A naturally occurring recombinant DNA-A of a typical bipartite begomovirus does not require the cognate DNA-B to infect *Nicotiana benthamiana* systemically

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Species of the genus *Begomovirus* (family *Geminiviridae*) found in the western hemisphere typically have a bipartite genome that consists of two 2-6 kb DNA genomic components, DNA-A and DNA-B. We have identified and cloned genomic components of a new tomato-infecting begomovirus from Brazil, for which the name *Tomato crinkle leaf yellows virus* (TCrLYV) is proposed, and a DNA-A variant of *Tomato chlorotic mottle virus* (ToCMV-[MG-Bt1]). Sequence analysis revealed that TCrLYV was most closely related to ToCMV, although it was sufficiently divergent to be considered a distinct virus species. Furthermore, these closely related viruses induce distinguishable symptoms in tomato plants. With respect to ToCMV-[MG-Bt1] DNA-A, evidence is presented that suggests a recombinant origin. It possesses a hybrid genome on which the replication compatible module (AC1 and replication origin) was probably donated by ToCMV-[BA-Se1] and the remaining sequences appear to have originated from *Tomato rugose mosaic virus* (ToRMV). Despite the high degree of sequence conservation with its predecessors, ToCMV-[MG-Bt1] differs significantly in its biological properties. Although ToCMV-[MG-Bt1] DNA-A did not infect tomato plants, it systemically infected *Nicotiana benthamiana*, induced symptoms of mottling and accumulated viral DNA in the apical leaves in the absence of a cognate DNA-B. The modular rearrangement that resulted in ToCMV-[MG-Bt1] DNA-A may have provided this virus with a more aggressive nature. Our results further support the notion that interspecies recombination may play a significant role in geminivirus diversity and their emergence as agriculturally important pathogens.

**INTRODUCTION**

Geminiviruses (family *Geminiviridae*) are a large and diverse group of plant viruses characterized by their unique icosahedral capsids (Lazarowitz, 1992). They have circular, single-stranded DNA genomes that replicate via double-stranded DNA intermediates in the nuclei of infected cells (Hanley-Bowdoin et al., 1999). The members of the *Geminiviridae* can be classified into four genera according to their host-range properties, insect vector and genome organization, which may be in either single or double-component configuration (Rybsicki et al., 2000). The *Begomovirus* genus is the largest genus of this family and comprises the whitefly-transmitted geminiviruses that infect dicotyledonous plants. Begomoviruses found in the western hemisphere typically have bipartite genomes, whereas several monopartite begomoviruses have been identified in the eastern hemisphere, such as *Tomato yellow leaf curl virus* (TYLCV; Kheyr-Pour et al., 1991; Navot et al., 1991) and *Tomato leaf curl virus* (ToLCV; Dry et al., 1993), among others.

The genome of the bipartite begomoviruses is split between two genomic components, designated DNA-A and DNA-B (Lazarowitz, 1992). Both components are organized into divergent transcription units separated by an intergenic region (IR) of about 200 bp, which contains the replication origin and two divergent promoters (Hanley-Bowdoin et al., 1999). DNA-A has the potential to code for five gene products (AV1, AC1, AC2, AC3, AC4) and DNA-B encodes two gene products (BV1 and BC1). Genetic and biochemical studies of viral genes and proteins have provided insights into their function. The AV1 gene product or Rep (replication-associated protein) is a sequence-specific DNA-binding protein essential for replication of both DNA-A and DNA-B genomic components (Elmer et al., 1988;
Fontes et al., 1992, 1994a, b). The AC2 gene product or TrAP is a trans-acting factor needed for the expression of both AV1 (coat protein) and BVI genes (Sunter & Bisaro, 1992). The AC3 protein enhances the accumulation of viral DNA (Sunter et al., 1990) and is also designated Ren (replication enhancer protein). The BVI protein, also named nuclear shuttle protein (NSP), facilitates movement of the virus from the nucleus to the cytoplasm, whereas the BCI protein functions as a classic viral movement protein (MP) as it promotes an increase in the size exclusion limit of the plasmodesmata (Sanderfoot & Lazarowitz, 1996).

Begomoviruses are considered one of the largest and most successful groups of plant viruses that infect a wide range of crops, particularly in tropical and subtropical regions. They are responsible for numerous diseases of economically important crops, such as cassava, cotton, bean, pepper and tomato (Simone et al., 1990; Brown & Bird, 1992; Polston & Anderson, 1997; Moriones & Navas-Castillo, 2000). Recently, diseases caused by begomoviruses have become an even greater threat to Brazilian agriculture (Ambrozevicius et al., 2002; Ribeiro et al., 2002) due to the introduction of a new biotype of the whitefly, Bemisia tabaci, that colonizes tomato plants with high efficiency (Ribeiro et al., 1998) and to the high recombogenic properties of the virus (Padidam et al., 1999). Interspecies recombination events have probably contributed significantly to the diversity of begomoviruses and their emergence as economically important pathogens (Deng et al., 1997; Zhou et al., 1997; Fondong et al., 2000).

