Molecular Characterization of a New Sap-Transmissible Bipartite Genome Geminivirus Infecting Tomatoes in Mexico

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ABSTRACT

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A bipartite genome geminivirus infecting tomatoes in northwestern Mexico was sap transmitted to tomato, *Nicotiana benthamiana,* and common bean and induced leaf crumpling, epinasty, and mottling. Geminivirus DNA-A and DNA-B components were each cloned from infected *N. benthamiana* and bean leaves. The *N. benthamiana* DNA-A and DNA-B components were infectious but induced only mild symptoms in *N. benthamiana* plants. The reduction in symptom severity was not the result of impaired replication of either component but was associated with the DNA-B component. In contrast, the bean DNA-A and DNA-B components were highly infectious and induced disease symptoms in *N. benthamiana,* bean, and tomato plants indistinguishable from those induced by the sap-transmissible geminivirus. Molecular characterization of the *N. benthamiana* and bean DNA components indicated that the DNA-A components were identical, whereas the DNA-B components were different but closely related. The original tomato sample was shown to be infected with both DNA-B components by polymerase chain reaction analysis. The bean DNA-A and DNA-B components comprise the genome of a new sap-transmissible bipartite genome geminivirus. This geminivirus is different from previously characterized geminiviruses, and the name tomato leaf crumple (TLCrV) is proposed. TLCrV is a member of the Abutilon mosaic cluster of Western Hemisphere bipartite genome geminiviruses and is most closely related to tomato mottle, Abutilon mosaic, and bean dwarf mosaic geminiviruses.

Additional keywords: *Bemisia tabaci, Lycopersicon esculentum,* pseudorecombination, viral evolution, whitefly-transmitted geminiviruses.

Whitefly-transmitted geminiviruses cause economically important diseases such as bean golden mosaic, tomato yellow leaf curl, and African cassava mosaic (2). Recent increases in whitefly (*Bemisia tabaci* Gennadius) populations throughout tropical and subtropical regions of the Americas and the Caribbean Basin have resulted in serious crop losses caused by whitefly-transmitted geminiviruses (3,4). One of the most striking examples has been in tomatoes in Central America (e.g., Guatemala, Honduras, and Costa Rica), the Caribbean Basin (e.g., the Dominican Republic), and the southern United States (e.g., Florida [38]). In Mexico, whitefly-transmitted geminiviruses have been implicated in several tomato and pepper diseases, such as chino del tomate (6), serrano golden mosaic (8), pepper tigré (5,7), and rizado amarillo (15). Because of the increasing economic importance of these diseases and the lack of natural resistance in tomato and pepper to geminivirus infection, it is important to determine how many viruses are involved and their geographic distribution. Disease symptoms alone are not suitable for identification or differentiation of geminiviruses in the field because they vary as a result of factors such as time of infection (plant age), host plant cultivar, environmental conditions (e.g., daylight and nutrition), and mixed viral infections. Thus, to establish the role of a geminivirus in causing a disease, it is necessary to clone the viral DNA, introduce it into plants, and compare the resulting symptoms with those in the plants from which the viral DNA was cloned. This approach has been used to establish unequivocally that geminiviruses cause diseases such as bean golden mosaic (17), bean dwarf mosaic (17), squash leaf curl (28), and tomato yellow leaf curl (30).

Here we describe the cloning, molecular characterization, and infectivity of the DNA-A and DNA-B components of a newly described sap-transmissible bipartite genome geminivirus infecting tomatoes in the Culiacan Valley of northwestern Mexico (33). We demonstrate that this virus can infect tomato, *Nicotiana benthamiana,* and common bean and propose that it be named tomato leaf crumple geminivirus (TLCrV).

MATERIALS AND METHODS

Virus isolate and sap transmission. Diseased tomato leaves were collected in the Culiacan Valley (Sinaloa state) of Mexico during the fall of 1990. Sap transmission to *N. benthamiana,* common bean (*Phaseolus vulgaris* 'Topcrop'), tomato (*Lycopersicon esculentum* 'Peto VF 6203,' 'Florida MH-1,' 'Empire,' or line Geneva 80), and pepper (*Capsicum annuum* 'Yolo Wonder') plants was carried out as previously described (18,19).

Molecular biology techniques. Restriction enzyme digests, Southern blot hybridization analysis, labeling of DNA with [α-^{32}P]dATP by nick translation, ligation reactions, transformation of *Escherichia coli,* and other molecular biology techniques were done according to standard procedures (29). DNA sequencing was done by the dideoxynucleotide chain termination method with Sequenase (USB, Cleveland, OH) according to manufacturer's instructions. DNA sequences were assembled and analyzed with the software of the Genetics Computer Group, University of Wisconsin, Madison (11). Relationships among geminivirus sequences were determined with the GAP program

with a gap weight of 5 and a gap length weight of 0.3. Phylogenetic analyses were carried out with the computer program Phylogenetic Analysis Using Parsimony (PAUP), version 3.1, developed by D. L. Swofford and distributed by the Illinois Natural History Survey, Champaign. Optimum trees, branch strengths, and geminivirus clusters were determined as previously described (12).

Detection and cloning of geminiviral DNA. Geminivirus nucleic acids were detected in infected plant tissues by squash blot hybridization with general or specific DNA probes for whitefly-transmitted geminiviruses (18). Geminivirus nucleic acids were extracted from infected plant tissues and purified from agarose gels with glass matrix (Bio 101, LaJolla, CA) as previously described (17). To identify unique restriction enzyme sites in geminivirus replicative form (RF) DNA, viral DNA was digested with various restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes, and analyzed by Southern blot hybridization with a mixture of full-length cloned DNA-A or DNA-B components of bean golden mosaic geminivirus (BGMV) isolates from Brazil (BGMV-BZ) (16) or Guatemala (BGMV-GA) (17), bean dwarf mosaic geminivirus (BDMV) (24), and tomato mottle geminivirus (ToMoV) (19). Putative full-length DNA-A and DNA-B components were cloned by ligating digested RF DNA into digested pSP72 (Promega, Madison, WI) or pBluescript II KS+ (Stratagene, LaJolla, CA) plasmids. Recombinant plasmids were analyzed by restriction enzyme digestion, Southern blot hybridization, and DNA sequencing.

Production of plasmids having 1.5 copies of geminivirus DNA components. Recombinant plasmids containing full-length DNA-A or DNA-B clones were double digested with restriction endonucleases to release a fragment that included approximately 1.0–2.0 kb of the DNA component and the cloning site of the DNA component. This fragment was recloned into pSP72 or pKS+ double digested with the same enzymes, resulting in a recombinant plasmid that contained an approximately 0.5 copy of the DNA component (0.5-mer) and the cloning site of the full-length DNA component. The 0.5-mer was linearized at the cloning site with the appropriate restriction endonuclease and then ligated with the excised full-length DNA component to produce a recombinant plasmid containing approximately 1.5 copies of the DNA component (1.5-mer). Recombinant plasmids were analyzed by restriction enzyme digestion to determine the orientation of the two fragments in the 1.5-mer.

