistas de begomovírus geralmente depende da conservação dessas regiões (Arguello-Astorga et al., 1994; Arguello-Astorga e Ruiz-Medrano, 2001; Ruiz-Medrano et al., 2001; Bull et al., 2007).

Os begomovírus são transmitidos naturalmente por moscas-brancas pertencentes ao complexo de espécies crípticas Bemisia tabaci (De Barro et al., 2011). As espécies B. tabaci Middle East-Asia Minor 1 (BtMEAM1, antigo biótipo B) e B. tabaci Mediterranean (BtMED, antigo biótipo Q) destacam-se por serem mais invasivas e apresentarem distribuição mundial (Barbosa et al., 2015). A emergência de begomovírus em tomateiro no Brasil parece ter sido reflexo da dinâmica das populações do inseto vetor, as quais aumentaram significativamente

após a introdução de BtMEAM1 na década de 1990 (Ribeiro et al., 2003). A hipótese mais aceita é que B. tabaci MEAM1 tenha sido capaz de transmitir para o tomateiro espécies virais que anteriormente eram restritas a hospedeiros não-cultivados, o que pode ser justificado pelo comportamento polífago dessa espécie (Rocha et al., 2013; Inoue-Nagata et al., 2016).

Tomato rugose mosaic virus (ToRMV) e Tomato severe rugose virus (ToSRV) pertencem ao complexo de espécies de begomovírus que infectam o tomateiro no Brasil. Em um estudo realizado por Silva et al. (2014), plantas de tomateiro foram inoculadas com clones infecciosos de ToRMV e ToSRV em todas as combinações possíveis de infecção simples e mista entre o DNA-A e DNA-B de ambos os vírus. Curiosamente, embora o ToRMV e ToSRV formem pseudo-recombinantes viáveis em seu hospedeiro natural e possuam iterons e SPDs idênticos, os DNAs A e B do ToRMV foram detectados preferencialmente em relação aos de ToSRV quando ambos os vírus foram inoculados simultaneamente, e o acúmulo de ToSRV foi muito reduzido, sugerindo uma interferência negativa na replicação de ToRMV em relação ao ToSRV em infecção mista (Silva et al., 2014). Assim, eficiência na replicação de begomovírus em infecção mista pode ser resultante não apenas da sequência dos iterons e SPDs localizados na IRD.

Embora o ToRMV tenha replicado preferencialmente em relação ao ToSRV em condições experimentais (Silva et al., 2014), no campo o ToSRV é o vírus predominante (Lima et al., 2006; Cotrim et al., 2007; Fernandes et al., 2008; González-Aguilera et al., 2012; Rocha et al., 2013). Isso pode ser devido a diferenças na eficiência de transmissão pelo inseto vetor, uma vez que a proteína capsidial, distinta entre os dois vírus, é o principal determinante da transmissão.

Em campos de produção de tomateiro no Brasil e em outros países, infecções mistas com dois ou mais begomovírus são comuns (Fernandes et al., 2008; Diaz-Pendon et al., 2010; Banuelos-Hernandez et al., 2012; Rocha et al., 2013; Naseem e Winter, 2016). Entretanto, os

mecanismos responsáveis pelo processo de replicação, preferência e eficiência de transmissão pela mosca-branca nos casos em que dois ou mais begomovírus encontram-se presentes na mesma planta são pouco compreendidos.

Nos últimos anos, alfassatélites associados a begomovírus do NM foram relatados infectando plantas não-cultivadas no Brasil e em Cuba (Paprotka et al., 2010; Jeske et al., 2014; Ferro et al., 2017; Mar et al., 2017) e em cultivos de melancia na Venezuela (Romay et al., 2010). Alfassatélites são moléculas de DNA circular de fita simples que replicam independentemente, no entanto dependem de um vírus auxiliar (um begomovírus ou um membro da família Nanoviridae) para infecção sistêmica e transmissão pelo inseto vetor (Saunders et al., 2002; Briddon et al., 2018). Apesar de terem sido descobertos há mais de quinze anos, pouco se conhece sobre o efeito da presença de alfassatélites na infecção por begomovírus. A maioria dos estudos tem mostrado que alfassatélites não alteram os sintomas causados pelos begomovírus (Saunders e Stanley, 1999; Saunders et al., 2000; Saunders et al., 2002). No entanto, foi demonstrado que a proteína alpha-Rep pode atuar como supressora do silenciamento gênico transcricional para Cotton leaf curl Multan alphasatellite (Abbas et al., 2016) e como supressora pós-transcricional para dois alfassatélites encontrados no Paquistão (Nawaz-Ul-Rehman et al., 2010), favorecendo a patogenicidade. No Brasil, a detecção de alfassatélites associados a begomovírus em plantas não-cultivadas (Paprotka et al., 2010; Ferro et al., 2017; Mar et al., 2017) sugere a possibilidade de transmissão para plantas cultivadas pelo inseto vetor.

Os objetivos desta tese foram: i) avaliar a eficiência de transmissão de ToSRV e ToRMV em infecções simples e mistas por BtMEAM1 e BtMED; ii) verificar se nucleotídeos divergentes na RC e na região correspondente a região IRD da Rep de ToSRV e ToRMV são responsáveis pela interferência negativa que ToRMV exerce sobre o ToRSV; iii) verificar se o alfassatelite Euphorbia yellow mosaic alphasatellite (EuYMA), detectado em associação a um

begomovírus em plantas não-cultivadas, pode interagir com begomovírus que infectam o tomateiro. Cada objetivo está relacionado a um capítulo nesta tese.

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CHAPTER 1

TRANSMISSION OF TWO BIPARTITE BEGOMOVIRUSES IN SINGLE AND MIXED INFECTIONS BY TWO SPECIES OF THE *Bemisia tabaci* CRYPTIC SPECIES COMPLEX

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Transmission of two bipartite begomoviruses in single and mixed infections by two species of the Bemisia tabaci cryptic species complex

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Abstract

The genus Begomovirus (family Geminiviridae) includes viruses that infect dicotyledonous plants, have a single-stranded, circular DNA genome and are transmitted by whiteflies of the Bemisia tabaci cryptic species complex. Begomoviruses cause diseases in several economically important crops around the world. Tomato severe rugose virus (ToSRV) and Tomato rugose mosaic virus (ToRMV) belong to a complex of begomoviruses that infects tomato in Brazil. ToRMV is not widely disseminated in the field, unlike ToSRV, which predominates in tomato fields in southeastern Brazil. This work was conducted to compare the transmission efficiency of ToSRV and ToRMV by B. tabaci Middle East-Asia Minor 1 (BtMEAM1) and Mediterranean (BtMED) in single and mixed infections. Following biolistic inoculation, plants with each virus in single or mixed infection were used as inoculum sources for the transmission experiments. When plants with single infections were used as sources, the rates of transmission of ToSRV and ToRMV by BtMEAM1 were similar. However, ToSRV was more efficiently transmitted by BtMED than ToRMV. When plants with mixed infection were used as sources of inoculum, the genomic components of ToRMV were always detected in the inoculated plants, while the ToSRV components were rarely detected. These results suggest that although ToSRV is transmitted with the same efficiency in relation to ToRMV by BtMEAM1 and even at higher rates by BtMED in single infections, efficiency or success in the transmission of two (or more) begomoviruses in mixed infection may be more related to the viral concentration in the source host and/or the interference that one virus may exert in the replication of the other. This is consistent with previous results indicating that ToRMV exerts a negative interference on the accumulation of ToSRV at the early stages of the infection cycle.

Introduction

Whiteflies of the Bemisia tabaci species complex (order Hemiptera, family Aleyrodidae) cause enormous damage to agricultural and ornamental crops due to their polyphagous food habit and the fact that they transmit viruses belonging to the genera Begomovirus, Carlavirus, Crinivirus, Ipomovirus and Torradovirus [1, 2].

B. tabaci comprises a cryptic species complex with at least 43 morphologically indistinguishable species [3-5] which are genetically defined based on the sequence of the mitochondrial cytochrome oxidase I (mtCOI) gene [3, 5, 6]. B. tabaci species differ in their biological characteristics, such as host range, resistance to insecticides, endosymbiotic composition, dispersion capacity and virus transmission efficiency [2, 5, 7, 8].

The species B. tabaci Middle East-Asia Minor 1 (BtMEAM1, formerly "biotype B") and B. tabaci Mediterranean (BtMED, formerly "biotype Q") are more invasive and have the greater global distribution [9]. BtMEAM1 has been the predominant species in Brazil since the mid-1990's [2, 10] and is responsible for damage to various crops. The BtMED species was detected recently in the states of Rio Grande do Sul, São Paulo and Paraná [9, 11], leading to concerns about its spread as it displays lower susceptibility to insecticides, a capacity to survive at different temperatures [7, 12] and the ability to replace other whiteflies species, which occurred in different regions of the world [13-16]. Due to its recent introduction, studies of virus transmission by BtMED in Brazil are scarce.

The majority of viruses transmitted by whiteflies belong to the genus Begomovirus of the family Geminiviridae [1, 17]. Begomoviruses have a genome composed of one or two molecules of circular, single-stranded DNA encapsidated in geminated icosahedral particles [18]. The two genomic components of bipartite begomoviruses are named DNA-A and DNA-B. The DNA-A encodes proteins involved in viral replication, transactivation of viral genes,

suppression of host defense responses and encapsidation [19, 20], while the DNA-B encodes proteins associated with intra- and intercellular movement, determination of host range and suppression of defense responses [19-22].

Begomoviruses are transmitted by B. tabaci in a persistent circulative manner. Viral particles are acquired via the stylet during the process of feeding in the phloem of infected plants, enter the esophagus and the filter chamber and subsequently are transported through the wall of the gut to the haemolymph, where they circulate until entering the salivary gland, from which they are transmitted to new plants during the feeding of the insect [23, 24]. Translocation of begomoviruses along the body of the insect is dependent on the interaction of the virus with putative receptors on the insect tissues [25]. The capsid protein is responsible for recognizing these putative receptors [26], and is the essential viral protein for the acquisition and transmission of begomoviruses by the insect vector [26, 27]. The viral CP also interacts with GroEL homologue proteins, which are secreted into the haemolymph by bacterial endosymbionts [28, 29]. This interaction protects viral particles from degradation by proteases and nuclease which are present in the haemolymph, and is essential for virus transmission [29]. Different species of bacterial endosymbionts secrete different GroEL homologs, which in turn differ in the efficiency of their interaction with the CPs of different begomoviruses. Thus, the composition of their endosymbiont population affects the transmission efficiency of begomoviruses by B. tabaci species [30, 31].

In the early 1990, a begomovirus complex emerged in tomato plants in Brazil, coinciding with the introduction of BtMEAM1 [32], which quickly displaced the previously existing species B. tabaci New World (NW, formerly "biotype A"). Since then, a large number of new begomoviruses infecting tomato have been characterized [33-39]. It is possible that BtMEAM1 was able to transmit viruses that were previously restricted to uncultivated hosts to

tomato, which may be explained by the polyphagous behavior of this species, unlike that of BtNW.

Currently, begomoviruses affect tomato crops in all producing states. However, some viruses are widely distributed throughout the country, while others are restricted to certain regions [32, 34, 35]. Tomato severe rugose virus (ToSRV) is the begomovirus that predominates in southern and southeastern Brazil [35, 39-42]. Other begomoviruses, including Tomato chlorotic mottle virus (ToCMoV), Tomato common mosaic virus (ToCmMV), Tomato mottle leaf curl virus (TMoLCV) and Tomato yellow vein streak virus (ToYVSV), have also been found frequently in tomato growing fields [38, 39, 42-44]. However, viruses such as Tomato leaf distortion virus (ToLDV), Tomato yellow spot virus (ToYSV) and Tomato rugose mosaic virus (ToRMV) have been reported sporadically [33, 37, 39]. This biogeographic pattern may be the result of virus-host coevolution, viral adaptation to different climatic conditions or differences in vector transmission efficiency [37, 39, 45]. It is important to emphasize that these possibilities are not mutually exclusive.

Although mixed infections (two or more begomoviruses simultaneously infecting the same plant) are common in tomato plants in Brazil and in other countries [35, 39, 46-48], the mechanisms that favor the predominance and adaptation of a virus in relation to others are poorly understood. Silva et al. [49] showed that under experimental conditions (biolistic inoculation) ToRMV and ToSRV form viable pseudorecombinants in all possible combinations between DNA-A and B. However, in most plants inoculated with both viruses simultaneously, only the ToRMV DNA-A and DNA-B components were detected, and ToSRV accumulation was very reduced. This suggested that ToRMV exerts a negative interference on the replication of ToSRV, and was an intriguing result considering the predominance of ToSRV over ToRMV in the field. It was proposed that this predominance could be due to differences in the efficiency of transmission by the insect vector, which may favor ToSRV, since the capsid proteins are

distinct between the two viruses [49]. In support of this hypothesis, Macedo et al. [45] evaluated the transmission efficiency of two begomoviruses (ToSRV and Tomato golden vein virus, TGVV) by BtMEAM1 in single and mixed infections, and found that ToSRV was transmitted more efficiently in all situations.

In this study, we analyzed the transmission efficiency of ToSRV and ToRMV by BtMEAM1 and BtMED, both in single and mixed infections. Our results indicate that in single infections ToSRV is transmitted with the same efficiency in relation to ToRMV by BtMEAM1, but at higher rates by BtMED. Surprisingly, ToRMV was transmitted more efficiently than ToSRV in mixed infections. However, previous studies [47] and our own results (Chapter 2) suggest that this preferential transmission of ToRMV over ToSRV is due to its higher rate of replication. Virus-vector-plant interactions that contribute to the adaptation and predominance of begomoviruses in the field are discussed.

Material and Methods

Inoculum sources

Tomato (Solanum lycopersicum) plants cv. Santa Clara were biolistically inoculated [50] with infectious clones corresponding to the DNA-A and DNA-B of the viral isolates ToRMV-[BR:Ub1:96] [33] and ToSRV-[BR:PG1:Pep:03] [51]. The plants were inoculated with the following combinations of DNA components: (i) ToRMV DNA-A + DNA-B (single infection with ToRMV); (ii) ToSRV DNA-A + DNA-B (single infection with ToSRV); (iii) ToRMV DNA-A + DNA-B together with ToSRV DNA-A + DNA-B (mixed infection). Tomato plants bombarded with tungsten particles without DNA were used as negative controls. These inoculum source plants were kept in a temperature-controlled growth chamber at 22°C to maintain uniformity in the concentration of the viral inoculum during the experiments.

To confirm the infection, total DNA of all plants was extracted as described by Doyle and Doyle [52]. Plants with single infection were confirmed by PCR using primers to amplify DNA-A fragments of ToRMV (ToRMV/For: 5'-CAG TAG TTG CCT TCG AAT TGA AG-3'; ToRMV/Rev: 5'-CAC GTG TAG CAA TCT CCT TAA AGG-3') or ToSRV (ToSRV/For: 5'-CAG TAG TTG CCC TCA AAT TGA AG-3'; ToSRV/Rev: 5'-CAC GTG TAG CAA TCT CCT TAA AGA G-3'). Reactions contained 12.5 µL of PCR master mix (2x) (GoTaq Green Master Mix, Promega), 2 µL of total DNA and 0.4 µM of each primer in a final volume of 25 µL. The PCR parameters consisted of an initial denaturing step at 95°C for 2 min, followed by 38 cycles at 95°C for 1 min, 66°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Plants with mixed infection were confirmed by rolling-circle amplification (RCA) [53] followed by digestion with restriction enzymes that specifically cleave the DNA-A and DNA-B components of each virus (RCA-RFLP; Silva et al. [49]) (Supplementary Figure S1). Transmission assays were performed using inoculum source plants at 21 days post-inoculation (dpi). Confirmation of mixed infection was always performed one day before the transmission experiment to ensure that inoculum source plants were infected with the four genomic components. Four independent experiments were conducted.