In this investigation, we report the identification and characterization of new species and strains of tomato-infecting geminivirus that belong to the genus Begomovirus, provisionally designated Tomato crinkle leaf yellows virus (TCrLYV) and Tomato chlorotic mottle virus, isolate MG-Bt1 (ToCMV-[MG-Bt1]). Remarkably, the DNA-A of ToCMV-[MG-Bt1] retains a high degree of sequence conservation with other species from the western hemisphere but differs significantly in its biological properties. The recombinant nature of this DNA-A variant of ToCMV is discussed.

**METHODS**

**Molecular cloning of viral DNA.** Tomato (*Lycopersicon esculentum*) plants with symptoms typical of begomovirus infection were collected in fields located in Minas Gerais and Rio de Janeiro States. Total DNA was extracted from systemically infected tissues, as described by Gilbertson et al. (1991), digested with various restriction endonucleases and analysed by Southern blot to determine single cutting sites suitable for cloning the full-length genome. Total DNA was then digested with the appropriate enzyme, separated by electrophoresis and virus-specific DNA in the range 2.5–3.0 kb was recovered from the agarose gel and inserted into the corresponding site in pUC118. Full-length copies of the viral genome components were identified by dot blot and sequencing. This strategy allowed the cloning of a DNA-A-like component of a geminivirus detected in tomato plants growing in Minas Gerais State and designated Tomato chlorotic mottle virus, isolate Minas Gerais (ToCMV-[MG-Bt1]) and the DNA-B of a tomato-infecting geminivirus detected in Rio de Janeiro State, designated Tomato crinkle leaf yellows virus (TCrLYV). For cloning ToCMV-[MG-Bt1] DNA-A, a SacI-linearized replicative form dsDNA was inserted into pUC118 to yield pUFV290, also called pToCMV-A. A full-length copy of TCrLYV DNA-B was inserted into the EcoRI site of pUC118, generating pUFV248 (pTCrLYV-B).

The DNA-A of TCrLYV was cloned by a PCR-based method. Fragments of the viral genomes were PCR-amplified using total DNA from infected tomato leaves and a set of begomovirus DNA-A-specific degenerate primers, as previously described (Rojas et al., 1993). The amplified 1–1 kb TCrLYV DNA-A fragment was cloned into pUC118 and sequenced. Sequence analysis of the cloned fragment confirmed that it had originated from a 1 kb DNA and allowed the design of overlapping DNA-A-specific primers that were used to generate full-length Clal clones of the genomic component. Attempts to clone the full-length Clal A component were unsuccessful, using a diversity of PCR-amplification cloning strategies. Nevertheless, cloning of overlapping fragments of TCrLYV DNA-A allowed the sequencing of the entire TCrLYV DNA-A.

**Sequence analysis.** Phylogenetic analysis was done on matrices of aligned sequences using the neighbour-joining and bootstrap (1000 replications) options of DNAMAN 3.0 software. The sources and GenBank accession numbers of geminivirus DNA-A and DNA-B sequences used are provided in the supplementary table (available at http://vir.sgmjournals.org).

**Construction of infectious DNA clones.** Infectivity assays were performed with plasmids containing partial tandem repeats of the genomic components of the viruses under investigation. To construct a partial repeat of ToCMV-[MG-Bt1], the full-length SacI insert of pUFV290 (pToCMV-A) was transferred to the unique SacI site of pUFV310, which contained a 1–1 kb SacI-XhoI fragment of pToCMV-A inserted into the SacI and Sall sites of pUC118. The resulting clone contains 1.45 copies of ToCMV-[MG-Bt1] DNA-A, flanked by identical sequences of the ACI gene and was designated pToCMV-A-1.45 (pUFV398). Tandemly arranged partial repeats of TCrLYV DNA-B were constructed by moving the full-length EcoRI insert of pUFV248 (pTCrLYV-B) into the unique EcoRI site of pUFV267, which contained a 900 bp EcoRI–BamHI fragment of pTCrLYV-B. The resulting clone, pUFV393 (also designated pTCrLYVB-1.34), harbours 1.34 copies of TCrLYV DNA-B and has a duplicated origin of replication.

**Plant inoculation and viral DNA detection.** Nicotiana benthamiana plants were inoculated with plasmids containing partial tandem repeats of viral DNA components by bioinfiltration as previously described (Schaffer et al., 1995). Between 14–21 days post-inoculation, both inoculated and apical leaves were harvested. Total nucleic acid was extracted as described (Fontes et al., 1994a) and viral DNA was detected by a PCR-based assay using DNA-A- and DNA-B-begomovirus specific primers (Rojas et al., 1993) and/or Southern blot analysis using radio-labelled DNA-A or DNA-B probes.

