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Tomato yellow spot virus, a tomato-infecting begomovirus from Brazil with a closer relationship to viruses from *Sida* sp., forms pseudorecombinants with begomoviruses from tomato but not from *Sida*

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Geminiviruses are characterized by a circular, single-stranded DNA genome and twinned icosahedral particles. Begomoviruses (whitefly-transmitted geminiviruses) are a major constraint to crop production worldwide. In Brazil, tomato-infecting begomoviruses emerged as serious pathogens over the last 10 years, due to the introduction of a new biotype of the insect vector. Tomato yellow spot virus (ToYSV) is a newly described begomovirus originally isolated from tomato, but phylogenetically closer to viruses from *Sida* sp. A study was performed to determine the viability of pseudorecombinants formed between the DNA components of ToYSV and other weed- and tomato-infecting begomoviruses from Brazil. Despite its closer relationship to weed-infecting viruses, ToYSV was only capable of forming viable pseudorecombinants with tomato viruses. An infectious pseudorecombinant formed between ToYSV DNA-A and tomato crinkle leaf yellows virus (TCrLYV) DNA-B induced severe symptoms in *Nicotiana benthamiana*. This was attributed, at least in part, to the fact that the origins of replication of both components had identical Rep-binding sequences. However, this was not the case for another infectious pseudorecombinant formed between tomato golden mosaic virus (TGMV) DNA-A and ToYSV DNA-B, which have different Rep-binding sequences. These results reinforce the notion that pseudorecombinant formation cannot be explained solely on the basis of phylogenetic relationships and conserved iteron sequences, and suggest that the TGMV Rep protein may be more versatile in terms of recognizing heterologous DNA components than that of ToYSV.

INTRODUCTION

Plant viruses of the family *Geminiviridae* are characterized by possessing a genome composed of one or two circular, single-stranded DNA (ssDNA) molecules, encapsidated in twinned, incomplete, icosahedral virions. The family is divided into four genera based on host range, insect vector and genome organization. Species in the genus *Begomovirus* have a genome composed of one (monopartite) or two (bipartite) ssDNA molecules of approximately 2·6 kb each and are transmitted by the whitefly *Bemisia tabaci* to dicotyledonous plants (Rojas *et al.*, 2005; Stanley *et al.*, 2005). For bipartite begomoviruses, the two genomic components are referred to as DNA-A and DNA-B. Gene products encoded by DNA-A are responsible for virus replication (Rep and REn), regulation of gene expression, and suppression of RNA silencing (TrAP) and particle encapsidation (CP) (Rojas *et al.*, 2005). DNA-B encodes two proteins, a movement protein (MP) and a nuclear-shuttle protein (NSP), involved in inter- and intracellular trafficking within the plant, host range and symptom modulation (Ingham *et al.*, 1995; Noueiry *et al.*, 1994; Schaffer *et al.*, 1995).

The two cognate DNA components have little sequence similarity, sharing only a 'common region' (CR) of approximately 200 bp with high sequence identity (usually $>90\,\%$). The CR contains the origin of replication (*ori*) and several sequence elements required for DNA replication (Chatterji *et al.*, 1999; Eagle *et al.*, 1994; Hanley-Bowdoin *et al.*, 1999; Laufs *et al.*, 1995). One of these elements consists of two tandemly repeated sequences (iterons) located near *ori*. The iterons are virus-specific binding sites for the cognate Rep protein and help to initiate virus replication (Argüello-Astorga *et al.*, 1994a; Fontes *et al.*, 1994b). The specificity of the Rep–iteron binding is considered to be a major determinant in the formation of viable pseudorecombinants between different species/strains of

begomoviruses (Argüello-Astorga *et al.*, 1994a; Chatterji *et al.*, 1999; Eagle *et al.*, 1994; Fontes *et al.*, 1994b). The formation of viable pseudorecombinants indicates that factors responsible for replication and movement are interchangeable between non-cognate DNA components and is a useful measure of relationships among bipartite begomoviruses.

Begomoviruses are one of the most economically important groups of plant viruses, due to their high incidence and disease severity in vegetable and field crops in tropical and subtropical areas of the world (Briddon, 2003; Morales & Anderson, 2001; Moriones & Navas-Castillo, 2000; Were *et al.*, 2004). In Brazil, begomoviruses have been a limiting factor for bean production since the 1970s (Costa, 1976) and in tomatoes since the mid-1990s (Ribeiro *et al.*, 2003). The first report of a tomato-infecting geminivirus in Brazil was made in 1975. The virus was purified and named *Tomato golden mosaic virus* (TGMV) (Matyis *et al.*, 1975). TGMV was one the first geminiviruses to be cloned as infectious DNA (Hamilton *et al.*, 1984) and became one of the prime model systems for the study of geminivirus molecular biology and host interactions (Hanley-Bowdoin *et al.*, 1999). However, tomato golden mosaic was never an economically important disease, probably because the A biotype of the insect, which was prevalent in Brazil at that time, colonizes tomatoes at low efficiency (Bedford *et al.*, 1994). Following the introduction of the B biotype in the early 1990s (Lourenção & Nagai, 1994), tomato-infecting begomoviruses emerged and spread rapidly. Preliminary molecular studies indicated the presence of several putative novel begomovirus species (Ribeiro *et al.*, 1998), with subsequent studies revealing at least seven novel species (Ambrozevicius *et al.*, 2002; Fernandes *et al.*, 2006; Ribeiro *et al.*, 2003). The source of these novel viruses is thought to have been indigenous begomoviruses infecting weed genera, such as *Malva* and *Sida*, that have been introduced into tomato by the B biotype of the insect vector. In the new host, mixed infections would have facilitated recombination or pseudorecombination events, leading to the formation of novel, better-adapted species. Evidence in support of this hypothesis includes the finding of several interspecies recombination events in natural mixed infections (Monci *et al.*, 2002; Rothenstein *et al.*, 2006), the formation of infectious pseudorecombinants naturally (Pita *et al.*, 2001; Zerbini *et al.*, 2002) or under laboratory conditions via the exchange of cloned DNA components from distinct species of bipartite begomoviruses (Garrido-Ramirez *et al.*, 2000; Gilbertson *et al.*, 1993; Hou *et al.*, 1998; Ramos *et al.*, 2003; Unseld *et al.*, 2000).