Transmission assays and confirmation of infection

Transmission assays to evaluate the transmission efficiency of ToRMV and ToSRV by BtMEAM1 from source plants with single and mixed infections were conducted at UFV. BtMEAM1 populations were obtained from colonies maintained in cabbage plants (Brassica oleracea var. capitata; a non-host for tomato begomoviruses) kept inside whitefly-proof screened cages in a growth chamber with controlled temperature of 25°C and a photoperiod of 14 h light and 10 h dark. Source plants (with ToRMV and ToSRV in single or mixed infections)

were placed in separate screened cages with about 1000 aviruliferous whiteflies for an acquisition access period (AAP) of 48 h. After the AAP, whiteflies were transferred to plastic cups containing healthy plants (30 adult insects/plant) using a mouth aspirator for an inoculation access period (IAP) of 48 h. After the IAP, whiteflies were eliminated mechanically and by the application of acetamiprid (80 mg A.I./L). The plants were kept in a greenhouse in protected cages separated by treatment to avoid contamination, with weekly applications of insecticides (alternating tebuconazole, 1 mL A.I./L, acetamiprid, 0.4 g A.I./L and abamectin, 1 mL A.I./L with spiromesifene, 2 mL A.I./L and copper hydroxide, 3.5 mL A.I./L). Aviruliferous whiteflies transferred to healthy tomato plants for AAP and IAP of 48 h were used as negative controls. The appearance of symptoms was evaluated up to 35 dpi. Total DNA was extracted from all tested plants as described by Doyle and Doyle [52]. Confirmation of single infections was performed at 28 dpi by PCR using primers for detection of the DNA-A, as described above. Confirmation of mixed infection was performed at 14 and 28 dpi by RCA-RFLP, as described above. Four independent experiments were conducted using plants with single infection as inoculum sources, and three experiments were conducted using plants with mixed infection as inoculum sources. The number of plants tested varied by treatment and in each experiment (Table 1).

Comparative assays of transmission of ToRMV and ToSRV using plants with single infection as sources, mediated by populations of BtMEAM1 and BtMED, were conducted at the Virology Laboratory of UNESP Botucatu. The populations of BtMEAM1 and BtMED were maintained in cabbage and cotton plants, respectively, in separate environments with controlled temperature of 26°C and a photoperiod of 12 h light and 12 h dark. The inoculum sources were tomato plants inoculated with either ToRMV or ToSRV (single infections). The transmission efficiencies of ToRMV and ToSRV by BtMEAM1 and BtMED were tested in 30 plants. Adult, aviruliferous insects were transferred using a mouth aspirator to small cages containing leaves

of plants infected with one of the viruses for an AAP of 48 h. The insects were then transferred to cages containing healthy plants for an IAP of 48 h (10 insects/plant). Following the IAP, whiteflies were eliminated by the application of insecticides (cartap hydrochloride, 2.5 g A.I./L and spiromesifene, 1.25 mL A.I./L). The plants were kept in a greenhouse inside protected cages separated by treatment. Infection of the tested plants was confirmed by PCR using primers for detection of the DNA-A, as described above.

Results

Symptoms in tomato plants inoculated with ToSRV and ToRMV in single and mixed infections appeared at 10-14 days post-inoculation (dpi), and consisted of yellow mosaic. There were no significant differences in symptoms displayed by plants with single or mixed infection (Figure 1).

ToSRV and ToRMV were transmitted with the same efficiency in single inoculations mediated by B. tabaci Middle East Asia Minor 1 (BtMEAM1), either from colonies kept at UFV (98% for both viruses; Table 1) or at Unesp Botucatu (90% and 86.7% for ToSRV and ToRMV, respectively; Table 2). Conversely, B. tabaci Mediterranean (BtMED) transmitted ToSRV more efficiently than ToRMV (96.7% and 76.7%, respectively; Table 2). Confirmation of the percentage of infected plants was performed at 28 dpi by PCR using primers to detect the DNA-A from each virus. These results indicate that different species of the Bemisia tabaci complex can play distinct roles in the transmission and epidemiology of begomoviruses infecting tomato.

Although both viruses were detected in the plants used as inoculum source for mixed inoculations mediated by BtMEAM1, the genomic components of ToRMV were detected preferentially in relation to the ToSRV components in the inoculated plants, in the three

independent experiments (Table 1). Confirmation of infection was performed by RCA-RFLP to determine the percentage of transmission of each genomic component. Evaluations were performed at 14 and 28 dpi to eliminate the interference in replication that one virus may exert on the other in mixed infection, as previously demonstrated [49].

At 14 dpi, the average percentage of plants inoculated with both viruses but in which only ToRMV DNA-A and DNA-B were detected were 82.1% and 76.9% respectively. At 28 dpi these values increased to 89.7% and 87.2%, respectively. Of the 39 plants inoculated with both viruses, none were infected only with ToSRV DNA-A and B. ToRMV and ToSRV DNA-As were detected together with ToRMV DNA-B in one plant (2.6%) and only at 14 dpi. ToRMV and ToSRV DNA-Bs were detected together in three plants (7.7%) at 14 dpi and in one plant (2.6%) at 28 dpi.

Considering each experiment separately, ToRMV DNA-A and DNA-B were detected in 100% the plants of the first experiment at 14 dpi and 28 dpi. The ToSRV DNA-B was detected in only 20% of the plants and only at 14 dpi, with no ToSRV components being detected at all at 28 dpi. In the second experiment, the transmission rate of ToRMV was 100% for both components at both 14 and 28 dpi, while the ToSRV DNA-B was detected in only one plant at 28 dpi and the DNA-A was not detected at all at either time point. In the third experiment, at 14 dpi, the two ToRMV components were detected in 53.3% of the inoculated plants, and in one plant (6.7%) in mixed infection with ToSRV DNA-A. Detection performed at 7 dpi confirmed the presence of ToSRV DNA-A in mixed infection with ToRMV in this single plant (data not shown). Likewise, the ToSRV DNA-B was detected in only one plant together with both ToRMV components. At 28 dpi, the two ToRMV components were detected in 73.3% of the inoculated plants, while the ToSRV components were not detected at all. These results clearly indicate that the ToRMV genomic components were transmitted at a higer rate by BtMEAM1 in relation to the ToSRV components from tomato plants with mixed infection.

Discussion

The high transmission efficiency of ToRMV and ToSRV in single inoculations mediated by two populations of BtMEAM1 is further indication that this whitefly species is an excellent vector for the transmission of these begomoviruses. The capsid proteins of ToRMV and ToSRV have about 81% amino acid sequence identity, a considerable degree of divergence considering that the CP is the most conserved protein among begomoviruses. Nevertheless, these two viruses have the same rates of transmission by BtMEAM1, indicating that their CPs are capable of interacting equally efficiently with potential receptors in the whitefly tissues, ensuring their acquisition, translocation and transmission in a similar way. Conversely, differences in the transmission efficiency of ToRMV and ToSRV by BtMED were observed, with ToSRV being transmitted with greater efficiency. This is a worrisome result, considering the predominance of ToSRV in the field and the high capacity of adaptation to different environmental conditions displayed by BtMED [7, 12]. The transmission efficiency of ToSRV by the indigenous species B. tabaci NW2 has also been reported to be high [54]. Distinct whitefly species can transmit the same begomovirus with different efficiencies. For example, B. tabaci MEAM1 acquires and transmits Papaya leaf curl China virus (PaLCuCNV) with greater efficiency compared to B. tabaci MED, Asia 1 and Asia II 7 [55]. The introduction and dissemination of different B. tabaci species in a new geographical region may therefore cause relevant changes in the distribution and prevalence of tomato-infecting begomoviruses in the field.

ToSRV and ToRMV were transmitted by BtMEAM1 with the same efficiency in single infections. However, when whiteflies fed on plants containing both viruses, the genomic components of ToRMV were always detected in all inoculated plants, unlike the ToSRV

components which were rarely detected. We propose three hypotheses to explain the preferential whitefly transmission of ToRMV in relation to ToSRV in mixed infections.

Silva et al. [49] showed that the accumulation of ToSRV is lower than that of ToRMV in plants with mixed infection. Thus, the first hypothesis is that the number of ToRMV particles is more abundant in the plant phloem sap in relation to ToSRV, favoring the acquisition of ToRMV by the whitefly during ingestion and consequently ensuring greater transmission efficiency. Consistent with this hypothesis, Kollenberg et al. [56] found that the highest uptake of Watermelon chlorotic stunt virus (WmCSV) in relation to Tomato yellow leaf curl virus (TYLCV) by BtMEAM1, as well as the higher accumulation of WmCSV compared to TYLCV in whitefly tissues, was due to a higher concentration of WmCSV DNA in watermelon than that of TYLCV DNA in tomato. After supplying the same concentration of each virus via artificial media, similar amounts of WmCSV and TYLCV were acquired by the whiteflies. To evaluate whether the higher whitefly transmission efficiency of ToRMV compared to ToSRV may be due to a higher acquisition rate of this begomovirus, experiments to determine the rate of acquisition of both viruses by the whitefly based on the detection of viral particles in the salivary glands of the insects after feeding in inoculum sources with single and mixed infections, could be performed.

Macedo et al. [45] showed that ToSRV and Tomato golden vein virus (TGVV) were detected at a similar rate in whiteflies after feeding in plants with single and mixed infections, showing that the transmission efficiency is possibly more related to inoculation efficiency than acquisition. Thus, a second hypothesis would be that whiteflies acquire both viruses equally efficiently during feeding, however they inoculate ToRMV preferably in relation to ToSRV. In support of this hypothesis, Sánchez-Campos et al. [57] reported that differences in the transmission efficiency of Tomato yellow leaf curl Sardinia virus (TYLCSV) and TYLCV by BtMEAM1 and BtMED were not related to differences in acquisition. There are a number of

receptors in the tissues of the insect with which the viral CP must interact to cross the barriers along the body of the whitefly, allowing the virus to be transmitted [25]. After ingestion by the whitefly, ToRMV could compete and bind with higher affinity to the receptors in the whitefly, facilitating its translocation and transmission in relation to ToSRV. However, the high transmission efficiency of both viruses in single infection makes this hypothesis unlikely.

The third hypothesis states that both viruses are acquired and transmitted efficiently, however, as demonstrated by Silva et al. [49], ToRMV exerts a negative interference on the accumulation of ToSRV during the initial stages of infection in tomato, suggesting a preferential replication of the ToRMV genomic components in relation to ToSRV. In support of this hypothesis, it was shown that in plants inoculated with both viruses the ToSRV DNA-A could not be detected at 14 dpi, with the exception of one plant in the third replication of the experiment. Out of the 39 plants tested, no plant inoculated with both viruses was infected with the four components or with the ToSRV DNA-A and DNA-B together. Thus, after inoculation, possibly at the early stages of the infection cycle, there could be a competition between the two viruses and ToRMV prevails. Coexistence between the two viruses is rare even under artificial conditions. Silva et al. [49] showed that out of 13 plants biolistically inoculated with all four components, the DNA-As of ToRMV and ToSRV were detected together in only two plants. Another interesting aspect is the predominance of the DNA-B. The ToRMV and ToSRV DNA-Bs are virtually identical, displaying 98.2% nucleotide sequence identity. However, a decrease in the number of plants in which the ToSRV DNA-B was detected was verified between 14 and 28 dpi. High detection rates of ToRMV DNA-B and the decrease of ToSRV reinforce the evidence of a negative effect of ToRMV on ToSRV replication. Negative interference caused by ToRMV has also been reported in mixed infection with another begomovirus, Tomato yellow spot virus (ToYSV) [58]. During the initial events of the infection cycle, ToRMV negatively affects the accumulation of ToYSV, however after the establishment of systemic infection this

negative interference ceases to occur. Thus, it is reasonable to suggest that ToRMV may interfere in the replication of ToSRV, rather than in its transmission by the insect vector.

ToSRV is the predominant begomovirus found in tomato fields in southern and southeastern Brazil, while ToRMV is rarely found [35, 39-42]. Whitefly transmission assays using ToSRV-infected tomato plants as an inoculum source have shown that this virus can cause a change in the composition of some volatiles in tomato and in the behavior of the whitefly, increasing its preference for volatiles emitted by healthy plants and consequently favoring its dissemination [59]. In addition to this adaptive advantage in the field, ToSRV has shorter acquisition and inoculation access periods than those reported for other begomoviruses [60]. Although the specific location of ToSRV in tomato leaf tissue has not been determined, Toloy et al. [60] suggest that its short AAP is due to its presence in mesophyll cells. Tomato-infecting begomoviruses have been shown to be phloem-restricted in this host [58, 61, 62], although some of them can infect mesophyll cells of Nicotiana benthamiana [58, 63]. The tissue tropism of ToSRV in tomato and other hosts remains to be determined.

Due to the prevalence of ToSRV in the field, it was expected that ToSRV would be transmitted more efficiently than ToRMV in both single and mixed infections. ToSRV may prevail in nature by infecting plants in the absence of ToRMV. ToSRV has been found to naturally infect several cultivated hosts, including potato, common bean, pepper, eggplant and soybean [64-68] as well as ubiquitous non-cultivated plants such as Chenopodium album, C. ambrosioides, Crotalaria spp., Euphorbia heterophylla, Nicandra physaloides and Sida spp [69-71]. In comparison, the host range of ToRMV seems to be much narrower, including only tomato and N. physaloides [33]. Studies have shown that non-cultivated plants growing in close proximity to tomato crops may serve as reservoirs for begomoviruses [69, 70], conferring an advantage to begomoviruses with wide host ranges such as ToSRV. Thus, the limited ability to

colonize different hosts may be a factor responsible for restricting the maintenance and propagation of ToRMV in the field.

The predominance and adaptative advantage of some begomoviruses in crop species such as tomato are still poorly understood phenomena. Our results indicate that although individual begomoviruses may be transmitted with the same efficiency in single infections, success in the transmission of each begomovirus in mixed infections may be dependent on the viral concentration in the source host and/or the interference that one virus may exert in the replication of the other. They also suggest that the prevalence of certain begomoviruses in Brazilian tomato fields may not be related to differences in transmission efficiency by the insect vector.

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Table 1. Detection of begomoviruses Tomato severe rugose virus (ToSRV) and Tomato rugose mosaic virus (ToRMV) transmitted by Bemisia tabaci Middle East Asia Minor 1 (BtMEAM1) in single or mixed infection.

Virus	Genome component	Infected/inoculated (%)*									
		Experiment 1		Experiment 2		Experiment 3		Experiment 4		Average (%)	
		14 dpi	28 dpi	14 dpi	28 dpi	14 dpi	28 dpi	14 dpi	28 dpi	14 dpi	28 dpi
ToSRV[#]	ToS-A	n.a.[†]	10/10 (100)	n.a.	15/15 (100)	n.a.	15/15 (100)	n.a.	10/11 (90.9)	-	98.0
ToRMV[#]	ToR-A	n.a.	10/10 (100)	n.a.	15/15 (100)	n.a.	15/15 (100)	n.a.	12/13 (92.3)	-	98.1
ToSRV + ToRMV	ToS/R-A[&]	0/10 (0)	0/10 (0)	0/14 (0)	0/14 (0)	1/15 (6.7)	0/15 (0)	n.p.[‡]	n.p.	2.6	0
	ToS/R-B	2/10 (20)	0/10 (0)	0/14 (0)	1/14 (7.1)	1/15 (6.7)	0/15 (0)	n.p.	n.p.	7.7	2.6
ToSRV + ToRMV	ToS-A[¶]	0/10 (0)	0/10 (0)	0/14 (0)	0/14 (0)	0/15 (0)	0/15 (0)	n.p.	n.p.	0	0
	ToS-B	0/10 (0)	0/10 (0)	0/14 (0)	0/14 (0)	0/15 (0)	0/15 (0)	n.p.	n.p.	0	0
ToSRV + ToRMV	ToR-A[¶]	10/10 (100)	10/10 (100)	14/14 (100)	14/14 (100)	8/15 (53.3)	11/15 (73.3)	n.p.	n.p.	82.1	89.7
	ToR-B	8/10 (80)	10/10 (100)	14/14 (100)	13/14 (92.9)	8/15 (53.3)	11/15 (73.3)	n.p.	n.p.	76.9	87.2

[*] Number of infected plants/number of inoculated plants, confirmed by RCA following digestion using restriction enzymes that specifically cleave each component, performed at 14 and 28 days post-inoculation. Transmission experiments conducted at UFV.

[#] Plant with single infection.

[&] Plants inoculated with both viruses in which both viruses were detected.

[¶] Plants inoculated with both viruses in which only ToSRV or ToRMV were detected.

[†] n.a., not analyzed.

[‡] n.p., not performed.

Table 2. Transmission of begomoviruses Tomato severe rugose virus (ToSRV) and Tomato rugose mosaic virus (ToRMV) by Bemisia tabaci Middle East Asia Minor 1 (BtMEAM1) and Mediterranean (BtMED) in single infection.