**Replication assays of viral DNA.** Callus cultures were initiated from the pith of tomato plants as previously described (Cascardo et al., 2000; Alvim et al., 2001). Cell culture lines were generated by transferring 2 g of friable calli to 25 ml of medium (MS salts supplemented with 3% (w/v) sucrose, 0.0001% (w/v) thiamin.HCl, 0.01% (w/v) inositol, 0.2 μg 2,4-dichlorophenoxacyclic acid ml⁻¹, 1.32 mM KH₂PO₄). The cell culture was established after four subcultures in liquid medium prior to replication assays. Protoplast preparation, transfection conditions and DNA extraction were according to Fontes et al. (1994a, b). Total DNA was isolated from protoplasts 48 h post-transfection, digested overnight with Sall and Dspl (ToCMV-[MG-Bt1]) or EcoRI and Dspl (TGVM DNA-A, -
TGMV DNA-B, TCrLYV DNA-B), separated on a 1 % (w/v) agarose gel, blotted and hybridized with 32P-labelled DNA-A and DNA-B specific fragments as described (Sambrook et al., 1989).

RESULTS

Nucleotide sequence analysis of ToCMV-[MG-Bt1] and TCrLYV

Begomovirus-infected tomato plants were collected from a field near Betim, MG (ToCMV-[MG-Bt1]) and Campos, RJ, Brazil (TCrLYV) and grafted onto tomato stocks. Leaf crumpling, mottle, and mild leaf distortion symptoms were observed on host plants 4–6 weeks after grafting. Both DNA-A and DNA-B components were detected in all infected plants by PCR analysis with begomovirus-specific primers and the genomic components were cloned and sequenced.

The complete nucleotide sequences of clones pUFV290 (ToCMV-[MG-Bt1] DNA-A), pUFV70+pUFV245+pUFV246 (TCrLYV DNA-A) and pUFV248 (TCrLYV DNA-B) were determined. The genome organization of their viral components was similar to other western hemisphere begomoviruses. The intergenic region separating the divergent transcription units on both components harbours the conserved 30 bp stem–loop structure found in all geminiviruses and the conserved TAATATTAC nonanucleotide sequence (Fig. 1) that contains the nicking site for initiation of virion-sense DNA replication (Laufs et al., 1995; Stanley, 1995). The DNA-A of the viruses encodes one virion-sense (AV1) and

Table 1. ORFs of the tomato-infecting begomoviruses

<table>
<thead>
<tr>
<th>ORF</th>
<th>Sequence coordinates (nt)*</th>
<th>Amino acid residues</th>
<th>Deduced mol. mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCrLYV</td>
<td>ToCMV-[MG-Bt1]</td>
<td>TCrLYV</td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td>AV1</td>
<td>189–944</td>
<td>198–953</td>
<td>251</td>
</tr>
<tr>
<td>AC1</td>
<td>1416–2474</td>
<td>1426–2484</td>
<td>352</td>
</tr>
<tr>
<td>AC2</td>
<td>1085–1474</td>
<td>1095–1484</td>
<td>129</td>
</tr>
<tr>
<td>AC3</td>
<td>787–1338</td>
<td>950–1348</td>
<td>132</td>
</tr>
<tr>
<td>AC4</td>
<td>2024–2323</td>
<td>2034–2327</td>
<td>99</td>
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<tr>
<td>AC5</td>
<td>96–848</td>
<td>–</td>
<td>250</td>
</tr>
<tr>
<td>BV1</td>
<td>455–1222</td>
<td>ND</td>
<td>255</td>
</tr>
<tr>
<td>BC1</td>
<td>1314–2198</td>
<td>ND</td>
<td>297</td>
</tr>
</tbody>
</table>

*Numbering scheme according to current convention (base 1 = underlined A of the conserved TAATATT1AC motif). ND, Not determined.
four complementary-sense (AC1, AC2, AC3, AC4) ORFs and TCrLYV DNA-B encodes one virion-sense (BV1) and one complementary-sense (BC1) ORF (Table 1).

ToCMV-[MG-Bt1] DNA-A encodes an additional ORF, designated AC5, with the potential to encode a protein of 250 amino acid residues.

Among begomoviruses, the TCrLYV nucleotide sequence is most closely related to Tomato chlorotic mottle virus, isolate BA-Se1, (ToCMV-[BA-Se1]; DNA-A, 84 % identity and DNA B, 77 % identity), although it is sufficiently dissimilar to be considered a distinct virus. The common region of TCrLYV is highly conserved between its components with 95 % sequence identity over 140 bp upstream of the stem–loop (Fig. 1). This region contains functional domains for AC1 specific origin recognition that have been shown to confer species-specific replication competence (Lazarowitz et al., 1992; Fontes et al., 1994b). Both components contain the duplicated iteron motif GGAG, which is predicted to be the primary high-affinity AC1 binding motif (Fontes et al., 1994a; Chatterji et al., 2000).