DNA extraction and PCR. DNA extraction for the polymerase chain reaction (PCR) and PCR conditions used to amplify geminivirus fragments were carried out as previously described (21,37). To amplify a DNA-A fragment, primers PAL1v1978 and PAR1c496 were used; to amplify a DNA-B fragment, primers PBL1v2040 and PCRc1 were used (37).

Inoculation of plants with cloned viral DNAs. Full-length excised DNA-A and DNA-B components (monomers) or undigested plasmids (1.5-mers) were used as inocula. For rub inoculation of *N. benthamiana,* approximately 5–10 μg of each DNA component or recombinant plasmid was coinoculated onto Celite-dusted leaves of plants at the five- to seven-leaf stage.

For inoculation of plants by the biolistic procedure, monomers or undigested 1.5-mers were coated onto gold particles (Aldrich Chemical Co., Milwaukee, WI) that had been washed with ethanol and resuspended in sterile distilled water. DNA was coated onto gold particles by sequentially adding 5 μl each of DNA solutions containing 1 μg/μl of each component, 50 μl of gold particles, 50 μl of 2.5 M $CaCl_2$, and 20 μl of 0.1 M spermidine. The suspension was vortexed for 3 min, and the gold particles were recovered by centrifugation. The gold particles were washed with 250 μl of absolute ethanol, recovered by centrifugation, and resuspended in 60 μl of absolute ethanol. Five or 10 μl of this suspension was spread onto a macrocarrier membrane for each particle bombardment. Gold particles were accelerated with helium pressure (approximately 1,500–1,600 psi) in a PDS-1000 particle acceleration device (Du Pont, Wilmington, DE). Bean and tomato radicles and tomato and pepper plants at the two- to three-true-leaf stage were inoculated. Inoculated plants were maintained in a growth chamber at 23–28 C with 16 h of light.

RESULTS

Sap transmission. Tomato leaves collected in the Culiacan Valley, Mexico, during 1990 had crumpling, distortion, and yellow mottle symptoms, and geminivirus nucleic acids were detected in these leaves by squash blot hybridization analysis with a general geminivirus DNA probe (18). *N. benthamiana* plants rub inoculated with sap prepared from these tomato leaves developed severe leaf distortion, epinasty, and mottling symptoms, and geminivirus nucleic acids were detected in these plants. These results established the presence of a bipartite geminivirus in the original tomato sample and its sap transmission to *N. benthamiana* plants. The geminivirus was readily sap transmitted from infected *N. benthamiana* plants to *N. benthamiana* and bean plants and, with more difficulty, to tomato plants. The following symptoms developed in leaves of plants infected by sap inoculation: *N. benthamiana,* severe leaf distortion, crumpling, and mottling; bean, leaf epinasty and vein clearing similar to symptoms induced by BDMV (24); and tomato (cultivars Empire and Florida MH-1), leaf crumpling and yellow mottling. The geminivirus was not sap transmissible to pepper plants.

Cloning, characterization, and infectivity of geminivirus DNAs from infected *N. benthamiana* plants. Single-stranded and double-stranded (RF-DNA) forms of geminivirus DNA-A and DNA-B components were detected in nucleic acid extracts prepared from infected *N. benthamiana* leaves. Restriction enzyme and Southern blot hybridization analyses with general DNA-A or DNA-B component probes revealed that the DNA-A and DNA-B RF DNA forms were linearized with the restriction endonucleases *Bgl*II and *Bam*HI, respectively, and that each was approximately 2.6 kb. *Bgl*II-digested DNA-A RF was ligated into *Bgl*II-digested pSP72, and *Bam*HI-digested DNA-B RF was ligated into *Bam*HI-digested

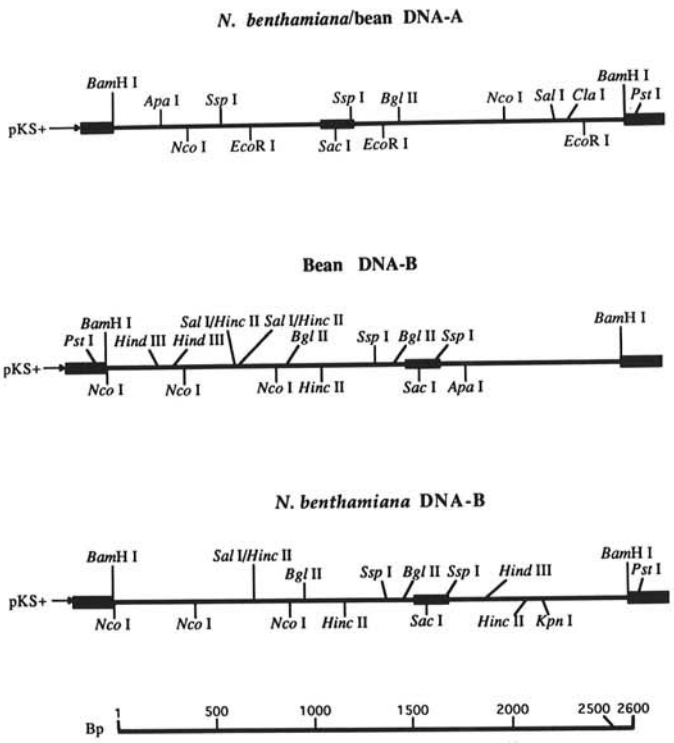

Fig. 1. Restriction maps of the geminivirus DNA-A and DNA-B components cloned from *Nicotiana benthamiana* and bean plants infected with a sap-transmissible geminivirus from Mexico. The restriction maps of the DNA-A components were identical and are both represented as a *Bam*HI fragment cloned in pKS+. Both DNA-B components were cloned as *Bam*HI fragments into pKS+ but in different orientations. The *N. benthamiana* DNA-B component map (including the *Pst*I site of pKS+) is shown in the reverse orientation to allow for comparison with the bean DNA-B component map. The thick lines within the component maps represent the location of the common region sequences. Bp = basepairs.

TABLE 1. Infection of *Nicotiana benthamiana*, bean, and tomato plants with geminivirus DNA-A and DNA-B components cloned from *N. benthamiana* or bean plants infected with a sap-transmissible geminivirus from Mexico

Inoculation method[a] Inoculum[b]	Host	Infectivity[c]	Symptoms[d]
Rub inoculation			
N. benthamiana A + B (excised)	*N. benthamiana*	4/15 (3)	Yes
N. benthamiana A + B (1.5-mers)	*N. benthamiana*	4/23 (4)	Yes
Bean A + B (1.5-mers)	*N. benthamiana*	5/5 (1)	Yes
Biolistic			
Bean A + B (excised)	Bean radicles	3/10 (2)	Yes
	Tomato radicles	1/15 (1)	Yes
Bean A + B (1.5-mers)	Tomato	8/20 (3)	Yes
Gold particles	Bean radicles	0/10 (2)	No
	Tomato radicles	0/10 (1)	No
Protoplast bioassay			
N. benthamiana A + B (1.5-mers)	*N. benthamiana*	0/10 (2)	No
Bean A + B (1.5-mers)	*N. benthamiana*	7/10 (2)	Yes
N. benthamiana A + bean B (1.5-mers)	*N. benthamiana*	9/10 (2)	Yes
Bean A + *N. benthamiana* B (1.5-mers)	*N. benthamiana*	0/10 (2)	No
Potassium phosphate buffer	*N. benthamiana*	0/10 (2)	No