Virus	**Infected/inoculated (%)**[*]	
	BtMEAM1	BtMED
ToSRV	27/30 (90)	29/30 (96,7)
ToRMV	26/30 (86,7)	23/30 (76,7)

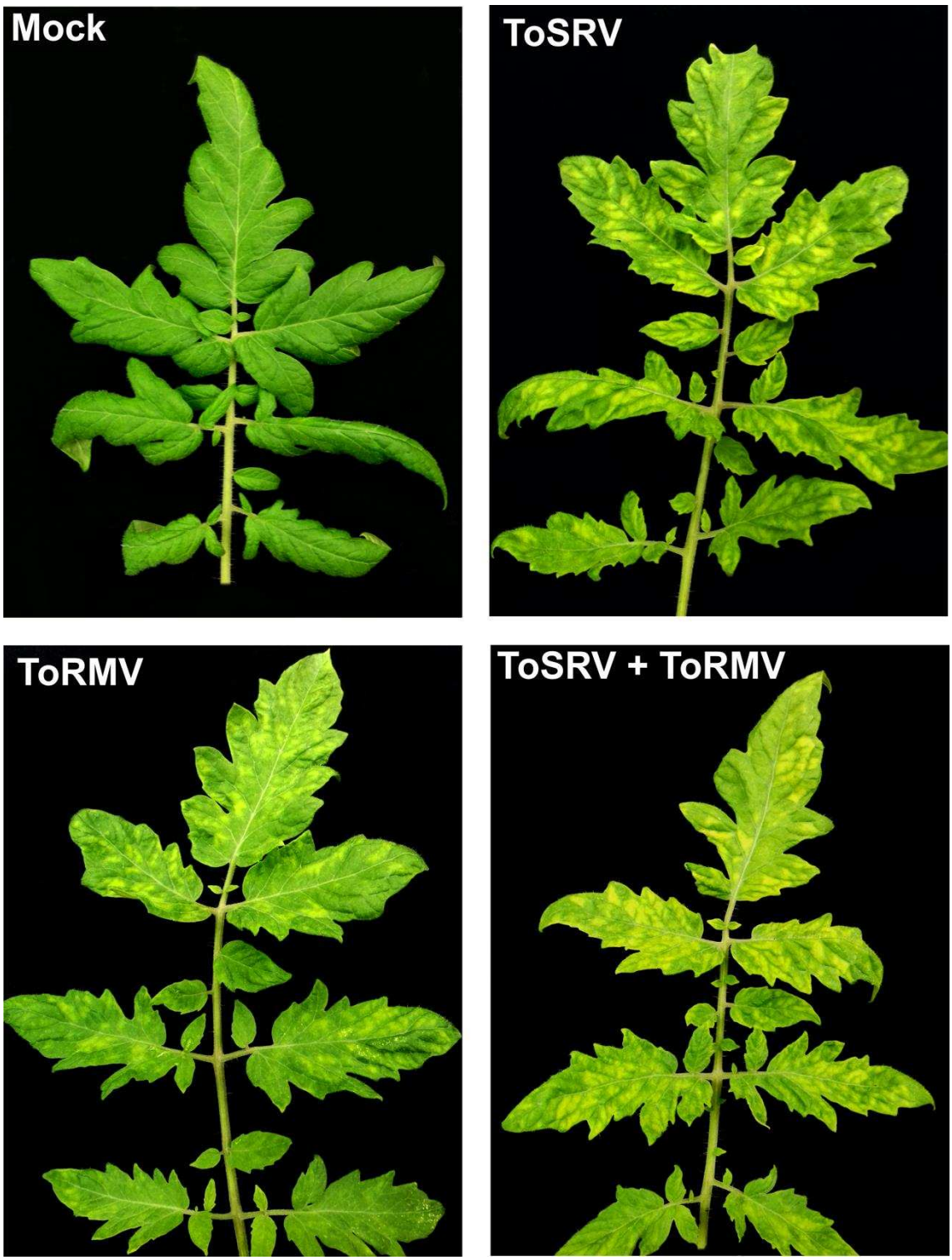
[*] Number of infected plants/number of inoculated plants, confirmed by PCR performed at 28 days post-inoculation. Transmission experiment conducted at Unesp.

Figure legends

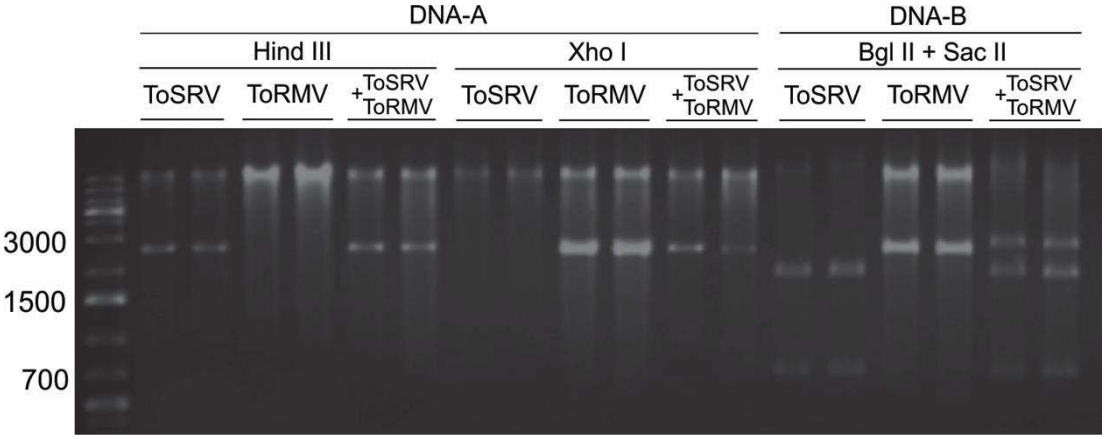
Figure 1. Symptoms in tomato plants inoculated with Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV) in single or mixed infections.

Supplementary Figure S1. Identification of plants with single or mixed infection by Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV) by rolling circle amplification (RCA) followed by digestion of the amplified DNA with restriction enzymes. Digestion with XhoI and BglII enable detection of ToRMV DNA-A and DNA-B, respectively. Digestion with HindIII and BglII + SacII enable detection of ToSRV DNA-A and DNA-B, respectively.

Figure 1



Supplementary Figure S1



CHAPTER 2

SPECIFIC NUCLEOTIDES IN THE COMMON REGION OF THE BEGOMOVIRUS Tomato rugose mosaic virus (ToRMV) ARE RESPONSIBLE FOR THE NEGATIVE INTERFERENCE OVER Tomato severe rugose virus (ToSRV) IN MIXED INFECTION

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Specific nucleotides in the common region of the begomovirus Tomato rugose mosaic virus (ToRMV) are responsible for the negative interference over Tomato severe rugose virus (ToSRV) in mixed infection

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Abstract

Natural mixed infections with two or more begomoviruses are common and may alter infectivity, symptom severity and viral accumulation in comparison to single infections. The begomoviruses Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV) have genomes with a high degree of sequence identity, including the cis-elements (iterons) in the common region (CR) and their specific recognition sites within the Rep gene (iteron-related domain, IRD), which are essential for initiating viral replication. Previous work has shown that the interaction between these begomoviruses in mixed infection is complex, with ToRMV negatively interfering in infectivity and in the accumulation of ToSRV. In this work we investigated if divergent sites in the CR and IRD of these begomoviruses are involved in this interference. ToSRV DNA-A mutants containing the same nucleotides of ToRMV DNA-A at the divergent positions of the CR (ToSRV-$A_{(CR)}$), of the Rep gene IRD (ToSRV-$A_{(IRD)}$) and in both regions (ToSRV-$A_{(CR+IRD)}$) were constructed. Infectivity and viral accumulation of ToSRV in single infection were not affected by mutations in either of the two regions. However, in mixed inoculation of ToRMV with ToSRV-$A_{(CR)}$, high infectivity of both viruses and high accumulation of ToSRV-$A_{(CR)}$ DNA in relation to wild-type ToSRV was observed. This was not observed when plants were inoculated with ToRMV and ToSRV-$A_{(IRD)}$. These results suggest that the mutated CR sites serve as specific recognition sites for Rep binding, increasing the viral replication rate and viral DNA accumulation. On the other hand, decreased viral accumulation in plants inoculated with ToSRV-$A_{(CR+IRD)}$ suggests that the divergent amino acids in the IRD do not offer an advantage for ToSRV replication efficiency.

Introduction

The genus Begomovirus (family Geminiviridae) is composed of viral species that have one (monopartite) or two (bipartite) components of circular single-strand DNA, each one encapsidated in geminate icosahedral particles [1]. The two genomic components of bipartite begomoviruses, denominated DNA-A and DNA-B, are essential for the establishment of systemic infection, with replication and encapsidation functions encoded exclusively by the DNA-A [2-4] and movement functions encoded by the DNA-B [3-7]. Cognate DNA-A and DNA-B components share low nucleotide sequence identity, except for a region of approximately 200 nucleotides within the intergenic region, denominated common region (CR), which has more than 85% identity [8].

Begomoviruses replicate in the nucleus of infected cells by rolling-circle and recombination-mediated replication mechanisms [2, 9-11]. Rep is the only viral protein essential for initiating replication [12, 13] and interacts with viral and host factors for the synthesis of new copies of viral DNA [14-16]. The Rep protein has a DNA binding domain and an oligomerization domain at the N-terminal region and a helicase domain at the C-terminal region. The N-terminal region of the Rep protein has four conserved motifs in the family Geminiviridae [17, 18]. Motif I is required for DNA-specific binding [17, 19], motif II is a metal binding site involved in DNA cleavage and protein conformation, motif III is involved in DNA binding and, as motif IV, is also involved in DNA cleavage [17, 18].

The origin of replication is comprised of a loop-like structure with an invariant nonanucleotide (TAATATT//AC) located within the CR [2]. This region also contains iterated cis-elements (direct and inverted repeats) named iterons, which are recognized by the iteron-related domain (IRD) of Rep to initiate replication [11, 20-22]. The IRD of the Rep protein is comprised of eight amino acids and together with motif I form the functional domain of Rep

[19]. The core sequence of begomovirus iterons is comprised of two guanines, followed by three nucleotides (N1, N2, and N3) that vary depending on the begomovirus [19, 20]. Generally, begomoviruses with identical iteron sequences encode Rep proteins with similar IRDs [19].

The correspondence between Rep and the iterons occurs between N1 and the eighth amino acid of the IRD, N2 with the sixth amino acid, and N3 with the first or third amino acids depending on the iteron sequence [19]. These specific amino acid residues within the IRD which are important for iteron recognition are named DNA binding specificity determinants (SPDs) [19, 23]. The presence of conserved SPDs in a number of begomoviruses has been mapped within the IRD [13, 23], and additional SPD are located adjacent to motif II [23, 24]. Some SPDs may confer greater flexibility to Rep and allow the recognition of non-cognate, divergent iteron sequences [25].

The Rep protein is a site-specific endonuclease [12, 19, 26] which confers specific recognition only of cognate DNA-A and DNA-B components. However, in mixed infections, an exchange of genomic components between bipartite begomoviruses may occur, and the DNA-B of one virus may be recognized by the Rep protein from another. This is known as pseudorecombination. Success in the formation of viable pseudorecombinants generally depends on conservation of iteron sequences [20, 21, 27] and of the amino acid sequence of the Rep protein, mainly the three SPDs within the IRD and the two SPDs near motif II, which control the affinity of the Rep by specific DNA sequences [19, 23, 28].

Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV) belong to a complex of begomoviruses that infect tomato in Brazil. Silva et al. [29] showed that ToRMV and ToSRV form viable pseudorecombinants in all possible combinations of single and mixed infections under experimental conditions (inoculation by biolistics). However, although these viruses possess the same iteron core and identical SPDs, in addition to almost identical DNA-B sequences (98.2% identity), the genomic components of ToRMV were

detected preferentially than those of ToSRV when three or four components were inoculated simultaneously. In addition, the accumulation of ToSRV was reduced, suggesting that ToRMV negatively interferes in the replication of ToSRV in a mixed infection. Thus, replication efficiency of two begomoviruses in mixed infection may depend on additional factors besides the sequences of the iterons and the SPDs.

When comparing the CR and Rep sequences of ToSRV and ToRMV, Silva et al. [29] observed nucleotide differences in nine positions in the CR and different amino acids at positions 2 and 3 of the IRD (Pro/Thr and Lys/Arg for ToSRV/ ToRMV, respectively). It is possible that one or more of these CR nucleotides and/or IRD amino acids play a role in the preferential replication of ToRMV in relation to ToSRV in a mixed infection.

Different mechanisms can favor the replication of one virus in relation to the other. The mechanisms responsible for the replication process of two or more begomoviruses present in the same cell are poorly understood. In this study we evaluated the effect of nucleotide changes in the CR and in the region corresponding to the Rep protein IRD in the infectivity and accumulation of ToSRV. Our results indicate that specific nucleotides in the CR, but not in the IRD, may be determinants for the efficiency of replication and responsible for the negative interference that ToRMV exerts on the accumulation of ToSRV in mixed infection.

Material and methods

Viral isolates and re-sequencing of isolates

Infectious clones corresponding to DNA-A and DNA-B of the viral isolates ToRMV-[BR:Ub1:96] [30] and ToSRV-[BR:PG1:Pep:03] [31] were used. These are the same clones used in the study of Silva et al. [29].

For confirmation of the sequences of the infectious clones, complete genome units were obtained by excision with BamHI (ToSRV DNA-A), EcoRI (ToRMV DNA-A) and KpnI (DNA-B from both viruses). After purification, these fragments were ligated into the pBLUESCRIPT-KS+ vector (Stratagene) previously linearized with the same enzymes. Recombinant plasmids were used for transformation into Escherichia coli by electroporation and three clones of each genomic component were sent for commercial sequencing by primer walking (Macrogen Inc, South Korea). Sequences were assembled using DNA BASER v. 3.5 (Heracle 616 Biosoft), and organized to begin at the nicking site in the invariant nonanucleotide at the origin of replication (5'-TAATATT//AC-3'). Pairwise sequence comparisons of DNA-A and DNA-B, CRs and ORFs between ToRMV and ToSRV were performed using Sequence Demarcation Tool (SDT) v.1.2 [32] using the MUSCLE alignment option [33].

Construction of mutants

After re-sequencing of the ToRMV and ToSRV infectious clones, nucleotide differences were observed at eight positions in the CR, and amino acid differences (due to two nucleotide differences) were observed in two positions within the IRD of the Rep protein (Figure 1).

To verify if these differences are responsible for the reduced accumulation of ToSRV and the predominance of ToRMV over ToSRV in a mixed infection, a full-length clone of the ToSRV DNA-A exchanging the nucleotides at the divergent positions in the CR and the IRD by those of ToRMV DNA-A was synthesized using the GenPlus service from the company GenScript (www.genscript.com) (Figure 2). In the ToSRV DNA-A CR, the adenine at position 1 (Figure 1) was replaced by thymine, the cytosine at position 2 by a guanine, and the thymine at position 5 was deleted. In the Rep gene IRD, the first base of the fifth codon (cytosine) was replaced by adenine, and the second base of the sixth codon (adenine) was replaced by guanine.

These two substitutions altered the amino acid sequence of the Rep protein (proline to threonine and lysine to arginine, respectively) (Figure 2). It is important to mention that as the Rep protein is expressed from the virion complementary strand (synthesized during viral DNA replication), the bases replaced on the virion strand of the infectious clone sequence are complementary bases, thus, in the region corresponding to the fifth codon of Rep in the virion strand, a guanine was replaced by thymine and in the sixth a thymine by a cytosine. After synthesis of the infectious clone, cloning was performed on the pUC57 vector, and sequencing was performed to confirm that only the mutations of interest were inserted.

To construct the infectious clone ToSRV-$A_{(CR+IRD)}$, containing the mutations in the CR and the IRD, the complete genome sequence was subjected to restriction analysis using the program ApE (A plasmid Editor) v. 2.0.53. We chose the restriction enzyme SacI, which cleaves at only one point in the genome and is located closer to the origin of replication, to simulate the site that would be used for cloning the full-length ToSRV DNA-A (2593 pb). To generate the partially redundant portion of the ToSRV clone that must contain the origin of replication, we used SacI and NcoI to generate a 476 bp fragment ($SacI_{2593}$ to $NcoI_{3069}$). This fragment also contains the region where the mutations were inserted (represented as green rectangles in Figure 2A). The presence of the same region where the mutations were inserted in both the 2593 bp and 476 bp fragments would hinder the site-specific mutagenesis process to be performed subsequently, because the primer containing the mutations could anneal in only one of two regions during PCR. Considering that for a clone to be infectious it needs to have only two origins of replication, the repeated region where the mutations were inserted and the one immediately upstream to it were excluded from the fragment corresponding to one copy of the viral genome, generating an infectious clone with 2829 bp (Figure 2B). After synthesis, the fragment was cloned into the pUC57 vector using the blunt end enzyme EcoRV.