The ToCMV-[MG-Bt1] DNA-A is most closely related to ToCMV-[BA-Se1] (92 % sequence identity) and may be considered a strain of this virus. In addition to exhibiting a high degree of sequence conservation, the two share near-identical intergenic regions (94 % identity) and identical putative AC1 binding motifs (TGTGAATGGTG) upstream of the TATA box (Fig. 1). Based on sequence conservation of their Rep (AC1) proteins (93 % identity at the amino acid level) and origin of replication, one may predict efficient AC1-mediated trans-replication of the heterologous DNA-B genomic component from these closely related isolates.

**Phylogenetic analysis and recombination events among recently identified tomato-infecting begomoviruses from Brazil**

Phylogenetic analyses based on nucleotide sequence conservation of the Geminiviridae family have demonstrated that members of the Begomovirus genus form clusters according to their geographical origin with distinct branches for viruses from the Americas, Asia and Africa (Padidam et al., 1999). The inclusion of recently characterized begomoviruses from Brazil in sequence comparison analyses reinforces a geographical origin-based relatedness, as they are clustered together under sub-branches of the viruses from the Americas (Fig. 2). A similar relationship holds...
when the deduced amino acid sequences of AV1, AC1, AC2 and AC3 served as the basis for comparison (data not shown). In contrast, based on AC4 phylogeny, ToCMV-[MG-Bt1] segregates more closely with clusters of the monopartite begomoviruses TYLCV and ToLCV from Africa and Asia (Fig. 3). This observation may be relevant as the C4 ORF from the monopartite begomoviruses and the AC4 ORF of bipartite begomoviruses appear not to be functional analogues (Jupin et al., 1994; Pooma & Petty, 1996; Rigden et al., 1994).

We have also observed that the relative positions of Brazilian tomato-infecting begomoviruses vary when different parts of the viral genome are used as the basis for phylogenetic analysis (data not shown). The differential clustering of parts of sequences from the same virus has been considered as indicative of intermolecular recombination among viral genomes. In view of this observation, we searched for recombination events on the DNA-A genomic component of recently identified tomato-infecting geminiviruses from Brazil, using the Recombination Detection Program (Martin & Rybicki, 2000), available at http://www.uct.ac.za/depts/microbiology/microdescription.htm with the same criteria as adopted by Martin et al. (2001). The recombination events detected are presented in Fig. 4(A).

We found two groups of recombinant sequences particularly interesting because they might be related to selection pressure towards enhanced fitness. The first one refers to a recombination event in which part of the AC1 coding region of Tomato rugose mosaic virus (ToRMV-[Ub1]) and Tomato rugose severe virus (ToRSV) was replaced with the equivalent region of Tomato golden mosaic virus (TGMV). The recombinant region corresponds to sequences that encode amino acid positions 34–163 of ToRMV-[Ub1] AC1 and 46–174 of ToSRV AC1 and encompasses the DNA-binding domain of the TGMV AC1 protein (Gladfelter et al., 1997). These recombinants might have been selected due to a more perfect fit between the AC1 DNA-binding domain and AC1 binding motifs in the origin of replication. A comparison of the minimal origin of replication of TGMV (Lazarowitz et al., 1992; Fontes et al., 1994a, b) with the corresponding region of ToRMV-[Ub1] and ToSRV revealed that they share 95 % and 97 % sequence identity, respectively (Fig. 1). Remarkably, both ToRMV-[Ub1] and ToSRV contain the duplicated iteron motif GGATGTAATGGATG, which differs by just a one nucleotide insertion from the TGMV AC1 high-affinity DNA-binding site GGATGTAAGGATG (Fontes et al., 1994a). Although TGMV and the closely related viruses ToRMV-[Ub1] and ToSRV are quite divergent in overall sequence and induce different symptoms in their hosts (F. M. Zerbini, unpublished), the recombinant nature of these viruses may render their A component competent for trans-replication of the heterologous B component. If this assumption holds true, the trans-replication properties of naturally occurring recombinants will further complicate the current taxonomic criteria for distinction of species and strains of the family Geminiviridae.

A second recombination event of particular interest might have occurred between ToRMV-[Ub1] and ToCMV-[BA-Se1], resulting in ToCMV-[MG-Bt1], which retains ToCMV-[BA-Se1]-like sequences corresponding to the AC1 coding region and intergenic region. This
A statistically significant recombination event might have enabled the recombinant ToCMV-[MG-Bt1] DNA-A to recruit the DNA-B of ToCMV-[BA-Se1] and therefore, it may be considered a DNA-A variant of the bipartite ToCMV species. In fact, sequencing of a 300 bp DNA-B fragment, which was PCR-amplified from the DNA samples used to clone ToCMV-[MG-Bt1] DNA-A, revealed that it was identical to the corresponding region of ToCMV-[BA-Se1] DNA-B (not shown). The apparent recombinant nature of ToCMV-[MG-Bt1] was further confirmed by using its

