Use of Degenerate Primers in the Polymerase Chain Reaction to Detect Whitefly-Transmitted Geminiviruses

MARÍA R. ROJAS, Department of Plant Pathology, University of Wisconsin, Madison 53706; ROBERT L. GILBERTSON, Department of Plant Pathology, University of California, Davis 95616; DAVID R. RUSSELL, Agracetus, Inc., 8520 University Green, Middleton, WI 53717; and DOUGLAS P. MAXWELL, Department of Plant Pathology, University of Wisconsin, Madison 53706

ABSTRACT

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Geminiviruses are widely recognized as a serious threat to vegetable production in many tropical and subtropical regions. This has increased the need for accurate identification of these viruses. Geminiviruses are well suited to polymerase chain reaction (PCR) methods because they replicate via a double-stranded, circular DNA form. Degenerate PCR primers were designed to anneal to highly conserved nucleotide sequences identified in the genomes of 10 whitefly-transmitted geminiviruses. The PCR primers were tested for their effectiveness in the amplification of viral DNA fragments from the DNA-A and/or DNA-B components of 15 previously uncharacterized geminiviruses from the Americas, the Caribbean Basin, and Africa. Symptomatic plant samples tested included tomato, bean, pepper, soybean, cassava, and four weed species. Two primer pairs designed to anneal to DNA-A amplified viral DNA from the monopartite, leafhopper-transmitted geminivirus, beet curly top geminivirus. PCR-amplified viral fragments were further characterized by Southern blot DNA-DNA hybridization analysis with geminivirus DNA probes, by restriction fragment length polymorphism analysis, and/or by cloning and sequencing. These methods also detected mixed geminivirus infections in two cases. PCR using these degenerate geminivirus primers offers a rapid means of geminivirus detection.

Additional keywords: bean dwarf mosaic virus, bean golden mosaic virus, cassava-infecting geminivirus, pepper-infecting geminivirus, Rhynchosia mosaic virus, soybean-infecting geminivirus, tomato-infecting geminiviruses, tomato yellow leaf curl virus

Geminiviruses are an economically important group of plant viruses characterized by their twinned (geminate) isometric virions and circular single-stranded DNA genomes. Geminiviruses are transmitted by either leafhoppers or whiteflies (*Bemisia tabaci* Gennadius). Whitefly-transmitted geminiviruses cause serious losses to many important food crops (e.g., bean, cucurbits, tomato, pepper, and cassava) in tropical and subtropical regions (3,4). Tomato yellow leaf curl geminivirus (TYLCV) seriously limits tomato production in the Middle East (21). Recently, tomato-infecting geminiviruses have caused substantial losses in Florida (40), Costa Rica (36,37), and other parts of the Americas (3,4). Bean golden mosaic disease is recognized as the most serious constraint to bean production in certain regions of Brazil, Central America, and the Caribbean Basin (12). In addition, numerous common weeds are infected with geminiviruses (2,3,6), but little is known of their relation to crop-infecting geminiviruses. Because of the increasing importance of whitefly-transmitted geminiviruses, rapid and accurate methods are needed for virus detection and subsequent identification. Such methods would greatly facilitate studies of the epidemiology and the genetic diversity of these viruses. This information would have important implications in designing strategies for breeding for disease resistance and disease control.

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The polymerase chain reaction (PCR) is an extremely sensitive and specific technique (24,39) for the detection and identification of plant pathogens, and it can be used to investigate precise questions about the composition of pathogen populations and the genetic diversity of viruses (16,35). The specificity of PCR is based on the use of oligonucleotide primers that are complementary to the regions flanking the DNA sequence to be amplified. Because PCR amplifies nucleic acids, the technique could be useful in overcoming many of the present difficulties associated with serological detection methods, e.g., low titer of antigen, cross-reaction of antibodies with heterologous antigens, and developmental or environmental regulation of antigen production. Furthermore, small amounts of plant samples, which can be fresh, frozen, or even dried, are suitable for PCR. PCR methods have been used for detection and to determine the genetic variability of plant viruses, including luteoviruses (35), potyviruses (26,45), leafhopper-transmitted geminiviruses infecting monocotyledonous plants (38), and the whitefly-transmitted geminiviruses, bean golden mosaic virus (BGMV; 16) and TYLCV (32).

Geminiviruses are well suited to detection and identification by PCR because they replicate via a double-stranded, circular DNA intermediate—the replicative form (41)—which can serve as a template for amplification by PCR. The genome of many well-characterized whitefly-transmitted geminiviruses is composed of two DNA components designated DNA-A and DNA-B (Fig. 1). The complete nucleotide sequences of five bipartite, whitefly-transmitted geminiviruses from the Western Hemisphere (7,11,19,22,28,44) and the more distantly related African cassava mosaic geminivirus (ACMV) from the Eastern Hemisphere (42) have been published, and we have determined the complete nucleotide sequences for four bipartite bean-infecting geminivirus isolates from the Western Hemisphere (*unpublished*). These sequences were aligned so that highly conserved regions could be identified for use in designing degenerate PCR primers. We report the use of PCR to detect and differentiate whitefly-transmitted geminiviruses from the Americas, the Caribbean Basin, and Africa.