Tomato yellow spot virus (ToYSV) is an emerging begomovirus described recently, infecting tomatoes in Brazil (R. F. Calegario, S. S. Ferreira, E. C. Andrade & F. M. Zerbini, unpublished data). The virus is phylogenetically closer to viruses from *Sida* sp. and causes severe symptoms in tomato and experimental hosts, such as *Nicotiana benthamiana* and *Nicotiana glutinosa* (Ambrozevicius *et al.*, 2002; R. F. Calegario and others, unpublished data). In contrast, *Tomato rugose mosaic virus* (ToRMV) and *Tomato chlorotic mottle virus* (ToCMoV), two other recently characterized species, induce relatively mild symptoms in the same hosts (Ambrozevicius *et al.*, 2002; Fernandes *et al.*, 2006; Galvão *et al.*, 2003; Ribeiro *et al.*, 2003). Also, ToYSV is readily sap-transmissible to several solanaceous hosts, such as tobacco and sweet pepper (but not to tomato) (R. F. Calegario and others, unpublished data), whereas ToRMV and ToCMoV are not sap-transmissible (Ambrozevicius *et al.*, 2002; Fernandes *et al.*, 2006). Analysing the properties of ToYSV, as well as its relationships with other tomato- and weed-infecting begomoviruses from Brazil, may help to understand the mechanisms involved in begomovirus emergence and evolution, as well as in symptom induction. Here, we studied the capacity to form viable pseudorecombinants between ToYSV and previously characterized Brazilian begomoviruses from tomato and *Sida rhombifolia*.

METHODS

Sequence comparisons and recombination analysis. Nucleotide and amino acid sequences of ToYSV and additional begomoviruses used in the pseudorecombination experiments (see Table 1 for the complete list and GenBank accession numbers) were aligned by using CLUSTAL W (Thompson *et al.*, 1994). Percentage nucleotide and amino acid sequence identities for the entire genome and for the Rep protein were calculated by using DNAMan ver. 4.0 (Lynnon BioSoft). Analyses of potential recombination events were carried out by using the Recombination Detection Program (RDP) ver. 2.0 (Martin & Rybicki, 2000), using all default parameters.

Construction of infectious ToYSV clones. The isolation, characterization and construction of full-length clones corresponding to DNA-A and DNA-B of the Bi2 isolate of ToYSV will be described elsewhere (R. F. Calegario and others, unpublished data); details are available from the authors on request. To construct partially redundant clones of the ToYSV genome, a *Sac*I–*Eco*RV fragment of pToYSV-A was inserted into the pUC118 vector, generating pToYSV-A0.2. A full-length *Sac*I fragment was then inserted into pToYSV-A0.2, generating pToYSV-A1.2. Likewise, a *Sac*I–*Xba*I fragment of pToYSV-B was inserted into pUC118, generating pToYSV-B0.2, and a full-length *Sac*I fragment was then inserted into pToYSV-B0.2 to generate pToYSV-B1.2.

Plant inoculations. *N. benthamiana* plants were inoculated biolistically (Aragão *et al.*, 1996) with different combinations of the cloned DNA-A and DNA-B of ToYSV and of the additional tomato- and *Sida*-infecting begomoviruses (Table 1). The clones of ToCMoV-[Se1] DNA-A and DNA-B are monomers, and the insert DNA was excised with *Kpn*I and *Hin*dIII, respectively, and religated prior to inoculation. All other virus genomes were cloned as partial tandem-repeated sequences. For SiMoV-[Vi1] and SiYMV-[Vi2], only DNA-A was cloned. For ToCMoV-[Ig1] and TCrLYV-[Vi3], only DNA-B was cloned. Approximately 2 μg of each recombinant plasmid DNA (1 μg of insert DNA in the case of ToCMoV-[Se1]) was used for the inoculations.

Detection of viral infection. Four weeks after inoculation, plants were evaluated visually for the presence of symptoms, photographed and total DNA was extracted from newly emerged leaves (Dellaporta *et al.*, 1983). Viral DNA was detected by PCR-restriction fragment-length polymorphism (RFLP) using universal begomovirus primers

Table 1. Viruses used for sequence comparisons, phylogenetic/recombination analyses and in the pseudorecombination assays