**Fig. 4.** (A) Schematic representation of the recombinant regions of Brazilian tomato-infecting begomoviruses. Potential recombination break points and the probability (P) that the indicated regions do not have a recombinant origin (Martin & Rybicki, 2000) are presented on the linear map of the DNA-A components. The shading patterns indicate the viral origin of the recombinant region. The solid arrows define the position, orientation and extension of the ORFs, as indicated. IR indicates the intergenic region. ToCMV-[BA-Se1], Tomato chlorotic mottle virus, isolate BA-Se1; ToCMV-[MG-Bt1], Tomato chlorotic mottle virus, isolate MG-Bt1; ToRMV-[Ub1], Tomato rugose mosaic virus, isolate Ub1; ToSRV, Tomato severe rugose virus; BDMV, Bean dwarf mosaic virus; TGMV, Tomato golden mosaic virus; SiGMV-Flo, Sida golden mosaic virus-Florida. (B) Phylogenetic tree obtained from aligned sequences of Brazilian begomoviruses and the ToRMV-[Ub1]-like genome fragment of ToCMV-[MG-Bt1]. The dendrogram was calculated using the neighbour-joining method. Numbers at nodes indicate the percentage bootstrap scores (1000 replications).
proposed recombined genome segments, such as the ToRMV-[Ub1]-like sequence (positions 100–1601 on the ToCMV-[MG-Bt1] genome), as the basis for phylogenetic analysis (Fig. 4B). This caused a variation in the relative position of ToCMV-[MG-Bt1] shown in Fig. 2, as it segregated more closely with ToRMV-[Ub1].

Table 2. Infectivity of ToCMV-[MG-Bt1] DNA-A and pseudorecombinants of ToCMV-[MG-Bt1], TCrLV and TGMV-[MG] in N. benthamiana

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Plants infected/inoculated (no. of experiments)</th>
<th>Symptoms</th>
<th>Detection of viral DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inoculated leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DNA-A</td>
</tr>
<tr>
<td>ToCMV-[MG-Bt1]-A</td>
<td>5/5 (2); 4/5 (1)</td>
<td>Systemic</td>
<td>+</td>
</tr>
<tr>
<td>TGMV-A</td>
<td>0/5 (3)</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>TGMV-B</td>
<td>0/5 (3)</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>TCrLYV-B</td>
<td>0/5 (3)</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>ToCMV-[MG-Bt1]-A + TCrLYV-B</td>
<td>5/5 (3)</td>
<td>Systemic</td>
<td>+</td>
</tr>
<tr>
<td>ToCMV-[MG-Bt1]-A + TGMV-B</td>
<td>3/5 (2); 5/5 (1)</td>
<td>Systemic</td>
<td>+</td>
</tr>
<tr>
<td>TGMV-A + TGMV-B</td>
<td>5/5 (3)</td>
<td>Systemic</td>
<td>+</td>
</tr>
<tr>
<td>TGMV-A + TCrLYV-B</td>
<td>2/5 (2); 3/5 (2)</td>
<td>Localized-A†</td>
<td>+</td>
</tr>
</tbody>
</table>

*PCR-based diagnosis.
†Attenuated.
ND, Not determined.

**Fig. 5.** Viral DNA detection in inoculated plants. Total DNA was isolated from greenhouse-grown plants 2–3 weeks after inoculation with tandemly repeated viral DNA and used as template in PCR assays with (A) DNA-A- and (B) DNA-B-specific primers. IN refers to DNA isolated from inoculated leaves, whereas UP indicates upper leaves. M corresponds to DNA standard markers whose sizes are shown on the left in kb. (A) PCR analysis of viral DNA accumulation in plants inoculated with plasmids containing partial tandem copies of either an A-component or a B-component, as indicated. Plants were inoculated by the biolistic method and viral DNA accumulation was analysed by PCR. Lanes 1, 2, 3 and 4 represent control reactions using plasmid DNAs pTGMV-A 1.3 (lane 1), pTGMV-B 1.45 (lane 2), pToCMV-A 1.45 (lane 3) and pTCrLYV-B 1.34 (lane 4) as DNA template in PCR assays with the corresponding primers. Control indicates the result of PCR reactions performed with DNA from mock-inoculated plants. (B) Pseudorecombination assays. Plants were inoculated with a combination of viral DNA-A and DNA-B, as indicated, by the biolistic method, and viral DNA accumulation was analysed by PCR 3–4 weeks post-inoculation.
Infectivity of ToCMV-[MG-Bt1] DNA-A in N. benthamiana