After the synthesis of ToSRV-$A_{(CR + IRD)}$, mutant clones containing only the CR mutations (ToSRV-$A_{(CR)}$) or only the IRD mutations (ToSRV-$A_{(IRD)}$) were constructed by site-directed mutagenesis (also by GenScript), using ToSRV-$A_{(CR+IRD)}$ as a template. To construct ToSRV-$A_{(CR)}$, the two mutations in the Rep gene were reversed to the original sequence. To construct ToSRV-$A_{(IRD)}$, the three mutations in the CR were reversed to the original sequence (Figure 2). Confirmation of all the mutants was performed by sequencing.

Plant inoculations

The infectious clones of ToSRV and ToRMV wild-type isolates and mutants (ToSRV-$A_{(CR+IRD)}$, ToSRV-$A_{(CR)}$ and ToSRV-$A_{(IRD)}$) were inoculated on tomato seedlings (S. lycopersicum cv. Santa Clara) using biolistics [34], with 10 µg of each genomic component in single (ToSRV_{wt}-A+B; ToRMV-A+B; ToSRV-$A_{(CR+IRD)}$+B; ToSRV-$A_{(CR)}$+B; ToSRV-$A_{(IRD)}$+B) and mixed inoculations (ToSRV_{wt}-A+B + ToRMV-A+B; ToSRV-$A_{(CR+IRD)}$+B + ToRMV-A+B; ToSRV-$A_{(CR)}$+B + ToRMV-A+B; ToSRV-$A_{(IRD)}$+B + ToRMV-A+B). ToSRV_{wt} DNA-B was inoculated with the mutants in single inoculations and in mixed inoculations together with the ToRMV DNA-B. Tomato plants bombarded with tungsten particles without DNA were used as healthy controls. Ten plants were inoculated per treatment and two independent experiments were conducted. Plants were kept in a greenhouse and expression of symptoms was evaluated up to 35 days post-inoculation (dpi).

Detection and quantification of genomic components

Total DNA from all plants was extracted after 28 dpi according to the method described by Doyle and Doyle [35]. Confirmation of plants with single infections was performed by PCR using primers ToRMV-$A_{(For)}$ (5'-CAG TAG TTG CCT TCG AAT TGA AG-3') and ToRMV-$A_{(Rev)}$ (5'-CAC GTG TAG CAA TCT CCT TAA AGG-3') for detection of ToRMV DNA-A,

and ToSRV-$A_{(For)}$ (5'-CAG TAG TTG CCC TCA AAT TGA AG-3') and ToSRV-$A_{(Rev)}$ (5'-CAC GTG TAG CAA TCT CCT TAA AGA G-3') for detection of ToSRV DNA-A. Reactions contained 1 µL of total DNA, 0.4 µM of each primer, 1x Colorless GoTaq Reaction Buffer (Promega), 0.2 mM of each dNTP and 1.25 U of GoTaq DNA Polymerase (Promega) in final volume of 25 µL. The PCR parameters consisted of an initial denaturing step at 95°C for 2 min, followed by 38 cycles at 95°C for 1 min, 66°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

Confirmation of plants with mixed infection was performed by amplification of the complete viral genome by rolling circle amplification (RCA) [36] followed by cleavage with specific restriction enzymes for detection of each component (RCA-RFLP), as described by Silva et al. [29] (Suppl. Figure S1).

After confirmation of plants with single and mixed infections, three plants in single infection and all plants in mixed infection were selected by treatment in each experiment to analyze the accumulation of ToSRV DNA by absolute quantification using quantitative real-time PCR (qPCR). In mixed infection, only plants in which the wild-type or mutant ToSRV DNA-A was detected simultaneously with the ToRMV DNA-A were selected.

To construct the standard curve for ToSRV DNA-A, serial dilutions (5×10^4 to 5×10^9 copies) were prepared from known quantities of a plasmid containing a single copy of the genomic component. Quantifications of the plasmid DNA used to construct the standard curve and the total DNA samples were performed using a Nanodrop 2000c (ThermoFisher Scientific). To detect the accumulation of ToSRV DNA-A, a pair of specific primers designed to anneal outside the mutated sites (qToSRV-$A_{(For)}$, 5'-AAA GTA AAG TGA TTG TCT GTG G-3' and qToSRV-$A_{(Rev)}$, 5'-GCC GTT CAA CAA ATT GGG-3'); [29] was used. Reactions were prepared in a final volume of 10 µL using Fast SYBR Green Master Mix (Bio-Rad) and

analyzes were conducted in a CFX96 thermocycler (Bio-Rad). Cycling conditions were an initial denaturing step of 95°C for 3 min followed by 40 cycles of 95°C for 10s and 66ºC for 30s, with a final dissociation stage. Viral accumulation was determined by interpolation of the Ct values of each tested sample within the standard curve. Several primers were tested for ToRMV DNA-A amplification, however, none of them were specific. Likewise, for the DNA-B, due to the high sequence identity between the two viruses, it was not possible to design specific primers for viral DNA quantification.

The data were submitted to analysis of variance and the differentiation of the means was performed using Student's t test in Microsoft Excel.

Results

To confirm the sequences of the ToSRV and ToRMV isolates and make sure that the correct nucleotides were modified in the site-directed mutagenesis procedure, three clones of each viral genomic component of the two viruses were resequenced. The sequencing results were consistent and of high quality, without variations in the three replications. In comparison to the original sequences of the isolates (ToRMV: AF291705 and AF291706; ToSRV: DQ207749 and EF534708), a small number of nucleotide changes were verified, one of them within the CR of both the DNA-A and DNA-B of ToRMV.

Pairwise comparisons of the complete genome nucleotide sequences and of coding and non-coding regions confirmed that ToRMV and ToSRV are very similar, with a DNA-A identity of 88.1%. In addition, the two DNA-Bs are practically identical (98.2% identity). The nucleotide identity of the Cp, Rep, Trap, Ren, Ac4, Mp and Nsp genes are also high, at 81.1%, 95.3%, 83.3%, 86.2%, 97.0%, 99.0% and 98.8% respectively. The identity between the CRs of ToSRV cognate DNAs is 98.7%, and that of all the other possible combinations between the

DNA-A and DNA-B CRs from ToRMV and ToSRV (ToRMV-A/ToRMV-B, ToRMV-A/ToSRV-A, ToRMV-A/ToSRV-B, ToSRV-A/ToRMV-B and ToRMV-B/ToSRV-B) is 97.5%.

The CR alignment of the four genomic components indicates a total of eight positions with nucleotide differences among them (Figure 1A). At positions 1 and 5 the differences are virus-specific, with the same nucleotides occurring in cognate DNAs. The nucleotide difference at position 2 is specific for ToSRV DNA-A, and at position 8 for ToSRV DNA-B. Divergent nucleotides at positions 4 and 6 are present only in ToRMV DNA-A, and at positions 3 and 7 only in ToRMV DNA-B (Figure 1A). In the IRD region of the Rep gene, the first base of the fifth codon and the second base of the sixth codon are different between the two viruses, and consequently the deduced amino acid sequence is also different (Figure 1B, C). Thus, the only difference between the original sequences analyzed by Silva et al. [29] and the new sequences determined in this study is that, based on the new sequences, the nucleotide position two nucleotides downstream from the G-box has the same sequence in all four genomic components (Figure 1A).

To verify if the observed changes in the CR and Rep protein IRD are responsible for the reduced accumulation of ToSRV and for the preferential detection of ToRMV genomic components over ToSRV in mixed infection, infectious clones of ToSRV DNA-A containing the ToRMV DNA-A sequence in the CR (ToSRV-$A_{(CR)}$), the IRD (ToSRV-$A_{(IRD)}$) and in both regions (ToSRV-$A_{(CR+IRD)}$) were inoculated in tomato in single (ie, together with wild-type ToSRV DNA-B) and mixed infections (ie, together with wt ToSRV DNA-B and wt ToRMV DNA-A and DNA-B). Symptoms began to appear at 10-14 dpi and consisted of yellow mosaic in all treatments (Figure 3). Thus, no synergistic interaction was observed between the two viruses.

The results of the infectivity assay are presented in Table 1. The overall rate of infectivity was low but similar for all treatments, ranging from 7 (35%) to 10 (50%) infected plants of a total of 20 inoculated plants per treatment. This was considerably lower than the infectivity reported in the study of Silva et al. [29] and in other infectivity assays performed in our laboratory (data not shown).

Results of single infections indicate that both wt and mutant ToSRVs as well as ToRMV infected tomato with equivalent efficiency.

The presence of each inoculated DNA component in mixed infection was verified by RCA-RFLP (one representative of each combination between DNA-A and B of both wild-type and mutant viruses detected in each treatment in mixed infection are shown in Suppl. Figure S1). Considering only the DNA-A detection, in mixed infections, the mean percentage of plants infected with only ToSRV-$A_{(CR+IRD)}$ or ToRMV-A was 15%, and of plants infected with both components was also 15%. In plants inoculated with ToSRV-$A_{(IRD)}$ and ToRMV, 15% of the plants were infected only with ToSRV-$A_{(IRD)}$, 30% only with ToRMV-A, and no plants were infected with both components. The highest percentage of plants infected with both DNA-A components (35%) was observed when ToSRV-$A_{(CR)}$ and ToRMV were inoculated . In this treatment, only one plant (5%) was infected with ToSRV-$A_{(CR)}$ alone and one plant with ToRMV-A alone. These results suggest that the nucleotide differences in the CR, but not the amino acid differences in the IRD, are responsible for the prevalence of ToRMV components over ToSRV components. Considering only the DNA-B detection, it is noteworthy that the wt ToSRV DNA-B component was detected at a much lower percentage than the ToRMV DNA-B component, in all treatments (Table 1). Compared to ToSRV-$A_{(CR)}$, the wt ToSRV DNA-B has three nucleotide differences in the CR, at positions 1, 5 and 8 (Figure 1). Thus, the results of the infectivity assay suggest that nucleotide positions 1 and 5 in the CR are responsible for the differential infectivity of the ToSRV and ToRMV components.

In plants inoculated with the wild-type begomoviruses, ToSRV-A was detected alone in 20% of the plants, ToRMV-A alone was also detected in 20% of the plants, and the two DNA-A components were detected together in 10% of the plants (Table 1). Thus, the negative interference of ToRMV over ToSRV infectivity was not observed in this treatment.

To verify the accumulation of wt and mutant ToSRV DNA-A in single and mixed inoculations, real-time quantitative PCR assays (qPCR) were performed using primers to detect the most divergent region of the ToSRV DNA-A. Several primers were tested for the ToRMV DNA-A, however, none of them were specific. In mixed infection, only plants in which wt or mutant ToSRV DNA-A were detected simultaneously with ToRMV DNA-A were selected for viral load quantification. Accumulation of wt ToSRV DNA-A and of mutants ToSRV-$A_{(CR + IRD)}$, ToSRV-$A_{(CR)}$ and ToSRV-$A_{(IRD)}$ in single inoculations was similar, with no statistical differences (Figure 4), thus confirming the infection efficiency of the mutants. In mixed infection with ToRMV, the accumulation of wt ToSRV DNA-A was strongly reduced (Figure 4), corroborating the results of Silva et al. [29]. However, in plants inoculated with ToSRV-$A_{(CR)}$ and ToRMV, the accumulation of ToSRV-$A_{(CR)}$ was approximately 100 times greater than that of wt ToSRV DNA-A (Figure 4). Although the accumulation of ToSRV-$A_{(CR + IRD)}$ was lower than that of ToSRV-$A_{(CR)}$, it was approximately 10 times greater than that of wt ToSRV DNA-A (Figure 4). It is possible that the reduction in the accumulation of ToSRV-$A_{(CR + IRD)}$ in relation to ToSRV-$A_{(CR)}$ is due to the changes in Rep protein IRD. However, due to the absence of plants in which ToSRV-$A_{(IRD)}$ and ToRMV-A were detected simultaneously, this hypothesis could not be tested. Nevertheless, the results suggest that the nucleotides differences in the CR are responsible for the negative interference of ToRMV on the replication of ToSRV.

The ToSRV isolate (BR:PG1:Pep:03) used in our study was obtained from a pepper plant growing next to tomato crops [31]. Comparison of the divergent positions in the CR and IRD sequences of ToSRV-[BR:PG1:Pep:03] with other 138 ToSRV isolates from different

hosts showed that the IRD nucleotide sequence is highly conserved in all isolates. All but two isolates have cytosine as the first base of the fifth Rep codon, like ToSRV-[BR:PG1:Pep:03]. Comparing the entire IRD region, only five isolates showed a single variable amino acid. However, comparing the CR of these isolates, the adenine in position 1 of ToSRV-[BR:PG1:Pep:03] is present in only four other isolates (BR:Pip1792:03, BR:ITA1274:14, BR:SOITA1014:14 and BR:DF607) obtained from tomato, common bean, soybean and tree tomato, respectively. Other isolates (as well as ToRMV) have a thymine in this position. In position 2, only ToSRV-[BR:PG1:Pep:03] has cytosine, with all other isolates (and ToRMV) having guanine. In the divergent position 5, 52.2% of the isolates have a thymine like ToSRV-[BR:PG1:Pep:03], 44.2% of the isolates do not have a nucleotide in this position (like ToRMV) and 3.6% have a cytosine.

Discussion

Mixed infections with two or more begomoviruses infecting the same host are very common in nature [37, 38]. However, the mechanisms controlling differences in viral accumulation and consequently the predominance of one virus over the other are unknown. In this study we showed that (apparently minor) nucleotide differences in the common region (CR) of the begomoviruses ToRMV and ToSRV may be involved in this process.

The genomes of ToRMV and ToSRV have a high degree of nucleotide sequence identity. Ribeiro et al. [39] and Silva et al. [29] suggest that ToRMV is a recombinant virus, with Tomato chlorotic mottle virus (ToCMoV) and ToSRV as parents. Recombination analysis indicated that part of the CR, including the iterons, and practically the entire Rep gene were transferred from ToSRV to ToRMV, and that ToRMV captured the ToSRV DNA-B. The presence of the same core iteron sequence, the high similarity among the CRs of the four

components and the formation of viable pseudorecombinants in all possible combinations between DNA-A and B in single and mixed inoculations [29] all support the hypothesis of both recombination and pseudorecombination being involved in the shared evolutionary histories of ToRMV and ToSRV.

Recombination and pseudorecombination are common and important in generating genetic variability in begomoviruses [3, 25, 40, 41]. Recombinant sites are not evenly distributed in the genome: the origin of replication and the 5'-terminal portion of the Rep gene are recombination hot spots, often exchanged during replication [2, 42]. Hou and Gilbertson [43] showed that, after five successive mechanical passages in Nicotiana benthamiana, a pseudorecombinant produced between the DNA-A of Tomato mottle virus (ToMoV) and the DNA-B of Bean dwarf mosaic virus (BDMV) had the DNA-B CR replaced almost entirely by the DNA-A CR, increasing DNA-B accumulation (and symptom severity) due to the higher efficiency of recognition of the DNA-B by the ToMoV Rep protein. The formation of viable pseudorecombinants is common among isolates of the same species [44-47] and can also happen between distinct species which exhibit high identity in the CR sequences of the heterologous components [23, 25, 48-51]. Thus, viral factors involved in replication can be transferred (by recombination) or shared (by pseudorecombination) between different species.

Although ToRMV and ToSRV form viable pseudorecombinants, ToRMV infects tomato with higher efficiency in relation to ToSRV in a mixed infection [29]. These authors showed that the CR sequences of ToRMV and ToSRV differed at nine positions (corrected to eight positions after our resequencing of the isolates) and that the IRD sequences of their Rep proteins differed at two positions, and hypothesized that one or more of these differences could be responsible for the predominance of ToRMV components over those of ToSRV in a mixed infection. We constructed ToSRV mutants containing the ToRMV nucleotides at the divergent positions in the CR and IRD to verify if these regions ensure higher replication efficiency. If

the CR or IRD sequences at the divergent positions were indeed determinants for viral replication, the rate of plants infected with the mutant ToSRVs and ToRMV would be similar. This was the case when plants were inoculated with the ToSRV-$A_{(CR)}$ and ToSRV-$A_{(CR+IRD)}$ mutants, but not with the ToSRV-$A_{(IRD)}$ mutant, indicating that specific nucleotides in the CR are at least partly responsible for the efficiency of ToRMV replication. Comparison of the CR sequences of several ToSRV isolates indicated that the presence of T and G at position 1 and 2 and of a deletion at position 5 (numbering according to the alignment in Figure 1A) serve as specific recognition sites for Rep binding, and suggest that isolates containing these nucleotides in this region have an adaptive advantage in tomato plants.