[a] Details of the inoculation protocols are provided in Materials and Methods.
[b] *N. benthamiana* DNA-A and DNA-B components were cloned from *N. benthamiana* plants infected by rub inoculation with sap prepared from an infected tomato sample from northwestern Mexico; bean DNA-A and DNA-B components were cloned from bean plants infected with sap prepared from the infected *N. benthamiana* plants. Excised = a linear monomeric double-stranded form of the DNA component released from a recombinant plasmid by restriction enzyme digestion was used; 1.5-mers = undigested recombinant plasmids containing approximately 1.5 copies of the DNA component(s) were used.
[c] Number of plants infected/number of plants inoculated. Infections were determined on the basis of detection of geminivirus nucleic acids by squash blot hybridization with a general geminivirus DNA probe (18). Numbers in parentheses represent the number of experiments.
[d] Plants were evaluated for disease symptoms 14 and 21 days after inoculation. Plants that were scored positive for symptoms showed leaf crumpling, distortion, and mottling and stunted growth, except for those infected with the *N. benthamiana* DNA-A and DNA-B components, which showed mild leaf crumpling and slightly stunted growth compared with mock-inoculated plants.

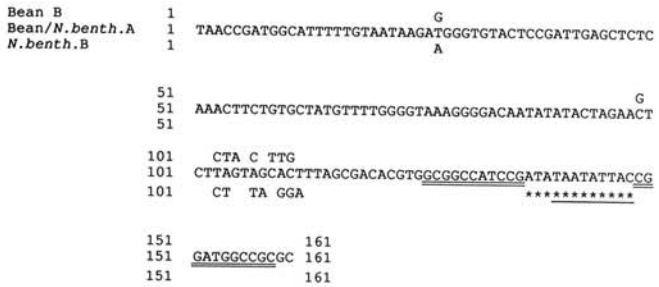

Fig. 2. Alignment of the nucleotide sequences of the common regions of the geminivirus DNA-A and DNA-B components cloned from *Nicotiana benthamiana* (*N. benth.*) and bean plants infected with a sap-transmissible geminivirus from Mexico. The DNA-A sequences were identical, and the complete sequence is shown. The differences in the sequences of the DNA-B components are shown above (for the bean DNA-B component) and below (for the *N. benthamiana* DNA-B component). The conserved geminivirus stem-loop sequence is indicated, with the stem sequence double underlined, the loop sequence indicated by asterisks, and the invariant nanonucleotide sequence single underlined.

pKS+ to obtain putative full-length DNA-A and DNA-B clones, respectively. One DNA-A clone, pTMX22A, and one DNA-B clone, pTMX44B, were identified as geminivirus DNA-A and DNA-B components, respectively, on the basis of comparisons with sequences of other bipartite geminiviruses and are referred to as *N. benthamiana* DNA-A and DNA-B components. The restriction maps of these components are shown in Figure 1 (the DNA-A component is presented as if it were cloned in pKS+ at its unique *Bam*HI site). A 1.5-mer of the DNA-A component was made by double digesting pTMX22A with *Bgl*II plus *Sal*I and cloning the approximately 2.0-kb DNA-A fragment into pSP72 digested with *Bgl*II plus *Sal*I. The resulting 0.5-mer was linearized with *Bgl*II and ligated with the full-length DNA-A insert from pTMX22A to generate the DNA-A 1.5-mer (pTMX22A1.5). A 1.5-mer of the DNA-B component was made by double digesting pTMX44B with *Sac*I plus *Pst*I (the *Pst*I site in pKS+) and cloning the approximately 1.0-kb *Sac*I-*Pst*I fragment into pKS+ digested with *Sac*I plus *Pst*I. The resulting 0.5-mer was linearized with *Bam*HI and ligated with the full-length DNA-B insert from pTMX44B to generate the DNA-B 1.5-mer (pTMX44B1.5).

None of the 15 *N. benthamiana* plants rub inoculated with the excised *N. benthamiana* DNA-A and DNA-B components developed the leaf crumpling, epinasty, and mottling symptoms (severe symptoms) that were evident in the plants from which these components were cloned. However, geminivirus DNA was detected in newly emerged leaves of four of the 15 inoculated plants, and these infected plants showed mild leaf crumpling and slightly stunted growth compared with mock-inoculated plants (Table 1). Similar results were obtained when the 1.5-mers of these components were rub inoculated onto *N. benthamiana* plants (Table 1), indicating that the failure of these components to induce severe disease symptoms was not caused by an effect of the cloning site location in the monomers. To determine whether both DNA components had spread systemically in the infected plants, PCR analyses with primers specific for DNA-A and DNA-B were conducted. One plant, which was infected after being inoculated with the 1.5-mers and had the mild disease symptoms, was analyzed, and similar levels of the DNA-A and DNA-B components were detected in newly emerged, uninoculated leaves (data not shown). Virus could not be sap transmitted from plants infected after inoculation with the *N. benthamiana* monomers or 1.5-mers to *N. benthamiana* plants. Thus, the DNA-A and DNA-B components cloned from *N. benthamiana* were infectious but did not comprise the genome of the sap-transmissible geminivirus from the original tomato sample.

To ascertain whether the failure of these components to induce severe disease symptoms was because these were components of two distinct geminiviruses, the common region sequences were determined. The common region sequences of the DNA components of a bipartite geminivirus are usually approximately 200 nucleotides (nt) in length and have greater than 90% sequence identity (similarity), whereas those of components of different geminiviruses have sequence identities of approximately 40–75% (23). The common region sequences of the *N. benthamiana* DNA-A and DNA-B components were 95% identical and had the characteristic geminivirus stem-loop sequence (Fig. 2), which suggest that these were not components of two distinct geminiviruses. However, the common region sequences were only 161 nt in length

and had a highly divergent 10-nt region located upstream of the stem-loop sequence (Fig. 2).

The possibility that a sap-transmissible RNA virus, present in the original tomato sample, was responsible for the severe disease symptoms in *N. benthamiana* was investigated by examination of leaf-dip preparations by electron microscopy and by inoculation of indicator plants. No identifiable virions (including geminivirus virions) were detected in the leaf-dip preparations, and the indicator plants did not develop any symptoms. Thus, there was no evidence of an RNA virus in the diseased *N. benthamiana* plants, which suggested that the severe symptoms were geminivirus induced.