The biolistic method was used to inoculate N. benthamiana and tomato plants with ToCMV-[MG-Bt1] DNA-A or TCrLYV DNA-B alone. A mock-inoculated control was included in each experiment. DNA-A components of ToCMV-[MG-Bt1] and TCrLYV exhibited similar genomic organization (Table 1) and were closely related to bipartite begomoviruses (Fig. 2) that require both components for systemic infection. In fact, N. benthamiana inoculated with TCrLYV DNA-B alone was not systemically infected and did not accumulate viral DNA (Table 2; Fig. 5A, lanes 13–16). Likewise, N. benthamiana inoculated with either infectious cloned TGMV DNA-A or TGMV DNA-B alone did not develop a systemic infection and viral DNA was not detected in upper leaves (Table 2; Fig. 5A, lanes 9–12). In contrast, inoculation of ToCMV-[MG-Bt1] DNA-A alone induced a systemic infection in N. benthamiana. The results of these analyses for three independent experiments are presented in Table 2. ToCMV-[MG-Bt1] DNA-A induced stunting, leaf crumpling and mottling in 90% of inoculated plants on average. In all ToCMV-[MG-Bt1] DNA-A-inoculated plants DNA-A accumulation was detected in inoculated (Fig. 5A, lane 5) and newly emerging leaves (lane 7). The absence of a contaminating DNA-B in inoculated plants was confirmed by the failure to detect viral DNA-B in infected tissues by PCR analysis (Fig. 5A, lanes 6 and 8) and Southern blotting (Fig. 6B, lane 4). Taken together, these results indicate that ToCMV-[MG-Bt1] DNA-A is able to systemically infect N. benthamiana in the absence of a cognate DNA-B. This result was also obtained when plasmid DNA-A was delivered by mechanical inoculation, albeit to a much lesser extent (2/24 inoculated plants), demonstrating that ToCMV-[MG-Bt1] DNA-A infection was not an artifact of the biolistic method of inoculation. Inclusion of a cognate DNA-B in the inoculation assays (Fig. 6, lane 5) caused a slight increase in ToCMV dsDNA-A accumulation (Fig. 6A, compare lanes 4 and 5), although it did not alter the symptomatology of ToCMV DNA-A infection. In contrast, ToCMV-[MG-Bt1] DNA-A was not infectious in tomato, as judged by the lack of symptom development and inability to detect viral DNA in emerging leaves (not shown).

Trans-replication in tomato protoplasts and pseudorecombination in N. benthamiana

We have not succeeded in recovering a full-length infectious TCrLYV DNA-A clone, such that the biological properties of the bipartite TCrLYV could not be examined. Nevertheless, based on sequence conservation of their putative minimal origin of replication (90% identity) and AC1 protein (85% similarity), we investigated the capacity of ToCMV-[MG-Bt1] DNA-A to trans-replicate the heterologous TCrLYV DNA-B component in tomato protoplasts. Infectious clones of TGMV DNA-A and DNA-B were also included in the assay as the most divergent replication module (origin of replication and AC1) among them. These investigations were also extended to pseudorecombination studies in plants inoculated with exchanged heterologous genomic components. For trans-replication assays, tomato protoplasts were transfected with the following viral DNA combinations: ToCMV-[MG-Bt1] DNA-A+TGMV DNA-B; ToCMV-[MG-Bt1] DNA-A+TCrLYV DNA-B; TGMV DNA-A+TGMV DNA-B; TGMV DNA-A+TCrLYV DNA-B. For pseudorecombination experiments, the same viral DNA combinations were introduced into N. benthamiana and tomato plants by biolistic delivery.

Fig. 6. DNA gel blot analysis of ToCMV DNA-A accumulation in infected plants. Total DNA was isolated from greenhouse-grown plants 2 weeks after inoculation with tandemly repeated viral DNA (ToCMV-A=TA or ToCMV-A+ToCMV-B=TA+TB), digested with SacI (TA, lane 4) or SacI/BamHI (TA+TB, lane 5), separated by electrophoresis, transferred to nylon membranes and hybridized with 32P-labelled probes specific for viral DNA-A or DNA-B as indicated on the right. Lanes 1 (CA) and 2 (CB) correspond to pToCMVA-1.45 and pToCMVB-1.4 tandemly repeated control DNAs. Lane 3 (Mo) corresponds to DNA from mock-inoculated plants. Migration positions of molecular mass standards are indicated on the left in kb.
amino acid level; Ori, 85% nucleotide sequence identity) than to that of TGMV (AC1, 71% identity; Ori, 34% identity). Consistent with the replication data, both DNA components were detected in inoculated and upper leaves of ToCMV-[MG-Bt1] DNA-A+TCrLYV DNA-B-infected plants (Fig. 5B, lanes 5–8). The accumulation of an apparently replication compatible DNA-B form in inoculated leaves was below the Southern blotting detection level (not shown), because the accumulation of DNA-B in inoculated leaves was below the Southern blotting detection level (not shown), the presence or absence of a released unit-length newly synthesized DNA B could not be determined with certainty.