Recognition of the iterons by the Rep protein is a key event for binding of the Rep/REn complex to the origin of replication and consequently for the initiation of viral replication [12, 20, 21]. According to the proposed model of recognition between Rep and the iterons, specific residues in the Rep IRD interact with nucleotides defined in the iteron sequence [19]. In the case of ToRMV and ToSRV, which have the same iteron sequence (GGTAG), the base T interacts with the amino acid lysine in the IRD, A with glutamine and G with alanine. According to Arguello-Astorga and Ruiz-Medrano [19], the sixth and eighth amino acids of the IRD are the major residues controlling the binding efficiency of Rep to the viral genome. However, it is important to point out that this model is based on in silico analysis, and that functional analyses have not been carried out. The second and third divergent amino acids in the IRD of ToRMV and ToSRV are not supposed to dictate preference for specific iteron sequences. Analysis of the Rep N-terminal region of other begomoviruses infecting tomato, such as Tomato yellow spot virus (ToYSV), Tomato golden vein virus (TGMV) and Tomato chlorotic mottle virus (ToCMoV) shows that, although they have different IRD sequences, they have the same amino acids as ToSRV in those divergent positions in the IRD [25], suggesting that the ToSRV Rep may be more versatile in the recognition of the iterons, while the ToRMV Rep may be more

specific. The smaller number of plants with mixed infection based on DNA-A detection after inoculation with ToRMV and $ToSRV_{(CR+IRD)}$ compared with plants inoculated with ToRMV and $ToSRV_{(CR)}$ suggests that although the ToRMV-derived CR sequence may favor replication, the ToRMV-derived IRD sequence provides a disadvantage. The absence of plants with mixed infection following inoculation with ToRMV and $ToSRV_{(IRD)}$ supports this hypothesis. In order to verify if amino acids in the Rep IRD determine higher binding specificity to ToRMV (ie, less versatility for its Rep protein), infectivity assays with ToRMV mutants exchanging amino acids at the divergent IRD sites by the amino acids of ToSRV should be conducted.

Co-existence between two or more viruses involves complex interactions and can modify several characteristics, such as transmission efficiency [52], severity of symptoms [37], resistance breaking of cultivated hosts [53, 54], cellular tropism [55, 56] and viral DNA titer [38, 57]. The effect of mixed infections on a virus can be beneficial, neutral or prejudicial. Results of viral load quantification showed that the accumulation of ToSRV is greatly reduced in mixed infection with ToRMV, reinforcing the results reported by Silva et al. [29]. Interestingly, ToRMV exerts a negative interference on the accumulation of another begomovirus, ToYSV, during the initial stages of infection in tomato [55]. Conversely, a beneficial effect of mixed infection involving begomoviruses has been reported for pepper plants infected with Pepper huasteco yellow vein virus (PHYVV) and Pepper golden mosaic virus (PepGMV) [37] and in cassava infected with African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) [58]. In both cases, plants with mixed infection showed higher viral concentration of both viruses and more severe symptoms in relation to single infections. In our study, despite differences in viral accumulation, synergism due to increased severity of symptoms in mixed infections compared to single infections were not detected.

According to Martin and Elena [38], different individuals in a population can be considered as different players of a biological game. The player who has the best strategy wins, and therefore will have the highest fitness and consequently greater frequency in the population. In our study, based on infectivity of genomic components and viral DNA accumulation, we have shown that the divergent nucleotides in the CR of ToRMV make this virus a better competitor in mixed infection with ToSRV. We suggest that these specific nucleotide positions serve as recognition sites for Rep binding to the viral DNA, and that although the nucleotides present in these positions in the ToSRV CR do not interfere with viral accumulation in single infections, in mixed infections the nucleotides present in the CR of ToRMV ensure increased replication rates and consequently greater viral DNA accumulation.

The disadvantage displayed by ToSRV in relation to ToRMV in mixed infections is somewhat unexpected, given the predominance of ToSRV over ToRMV in the field [59-61]. While factors unrelated to replication (such as vector transmission) could be involved, one interesting observation is that the vast majority of ToSRV field isolates have the same nucleotides as ToRMV in the three CR positions analyzed in our study. Thus, it is possible that the isolate used to construct the infectious clone (BR:PG1:Pep:03) is an atypical ToSRV isolate. Interaction studies between ToRMV and other ToSRV isolates that do not contain nucleotide differences in these positions would be helpful to evaluate whether the negative effect exerted by ToRMV prevails.

As a final observation, it may be noteworthy that this isolate was obtained from pepper rather than tomato. The differences observed in the CR sequence of ToSRV-[BR:PG1:Pep:03] may be a consequence of viral adaptation to a new host, and this adaptation may have incurred a fitness cost. Although ToSRV naturally infects both hosts, infection in tomatoes is much more common and severe than in peppers. Further studies comparing ToSRV isolates from different

hosts may provide valuable insights regarding viral evolution, in terms of adaptation to new hosts and possible effects on fitness.

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Table 1. Infectivity of wild-type and mutant clones of Tomato severe rugose virus (ToSRV) in single infection and in and mixed infection with Tomato rugose mosaic virus (ToRMV).

Treatment	Genomic component	Infected plants(%)*
$ToSRV_{wt}$	ToS-A[#]	7 (35)
ToRMV	ToR-A[#]	7 (35)
$ToSRV_{(CR)}$	$ToS\text{-}A_{(CR)}$ [#]	7 (35)
$ToSRV_{(IRD)}$	$ToS\text{-}A_{(IRD)}$ [#]	8 (40)
$ToSRV_{(CR+IRD)}$	$ToS\text{-}A_{(CR+IRD)}$ [#]	10 (50)
$ToSRV_{(CR)}$ + ToRMV	$ToS\text{-}A_{(CR)}$/ToR-A[&]	7 (35)
	$ToS\text{-}A_{(CR)}$ [¶]	1 (5)
	ToR-A[¶]	1 (5)
	ToS-B/ToR-B[&]	-
	ToS-B [¶]	-
	ToR-B[¶]	9 (45)
$ToSRV\text{-}A_{(IRD)}$ + ToRMV	$ToS\text{-}A_{(IRD)}$/ToR-A[&]	-
	$ToS\text{-}A_{(IRD)}$ [¶]	3 (15)
	ToR-A[¶]	6 (30)
	ToS-B/ToR-B[&]	1 (5)
	ToS-B [¶]	1 (5)
	ToR-B[¶]	7 (35)
$ToSRV_{(CR+IRD)}$ + ToRMV	$ToS\text{-}A_{(CR+IRD)}$/ToR-A[&]	3 (15)
	$ToS\text{-}A_{(CR+IRD)}$ [¶]	3 (15)
	ToR-A[¶]	3 (15)
	ToS-B/ToR-B[&]	1 (5)
	ToS-B [¶]	-
	ToR-B[¶]	8 (40)
$ToSRV_{wt}$ + ToRMV	ToS-A/ToR-A[&]	2 (10)
	ToS-A [¶]	4 (20)
	ToR-A[¶]	4 (20)
	ToS-B/ToR-B[&]	3 (15)
	ToS-B [¶]	1 (5)
	ToR-B[¶]	6 (30)

*Number of infected plants out of 20 inoculated plants, confirmed by PCR with virus-specific primers (single infections) or by RCA-RFLP (mixed infections). Results correspond to the sum of two independent experiments.
[#]Plants inoculated with viruses in single infection.
[&]Plants inoculated with both viruses in which DNA-As or DNA-Bs of both viruses were detected.
[¶] Plants inoculated with both viruses in which DNA-A or DNA-B of only one of them was detected.

Figure legends

Figure 1. A. Alignment of the nucleotide sequences of the common region (CR) of the DNA-A and DNA-B of Tomato rugose mosaic virus (ToRMV) and Tomato severe rugose virus (ToSRV). The arrowheads and numbers indicate the eight divergent positions of the CR among the four components. Asterisks represent conserved nucleotides among the aligned sequences. The horizontal arrows indicate the direction of the iterons. The TATA box and the G-box are boxed. The conserved nonanucleotide at the origin of replication is indicated in light gray. **B.** Alignment of the amino acid sequences of the N-terminal portion of the Rep proteins of ToRMV and ToRSV. Divergent amino acids are highlighted in black. The iteron-related domain (IRD), specificity determinants (SPDs) and protein motifs are boxed with dashed lines. **C.** Alignment of the nucleotide sequences of the region corresponding to the IRDs of ToRMV and ToSRV. Identical nucleotides are indicated by an asterisk.

Figure 2. Schematics of the construction of the $ToSRV_{(CR+IRD)}$ infectious clone, containing the ToRMV nucleotides at the divergent positions in the CR and the IRD. Altered nucleotides in the CR and IRD are indicated in the green and yellow boxes, respectively. In the CR (green box), the numbers 1, 2 and 5 represent divergent positions and underlined bases represent the mutations. In the IRD (yellow box), the numbers represent the amino acids encoded by the fifth and sixth codons of the Rep gene, and underlined bases represent the mutations. **A**. Representation of the ToSRV-A infectious clone containing a fragment corresponding to a full-length copy of the genome (2593 nt, gray line) and the fragment containing the partially redundant portion (476 nt, black line), both containing the origin of replication. **B**. Representation of the infectious clone after the deletion of the repeated region where the mutations were inserted (dotted line). The nonanucleotide at the origin of replication

(TAATATTAC) is indicated. The red and blue arrows represent the viral genes and the direction in which they are transcribed (viral and complementary sense, respectively).

Figure 3. Symptoms in tomato plants biolistically inoculated with wild-type (ToRMV, $ToSRV_{wt}$) and mutant (ToSRV-$A_{(CR + IRD)}$, ToSRV-$A_{(CR)}$ and ToSRV-$A_{(IRD)}$) infectious clones, in single and mixed infections.

Figure 4. Accumulation of wild-type (ToRMV, $ToSRV_{wt}$) and mutant (ToSRV-$A_{(CR+IRD)}$, ToSRV-$A_{(CR)}$, ToSRV-$A_{(IRD)}$) infectious clones in single and mixed infections in tomato plants. Means were compared using Student's t test. Letters represent statistically significant differences ($P<0.05$) and error bars indicate standard deviation. Results correspond to the sum of two independent experiments.

Supplementary Figure S1. Detection of all possible combinations between DNA-A and DNA-B of both ToSRV wild-type isolates and mutants (ToSRV-$A_{(CR+IRD)}$, ToSRV-$A_{(CR)}$ and ToSRV-$A_{(IRD)}$) in mixed infection with ToRMV by rolling circle amplification (RCA) followed by digestion of the amplified DNA with restriction enzymes (RCA-RFLP). The figure shows one representative of each combination in each treatment. Digestion with HindIII enables detection of $ToSRV_{wt}$ DNA-A and mutants. Digestion with XhoI enables detection of ToRMV DNA-A. Digestion with BglII + SacII enables detection and differentiation of ToRMV and ToSRV DNA-Bs. Detection of wild-type (ToRMV, $ToSRV_{wt}$) and mutants in single infection shown as controls. M, size marker (1 kb plus DNA ladder, Kasvi).

Figure 1

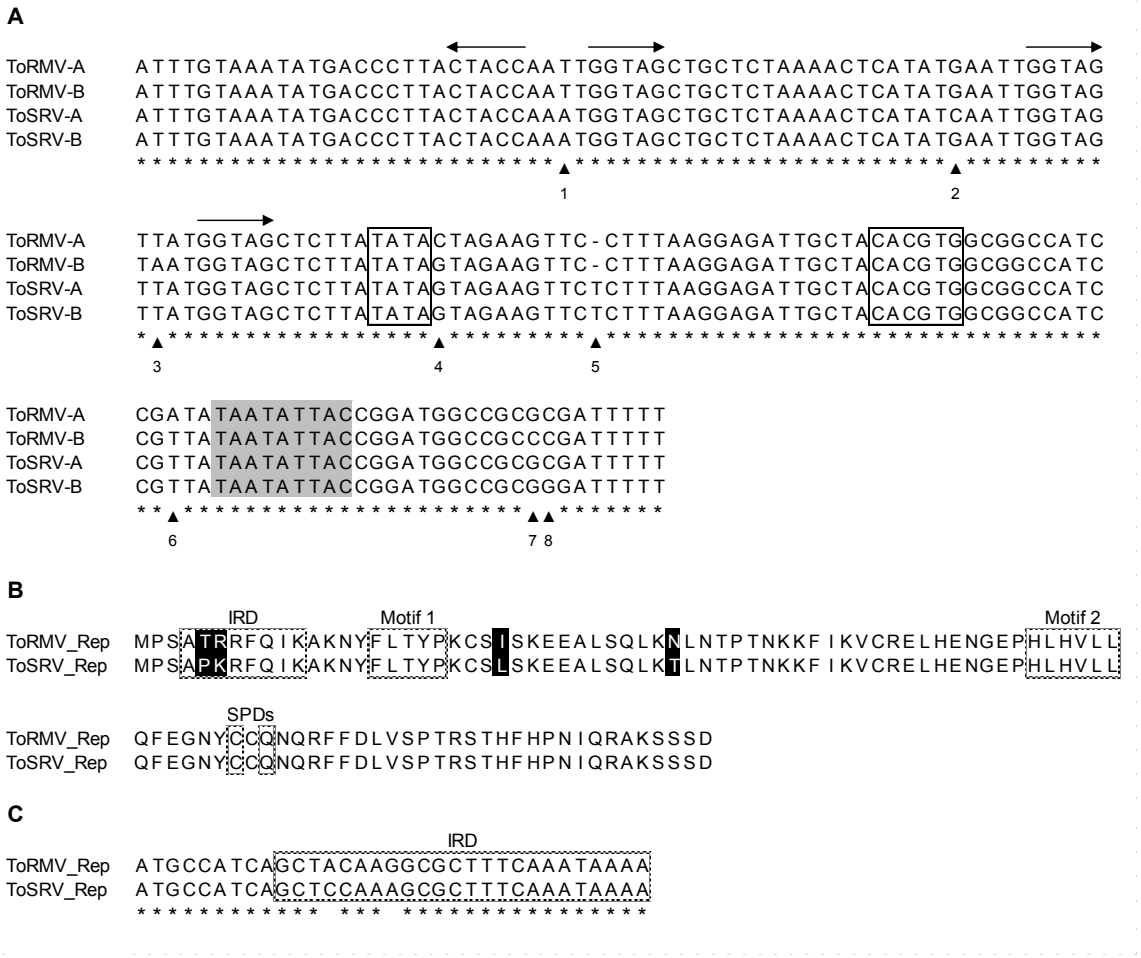

A
ToRMV-A ATTTGTAAATATGACCCTTACTACCAATTGGTAGCTGCTCTAAAACTCATATGAATTGGTAG
ToRMV-B ATTTGTAAATATGACCCTTACTACCAATTGGTAGCTGCTCTAAAACTCATATGAATTGGTAG
ToSRV-A ATTTGTAAATATGACCCTTACTACCAAATGGTAGCTGCTCTAAAACTCATATCAATTGGTAG
ToSRV-B ATTTGTAAATATGACCCTTACTACCAAATGGTAGCTGCTCTAAAACTCATATGAATTGGTAG
1
2
ToRMV-A TTATGGTAGCTCTTATATACTAGAAGTTC-CTTTAAGGAGATTGCTACACGTGGCGGCCATC
ToRMV-B TAATGGTAGCTCTTATATAGTAGAAGTTC-CTTTAAGGAGATTGCTACACGTGGCGGCCATC
ToSRV-A TTATGGTAGCTCTTATATAGTAGAAGTTCTCTTTAAGGAGATTGCTACACGTGGCGGCCATC
ToSRV-B TTATGGTAGCTCTTATATAGTAGAAGTTCTCTTTAAGGAGATTGCTACACGTGGCGGCCATC
3
4
5
ToRMV-A CGATATAATATTACCGGATGGCCGCGCGATTTTT
ToRMV-B CGTTATAATATTACCGGATGGCCGCCCGATTTTT
ToSRV-A CGTTATAATATTACCGGATGGCCGCGCGATTTTT
ToSRV-B CGTTATAATATTACCGGATGGCCGCGGGATTTTT
6
7 8
B
IRD
Motif 1
Motif 2
ToRMV_Rep MPSATRRFQIKAKNYFLTYPKCSISKEEALSQLKNLNTPTNKKFIKVCRELHENGEPHLHVLL
ToSRV_Rep MPSAPKRFQIKAKNYFLTYPKCSLSKEEALSQLKTLNTPTNKKFIKVCRELHENGEPHLHVLL
SPDs
ToRMV_Rep QFEGNYCCQNQRFFDLVSPTRSTHFHPNIQRAKSSSD
ToSRV_Rep QFEGNYCCQNQRFFDLVSPTRSTHFHPNIQRAKSSSD
C
IRD
ToRMV_Rep ATGCCATCAGCTACAAGGCGCTTTCAAATAAAA
ToSRV_Rep ATGCCATCAGCTCCAAAGCGCTTTCAAATAAAA