Cloning, characterization, and infectivity of geminivirus DNAs from bean plants. To clone the genome of the sap-transmissible geminivirus, DNA-A and DNA-B components were cloned from bean plants with leaf epinasty and vein clearing symptoms that had been inoculated with the sap-transmissible geminivirus. *Bam*HI was used to clone six putative full-length geminivirus DNA components from a nucleic acid extract prepared from infected bean leaves. On the basis of restriction enzyme analysis, three of the components were identical to the *N. benthamiana* DNA-A component, whereas the other three were different from the *N. benthamiana* DNA-A and DNA-B components. DNA sequence analysis confirmed that three clones had inserts identical to the *N. benthamiana* DNA-A component and that the other three clones had DNA-B inserts that were similar but not identical to the *N. benthamiana* DNA-B component. One DNA-A clone, pBMX5A, and one DNA-B clone, pBMX9B, were selected for further characterization and are referred to as bean DNA-A and DNA-B components. The restriction maps of these components are shown in Figure 1. To make 1.5-mers of these components, pBMX5A and pBMX9B were each double digested with *Sac*I plus *Pst*I (the *Pst*I site in pKS+), and the approximately 1.6- to 1.7-kb DNA-A and DNA-B fragments were individually cloned into pKS+ digested with *Sac*I plus *Pst*I. The resulting DNA-A 0.5-mer was linearized with *Bam*HI and ligated with the full-length DNA-A insert from pBMX5A to generate the DNA-A 1.5-mer (pBMX5A1.5). The resulting DNA-B 0.5-mer was linearized with *Bam*HI and ligated with the full-length DNA-B insert from pBMX9B to generate the DNA-B 1.5-mer (pBMX9B1.5).

To determine infectivity in bean, the excised DNA-A and DNA-B components were coinoculated into bean radicles by particle bombardment. Three of 10 plants developed leaf epinasty and stunting symptoms identical to those observed in plants infected with the sap-transmissible geminivirus (Table 1). The presence of the input DNA components in systemically infected bean leaves was confirmed by restriction enzyme digestion of PCR-amplified DNA-A and DNA-B fragments (data not shown). Virus from these infected bean plants was sap transmitted to *N. benthamiana,* bean, and tomato plants and induced severe disease symptoms in all three hosts. The excised bean DNA-A and DNA-B monomers were then coinoculated into tomato radicles (cultivar Peto VF 6203) by particle bombardment, and one of 15 plants developed leaf crumpling, distortion, and yellow mottling symptoms that were similar to those in the original tomato sample.

N. benthamiana plants rub inoculated with the bean DNA-A and DNA-B 1.5-mers developed severe leaf distortion, crumpling, and mottling symptoms that were identical to those observed in *N. benthamiana* plants inoculated with sap from the original tomato sample from Mexico (Table 1). Shoots from these infected *N. benthamiana* plants were grafted onto tomato plants, and leaf crumpling and yellow mottling symptoms developed on leaves of the newly emerged tomato shoots. Two-week-old tomato plants (cultivar Peto VF 6203) coinoculated with the 1.5-mers by particle bombardment developed leaf distortion, crumpling, and yellow mottling symptoms (Table 1 and Fig. 3). No symptoms developed in 3- to 4-wk-old pepper plants coinoculated with the 1.5-mers by particle acceleration.

These results establish that the bean DNA-A and DNA-B components comprise the genome of the sap-transmissible geminivirus from the original tomato sample. The common region sequences of these components were 94% identical and had the characteristic stem-loop sequence but were only 161 nt and had a highly divergent 10-nt region located upstream of the stem-loop sequence (Fig. 2). Thus, the bean DNA-A and DNA-B common region sequences were no more identical than were those of the *N. benthamiana* DNA-A and DNA-B components, and both sets of common region sequences had highly divergent 10-nt regions located upstream of the stem-loop sequence (Fig. 2). This suggests that the difference in the symptoms induced by the DNA components cloned from *N. benthamiana* and bean was not the result of bean components having more homologous common region sequences.

Comparison of the DNA-A components cloned from *N. benthamiana* and bean. The restriction maps and common region sequences of the *N. benthamiana* and bean DNA-A components were identical (Figs. 1 and 2). Furthermore, when either DNA-A component was inoculated with the bean DNA-B component onto *N. benthamiana* plants, severe disease symptoms developed (Table 1). Thus, these results indicate that differences in the DNA-B components were responsible for the different disease symptoms induced by the *N. benthamiana* and bean DNA-A and DNA-B components.

Comparison of the DNA-B components cloned from *N. benthamiana* and bean. The restriction maps of the *N. benthamiana* and bean DNA-B components were different (Fig. 1) but had large regions (between the *Nco*I site at the approximately 450-bp position and the *Ssp*I site at the approximately 1,700-bp position) (Fig. 1) that were nearly identical. The common region sequences of these components were 96% identical, and most of the differences were located in the highly divergent 10-nt region located upstream of the stem-loop sequence (Fig. 2). To further ascertain the relationship between these DNA-B components, the nucleotide sequences of the BR1 open reading frame (ORF), which is the most divergent ORF of the bipartite geminiviruses, and the DNA-B hypervariable region (HV), which is the most divergent

Fig. 3. Disease symptoms induced in tomato leaves by the cloned infectious DNA-A and DNA-B components of tomato leaf crumple geminivirus.

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BR1-Nb                      C                          Thr Thr Tyr
BR1-B  ATG TAT CCT TTT AGG TCT AAG CGT GGT GCC TCA TTT GTT GCA CGT CGT 48
       Met Tyr Pro Phe Arg Ser Lys Arg Gly Ala Ser Phe Val Ala Arg Arg 16

BR1-Nb                             Val
BR1-B  TCT TAT TCA CGT AAT AAT TTG TTT AAG CGA TCA ACC ATC TCC AAA CGA 96
       Ser Tyr Ser Arg Asn Asn Phe Phe Lys Arg Ser Thr Ile Ser Lys Arg 32

BR1-Nb                                                        Ser Val
BR1-B  GAT GAT GGG AGA CGT CGA TCT GTT AAT GCA ACT AAA CCC AAT GAC GAG 144
       Asp Asp Gly Arg Arg Arg Ser Val Asn Ala Thr Lys Pro Asn Asp Glu 48

BR1-Nb                Thr
BR1-B  CCC AAG ATG ATA GCC CAA CGC ATG CAC GAG AAT CAG TTT GGG CCA GAT 192
       Pro Lys Met Ile Ala Gln Arg Met His Glu Asn Gln Phe Gly Pro Asp 64

BR1-Nb                                    Ser
BR1-B  TTT GTA ATG GCC CAT AAT GCA GCC TTG GCA ACT TTC ATC AGC TTC CCT 240
       Phe Val Met Ala His Asn Ala Ala Leu Ala Thr Phe Ile Ser Phe Pro 80

       Gly
BR1-Nb G   C
BR1-B  TGT TTG GGT AAG ACT CAA CCG AAC CGA AGC AGG TCG TAT ATC AAG TTG 288
       Cys Leu Gly Lys Thr Gln Pro Asn Arg Ser Arg Ser Tyr Ile Lys Leu 96

BR1-Nb       T                                                    Thr
BR1-B  AAA CGG CTG CGT TTC AAA GGT ACG GTG AAG ATC GAA CGT GTC ATG TCT 336
       Lys Arg Leu Arg Phe Lys Gly Thr Val Lys Ile Glu Arg Val Met Ser 112

BR1-Nb                         Ile Pro
BR1-B  GAT ATG AAC ATG GAT GGT TCT ACT TCC AAG GTC GAA GGA GTT TTC TCA 384
       Asp Met Asn Met Asp Gly Ser Thr Ser Lys Val Glu Gly Val Phe Ser 128