### DISCUSSION

A current consensus prediction for the extent of begomovirus diversity holds that a high frequency of recombination resulted in the recent emergence of highly pathogenic virus genotypes causing a variety of serious begomovirus diseases (Zhou et al., 1997; Padidam et al., 1999). We have provided further evidence for the importance of interspecies recombination in begomovirus evolution and emergence as agriculturally relevant pathogens. Using the Recombination Detection Program software developed by Martin & Rybicki (2000), we were able to detect statistically significant recombination events among full-length DNA-A component sequences of recently identified tomato-infecting begomoviruses from Brazil. The recombinant progenies exhibited different biological and enhanced pathological properties as compared to their probable predecessors. A ToCMV DNA-A variant of recombinant origin was able to produce severe systemic symptoms in N. benthamiana in the absence of its cognate DNA-B and TGMV-derived recombinant progeny appeared to be more adapted to tomato plants, as they became dominant in that host over the last decade (Ribeiro et al., 1998, 2002).

The recent characterization of a new bipartite begomovirus from the eastern hemisphere, named Sri Lankan cassava mosaic virus (SLCMV), demonstrated that its DNA-A exhibits features of a monopartite geminivirus (Saunders et al., 1997; Padidam et al., 1999). We have provided further evidence for the importance of interspecies recombination in begomovirus evolution and emergence as agriculturally relevant pathogens. Using the Recombination Detection Program software developed by Martin & Rybicki (2000), we were able to detect statistically significant recombination events among full-length DNA-A component sequences of recently identified tomato-infecting begomoviruses from Brazil. The recombinant progenies exhibited different biological and enhanced pathological properties as compared to their probable predecessors. A ToCMV DNA-A variant of recombinant origin was able to produce severe systemic symptoms in N. benthamiana in the absence of its cognate DNA-B and TGMV-derived recombinant progeny appeared to be more adapted to tomato plants, as they became dominant in that host over the last decade (Ribeiro et al., 1998, 2002).

In tomato protoplasts TGMV DNA-A did not support replication of TCrLYV DNA-B (Fig. 7, lane 10). However, while TGMV DNA-A alone did not induce symptoms in N. benthamiana, TGMV DNA-A+TCrLYV DNA-B-inoculated plants developed mild localized symptoms and accumulated low levels of DNA-B in inoculated leaves (Fig. 5B, lane 14, and data not shown). Sequencing of the DNA-B intergenic region that was PCR-amplified from inoculated leaf DNA extracts confirmed that the DNA-B progeny retained its original sequence and eliminated the possibility that a recombination event had occurred to drive AC1-mediated replication of the heterologous DNA-B in N. benthamiana. Nevertheless, our results did not rule out the possibility that a subliminal AC1-supported trans-replication of the heterologous B component in N. benthamiana could account for the low accumulation of DNA-B, which was accompanied by symptom development in inoculated leaves. Because the accumulation of DNA-B in inoculated leaves was below the Southern blotting detection level (not shown), the presence or absence of a released unit-length newly synthesized DNA B could not be determined with certainty.

<table>
<thead>
<tr>
<th>DNA A</th>
<th>DNA B</th>
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<tbody>
<tr>
<td>ToCMV-A</td>
<td>+ - - - + + - -</td>
</tr>
<tr>
<td>TCrLYV-B</td>
<td>- + - - + - - +</td>
</tr>
<tr>
<td>TGMV-A</td>
<td>- - + + - + + +</td>
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<td>TGMV-B</td>
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![Fig. 7. ToCMV DNA-A-mediated replication of TCrLYV DNA-B in tomato protoplasts. Plasmids containing partial repeats of ToCMV-[MG-Bt1] DNA-A, TCrLYV DNA-B, TGMV DNA-A or TGMV DNA-B were electroporated into tomato protoplasts in the combinations indicated at the top. Total DNA was isolated 2 days post-transfection, digested with DpnI and SacI (lane 3), DpnI and EcoRI (lanes 4, 5, 6, 9 and 10) or DpnI, SacI and EcoRI (lanes 7 and 8). The digested DNA was separated by electrophoresis, transferred to nylon membranes and hybridized with 32P-labelled probes specific for viral DNA-A or DNA-B as indicated on the left. Lanes 1 and 2 correspond to pTGMV-A 1.3 and pTGMV-B 1.45 control DNAs.](http://vir.sgmjournals.org)
ToCMV-[MG-Bt1] and its WmCSV counterpart are genetically distinct, since they do not retain significant sequence conservation (32% identity at amino acid level). Genetic analysis of the AC5 ORF from ToCMV-[MG-Bt1] DNA-A will allow us to address this possibility.

An alternative explanation for the capacity of ToCMV-[MG-Bt1] DNA-A to move systemically and develop symptoms in *N. benthamiana* may be related to its AC4 protein, which is most closely related to the C4-deduced protein from eastern hemisphere monopartite begomoviruses (Fig. 3). While the function of the AC4 ORF from bipartite begomoviruses remains elusive (Pooma & Petty, 1996), the C4 gene product from monopartite begomoviruses, such as TLCV and TYLCV, has been proven to be an important determinant of symptom severity and to affect the systemic movement of the virus (Jupin et al., 1994; Rigden et al., 1994). The sequence relationship between the AC4 ORF of ToCMV-[MG-Bt1] and its counterpart of monopartite begomoviruses may imply similar functions.