Figure 2

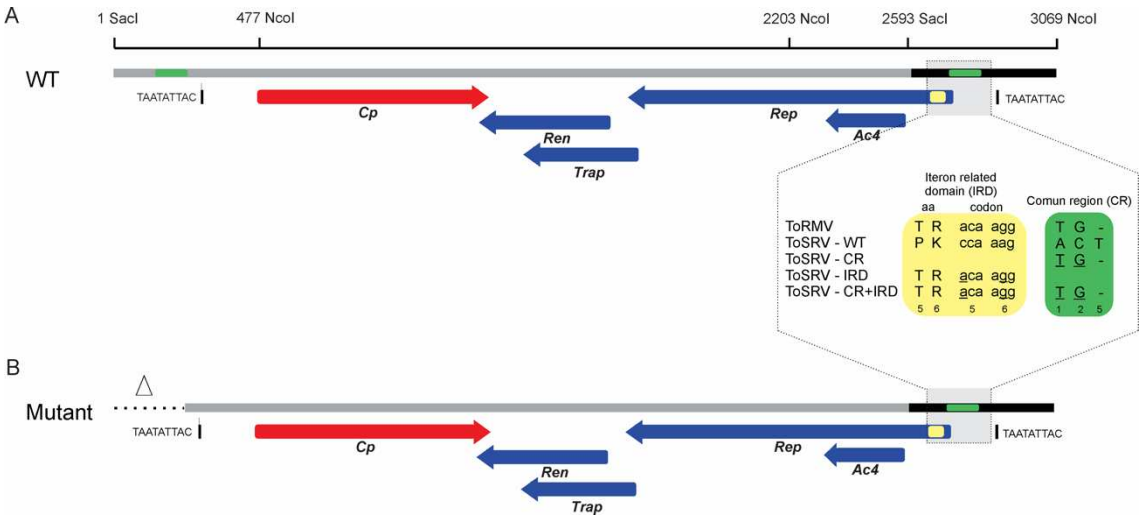

A
1 SacI
477 NcoI
2203 NcoI
2593 SacI
3069 NcoI
WT
TAATATTAC
Cp
Ren
Trap
Rep
Ac4
TAATATTAC
Iteron related domain (IRD)
aa
codon
Comun region (CR)
ToRMV
ToSRV - WT
ToSRV - CR
ToSRV - IRD
ToSRV - CR+IRD
B
Mutant
TAATATTAC
Cp
Ren
Trap
Rep
Ac4
TAATATTAC

Figure 3

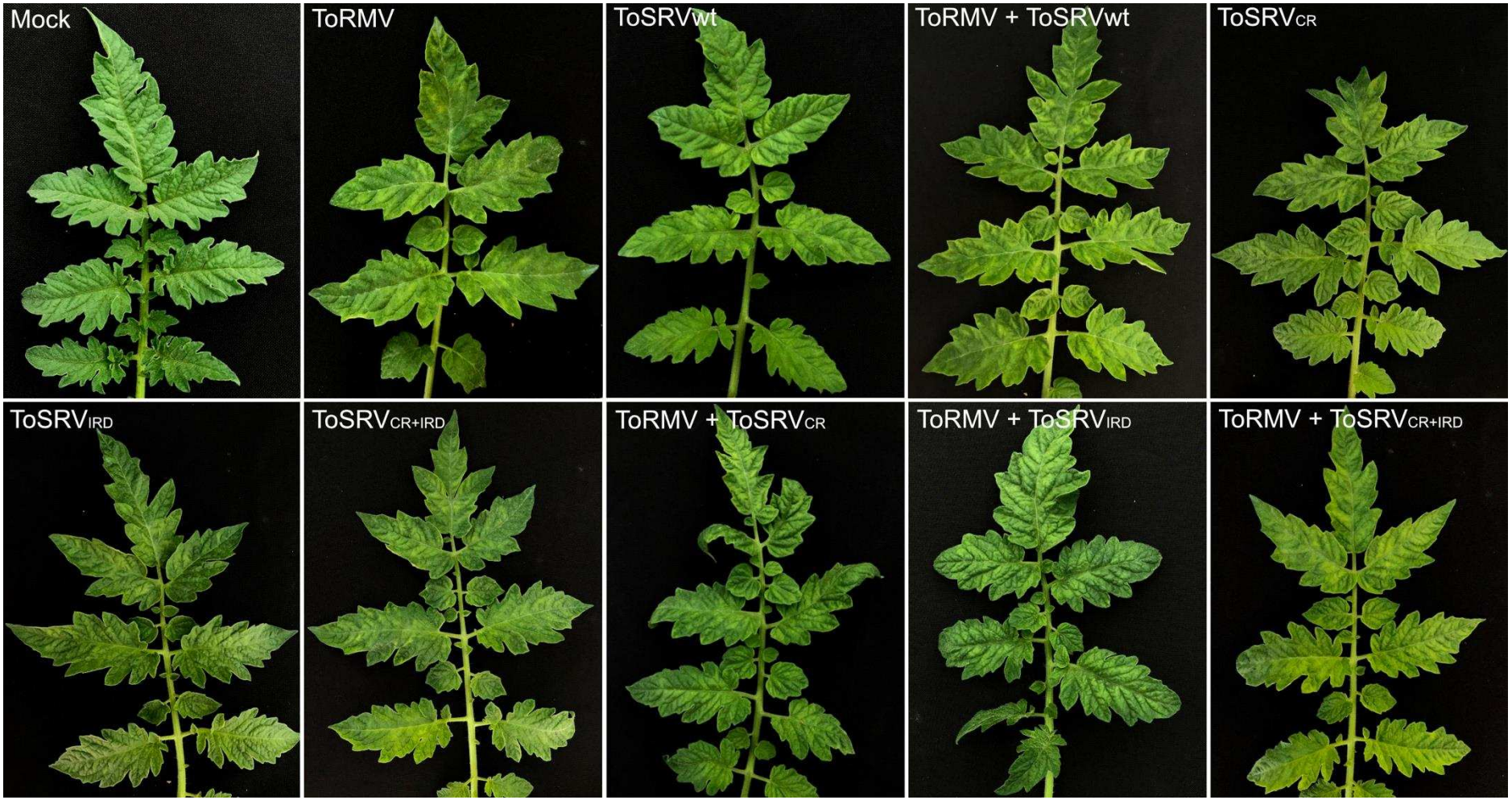
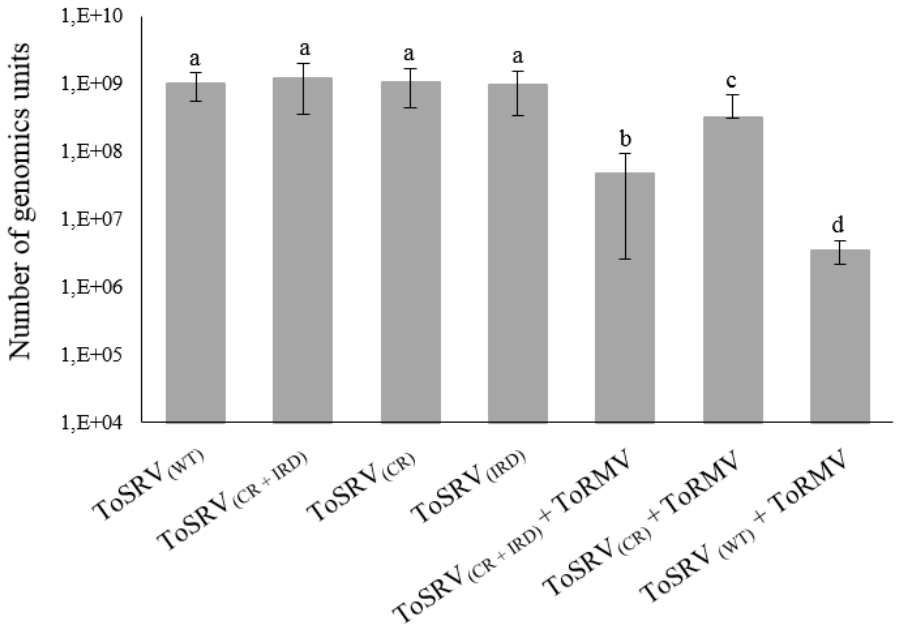

Mock
ToRMV
ToSRVwt
ToRMV + ToSRVwt
ToSRVCR
ToSRVIRD
ToSRVCR+IRD
ToRMV + ToSRVCR
ToRMV + ToSRVIRD
ToRMV + ToSRVCR+IRD

Figure 4


Number of genomics units
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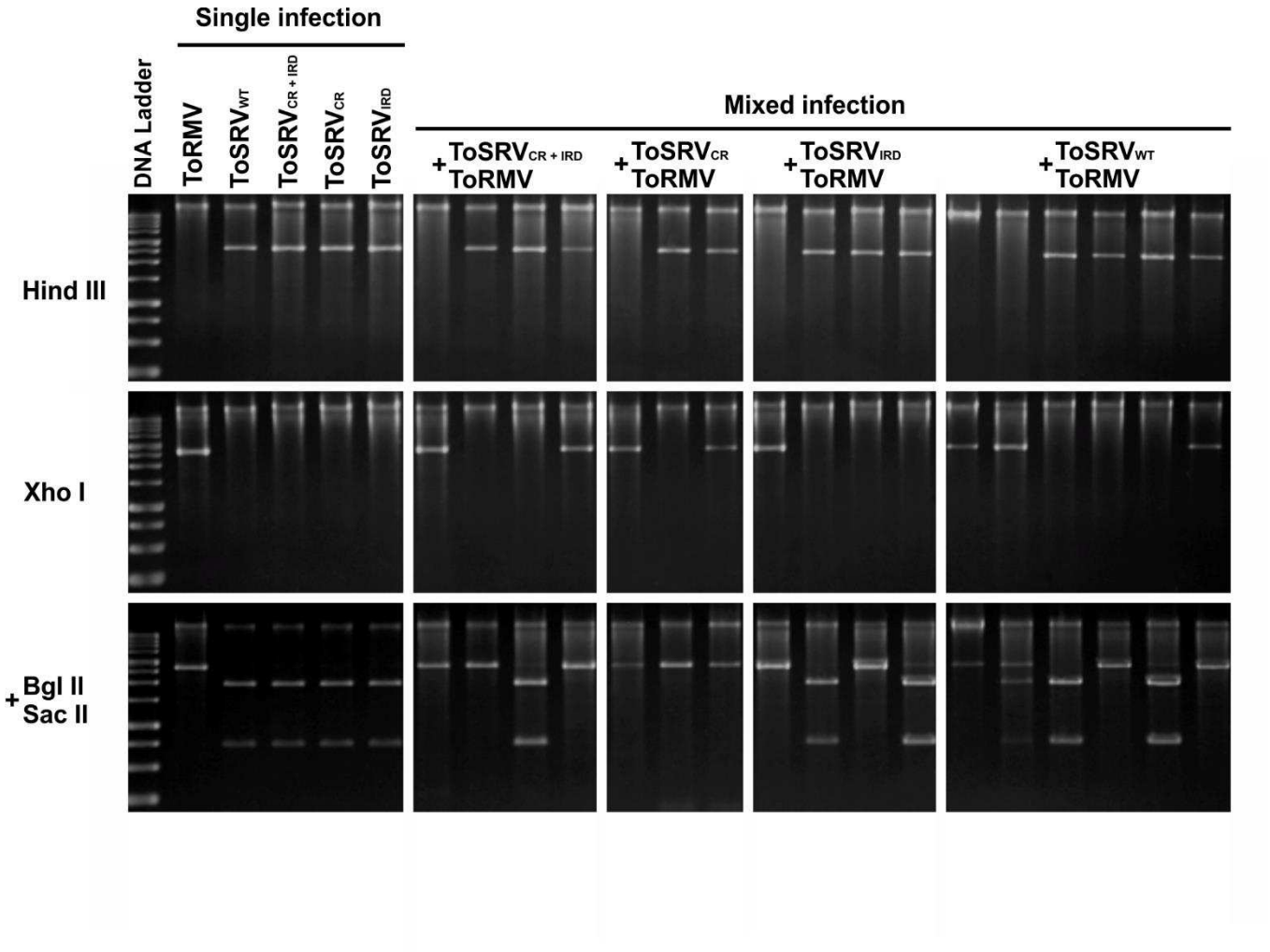
1,E+08
1,E+07
1,E+06
1,E+05
1,E+04
a
a
a
a
b
c
d
ToSRV (WT)
ToSRV (CR + IRD)
ToSRV (CR)
ToSRV (IRD)
ToSRV (CR + IRD) + ToRMV
ToSRV (CR) + ToRMV
ToSRV (WT) + ToRMV

Supplementary Figure S1



CHAPTER 3

AN ALPHASATELLITE INTERACTS WITH BIPARTITE BEGOMOVIRUSES AND INCREASES SYMPTOM SEVERITY IN TOMATO PLANTS

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An alphasatellite interacts with bipartite begomoviruses and increases symptom severity in tomato plants

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Abstract

Begomoviruses (family Geminiviridae) are whitefly-transmitted viruses with circular single-stranded (ss) DNA genomes that are frequently associated with DNA satellites. Alphasatellites are circular, ssDNA molecules, capable of autonomous replication but dependent on a helper begomovirus for systemic infection and plant-to-plant transmission. The presence of Euphorbia yellow mosaic alphasatellite (EuYMA) increases the severity of symptoms induced by Euphorbia yellow mosaic virus (EuYMV) in several hosts. The present work investigated whether EuYMA is capable of interacting with two tomato-infecting begomoviruses, Tomato yellow spot virus (ToYSV) and Tomato severe rugose virus (ToSRV) in tomato and N. benthamiana. The plants were inoculated with each virus in the presence or absence of the alphasatellite. Percentage of infectivity, viral accumulation and symptom development for each begomovirus alone or in the presence of EuYMA were evaluated. The association of EuYMA with ToYSV was less efficient in tomato than in N. benthamiana, as measured by a lower percentage of plants in which the presence of the alphasatellite was detected. The association between ToSRV and EuYMA was similar in both hosts, indicating differences in the interaction between the alphasatellite and different tomato-infecting begomoviruses. Quantification of ToYSV and ToSRV DNA-A accumulation indicated that EuYMA does not interfere in the accumulation these begomoviruses, however, symptoms were more severe in the presence of EuYMA for both viruses and hosts. Together, our results suggest that the association of EuYMA with begomoviruses is promiscuous, highlighting the potential of these DNA satellites to be widely propagated in the field. Whether the presence of alphasatellites affects begomovirus diseases in cultivated hosts remains to be demonstrated.

Introduction

Geminiviruses are non-enveloped plant viruses with genomes comprised of one or two single-stranded circular DNAs [1]. The Geminiviridae family consists of nine genera (Becurtovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus, Turncurtovirus and Begomovirus), defined according to the type of insect vector, host range, genomic organization and phylogenetic relationships [1-4]. Begomoviruses, which are transmitted by whiteflies of the Bemisia tabaci cryptic species complex, are the most economically important geminiviruses, and cause serious crop diseases in tropical and subtropical regions of the world [5].

Based on phylogenetic analysis and genomic features, begomoviruses are broadly divided into two groups: Old World (OW; Europe, Africa, Asia and Oceania) and New World (NW; the Americas) [6-8]. Begomoviruses in the NW are mostly bipartite, with the two genomic components named DNA-A and DNA-B. The DNA-A contains genes involved in replication, encapsidation of viral progeny and suppression of host defenses [9, 10], and the DNA-B contains genes required for intra- and intercellular movement in the plant, host range determination and suppression of host defenses [9-12]. The majority of OW begomoviruses are monopartite, with a genomic organization similar to the DNA-A of bipartite viruses [13, 14] plus the presence of an additional open reading frame (ORF) which partially overlaps the CP gene, named V2 in monopartite viruses or AV2 in bipartite viruses.