BR1-Nb                                                        Cys
BR1-B  CTC GTT GTC GTT GTG GAT CGA AAA CCC CAT CTG GGT GCG TCC GGC AGT 432
       Leu Val Val Val Val Asp Arg Lys Pro His Leu Gly Ala Ser Gly Ser 144

BR1-Nb   G           C
BR1-B  CTA CAT ACG TTT GAT GAA CTA TTT GGC GCT AGG ATC CAC AGC CAT GGA 480
       Leu His Thr Phe Asp Glu Leu Phe Gly Ala Arg Ile His Ser His Gly 160

BR1-Nb                                                 T
BR1-B  AAC CTC AGC ATA ACC CCT TCT TTG AAA GAC CGG TTT TAC ATA AGA CAC 528
       Asn Leu Ser Ile Thr Pro Ser Leu Lys Asp Arg Phe Tyr Ile Arg His 176

BR1-B  GTG TTC AAA CGT GTA TTG TCT GTG GAG AAG GAT AGT ATG ATG GTT GAT 576
       Val Phe Lys Arg Val Leu Ser Val Glu Lys Asp Ser Met Met Val Asp 192

BR1-B  GTT GAA GGG TCC ACA GCT CTC TCT AAC AGG CGT TTC AAT TGT TGG TCC 624
       Val Glu Gly Ser Thr Ala Leu Ser Asn Arg Arg Phe Asn Cys Trp Ser 208

                     Ile                                          Ala
BR1-Nb   T           A T                                          CC
BR1-B  ACG TTT AAG GAT TTG GAT CGT GAT TCA TGT AAC GGC GTT TAT GGT AAC 672
       Thr Phe Lys Asp Leu Asp Arg Asp Ser Cys Asn Gly Val Tyr Gly Asn 224

BR1-Nb                                  T
BR1-B  ATC AGC AAG AAC GCC CTG TTA GTC TAC TAT TGT TGG ATG TCA GAT ACT 720
       Ile Ser Lys Asn Ala Leu Leu Val Tyr Tyr Cys Trp Met Ser Asp Thr 240

BR1-Nb           A           T                       C               C
BR1-B  ATG TCT AGG GCG TCA AGC TTT GTA TCG TTT GAT CTC GAT TAT ATT GGA 768
       Met Ser Arg Ala Ser Ser Phe Val Ser Phe Asp Leu Asp Tyr Ile Gly 256

BR1-B  TGA 771
       *   257
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Fig. 4. Nucleotide and derived amino acid sequences of the BR1 open reading frames (ORFs) of the geminivirus DNA-B components cloned from *Nicotiana benthamiana* and bean. The entire nucleotide and derived amino acid sequences of the bean DNA-B BR1 ORF (BR1-B) are shown, and the differences in the nucleotide and derived amino acid sequences of the *N. benthamiana* DNA-B BR1 ORF (BR1-Nb) are indicated above.

sequence in the genome of the bipartite geminiviruses (21), were determined and compared. The BR1 sequences were 94% identical at the nucleotide and amino acid levels, with the differences distributed throughout the sequences (Fig. 4; *N. benthamiana* BR1, GenBank L34749, and bean BR1, GenBank L34746). The HV sequences also were 94% identical, and the HV sequence of the bean DNA-B component had a gap of 2 nt compared with that of the *N. benthamiana* DNA-B component (data not shown). These results, considered together with the differences in symptoms induced by these components, suggest that these are different but closely related DNA-B components.

Examination of replication of cloned geminiviral DNA-B components in protoplasts. To further characterize the DNA-B components, a protoplast bioassay (20,34) was used. In this assay, excised monomers or 1.5-mers are electroporated into *N. tabacum* protoplasts, and the protoplasts are incubated for 5 days in the dark at room temperature. Total DNA is extracted from an aliquot of protoplasts and analyzed by Southern hybridization analysis for replication of the DNA component or components. An extract prepared from a second aliquot of protoplasts is rub inoculated onto *N. benthamiana* plants to test for infectivity. When excised monomers or 1.5-mers of the *N. benthamiana* or bean DNA-A and DNA-B components were electroporated into protoplasts, the DNA-A and DNA-B components replicated. Furthermore, the *N. benthamiana* DNA-A component replicated the bean DNA-B component, and the bean DNA-A component replicated the *N. benthamiana* DNA-B component. For all these combinations, all the DNA components were replicated to similar levels. However, when protoplast extracts were rub inoculated onto *N. benthamiana* plants, only combinations having the bean DNA-B component induced disease symptoms (Table 1). These results indicate that the *N. benthamiana* DNA-B component can replicate efficiently in *N. tabacum* protoplasts in the presence of either DNA-A component but that only combinations having the bean DNA-B component induce severe symptoms in *N. benthamiana* plants.

Detection of the two distinct DNA-B components in the original infected tomato sample. To ascertain whether both DNA-B components were present in the original infected tomato sample, oligonucleotide primers were designed for the specific PCR amplification of HV region fragments from the *N. benthamiana* or bean DNA-B components. Primer PTBc3096 (5′ ATACAC-AAATTTAAAAAGAG 3′) is a complementary-sense sequence designed from the *N. benthamiana* DNA-B HV sequence with the 3′ nucleotide complementary to a C residue that is not present in the bean DNA-B HV sequence (because of the 2-nt gap). Primer PBBc3097 (5′ CAATACACATATTTAAAAGA 3′) is a complementary-sense sequence designed from the bean DNA-B component; the last 2 nt of the 3′ end are different from the corresponding nucleotides in the *N. benthamiana* DNA-B HV sequence. At an annealing temperature of 60 C, the primer pair PTBc3096 and PBL1v2040, a degenerate primer for whitefly-transmitted geminiviruses that anneals within the 5′ end of the BL1 ORF (37), directed the amplification of an approximately 375-bp fragment from the *N. benthamiana* DNA-B component (in pTMX44B) but not from the bean DNA-B component (in pBMX9B) (Fig. 5). Conversely, the primer pair PBBc3097 and PBL1v2040 directed the amplification of an approximately 375-bp fragment from the bean DNA-B component but not from the *N. benthamiana* DNA-B component (Fig. 5). Thus, these primer combinations could be used to specifically detect either the *N. benthamiana* or bean DNA-B component. DNA was extracted from approximately 25 mm^2 of leaf tissue of the original tomato sample, which had been stored at −80 C, as previously described (21,37) and resuspended in 200 μl of Tris-EDTA buffer. The DNA was concentrated by ethanol precipitation and resuspended in 10 μl of Tris-EDTA buffer, and 0.5 μl was used in the PCR analysis. At an annealing temperature of 60 C, the primer pair PTBc3096 and PBL1v2040 amplified the approximately 375-bp fragment from the original tomato sample and from the *N. benthamiana* DNA-B component but not from the bean DNA-B component (Fig. 5). Primer pair PBBc3097 and PBL1v2040 amplified the approximately 375-bp fragment from the original tomato sample and from the bean DNA-B component but not from the *N. benthamiana* DNA-B component (Fig. 5). With the same primer pairs, both DNA-B components also were detected in leaves of *N. benthamiana* plants that had been infected by rub inoculation with sap prepared from the original tomato sample. No DNA fragments were amplified from DNA extracts made from uninfected tomato or tobacco leaf tissue. These results show that both the *N. benthamiana* and bean DNA-B components were present in the original tomato sample and that they were both passaged into *N. benthamiana* by rub inoculation rather than being generated during passage through this host.