Species [isolate]	GenBank accession no.	Reference
Tomato yellow spot virus (ToYSV-[Bi2])	NC_007726 (DNA-A) NC_007727 (DNA-B)	Calegario *et al.* (unpublished data)
Tomato chlorotic mottle virus (ToCMoV-[Se1])	NC_003664 (DNA-A) NC_003665 (DNA-B)	Ribeiro *et al.* (2003)
Tomato chlorotic mottle virus (ToCMoV-[Ig1])	DQ336353 (DNA-A)* DQ336354 (DNA-B)	Andrade *et al.* (2002)
Tomato golden mosaic virus (TGMV-[Yellow vein])	NC_001507 (DNA-A) NC_001508 (DNA-B)	Fontes *et al.* (1994a)
Tomato rugose mosaic virus (ToRMV-[Ub1])	NC_002555 (DNA-A) NC_002556 (DNA-B)	Fernandes *et al.* (2006)
Tomato crinkle leaf yellows virus (TCrLYV-[Vi3])	AY090556 (DNA-B)	Galvão *et al.* (2003)
Sida micrantha mosaic virus (SimMV-[A2B2])*	NC_005330 (DNA-A) NC_005331 (DNA-B)	Jovel *et al.* (2004)
Sida mottle virus (SiMoV-[Vi1])	NC_004637 (DNA-A)	Fernandes *et al.* (1999)
Sida mottle virus (SiMoV-[A1B3])*	AJ557450 (DNA-A) AJ557454 (DNA-B)	Jovel *et al.* (2004)
Sida yellow mosaic virus (SiYMV-[Vi2])	NC_004639 (DNA-A)	Fernandes *et al.* (1999)

*Used only for sequence comparisons or phylogenetic/recombination analyses.

[PAL1v978/PAR1C715 for DNA-A and PBL1v2040/PCRc1 for DNA-B (Rojas *et al.*, 1993)], and amplicons were digested with diagnostic restriction enzymes specific for each virus species.

RESULTS

ToYSV is phylogenetically closer to viruses from *Sida* sp. than from tomato and has a possible recombinant origin

Sequence comparisons and phylogenetic analysis based on full-length DNA-A and DNA-B indicate a close relationship between ToYSV and the *Sida*-infecting begomoviruses *Sida mottle virus* (SiMoV), *Sida yellow mosaic virus* (SiYMV) and *Sida micrantha* mosaic virus (SimMV) (R. F. Calegario and others, unpublished data; Fig. 1a). In fact, the complete sequences of the DNA-A and DNA-B components of ToYSV display the highest nucleotide sequence identity to these three viruses (Fig. 1b) and the deduced amino acid sequence of the ToYSV Rep protein is more similar to those of *Sida*-infecting viruses than to those of other tomato-infecting viruses (Fig. 1c). The ToYSV CP amino acid sequence is 96 % identical to that of the SiMoV CP and <90 % identical to those from tomato-infecting begomoviruses (R. F. Calegario and others, unpublished data).

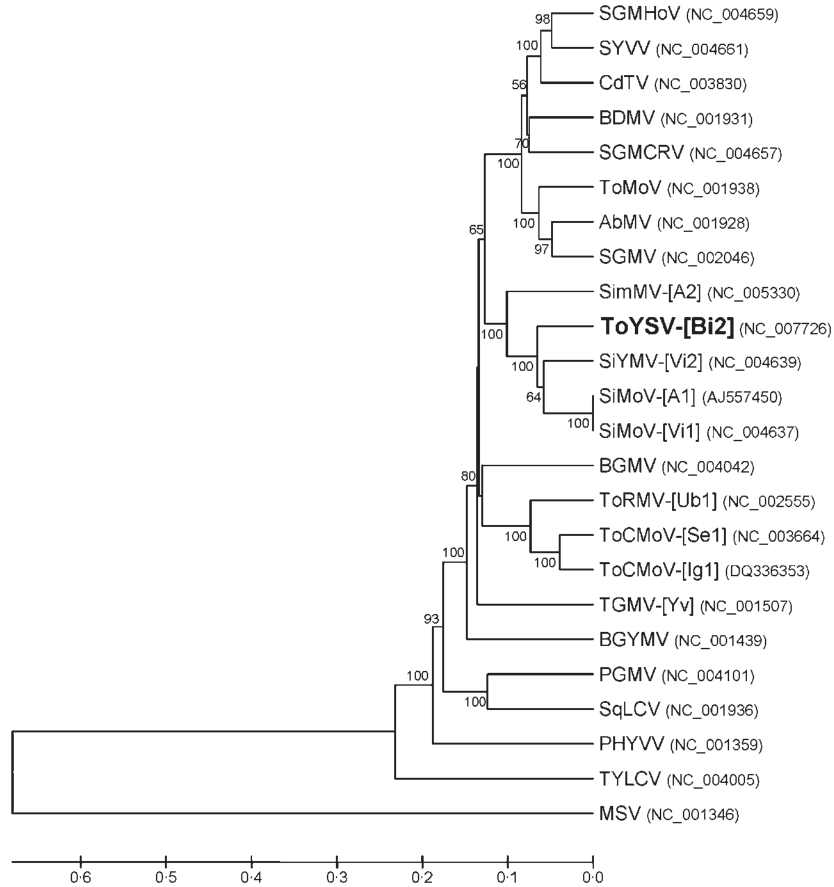
We performed a recombination-detection analysis by using complete DNA-A and DNA-B sequences of several tomato- and *Sida*-infecting begomoviruses from Brazil (listed in Table 1). This analysis indicated that ToYSV has a potential recombinant origin, with the fragment corresponding to nt 1–529 (the exact CP breakpoint varies, depending on the program module) probably being derived from SiMoV. This recombination pattern was detected by the RDP, GeneConv, Bootscan and Chimaera modules of the RDP package with confidence levels of $7{\cdot}3\times10^{-10}$, $4{\cdot}8\times10^{-5}$, $1{\cdot}5\times10^{-3}$ and $1{\cdot}7\times10^{-7}$, respectively. Together, results from sequence comparisons and phylogenetic and recombination analyses indicate that ToYSV is related more closely to *Sida*-infecting begomoviruses than to tomato-infecting begomoviruses.