Most OW begomoviruses infect plants in association with one or more types of DNA satellites, named alphasatellites (previously known as DNA-1), betasatellites (previously known as DNA β) and deltasatellites [15-19]. DNA satellites require a helper virus to complete one or more steps of their infection cycle. Recently, a number of NW begomoviruses were found to be associated with alphasatellites in natural infections [20-23].

Geminivirus-associated alphasatellites belong to the family Alphasatellitidae, subfamily Geminialphasatellitinae which includes four genera and 43 species defined based on pairwise nucleotide sequence identities [24]. Alphasatellite genomes have approximately 1.4 kb long and contain a stem-loop structure with a conserved nonanucleotide (5'-TAGTATTAC-3') that comprises the origin of replication, an adenine-rich region, and a single ORF encoding a replication-associated protein named alpha-Rep, which ensures replication autonomy [15, 24]. Nevertheless, alphasatellites still depend on the helper virus to systemically infect plants and be transmitted plant-to-plant by the whitefly vector [25]. Due to their great similarity with the DNA-R components of nanoviruses (family Nanoviridae), it is believed that alphasatellites evolved after a geminivirus captured one such component during co-infection of a common host [26, 27].

Effects of the association of alphasatellites with helper begomoviruses on infectivity, viral DNA accumulation and symptoms are poorly understood and appear to vary according with satellite, helper virus and host. Early studies with OW begomoviruses reported that alphasatellites did not alter the symptoms caused by the helper virus [25, 27, 28]. However, there is evidence that alphasatellites may be involved in pathogenicity, since the alpha-Rep protein could act as a suppressor of posttranscriptional [29] or transcriptional gene silencing [30]. In addition, an "atypical" (DNA-2-type) alphasatellite from Oman caused attenuation of the symptoms induced by a begomovirus/betasatellite complex, by reducing accumulation of the betasatellite [31].

In the NW, alphasatellites have been detected in association with bipartite begomoviruses infecting non-cultivated plants in Brazil and Cuba [23, 32] and watermelon crops in Venezuela [22]. In addition, alphasatellites were detected by metagenomics in insect samples from Guatemala and Puerto Rico. These alphasatellites are more closely related to DNA-2-type alphasatellites, and were classified in a third phylogenetically distinct group

named DNA-3-type [33]. In Brazil, DNA-3-type alphasatellites were found in association with bipartite begomoviruses in Euphorbia heterophylla, Cleome affinis, Leonurus sibiricus and Sida spp. [20, 21, 23]. Interestingly, and unlike DNA-2-type alphasatellites which attenuate the symptoms induced by the helper begomovirus [31], at least one of the DNA-3-type alphasatellites described in the NW, Euphorbia yellow mosaic alphasatellite (EuYMA), increases the severity of Euphorbia yellow mosaic virus (EuYMV) symptoms in plants of E. heterophylla and Nicotiana benthamiana. In Arabidopsis thaliana, symptoms of EuYMV infection were observed only in the presence of EuYMA, and in tomato there was no manifestation of symptoms of EuYMV infection either in the presence or absence of EuYMA [20, 23].

In addition to their ability to increase the severity of begomovirus-induced symptoms, the DNA-3-type alphasatellites described in the NW display a wide range of hosts and a flexibility in their association with begomoviruses in both experimental and natural conditions. EuYMA was detected in Euphorbia heterophylla (Euphorbiaceae) plants in association with EuYMV [20, 23] and also in Sida spp. (in this case the helper begomovirus was not identified) [21]; Cleome leaf crumple alphasatellite (ClLCrA) was found in plants of Cleome affinis (Cleomaceae) in association with Cleome leaf crumple virus (ClLCrV); Leonurus yellow spot alphasatellite (LeYSA) was detected in association with Tomato yellow spot virus (ToYSV) infecting plants of Leonurus sibiricus (Lamiaceae) [21, 23].

In this context, and considering the enormous diversity of begomoviruses found in cultivated and non-cultivated plants in Brazil [21, 34, 35], it is reasonable to assume that DNA-3 alphasatellites could interact with other begomoviruses in cultivated plants such as tomato. Since the main hypothesis for the emergence of begomovirus in cultivated plants in Brazil is the horizontal transfer of indigenous viruses from non-cultivated plants by the insect vector, the presence of alphasatellites adds an additional layer of complexity to this scenario, since the

effects on viral infection may be positive or negative depending on the begomovirus/alphasatellite/host combination.

The objective of this study was to verify the interaction capacity of the alphasatellite EuYMA with two begomoviruses that infect tomato in Brazil, ToYSV and Tomato severe rugose virus (ToSRV). We investigated effects on infectivity, symptom modulation and viral DNA accumulation in the presence and absence of the alphasatellite in two hosts, Nicotiana benthamiana and tomato. Variations in the detection rate of EuYMA associated with each helper begomovirus and host species were found. Although the presence of EuYMA increased the severity of symptoms induced by both begomoviruses, it did not increase viral DNA accumulation. Understanding the interaction of alphasatellites with bipartite begomoviruses is important to predict and monitor the possible emergence of diseases caused by new begomovirus-alphasatellite complexes.

Material and Methods

Tomato (Solanum lycopersicum) cv. Santa Clara and Nicotiana benthamiana plants were inoculated biolistically [36] using 10 µg of each genomic component (DNA-A and DNA-B) of viral isolates ToSRV-[BR:Pir1:05] [37] and ToYSV-[BR:Bic2:99] [38], alone or in the presence of alphasatellite EuYMA-[BRCha510-10] [20]. Inoculations in tomato plants and N. benthamiana with the begomovirus EuYMV-[BR:Cha510:10], to which EuYMA-[BRCha510-10] was originally found to be associated [20], were also performed. Fifteen tomato plants and 11 N. benthamiana plants were inoculated with each begomovirus in the presence or absence of the alphasatellite. Four plants were inoculated with tungsten particles without DNA as a negative control. The experiment was conducted once.

Plants were kept in a greenhouse and were evaluated for the appearance of symptoms up to 28 days post-inoculation (dpi). The youngest upper leaves were collected at 21dpi for total DNA extraction according to the method described by Doyle and Doyle [39]. Confirmation of infected plants was performed by conventional PCR. Information on the primers used for the detection of the two viruses and the alphasatellite is presented in Table 1. PCR amplifications were performed with 1 µL of total DNA, 0.4 µM of each primer, 1x Colorless GoTaq Reaction Buffer (Promega), 0.2 mM of each dNTP and 0.5 U of GoTaq DNA polymerase (Promega) in a final volume of 10 µL. The amplification program used to detect genomic fragments of ToSRV and ToYSV consisted of an initial denaturing step of 95°C for 2 min, followed by 38 cycles of 95°C for 1 min, 66°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplification program used to detect genomic fragments of EuYMV and of the EuYMA alpha-Rep gene consisted of an initial denaturing step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

DNA accumulation of each begomovirus and of the alphasatellite was determined by quantitative, real-time PCR (qPCR). A standard curve was obtained for ToSRV DNA-A, ToYSV DNA-A, EuYMV DNA-A and EuYMA by means of serial dilutions (5×10^0 to 5×10^7) of known quantities of plasmids containing one copy of the corresponding genomic component. Quantifications of plasmid DNAs used to construct the standard curve and of the total DNA samples were performed using a Nanodrop 2000c (ThermoFisher Scientific). The primers used for quantification (0.1 µM of each primer) of begomovirus and alphasatellite DNA accumulation are listed in Table 1. Reactions were prepared in a final volume of 10 µL using SYBR Green PCR Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems). Each sample was analyzed in triplicate by the amplification of 10 ng of total DNA.

Cycling conditions consisted of an initial denaturing step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60ºC for 1 min, with a final dissociation step to verify the specificity of amplification. The data generated were submitted to analysis of variance and the differentiation of means was performed using Student' s t test in Microsoft Excel.

Results and Discussion

The influence of EuYMA on the infectivity and symptoms of ToYSV and ToSRV infection in S. lycopersicum and N. benthamiana was evaluated by visual observation of symptoms up to 28 days post-inoculation and by PCR.

In tomato, the infectivity of ToSRV and ToYSV, either alone and when inoculated simultaneously with EuYMA, was 100% (Figure 1), indicating that the presence of the alphasatellite does not interfere with the infection process of these begomoviruses in this host. Such high efficiency of infection of these begomoviruses under experimental conditions has been reported before, and our results reinforce the high adaptability of these viruses in tomato [38, 40-42]. EuYMV was detected in seven plants (46.7%) when inoculated alone and in no plants when inoculated in mixed infection with EuYMA. Low detection rates of EuYMV in tomato both in the presence and absence of the alphasatellite is in agreement with the results obtained by Mar et al. [20] and indicates that EuYMV is not well adapted to tomato. Nevertheless, the ability of EuYMV to infect tomato indicates that it could be transferred to this host under natural conditions if BtMEAM1 and BtMED populations are capable of efficient transmission. The occurrence of host spillover infections is demonstrated by the fact that begomoviruses normally associated with non-cultivated plants, such as Sida mottle virus (SiMoV) and Sida micrantha mosaic virus (SiMMV), have been found infecting tomato in the

field [35, 43, 44], and viruses that are normally found in association with tomato, such as ToSRV and ToYSV, have also been found to infect non-cultivated hosts [21, 45-47].

In the experimental host N. benthamiana, the infectivity rate in plants inoculated with the virus alone was 100% for the three begomoviruses (Figure 1). In N. benthamiana plants inoculated with the begomoviruses and EuYMA the detection rates were 100%, 54.5% and 90.9% for ToYSV, ToSRV and EuYMV, respectively (Figure 1). These results suggest a possible negative effect of the presence of EuYMA on the infectivity of ToSRV in this host. However, the possibility of inoculation escapes cannot be ruled out, and the experiment should be repeated at least once before definitive conclusions can be established.

Considering that alphasatellites depend on a helper begomovirus to infect plants systemically, we can infer that the detection of EuYMA in non-inoculated leaves indicates an association with the helper begomovirus in question. We verified significant variations in the number of plants in which the alphasatellite was detected in non-inoculated leaves, depending on the helper begomovirus and the host. The detection rates were lower in tomato than in N. benthamiana when associated with ToYSV, with detection of EuYMA in only 20% of the plants (Figure 1). In N. benthamiana these values increased to 90.9% (Figure 1). When associated with ToSRV, EuYMA was detected at similar rates in both hosts (53.3% and 54.5% in tomato and N. benthamiana, respectively) (Figure 1).

Although EuYMA does not depend on a helper begomovirus to replicate, it depends on the capsid and movement proteins of the helper begomovirus for particle formation (which is essential for transmission by the insect vector) and cell-to-cell movement. We can infer that EuYMA interacts more efficiently with viral proteins and host factors in N. benthamiana compared to tomato, ensuring greater efficiency in movement of the begomovirus/alphasatellite complex and consequently greater adaptability and higher infection rates.

Although the infectivity of ToSRV in N. benthamiana inoculated with the virus and EuYMA was lower than in plants inoculated with the virus alone, the percentage of plants inoculated with ToSRV and EuYMA in which EuYMA was detected was similar in tomato and N. benthamiana. This raises concerns about the potential of this begomovirus/alphasatellite complex to disseminate in the field, considering the predominance of ToSRV in the field in different cultivated and non-cultivated hosts [45-52] and the efficiency of transmission of EuYMA by B. tabaci MEAM1 [20].

Saunders et al. [25] demonstrated that, albeit at low rates, an alphasatellite originally found in association with the Old World monopartite begomovirus Ageratum yellow vein virus (AYVV) can move systemically with the bipartite begomovirus Tomato golden mosaic virus (TGMV) and with the curtovirus Beet curly top virus (BCTV), but not with the mastrevirus Bean yellow dwarf virus (BYDV) in N. benthamiana. Although this study was performed with an experimental host, it demonstrated that alphasatellites can interact not only with different begomoviruses but even with viruses in different genera of the family Geminiviridae, highlighting the potential of these DNA satellites to be widely propagated in the field. Additional assays should be carried out to confirm if the variations in percentage of plants infected by EuYMA when associated with the three begomoviruses are significant.

All tomato plants infected with ToSRV and ToYSV were symptomatic. The symptoms in plants inoculated with ToSRV and ToYSV, both in the presence and absence of EuYMA, began to appear at 7 dpi and consisted of a yellow mosaic on ToSRV-infected plants and severe mosaic and leaf curling on ToYSV-infected plants (Figure 2). These symptoms have been previously reported for these viruses [38, 40-42]. Interestingly, the severity of symptoms increased for both viruses in the presence of EuYMA. Plants infected with ToSRV and EuYMA displayed more severe mosaic and leaf curling (Figure 2). Symptoms in plants infected with ToYSV and EuYMA were of the same nature compared to plants with ToYSV alone, but were

more severe (Figure 2). The infection of tomato by EuYMV was mostly asymptomatic, as previously reported [20] (Figure 2), with only a few plants showing faint chlorotic punctuations.

In N. benthamiana the first symptoms began to appear at 5 dpi and consisted of mosaic and leaf deformation for EuYMV (as reported by Mar et al. [20]) and ToSRV, and mosaic, leaf curling, rugosity and dwarfism for ToYSV (as reported by Andrade et al. [38], Calegario et al. [41] and Alves-Junior et al. [42]) (Figure 2). In all cases the symptoms were more severe in the presence of the alphasatellite compared to those induced by the virus alone. Mar et al. [20] showed that EuYMA increases the severity of symptoms caused by EuYMV in E. heterophylla and N. benthamiana and is a determinant of symptom development in Arabidopsis thaliana. The opposite effect was observed in N. benthamiana plants inoculated with Ageratum yellow vein Singapore alphasatellite simultaneously with begomovirus/betasatellite complexes. In this case, the presence of the alphasatellite led to the attenuation of symptoms by reducing the accumulation of the betasatellite (which encodes a strong RNA silencing suppressor protein) [31]. Possibly, reduction in the accumulation of the betasatellite occurs due to competition between the two DNA satellites by viral and host factors, which consequently leads to a dilution effect of available resources.

Differences in severity of symptoms can be related to variations in suppression of defense responses [53]. The RNA silencing suppressor activity of the Trap and AC4 proteins of begomoviruses [53-55], coupled with evidence that the alpha-Rep protein may act as a suppressor of post-transcriptional [29] or transcriptional RNA silencing [30], can lead to increased viral DNA accumulation and consequently increased severity of symptoms.

In N. benthamiana infected with ToYSV and EuYMA the symptoms were particularly severe. It has been shown that ToYSV infects mesophyll cells of N. benthamiana, which is related with the strong severity of symptoms [42]. Further studies should be performed to verify

if EuYMA also has the ability to invade mesophyll tissues in addition to vascular tissues in this host.

To evaluate whether EuYMA affects the accumulation of ToYSV and ToSRV in tomato and N. benthamiana, accumulation of the DNA-A from each virus in the presence and absence of alphasatellite was quantified at 21 dpi (Figure 3). The accumulation of the alphasatellite was also verified (Figure 3). The lack of tomato plants infected with EuYMV in the presence of EuYMA prevented the quantification of viral accumulation in this combination in this host.

The accumulation of ToYSV and ToSRV DNA-A in tomato plants did not present statistically significant differences when the virus was alone or in the presence of EuYMA (Figure 3). In both situations, the accumulation of EuYMA was statistically lower compared to accumulation of the helper virus (Figure 3), which may be due to a lower adaptability of EuYMA to the host in relation to ToYSV and ToSRV. These results indicate that the DNA-3-type alphasatellite EuYMA does not interfere in the accumulation of the DNA-A of these two begomoviruses in tomato.

In N. benthamiana there were no statistically significant differences in the accumulation of EuYMV and ToYSV alone and in the presence of EuYMA. Likewise, viral accumulation did not vary in the absence or presence of EuYMA in N. benthamiana plants inoculated with ToSRV, although EuYMA accumulated at higher levels compared to ToSRV in systemically infected leaves. Accumulation of EuYMV DNA-A did not increase in the presence of EuYMA, contrary to what was observed by Mar et al. [56]. However, the number of plants in mixed infection with EuYMV and EuYMA evaluated in our study was much lower. In general, the accumulation of EuYMA was higher in N. benthamiana than in tomato (Figure 3), in agreement with our data on higher infectivity (Figure 1) and adaptability of the alphasatellite in this host.