Relationship of the sap-transmissible geminivirus from Mexico to previously characterized whitefly-transmitted geminiviruses. The common region nucleotide sequence of the DNA-A component (GenBank L34748) and the nucleotide and derived amino acid sequences of the AR1 (GenBank L34747) and BR1 (GenBank L34746) ORFs of the sap-transmissible geminivirus (bean DNA-A and DNA-B components) were compared with respective se-

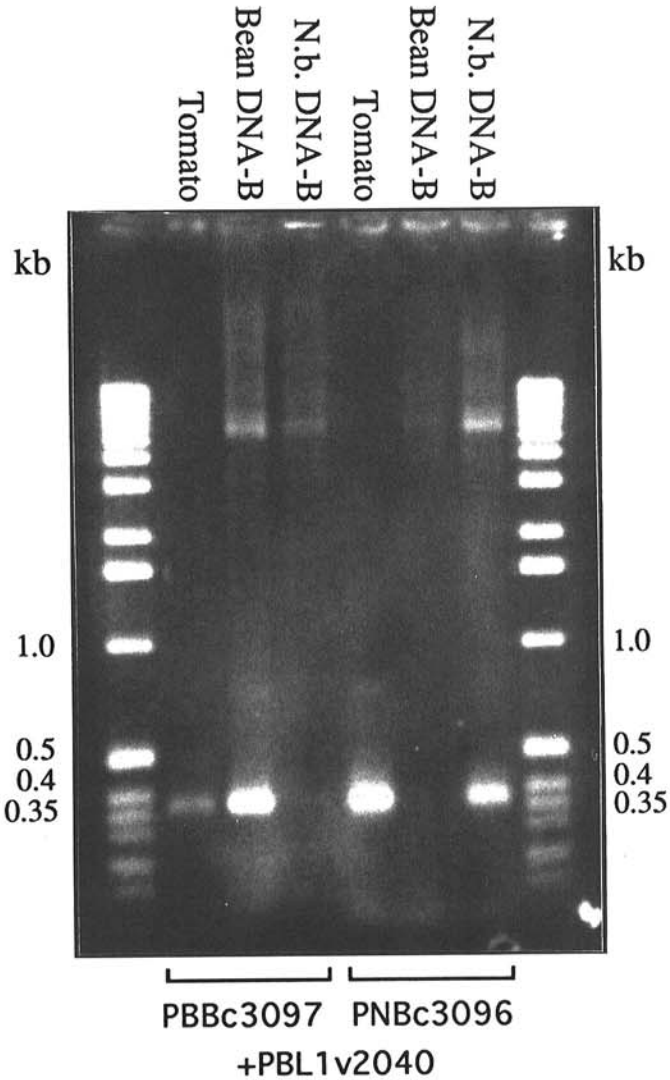

Fig. 5. Detection of two distinct DNA-B components in the original tomato sample collected in Mexico by polymerase chain reaction (PCR) amplification of an approximately 375-bp DNA fragment from the DNA-B hypervariable region. Primer pair PTBc3096 and PBL1v2040 directed the amplification of an approximately 375-bp fragment from the tomato sample (Tomato) and from the *Nicotiana benthamiana* DNA-B component (N.b. DNA-B) but not from the bean DNA-B component (Bean DNA-B). Primer pair PBBc3097 and PBL1v2040 directed the amplification of an approximately 375-bp fragment from the tomato sample and from the bean DNA-B component but not from the *N. benthamiana* DNA-B component.

quences from nine previously characterized whitefly-transmitted geminiviruses from the Western Hemisphere (New World): Abutilon mosaic (AbMV) (14), BDMV (24), type I BGMV-BZ (16), type II BGMV from Puerto Rico (BGMV-PR) (25), potato yellow mosaic (PYMV) (10), squash leaf curl (SqLCV) (28), tomato golden mosaic (TGMV) (22), ToMoV (1) (19), and pepper huasteco geminivirus (PHV) from Mexico (43); and two geminiviruses from the Eastern Hemisphere (Old World): African cassava mosaic from Kenya (ACMV-K) (40) and tomato yellow leaf curl from Israel (TYLCV-IR) (30). The results of these comparisons (Table 2) indicate that the sap-transmissible geminivirus from Mexico is a distinct bipartite genome geminivirus that is closely related to AbMV, BDMV, and ToMoV but not to PHV from Mexico. Phylogenetic trees generated by the PAUP program for the AR1 and BR1 ORF sequences revealed similar relationships (Fig. 6). On the basis of these results, we conclude that this is a newly identified bipartite genome geminivirus and propose the name tomato leaf crumple geminivirus (TLCrV).

DISCUSSION

In this report, we describe the infectious clones of a new sap-transmissible bipartite genome geminivirus infecting tomatoes in northwestern Mexico and present further evidence of the genetic complexity of geminivirus infections in plants. This geminivirus causes leaf crumpling, epinasty, and mottling symptoms and has a host range that includes tomato, *N. benthamiana,* and common bean. We propose the name tomato leaf crumple geminivirus (TLCrV) on the basis of its original isolation from tomato and distinctive symptomatology. The TLCrV genome is composed of DNA-A and DNA-B components that are each approximately 2.6 kb and share a common region of 161 nt in which they are 94% identical. The relatively short TLCrV common region, which ends immediately after the stem-loop sequence, and the highly divergent 10-nt region located upstream of the stem-loop region (Fig. 2) may indicate the recent and/or ongoing evolution of TLCrV, possibly from a pseudorecombinant bipartite genome geminivirus such as that recently made with the ToMoV DNA-A and BDMV DNA-B components (19). The common region sequence shared between the components of this pseudorecombinant geminivirus, which is 166 nt and has a highly divergent region located upstream of the stem-loop sequence (19), has some similarity to the TLCrV common region. It will be interesting

TABLE 2. Percent nucleotide (nt) identities and derived amino acid (aa) identities and similarities between the DNA-A component common region and the AR1 and BR1 open reading frames of the sap-transmissible geminivirus from Mexico and the respective sequences of other whitefly-transmitted geminiviruses

	Common region	AR1		BR1	
Geminivirus[a]	nt	nt	aa[b]	nt	aa
BDMV	80	87	96 (97)	78	80 (88)
ToMoV	74	88	96 (98)	75	78 (88)
AbMV[c]	76	86	93 (97)	76	75 (85)
BGMV-BZ	71	81	91 (94)	71	76 (87)
BGMV-PR	60	79	88 (93)	70	69 (83)
PHV	44	77	87 (92)	70	68 (81)
PYMV	56	85	96 (98)	70	68 (79)
TGMV	63	81	91 (94)	70	69 (79)
SqLCV	52	80	91 (96)	65	62 (76)
ACMV-K	45	66	69 (79)	44	33 (50)
TYLCV-IR[d]	46	67	73 (80)	...	...