ToYSV-B and *Sida*-infecting begomoviruses do not form viable pseudorecombinants

N. benthamiana plants bombarded with ToYSV DNA-B plus SiMoV or SiYMV DNA-A displayed no symptoms of viral infection, either local or systemic (Table 2). Viral DNA was not detected in newly emerged leaves of these plants (data not shown). These results indicate that, even though SiMoV and SiYMV are the phylogenetically closest viruses to ToYSV, the viral factors required for replication and/or movement are not exchangeable between these viruses. Alignment of part of the CR of the three viruses (Fig. 1b) reveals divergence among their iterons (ToYSV, GGTG; SiMoV, GGAG; SiYMV, GGGG). Similarly, the Rep proteins encoded by the three viruses have poorly conserved putative DNA-binding domains (Fig. 1c).

Analysis of the amino-terminal sequence of the Rep proteins of ToYSV, SiMoV and SiYMV, particularly the iteron-related domain (IRD), shows low conservation of the specific amino acids potentially responsible for iteron recognition (Fig. 1c). This observation, associated with the lack of iteron conservation between ToYSV, SiMoV and SiYMV, could explain the impediment for *trans*-replication

(a)



(b)

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                                                                                 % nt
                                                                            DNA-A  DNA-B
ToYSV-[Bi2]     AGTC---C--TTATGAATTGGTGACATTGGTGTACAATATATACTAGAAGTTCT
TCrLYV-[Vi3]    AACTCCGC--TAGTCAATTGGTGAC--TGGTGTACAATATATAGTAGAAGCCTC      NA     64
TGMV-[Yv]       AAAGT--T--ATATGAATTGGTAGTAA-GGTAGCTCTTATATATTAGAAGT-TC      76     62
ToRMV-[Ub1]     AAACT--C--ATATGAATTGGTAGTTATGGTAGCTCTTATATACTAGAAGTTCC      77     63
ToCMoV-[Ig1]    AACTTGCT--CTAGCAATTGGGGAC--TGGGGTCCTATATATACTAGAACCCTC      74     58
ToCMoV-[Se1]    AAACT-CT--AAAGCAATTGGGGAC--TGGGGTCCTATATATACTAGAACCCTC      75     60
SimMV-[A2B2]    ATAA-GTC--ATATGAATTGGGG-TAATGGGGAC-AATATATAGTAGAAGATCC      81     66
SiMoV-[A1B3]    ATAA-GTCTC-TATGAATTGGAGT--ATGGAGTACAATATATATGAGGAGTTCT      87     79
SiMoV-[Vi1]     ATAA-GTCTC-TATGAATTGGAGT--ATGGAGTACAATATATAGTAGGAGTTCC      87     NA
SiYMV-[Vi2]     ACTCTGTG--ATATGAACTGGGGAC--TGGGGTCTTATTTATAGCAG-AC--TC      83     NA
                *              ** ***       **       * ****  ** *
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(c)

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                          Motif 1
                    IRD     {                                                   % aa
ToYSV-[Bi2]     MPSAPKRFRISSKNYFLTYPRCSLSKEETLSQLQTINTPTNKKFIKICRELHEDGEPHLH
TGMV-[Yv]       MPSHPKRFQINAKNYFLTYPQCSLSKEESLSQLQALNTPINKKFIKICRELHEDGQPHLH      80
ToRMV-[Ub1]     MPSATRRFQIKAKNYFLTYPKCSISKEEALSQLKNLNTPTNKKFIKVCRELHENGEPHLH      80
ToCMoV-[Ig1]    MP-PPKHFRVNSKNYFLTYPHCSLSKEEALSQLLAIQIPVNKLFIRVARELHEDGQPHLH      74
ToCMoV-[Se1]    MP-LPRHFRVNSKNYFLTYPHCSLSKEEALSQLLALNTPTNKLFIRVSRELHENGEPHLH      74
SimMV-[A2B2]    MP-PPKRFKISAKNYFLTYPQCSLTKEETLSQLQSLSTPVNKKFIKICRELHENGEPHLH      84
SiMoV-[A1B3]    MPSKPRRFRVQAKNIFLTYPQCSLTKEEALSQLQAIQLPSNKKFIKICRELHEDGQPHLH      86
SiMoV-[Vi1]     MPSKPRRFRVQAKNIFLTYPQCPLTKEEALSQLQAIQLPSNKKFIKICRELHEDGQPHLH      77
SiYMV-[Vi2]     MP-SPRAFRINSKNYFLTYPKCSLTKEEALSQLKELNTPTSKKFIRVSRELHEDGSPHLH      75
                **  .: *::.:** *****:*.::***:****  :: * .* **::.*****:*.****
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Table 2. Infectivity of pseudorecombinants formed among ToYSV and begomoviruses from tomato and *Sida* sp.