ToCMV-[MG-Bt1] DNA-A possesses a hybrid genome on which the replication compatible module (AC1 and origin of replication sequences) was probably donated by ToCMV-[BA-Se1] and the remaining sequences appear to have originated from ToRMV-[UB1] (Fig. 4). This modular organization of ToCMV-[MG-Bt1] DNA-A may accommodate the argument that the recombinant regions can act as composite interacting modules to confer distinct biological properties to the virus progeny. In this case, one candidate for participating with AC4 in the architecture of functional complexes involved in replacing DNA-B-encoded movement functions would be the coat protein. In fact, the coat protein from monopartite begomoviruses participates actively in the systemic movement of the virus (Ridgen et al., 1993; Wartig et al., 1997). Likewise, in non-adapted hosts, bipartite begomovirus-mediated systemic infection requires a functional coat protein (Pooma et al., 1996). Although we do not yet know the effective contribution, if any, of interacting recombinant modules to the pathological properties of ToCMV-[MG-Bt1] DNA-A, such a model remains an attractive hypothesis that may explain the unique mechanism of ToCMV-[MG-Bt1] DNA-A-mediated disease in a permissive host. Genetic analysis of ToCMV-[MG-Bt1] recombinant DNA-A is currently in progress to investigate whether the predicted AC4 ORF performs similar function to the closely related C4 ORF from monopartite begomoviruses and to determine the contribution of the coat protein and/or AC5 ORF to virus pathogenesis.

We have also characterized a distinct tomato-infecting geminivirus, TCrLYV, that may be considered a new species of the *Begomovirus* genus, on the basis of DNA-A sequence identity (up to 84% identity with ToCMV-[BA-Se1] DNA-A) and difference in phenotype when compared to the most closely related virus, ToCMV-[BA-Se1]. Nevertheless, TCrLYV DNA-B was efficiently *trans*-replicated by ToCMV DNA-A, isolate MG, indicating that TCrLYV does not fulfill all requirements for its classification as distinct virus, according to the current criteria that dictate geminivirus taxonomy. In fact, DNA-A-mediated *trans*-replication of heterologous DNA-B components is generally limited to isolate/stains of a particular virus. Several exceptions in the literature indicate, however, that this criterion can not be considered as absolute for the taxonomic classification of distinct species of *Begomovirus* (Gilbertson et al., 1993; Frischmuth et al., 1997).

Replication assays in tomato protoplasts indicated that compatibility between heterologous DNA-A and DNA-B was correlated with sequence conservation of the replication module (AC1 and replication origin) of the viruses under investigation, as would be predicted from previous studies of AC1 specificity for replication origin recognition (Fontes et al., 1992, 1994a, b; Lazarovitz et al., 1992; Jupin et al., 1995; Chatterji et al., 2000). In fact, TCrLYV DNA-B was *trans*-replicated by ToCMV-[MG-Bt1] DNA-A, but not by TGMV DNA-A in tomato protoplasts. Nevertheless, inoculation of *N. benthamiana* plants with TGMV DNA-A + TCrLYV DNA-B induced mild localized symptoms. We consider it unlikely that intermolecular recombination had occurred to drive AC1-mediated replication of the heterologous DNA-B, because sequencing of PCR-amplified fragments from total DNA of symptomatic leaves indicated that the DNA-B progeny kept its original replication origin sequence. In contrast to tomato protoplasts, the permissive nature of the *N. benthamiana* host may allow high levels of viral gene expression, which may compensate for a defective interaction between AC1 and origin of replication to support low levels of DNA-B replication. In fact, replication assays of a TGMV-derived replicon in tobacco protoplasts have demonstrated that AC1 is able to overcome a mutation in its high-affinity binding site if AC3 is provided in *trans* (Fontes et al., 1994b). Therefore, the sequence-specific requirement for AC1-mediated *trans*-replication may vary to different extents depending on the efficiency of viral gene expression and host–virus interactions.

In summary, we have identified and characterized two distinct tomato-infecting geminiviruses and presented evidence that a DNA-A variant of ToCMV exhibits unique pathological properties, despite its similar genomic organization to the DNA-A of western hemisphere bipartite geminiviruses. Our results provided further support for the significant role of recombination in begomovirus evolution, diversity and emergence as economically important pathogens. Until recently, TGMV was the only geminivirus known to be associated with tomato plants grown in Brazilian territory and was never considered a serious threat to the Brazilian agriculture. However, geminivirus-associated epidemics are currently threatening tomato production in Brazil, with the proliferation of new virus species. The Brazilian tomato-infecting geminiviruses described here and by others carry recombinant regions on their genome that are likely to be responsible for the more aggressive nature and enhanced fitness of the recombinant progenies.
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