[a]BDMV = bean dwarf mosaic virus; ToMoV = tomato mottle virus; AbMV = Abutilon mosaic virus; BGMV-BZ = bean golden mosaic virus from Brazil; BGMV-PR = bean golden mosaic virus from Puerto Rico; PHV = pepper huasteco virus; PYMV = potato yellow mosaic virus; TGMV = tomato golden mosaic virus; SqLCV = squash leaf curl virus; ACMV-K = African cassava mosaic virus from Kenya; and TYLCV-IR = tomato yellow leaf curl virus from Israel.

[b]Numbers in parentheses are percent similarities.

[c]AbMV BR1 sequence was amended as previously described (19).

[d]TYLCV-IR does not have a DNA-B component.

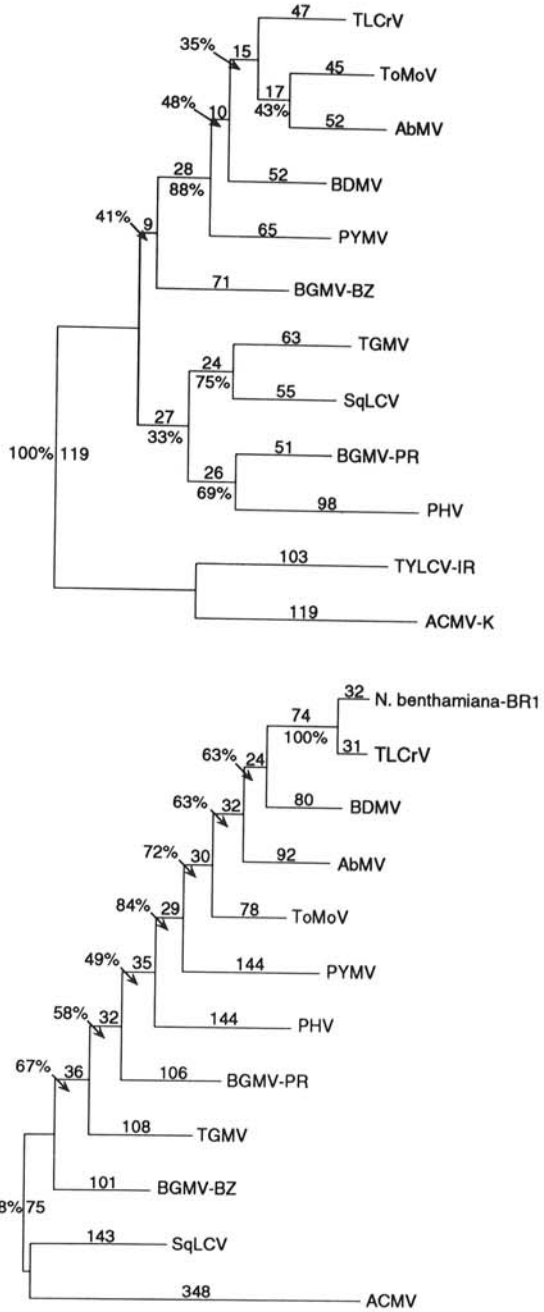

Fig. 6. Phylogenetic trees showing the relationship of tomato leaf crumple geminivirus (TLCrV) with other whitefly-transmitted geminiviruses on the basis of alignments of the **A,** AR1 and **B,** BR1 nucleotide sequences. Sequences were analyzed by the computer program Phylogenetic Analysis Using Parsimony (PAUP, version 3.1) with heuristic tree construction. Branch strengths were evaluated by constructing 100 trees by bootstrap and branch swapping. The number of nucleotide changes is shown above the horizontal line, and the percentage of trees having the given branch is shown below the horizontal line. Vertical distances are arbitrary, and horizontal distances are in proportion to the number of nucleotide differences between branch nodes. AbMV = Abutilon mosaic virus; ACMV-K = African cassava mosaic virus from Kenya; BDMV = bean dwarf mosaic virus; BGMV-BZ = bean golden mosaic virus from Brazil; BGMV-PR = bean golden mosaic virus from Puerto Rico; PHV = pepper huasteco virus; PYMV = potato yellow mosaic virus; SqLCV = squash leaf curl virus; TGMV = tomato golden mosaic virus; *N. benthamiana*-BR1 = DNA-B component cloned from *Nicotiana benthamiana* in this study; ToMoV = tomato mottle virus; and TYLCV-IR = tomato yellow leaf curl virus from Israel.

to determine for TLCrV and the ToMoV DNA-A and BDMV DNA-B pseudorecombinant whether this divergent region is maintained during serial passage through different host plants.

The definitive evidence for the role of a geminivirus in a disease is the introduction of cloned viral DNAs back into the host plant from which the DNAs were cloned and the subsequent production of the symptoms associated with the disease (i.e., completion of Koch's postulates). A number of methods have been used to introduce cloned geminivirus DNAs into plants and establish causality of disease. These include rub inoculation (39), agroinoculation (39), and particle acceleration (15,17). In this study, the bean DNA-A and DNA-B components were introduced into bean and tomato by a biolistic method and into *N. benthamiana* by rub inoculation. The development of characteristic disease symptoms in all three hosts and the subsequent sap transmission of virus from these infected plants established that the bean DNA-A and DNA-B components comprised the genome of the sap-transmissible bipartite geminivirus originally derived from tomato. Biolistic approaches have proved to be effective ways to inoculate plants with cloned geminivirus DNA components and have been used to establish that the cloned DNA components of BGMV and BDMV induce characteristic disease symptoms in bean (17) and that those of PHV induce disease symptoms in pepper (15). Interestingly, the PHV DNA-A and DNA-B components were found to induce only some of the disease symptoms associated with the pepper rizado amarillo disease, and it was suggested that PHV and an uncharacterized geminivirus were responsible for this disease.

Numerous whitefly-transmitted geminiviruses have been described from Mexico, but only PHV, which is from northeastern Mexico (Tamaulipas State) and is not sap transmissible, has been characterized at the molecular level. TLCrV was readily differentiated from PHV on the basis of comparisons of restriction maps and nucleotide sequences and differences in host range and sap transmissibility (15,43). The phylogenetic analyses (Fig. 6) indicated that TLCrV and PHV are not closely related and are found within distinct geminivirus phylogenetic clusters (12). Furthermore, the TLCrV DNA-A common region sequence was no more similar to that of PHV (44% identical) than it was to those of ACMV and TYLCV (44 and 46% identity, respectively), two Old World whitefly-transmitted geminiviruses. These findings show a remarkable amount of divergence between these two bipartite geminiviruses from Mexico and are consistent with the hypothesis that PHV might represent a hybrid or transition geminivirus between Eastern and Western Hemisphere types (43). TLCrV can also be distinguished from other previously described geminiviruses from Mexico on the basis of biological properties. Because TLCrV is sap transmissible but does not infect pepper, it is different from the geminiviruses chino del tomate (CdTV) (6) and pepper mild tigré (PMTV) (5,7) (which are not sap-transmissible) and the sap-transmissible serrano golden mosaic geminivirus (SGMV) (8); CdTV, PMTV, and SGMV all infect pepper. TLCrV is also different from the Texas pepper geminivirus (TPGV) (41) from southern Texas on the basis of comparisons of restriction maps and host range (TPGV infects pepper but not common bean). TLCrV appears most similar to CdTV because both viruses have similar host ranges, occur in northwestern Mexico, and had a similar reaction to a panel of geminivirus monoclonal antibodies (B. D. Harrison, *personal communication*). However, the symptoms induced in tomato by TLCrV are less severe than those described for CdTV, and TLCrV may be one of a complex of geminiviruses associated with the chino del tomate disease. Further determination of the relationship of TLCrV to CdTV and other geminiviruses infecting crop plants in Mexico awaits the availability of the nucleotide sequences of infectious clones.