Combination*	Plants infected/inoculated (no. independent experiments)	Virus detection†		Symptoms‡
		DNA-A	DNA-B	
ToYSV-A + ToCMoV-[Se1]-B	0/12 (2)	0	0	NA
ToCMoV-[Se1]-A + ToYSV-B	0/12 (2)	0	0	NA
ToYSV-A + ToCMoV-[Ig1]-B	2/12 (2)	2	0	Asymptomatic
ToYSV-A + TGMV-B	0/12 (2)	0	0	NA
TGMV-A + ToYSV-B	4/12 (2)	4	4	ep, lc, m
ToYSV-A + ToRMV-B	0/18 (3)	0	0	NA
ToRMV-A + ToYSV-B	1/18 (3)	1	0	Asymptomatic
ToYSV-A + TCrLYV-B	8/12 (2)	8	8	dc, ep, m
SiMoV-A + ToYSV-B	0/12 (2)	0	0	NA
SiYMV-A + ToYSV-B	0/12 (2)	0	0	NA
SiYMV-A + ToCMoV-[Se1]-B	7/18 (3)	7	7	ep, mm
ToYSV-A	0/15 (1)	0	0	NA
TGMV A + B	18/18 (3)	18	18	dc, lc, sm
ToCMoV-[Se1] A + B	12/18 (3)	12	12	ep, mm
ToCMoV-[Se1]-A + ToCMoV-[Ig1]-B	11/12 (2)	11	11	ep, mm
ToRMV A + B	17/18 (3)	17	17	lc, mm
ToYSV A + B	15/15 (1)	15	15	lc, sm
Empty vector	0/18 (3)	0	0	NA

*Virus abbreviations as in Table 1.

†Virus detection by PCR-RFLP, using universal begomovirus primers followed by digestion with species-specific restriction endonucleases.

‡dc, Down cupping; ep, epinasty; lc, leaf curling; m, mosaic; mm, mild mosaic; sm, severe mosaic; NA, not applicable.

of ToYSV DNA-B by the Rep proteins encoded by SiMoV and SiYMV.

Interestingly, ToCMoV-[Se1] DNA-B formed viable pseudorecombinants with both SiMoV and SiYMV DNA-A (Table 2). Iterons are conserved between ToCMoV and SiYMV (GGGG), but not between ToCMoV and SiMoV (GGGG and GGAG, respectively).

Pseudorecombination between ToYSV and tomato-infecting begomoviruses

Infectivity of pseudorecombinants produced between ToYSV and tomato-infecting begomoviruses could be divided into three groups. The first group includes non-viable pseudorecombinants, which did not induce symptoms and for which no viral DNA could be detected in newly emerged leaves (Table 2 and data not shown). This group includes pseudorecombinants between ToYSV DNA-A and ToRMV DNA-B, ToYSV DNA-A and TGMV DNA-B, and both combinations of ToYSV and ToCMoV-[Se1] DNAs A and B. There is no conservation among the iterons of ToYSV (GGTG) and ToRMV (GGTAG), ToCMoV (GGGG) or TGMV (GGTAG) (Fig. 1b). Likewise, the IRD sequences show poor conservation, with the specific amino acids potentially responsible for iteron recognition not fully conserved among these four viruses (Fig. 1c).

The second group comprises two pseudorecombinants: ToRMV DNA-A/ToYSV DNA-B and ToYSV DNA-A/ToCMoV-[Ig1] DNA-B. These combinations did not induce symptoms, but viral DNA (DNA-A only) was detected in newly emerged leaves of a small number of plants (Table 2; Fig. 3). The capacity of ToRMV DNA-A to infect

Fig. 1. (a) Phylogenetic tree based on a multiple sequence alignment of the complete DNA-A of selected New World begomoviruses, using the UPGMA method. Branches were bootstrapped with 1000 replications. Bar, number of substitutions per site. (b) Alignment of partial CR sequences and percentage nucleotide sequence identity (% nt; NA, not applicable) between the complete DNA-A and DNA-B components of ToYSV and those of the viruses used in the pseudorecombination assays. The Rep-binding sites (iterons), relevant for *trans*-replication of begomovirus DNA components, are double-underlined. The TATA box of the Rep gene is underlined. Asterisks indicate nucleotide positions that are conserved among all of the aligned sequences. (c) Rep alignment and percentage amino acid sequence identity (% aa; NA, not applicable) between the Rep protein of ToYSV and those of the viruses used in the pseudorecombination assays. The sequence identified as 'motif 1' is conserved in rolling-circle replication-initiator proteins, including geminivirus Rep proteins. Underlined sequences correspond to the domain associated with sequence-specific recognition of iterons, named 'iteron-related domain' (IRD) according to Argüello-Astorga & Ruiz-Medrano (2001). Amino acids within the IRD that are predicted to be responsible for recognition of the variant nucleotides in the iterons are in bold.