The impact of alphasatellites on the accumulation of viral DNA appears to vary according with the species of alphasatellite, host and helper begomovirus. The DNA-2-type

alphasatellite from Oman reduces betasatellite DNA accumulation, but not the accumulation of the helper begomovirus in N. benthamiana [31]. However, a DNA-1-type alphasatellite from Mali reduces the accumulation of the helper begomovirus in this host [57]. The presence of the same EuYMA isolate used in our study increased the accumulation of EuYMV in N. benthamiana and E. heterophylla [56]. This shows that although this alphasatellite has the ability to interact with ToYSV and ToSRV, the effect on increased viral accumulation seems to be a feature restricted to the EuYMV/EuYMA complex in these hosts.

Our results confirm the interaction between two tomato-infecting begomoviruses and a DNA-3-type alphasatellite which was initially found in association with a helper begomovirus in a non-cultivated host [56]. Thus, the begomovirus-alphasatellite interaction does not seem to be species-specific, which raises concerns about the dispersion, diversity and evolution of these subviral agents in the field. Furthermore, although the presence of the alphasatellite did not cause an increase in the accumulation of ToSRV and ToYSV DNA-A, it increased symptom severity independently of host and helper virus, possibly as a consequence of the RNA silencing suppressor activity of the alpha-Rep protein. ToYSV in the presence of the alphasatellite was better adapted to N. benthamiana than tomato. Higher detection rates of ToSRV plus EuYMA compared to ToYSV plus EuYMA in tomato suggests differences in the interaction between the alphasatellite and different tomato-infecting begomoviruses.

Currently, little is known about the effects of alphasatellites on begomovirus infection. Further studies are needed to verify if differences in the rate of detection and accumulation of EuYMA in association with ToSRV and ToYSV are significant, and if so, to better understand the mechanisms involved in the interaction of the alphasatellite with the two begomoviruses. This study is the first report that shows the ability of an alphasatellite detected in a non-cultivated host to interact with other begomoviruses in tomato plants.

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Table 1. Primers used for conventional and quantitative, real-time PCR.

Primer name	Sequence (5'-3')	C/Q*	Detection
ToSRV-$A_{(For)}$	CAGTAGTTGCCCTCAAATTGAAG	C	ToSRV DNA-A
ToSRV-$A_{(Rev)}$	CACGTGTAGCAATCTCCTTAAAGAG	C	ToSRV DNA-A
ToYSV-$A_{(For)}$	CACTTCGTTCGAGAAAACCTTTGAG	C	ToYSV DNA-A
ToYSV-$A_{(Rev)}$	CATGAGGGGACCATCAAGG	C	ToYSV DNA-A
EuYMV-$A_{(For)}$	GCCTAAGCGAGATGCCC	C	EuYMV DNA-A
EuYMV-$A_{(Rev)}$	GATTCTCGTATTTCCCTGCC	C	EuYMV DNA-A
$EuYMA_{(For)}$	GATCGGATCCATATGGGCTCAGCC	C	EuYMA alpha-Rep
$EuYMA_{(Rev)}$	GATCGAGCTCTTATTCCATATACGC	C	EuYMA alpha-Rep
qToSRV-$A_{(For)}$[#]	AAAGTAAAGTGATTGTCTGTGG	Q	ToSRV DNA-A
qToSRV-$A_{(Rev)}$[#]	GCCGTTCAACAAATTGGG	Q	ToSRV DNA-A
qToYSV-$A_{(For)}$	CCACGATTTTAAAGCTGCATTCT	Q	ToYSV DNA-A
qToYSV-$A_{(Rev)}$	CAATCCTGGTGAGGGAGTCAGT	Q	ToYSV DNA-A
qEuYMV-$A_{(For)}$[&]	AAGGCCTCTTCATGGGTGAA	Q	EuYMV DNA-A
qEuYMV-$A_{(Rev)}$ [&]	TTCGGTACATCTGGGCCTCTA	Q	EuYMV DNA-A
qEuYMA $_{(For)}$ [&]	CAAGAGGAAGACGATGGAACGT	Q	EuYMA alpha-Rep
qEuYMA$_{(Rev)}$ [&]	AGCAGCGACGATACAGCTTAGG	Q	EuYMA alpha-Rep

*C, conventional PCR; Q, quantitative, real-time PCR

[#]From Silva et al. [40]

[&]From Mar et al. [20]

Figure legends

Figure 1. Percentage of detection in systemic leaves of the begomoviruses Tomato yellow spot virus (ToYSV), Tomato severe rugose virus (ToSRV) and Euphorbia yellow mosaic virus (EuYMV) in the absence or presence of the alphasatellite Euphorbia yellow mosaic alphasatellite (EuYMA), and percentage of detection of EuYMA, in tomato and Nicotiana benthamiana plants. EuYMV was not detected when inoculated in mixed infection with EuYMA in tomato.

Figure 2. Symptoms in tomato and Nicotiana benthamiana plants infected with the begomoviruses Tomato yellow spot virus (ToYSV), Tomato severe rugose virus (ToSRV) and Euphorbia yellow mosaic virus (EuYMV) in the absence or presence of the alphasatellite Euphorbia yellow mosaic alphasatellite (EuYMA) at 14 and 28 days post-inoculation. Symptoms in tomato with EuYMV and EuYMA in mixed infection were not observed due to absence of plants infected with this combination of begomovirus and alphasatellite.

Figure 3. Accumulation of Tomato yellow spot virus (ToYSV), Tomato severe rugose virus (ToSRV) and Euphorbia yellow mosaic virus (EuYMV) DNA-A in the absence or presence of Euphorbia yellow mosaic alphasatellite (EuYMA), and accumulation of EuYMA, in tomato and N. benthamiana plants. Viral accumulation is presented as the log of the number of molecules. Means were compared using Student's t test ($P < 0.05$). Means with standard errors are indicated.

Figure 1

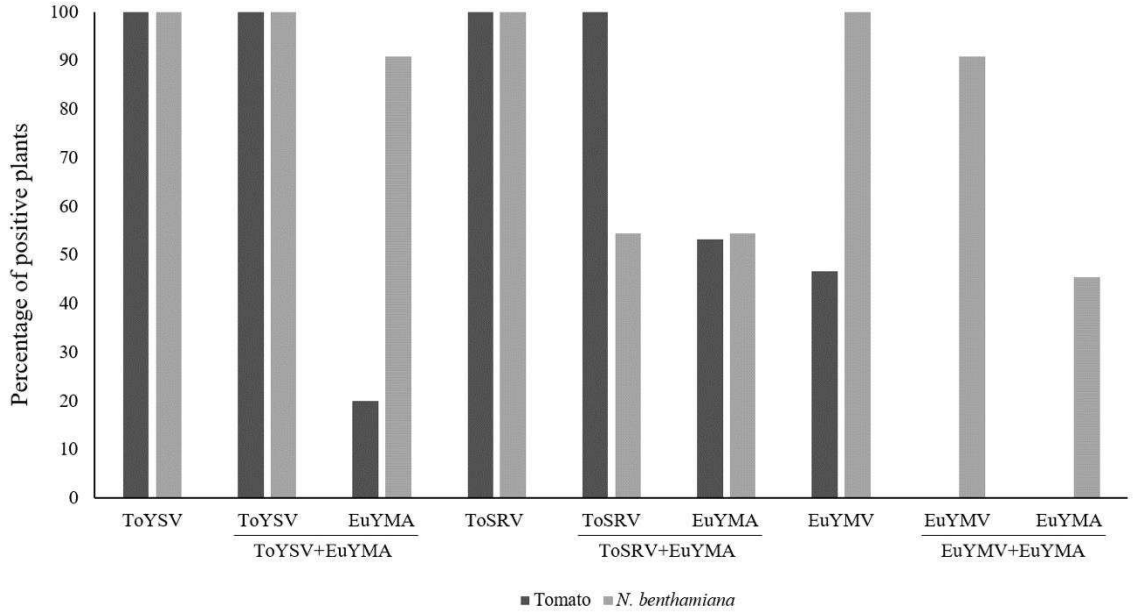

Percentage of positive plants
100
90
80
70
60
50
40
30
20
10
0
ToYSV
ToYSV
EuYMA
ToYSV+EuYMA
ToSRV
ToSRV
EuYMA
ToSRV+EuYMA
EuYMV
EuYMV
EuYMA
EuYMV+EuYMA
Tomato
N. benthamiana

Figure 2

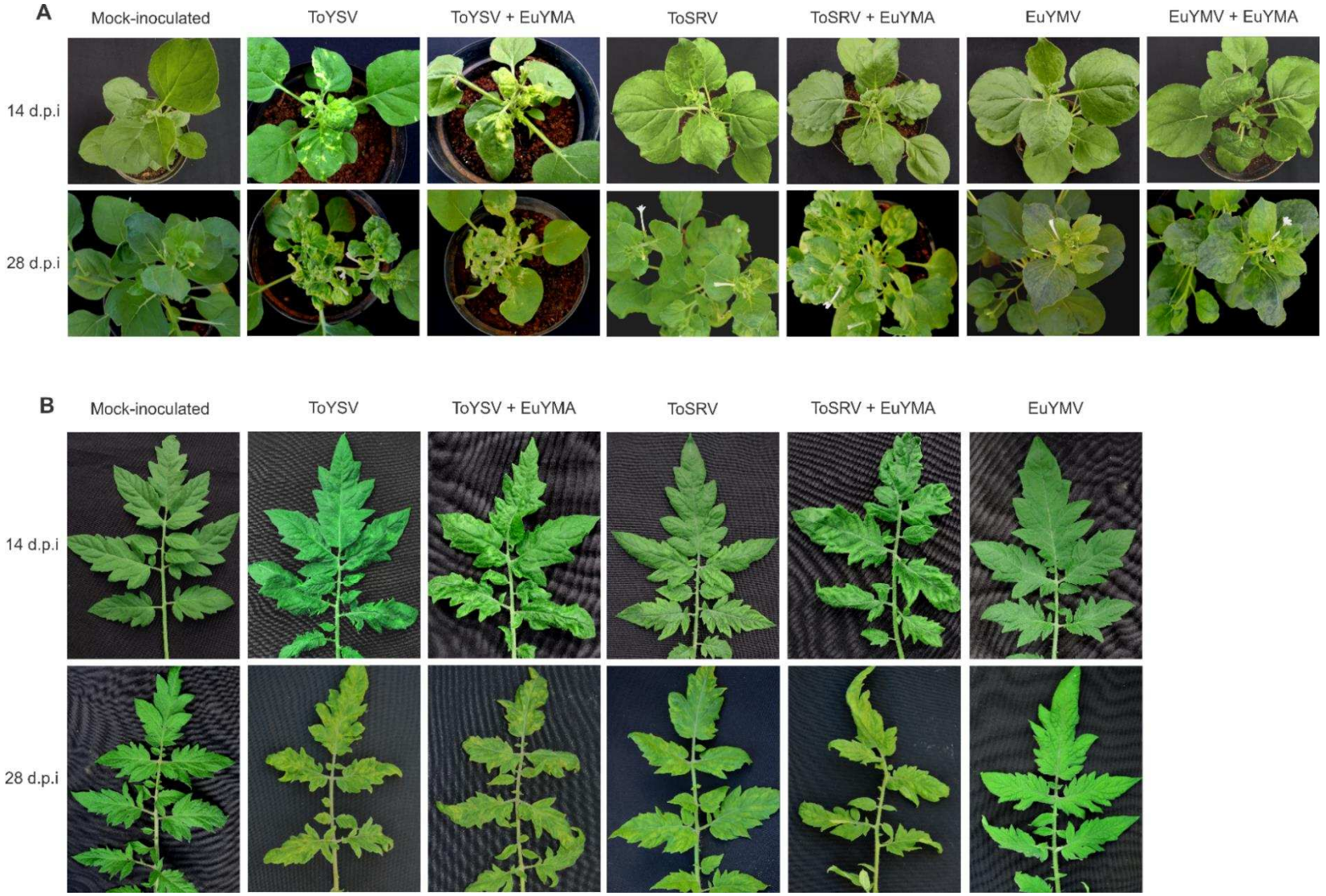
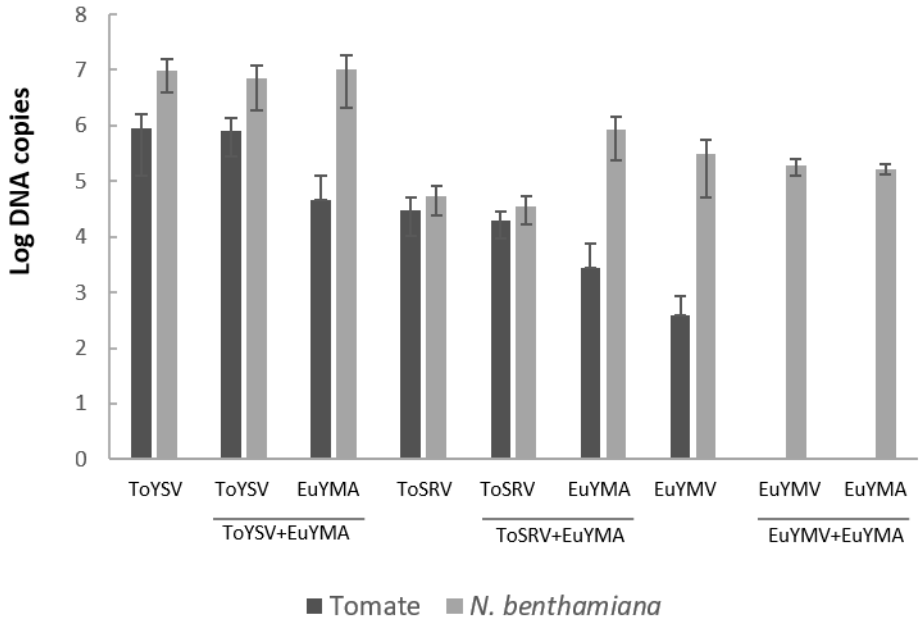

A
Mock-inoculated
ToYSV
ToYSV + EuYMA
ToSRV
ToSRV + EuYMA
EuYMV
EuYMV + EuYMA
14 d.p.i
28 d.p.i
B
Mock-inoculated
ToYSV
ToYSV + EuYMA
ToSRV
ToSRV + EuYMA
EuYMV
14 d.p.i
28 d.p.i

Figure 3


Log DNA copies
8
7
6
5
4
3
2
1
0
ToYSV
ToYSV
EuYMA
ToSRV
ToSRV
EuYMA
EuYMV
EuYMV
EuYMA
ToYSV+EuYMA
ToSRV+EuYMA
EuYMV+EuYMA
Tomate
N. benthamiana

GENERAL CONCLUSIONS

- ToSRV was transmitted with the same efficiency in relation to ToRMV by BtMEAM1 and at higher rates by BtMED in single infections;

- ToRMV genomic components were transmitted by BtMEAM1 at a higher rate in relation to ToSRV components in mixed infection, indicating that ToRMV predominance occurs most likely due to negative interference on ToSRV replication rather than in its transmission by the insect vector;

- In single infection, infectivity and viral accumulation of wild-type ToSRV and ToSRV mutants were similar;

- In mixed inoculation with the DNA-A and DNA-B of ToRMV and ToSRV-$A_{(CR)}$, high infectivity of both viruses and high accumulation of ToSRV-$A_{(CR)}$ DNA in relation to wild-type ToSRV indicate that only a three nucleotide difference in the CR serves as specificity determinant for Rep binding and is responsible for the negative interference of ToRMV on the replication of wild-type ToSRV;

- In mixed inoculation with the DNA-A and DNA-B of ToRMV and ToSRV-$A_{(CR+IRD)}$, decreased viral accumulation suggests that the divergent amino acids in the IRD do not offer an advantage for ToSRV replication;

- Considering only the DNA-B detection, wild-type ToSRV DNA-B was detected at a much lower percentage than the ToRMV DNA-B, highlighting the importance of changes in the CR for the differential infectivity of ToSRV and ToRMV components;

- The alphasatellite EuYMA, which was initially found in association with a helper begomovirus in a non-cultivated host, can interact with two tomato-infecting begomoviruses (ToYSV and ToSRV) in tomato and N.benthamiana, indicating that begomovirus-alphasatellite interaction is not host-specific;

- Higher detection rates of ToSRV plus EuYMA compared to ToYSV plus EuYMA in tomato suggests differences in the interaction between the alphasatellite and different tomato-infecting begomoviruses;

- The presence of the alphasatellite increased the severity of symptoms induced by ToYSV and ToSRV in tomato and N.benthamiana, however it did not interfere in the accumulation of ToSRV and ToYSV in both hosts.