Nucleotide sequence comparisons indicated that TLCrV is different from previously characterized whitefly-transmitted geminiviruses and is most closely related to BDMV, ToMoV, and AbMV. The TLCrV sequences were most similar to those of BDMV, which is consistent with the similarity in symptoms induced in common bean by these viruses. These results agree with those of a previous phylogenetic analysis conducted with common region and AL1 ORF sequences of 21 whitefly-transmitted geminiviruses in which BDMV, ToMoV, AbMV, TLCrV (then referred to as TGV-MX1), tomato-infecting geminiviruses from the Dominican Republic (TGV-DR) and Costa Rica (TGV-CR), and Sida golden mosaic (SiGMV) from Costa Rica were shown to be closely related and were placed in an "Abutilon mosaic cluster" (36). These geminiviruses induce leaf crumpling, distortion, and mottling symptoms in various plant species and are distributed over a large geographic region that includes Central America, the Caribbean Basin, and the southern United States. The recent appearance of some of these geminiviruses in geographically separate regions has been associated with increased whitefly populations (e.g., TGV-DR in the Dominican Republic and ToMoV in Florida) and suggests that crop-infecting geminiviruses, such as TLCrV, may have evolved from indigenous weed-infecting geminiviruses. A similar hypothesis has been proposed to explain the existence of genetically distinct types of BGMV (16). The ubiquitous SiGMV, which is widely distributed in weeds throughout Central America, the Caribbean Basin, and the southern United States (e.g., Florida and Texas) and is related to AbMV (9,36), may be the progenitor virus from which TLCrV and other members of the Abutilon mosaic cluster have evolved.

The initial effort to clone the TLCrV genome from *N. benthamiana* revealed the genetic complexity of geminivirus infections in field-infected plants. The original tomato sample was infected by at least three geminivirus components, all of which were sap transmitted to *N. benthamiana*. The DNA-A and DNA-B components cloned from *N. benthamiana* plants did not comprise the TLCrV genome, because they failed to induce severe symptoms in *N. benthamiana* plants and virus was not sap transmissible from plants infected with these components. Because *N. benthamiana* is permissive to infection by most bipartite geminiviruses and by component mixtures of different bipartite geminiviruses, e.g., TGMV and AbMV (13) or TGMV and BGMV (I. T. D. Petty, *personal communication*), TLCrV was sap transmitted from *N. benthamiana* into a less permissive host, common bean, and new geminivirus components were cloned. Although it is not known whether the bean host provided the intended selective effect, the infectious TLCrV DNA-A and DNA-B components were cloned from the infected bean plants.

The *N. benthamiana* and bean DNA-A components had identical restriction maps, common region sequences, and infectivity properties and are both TLCrV DNA-A clones. On the other hand, the *N. benthamiana* and bean DNA-B components had different restriction maps, nucleotide and amino acid sequences, and infectivity properties and are different DNA-B components. However, on the basis of nucleotide sequence similarities, the phylogenetic analysis of the BR1 ORF sequence (Fig. 6), and the replication of both DNA-B components by the TLCrV DNA-A component, these two DNA-B components are closely related. These components may belong to an evolving group of geminiviruses or geminivirus strains, with the *N. benthamiana* DNA-B component belonging to a bipartite genome geminivirus that is related to TLCrV or to a distinct TLCrV strain. Alternatively, the *N. benthamiana* DNA-B may be a recombinant DNA-B component, possibly generated through an intermolecular recombination event between the DNA-B components of TLCrV and another bipartite genome geminivirus. This latter hypothesis is supported by the fact that significant portions of the *N. benthamiana* and bean DNA-B component restriction maps are nearly identical. A similar complex situation has been previously reported for SqLCV, in which various combinations of the DNA components of two similar strains, SqLCV-E and SqLCV-R, that differ in host range properties were detected in field-infected squash plants by the use of component-specific DNA probes (35). Subsequent molecular characterization of the DNA components of these strains revealed that they had similar common region sequences and that the SqLCV-E DNA-A could replicate the SqLCV-R DNA-B in a leaf-disk assay (27).

A protoplast bioassay (20,34) was used to further investigate the replication, infectivity, and pathogenicity properties of the

N. benthamiana and bean DNA components. This method, which allows for the independent assay of geminivirus replication and systemic spread functions (20), was used to show that the *N. benthamiana* and bean DNA-B components were replicated to similar levels by the TLCrV DNA-A component. Thus, the failure of the *N. benthamiana* DNA-B component to induce severe symptoms in *N. benthamiana* was not caused by a reduced level of DNA-B replication, as was previously reported for the ToMoV DNA-A and BDMV DNA-B pseudorecombinant (19). However, the lack of disease symptoms was associated with the *N. benthamiana* DNA-B component. On the basis of the results of this study, it is not possible to determine precisely why the *N. benthamiana* DNA-B component did not induce severe disease symptoms. The DNA-B component of the bipartite geminiviruses encodes the viral movement proteins (31,39) and symptom determinants (44). It is possible that the differences in infectivity and symptoms between the *N. benthamiana* and bean DNA-B components are the results of differences in DNA-B–encoded proteins. A defective DNA-B component of SqLCV-R that does not infect *N. benthamiana* has been recently characterized, and the defect was mapped to a single missense mutation in the BR1 ORF that resulted in a single amino acid change (26). Alternatively, the TLCrV DNA-A component-encoded *trans*-acting factors involved in gene expression from the DNA-B component (e.g., transactivation of BR1 expression by the AL2 protein) (42) may not be fully compatible with the *N. benthamiana* DNA-B component. This would imply that the *N. benthamiana* DNA-B component may be able to induce severe symptoms in *N. benthamiana* and/or in other hosts in the presence of its homologous DNA-A component.

The role of TLCrV in geminivirus diseases of tomato, common bean, and possibly other hosts in Mexico can not be precisely determined on the basis of observation of disease symptoms in the field because of the effects of mixed viral infections, different cultivars, and other factors. Because TLCrV has been detected in tomato and common bean samples from various geographical regions in Mexico (32), it may play a role in a number of diseases, possibly as one of a complex of geminiviruses. Rojas et al (37) used PCR and degenerate primers to show that TLCrV (then referred to as TGV-MX1) and a distinct but related geminivirus, TGV-MX2, were present in a tomato plant from northwestern Mexico, which was showing severe leaf curl symptoms that were distinct from TLCrV symptoms. Much work remains to be done to sort out the complexity of geminiviruses infecting crop plants in Mexico, and the molecular characterization of TLCrV is another step towards understanding and managing this viral complex.

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