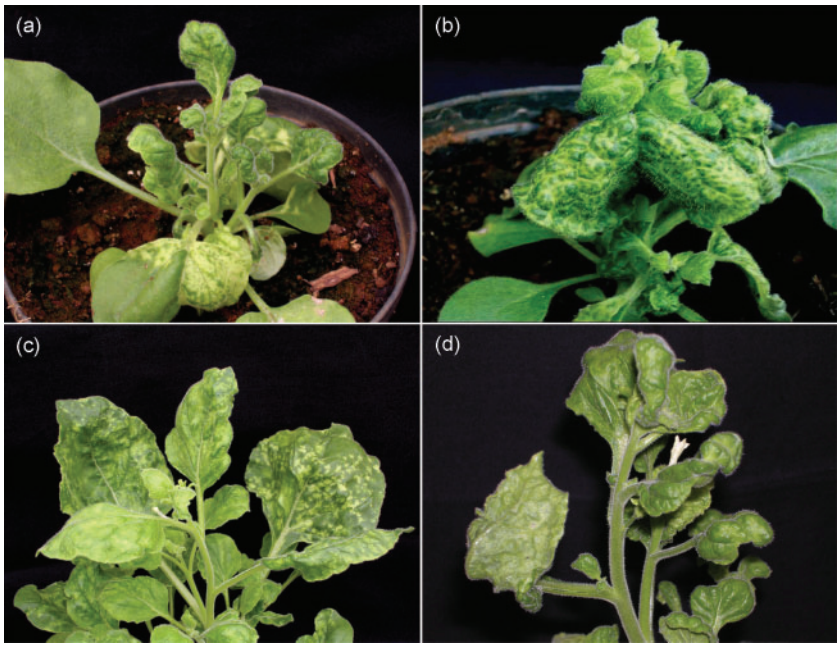

Fig. 2. Symptoms in *Nicotiana benthamiana* plants infected with ToYSV (a), TGMV (b), the pseudorecombinant ToYSV DNA-A/TCrLYV DNA-B (c) and the pseudorecombinant TGMV DNA-A/ToYSV DNA-B (d). Plants were inoculated biolistically with 2 μg of each DNA component and were photographed 28 days after inoculation.

N. benthamiana in the absence of the cognate DNA-B has been reported previously (Andrade *et al.*, 2004). However, ToYSV DNA-A alone did not infect *N. benthamiana* (Table 2), indicating that a very low accumulation of ToCMoV-[Ig1] DNA-B, below the limit of detection of the PCR-RFLP assay, may be sufficient to allow the DNA-B-encoded proteins to move ToYSV DNA-A from cell to cell. Our finding that plants inoculated with these two combinations do not display any symptoms is in agreement with previous studies demonstrating that symptom determinants in begomovirus infections are encoded by DNA-B (Ingham *et al.*, 1995; Schaffer *et al.*, 1995). The lack of iteron and IRD conservation between ToYSV and ToCMoV-[Ig1] could be a barrier to efficient *trans*-replication of the heterologous DNA-B, explaining its low level in non-inoculated leaves.

The third group comprises two pseudorecombinants: ToYSV DNA-A/TCrLYV DNA-B and TGMV DNA-A/ToYSV DNA-B. The pseudorecombinant formed by ToYSV DNA-A and TCrLYV DNA-B induced severe mosaic, leaf curling and epinasty in *N. benthamiana* (Table 2; Fig. 2c), similar to the symptoms induced by ToYSV. The identity of each viral component present in plants inoculated with the pseudorecombinant was confirmed by PCR-RFLP. Fragments corresponding to both DNAs were PCR-amplified from newly emerged, symptomatic leaves (Fig. 3a) and cleaved with diagnostic restriction enzymes to distinguish the fragments of the respective virus (*Sac*I for ToYSV DNA-A and *Eco*RI for TCrLYV DNA-B) (Fig. 3b). Equivalent results were obtained for the other pseudorecombinant (TGMV DNA-A/ToYSV DNA-B), which also induced severe mosaic, leaf curling and epinasty (Table 2; Fig. 2d). In this case, symptoms were as severe as those induced by ToYSV, but slightly less severe than those induced by TGMV, again confirming that symptom induction is governed by DNA-B. Both DNAs were PCR-amplified (Fig. 3a), and PCR-RFLP (using *Pst*I for TGMV DNA-A and *Hin*dIII for ToYSV DNA-B) confirmed the identity of each DNA component (Fig. 3b).

The CR alignment (Fig. 1b) revealed that ToYSV and TCrLYV have identical iterons, which probably contributed to the viability of this pseudorecombinant. However, ToYSV and TGMV iterons are not identical (GGTG and GGTAG, respectively). Nevertheless, the Rep protein encoded by TGMV was capable of mediating replication of ToYSV DNA-B. Analysis of the amino acid sequence of the Rep proteins indicates that the IRD domains of TGMV and ToYSV are the most similar among all begomoviruses analysed in this study (five out of eight amino acids), including residues predicted to be involved in Rep–iteron recognition (Fig. 1c). This sequence conservation could allow iteron recognition by the heterologous Rep protein.

DISCUSSION

Studying the relationship between ToYSV, which induces extremely severe symptoms in tomato, and ToRMV and ToCMoV, both of which induce milder symptoms, could provide an insight into the mechanisms governing symptom induction and host adaptation in begomoviruses. ToYSV was isolated from tomato (Ambrozevicius *et al.*, 2002) and is a distinct species in the genus *Begomovirus* (R. F. Calegario and others, unpublished data). Interestingly, sequence comparisons and phylogenetic analyses based on full-length DNA-A and DNA-B indicated that ToYSV is related more closely to the *Sida*-infecting begomoviruses SiMoV, SimMV and SiYMV than to other Brazilian tomato-infecting viruses such as TGMV, ToRMV or ToCMoV. It

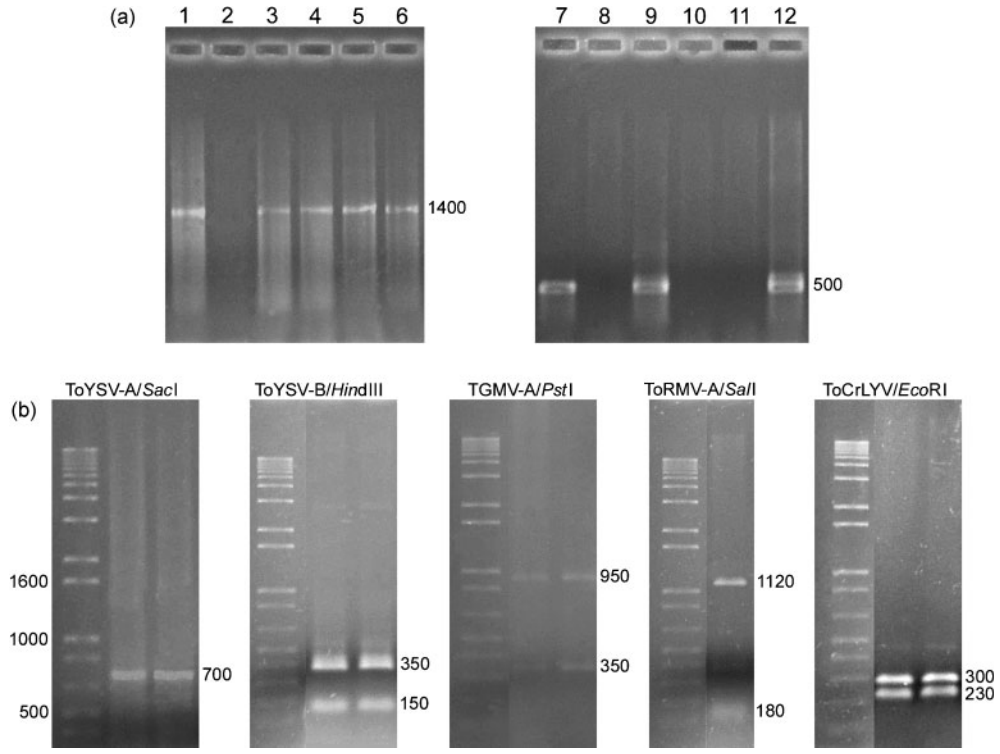

Fig. 3. Detection of viral infection in *Nicotiana benthamiana* plants by PCR-RFLP. Plants were inoculated biolistically with 2 μg of each DNA component, using different combinations of the cloned DNA-A and DNA-B of ToYSV and other tomato- and *Sida*-infecting begomoviruses. Total DNA was extracted at 28 days post-inoculation from non-inoculated leaves. (a) Viral DNA-A (lanes 1–6) and DNA-B (lanes 7–12) were detected by PCR using universal begomovirus primers. Lane pairs 1, 2 and 7, 8 are positive and negative controls, respectively. Lanes 3 and 9, PCR amplification from plant inoculated with the pseudorecombinant ToYSV DNA-A/TCrLYV DNA-B; lanes 4 and 10, PCR amplification from plant inoculated with the pseudorecombinant ToYSV DNA-A/ToCMoV-[Ig1] DNA-B; lanes 5 and 11, PCR amplification from plant inoculated with the pseudorecombinant ToRMV DNA-A/ToYSV DNA-B; lanes 6 and 12, PCR amplification from plant inoculated with the pseudorecombinant TGMV DNA-A/ToYSV DNA-B. (b) PCR products were cleaved with specific restriction enzymes for each viral component, as indicated. Sizes of the expected fragments (in bp) are indicated on the right. Sizes of markers (also in bp) are indicated on the left.

must be pointed out that the three aforementioned *Sida*-infecting viruses have never been detected infecting tomatoes, and that the three tomato viruses have never been detected in *Sida* or any host other than tomato. Here, we report that ToYSV has a possible recombinant origin, with a large part of its CP gene derived from SiMoV.

In several previous scenarios of begomovirus emergence, it was suggested that indigenous viruses infecting weeds and wild hosts had been transferred to the new host, generating novel species after recombination and/or pseudorecombination events (Monci *et al.*, 2002; Padidam *et al.*, 1999; Paplomatas *et al.*, 1994; Pita *et al.*, 2001; Saunders *et al.*, 2002; Unseld *et al.*, 2000; Zhou *et al.*, 1997). Among the Brazilian viruses, a recombinant origin has been indicated for ToCMoV (Galvão *et al.*, 2003), and possible pseudorecombination between ToRMV and uncharacterized viruses was observed under natural conditions based on PCR detection (Zerbini *et al.*, 2002).

The formation of viable pseudorecombinants is one of the easiest and most reliable ways to detect relationships among bipartite begomoviruses (Gilbertson *et al.*, 1993; Pita *et al.*, 2001; Sung & Coutts, 1995). In principle, pseudorecombination involves one viral protein (Rep) and sequences in the CR (e.g. the iterons). However, a number of recent studies have demonstrated that Rep-mediated binding can be more relaxed than thought previously (Garrido-Ramirez *et al.*, 2000; Lin *et al.*, 2003), suggesting a higher degree of complexity in begomovirus origin recognition. A critical factor determining the viability of the pseudorecombinants between ToYSV DNA-B and the DNA-A of the other tomato viruses would be the capacity of the DNA-A-encoded Rep proteins to recognize and mediate replication of both DNA components, because the interaction between DNA-B-encoded proteins and viral DNA is much less specific (Briddon & Markham, 2001; Frischmuth *et al.*, 1993; Rojas *et al.*, 1998). The severity and nature of the disease symptoms would be functions of the levels of DNA-B

replication and the properties of the DNA-B-encoded proteins (Ingham *et al.*, 1995; Petty *et al.*, 2000; Schaffer *et al.*, 1995).

At least two factors are involved directly in pseudorecombinant viability: (i) conservation of iteron sequence (Argüello-Astorga *et al.*, 1994b) and (ii) conservation of Rep protein amino acid sequence, particularly the 3 aa predicted to be involved in pairing with the iteron nucleotides (Argüello-Astorga & Ruiz-Medrano, 2001). The virus-specific recognition domain of the Rep protein has been mapped to its N-terminal region (Choi & Stenger, 1995; Gladfelter *et al.*, 1997) and includes the conserved 'motif 1' of rolling-circle replication-initiator proteins (Ilyina & Koonin, 1992). A detailed analysis of this region revealed an amino acid stretch (8–10 aa) located upstream of motif 1, designated IRD (Fig. 1c), that was proposed to contain the major DNA-binding specific determinants (Argüello-Astorga & Ruiz-Medrano, 2001). This analysis revealed a specific Rep protein–DNA sequence association that allows for the prediction of potentially compatible interactions (i.e. Rep protein binding and viral DNA replication). All begomovirus iterons are composed of an invariable GG sequence followed by 3 nt (named N1, N2 and N3) that vary among species (Fig. 1b). The IRD sequence is conserved among begomoviruses with identical iterons, but varies among species with different iteron sequences, with exception of an invariant Phe residue at the centre of the IRD. Predicted amino acid–nucleotide pairing would occur between iteron nucleotide N1 and the last amino acid of IRD, N2 with the sixth IRD amino acid and N3 with the first or third IRD amino acid, depending on the iteron sequence (Argüello-Astorga & Ruiz-Medrano, 2001). Among these 3 aa, none is conserved between ToYSV and ToRMV, only one is conserved among ToYSV, SiMoV, SiYMV and ToCMoV-[Se1] (an arginine at position 6) and two are conserved among ToYSV and ToCMoV-[Ig1] (lysine and arginine at positions 3 and 6, respectively). These two conserved amino acids may have been sufficient to warrant recognition, albeit inefficient, of ToCMoV-[Ig1] iterons by the Rep protein encoded by ToYSV. This inefficient interaction could allow for a low level of DNA-B replication, thereby providing sufficient expression of the DNA-B-encoded MPs to allow the pseudorecombinant to infect *N. benthamiana* systemically, as suggested by Hou *et al.* (1998) to explain infectivity of certain begomovirus pseudorecombinants in agroinoculated plants. The low DNA-B accumulation is consistent with the lack of symptoms in inoculated plants. It is possible that the maintenance of this pseudorecombinant in the plant might eventually yield intermolecular recombinants with an improved Rep–*ori* interaction, similar to those reported for *Bean dwarf mosaic virus* and *Tomato mottle virus* (Hou & Gilbertson, 1996).

The conservation between the iterons of ToYSV and TCrLYV is consistent with the viability of the pseudorecombinant formed between the components of these viruses. Symptoms induced by the pseudorecombinant are attenuated slightly in comparison to those induced by wild-type ToYSV, but, unfortunately, it is impossible to compare them with those induced by TCrLYV, as DNA-A of this virus has not been cloned. Interestingly, symptoms induced by the ToYSV DNA-A/TCrLYV DNA-B pseudorecombinant are more severe than those induced by a previously described ToRMV DNA-A/TCrLYV DNA-B pseudorecombinant (Andrade *et al.*, 2004), possibly due to the better iteron conservation between ToYSV and TCrLYV compared with ToRMV and TCrLYV.

Conversely, there is iteron divergence between ToYSV and TGMV, which in theory would not allow pseudorecombination between these viruses. Nevertheless, the TGMV DNA-A/ToYSV DNA-B pseudorecombinant was viable and induced symptoms that were attenuated only slightly compared with those induced by the wild-type viruses. This result suggests that the TGMV Rep might be more versatile and recognize a heterologous DNA component with different iterons, and that the gene products encoded by the two heterologous components were capable of interacting efficiently, allowing the development of a systemic infection with severe symptoms. Although they are not identical, the iterons of ToYSV and TGMV differ by only 1 nt, and ToYSV and ToRMV have the highest conservation among the viruses used in the assay in the IRD (five out of eight residues). The asymmetry in this combination (ToYSV DNA-A/TGMV DNA-B was not infectious) supports the hypothesis that the TGMV Rep is more versatile than that of ToYSV. A very similar result was obtained with pseudorecombinants between *Bean golden yellow mosaic virus* (BGYMV) and *Chino del tomate virus* (CdTV), which also have divergent CRs and iterons. A pseudorecombinant between CdTV DNA-A and BGYMV DNA-B was viable in *N. benthamiana* and induced symptoms that were attenuated slightly compared with those induced by the wild-type parents (Garrido-Ramirez *et al.*, 2000). The asymmetry observed between the reciprocal pseudorecombinants led the authors to conclude that the CdTV Rep protein is more versatile and can recognize and replicate a DNA component with a divergent CR.

In summary, our results demonstrate the potential for pseudorecombination among different begomovirus species in tomato in Brazil and reinforce the notion that phylogenetic relationship and iteron conservation are not the sole requirements for pseudorecombinant viability. They also demonstrate the intricate nature of the begomovirus complex infecting tomatoes in Brazil, with a virus that is phylogenetically closer to viruses infecting wild hosts, but nonetheless closer in terms of *trans*-replication to the tomato viruses. Considering the explosive emergence of tomato begomoviruses in the country in recent years and the existence of natural reservoirs in wild/weed species, it is likely that pseudorecombination also takes place in the field, leading to the surfacing of novel begomovirus species better adapted to tomato. Finally, the results also support the view

(Rothenstein *et al.*, 2006) that sequence comparisons and phylogeny of begomoviruses can be misleading if recombination is not taken into account.

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