MATERIALS AND METHODS

Geminiviruses and virus-infected plant samples. Three bean-infecting geminiviruses (13), BGMV from Guatemala (BGMV-GA), BGMV from the Dominican Republic (BGMV-DR), and bean dwarf mosaic geminivirus (BDMV), were maintained in *Phaseolus vulgaris* L. 'Topcrop' by sap inoculation (14). Bean tissue infected with a BGMV isolate from Brazil (BGMV-BZ; 14) was obtained by inoculating beans with cloned viral DNA by use of electric discharge particle acceleration (13). Tomato-infecting geminiviruses from Florida, Mexico, and Costa Rica were maintained in *Nicotiana benthamiana* Domin. by sap inoculation. Samples of other known or suspected geminiviruses were obtained in fresh or dried tissue (Table 1). Dried tissue was stored at room temperature prior to DNA extraction.

Extraction of viral DNA. Two methods were used for extraction of DNA from plant tissue. In the first method (D. R.

Russell and J. Cooley, *personal communication*), young leaf tissue (5–30 mg) was ground with 300 μl of extraction buffer (100 μM EDTA, 2.5 M NH_4Ac, 100 mM Tris buffer, pH 8) in a 1.5-ml microfuge tube and then centrifuged for 10 min at about 10,000 *g*. The supernatant fluid (approximately 250 μl) was transferred to a clean microfuge tube, and 500 μl of isopropanol was added. The tube was vortexed and centrifuged for 10 min at 10,000 *g*, and the supernatant fluid was removed. The pellet was washed with 200 μl of 70% ethanol, the tube centrifuged for 3 min at 10,000 *g*, the fluid removed, and the pellet dried for 5 min in a Speed-Vac dryer prior to resuspension in 100–300 μl of distilled water. The second DNA extraction method was developed by Dellaporta et al (8) and used as previously described (16). Between 20 and 40 μl of the DNA extract was used per 100 μl of PCR reaction mixture.

For some plant samples, an additional purification step was added to obtain suitable viral DNA. The DNA extracted by the Dellaporta et al (8) method was suspended in 500 μl of deionized water from the Milli-Q system, then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and centrifuged at 10,000 *g* for 5 min. The aqueous layer was removed to a clean microfuge tube. One milliliter of 100% ethanol was added, the sample was centrifuged for 5 min at 10,000 *g*, the liquid phase was removed, and the precipitated nucleic acid pellet was washed twice with 500 μl of 70% ethanol. The pellet was dried under a partial vacuum and resuspended in 50 μl of distilled water. Twenty microliters of this DNA extract was used in 100-μl PCR reaction mixtures.

Because the final concentration of viral DNA in the extract from the various tissue samples was unknown, it was sometimes necessary to test various amounts of DNA extract in the PCR reaction mixtures.

PCR. Primers were synthesized by the University of Wisconsin-Madison Biotechnology Center and were adjusted to 10 μM with filtered water (Milli-Q filter). PCR reaction mixtures of 100 μl contained 20–40 μl of sample DNA solution and dNTP and primer concentrations of 25 μM for each nucleotide and 0.2 μM for each primer. The Mg^{2+} concentration was between 0.5 and 2.5 mM, and this concentration could greatly influence the success of the viral DNA amplification. *Taq* polymerase (Promega, Madison, Wisconsin) was used according to manufacturer's instructions. Each reaction mixture was covered with 50 μl of mineral oil to prevent evaporation. Presumed viral DNA was amplified in a Coy Thermal Cycler by 30 cycles of melting, annealing, and DNA extension conditions of 1 min at 94 C, 1 min at 50 C, and 3 min at 72 C, or 1 min at 94 C, 2 min at 55 C, and 2 min at 72 C. For the last cycle, the extension time was increased to 3 min, and then the temperature was decreased to 4 C until the reaction mixtures were removed. Amplified DNA

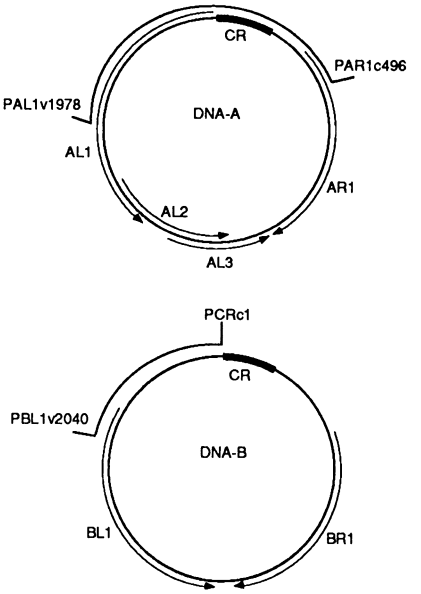

Fig. 1. Diagrammatic representation of the replicative forms for DNA-A (top, 2,650 bp) and DNA-B (bottom, 2,600 bp) of a typical bipartite, whitefly-transmitted geminivirus. Open reading frames (AR1, BR1, etc.) are represented by arrows and the common regions (CR) by a solid bar. The viral DNA region amplified by polymerase chain reaction with the primer pair for DNA-A (PAL1v1978 and PAR1c496) and with the primer pair for DNA-B (PBL1v2040 and PCRc1) are shown.

Table 1. Geographic origin and host symptoms of tissue samples used for polymerase chain reaction

Geminivirus[a]	Host	Family	Location	Symptoms[b]	Collector
TGV-CR	*Lycopersicon esculentum*	Solanaceae	Alajuela, Costa Rica	MY, St, Cr	Authors
TGV-MX1	*L. esculentum*	Solanaceae	Sinaloa, Mexico	MY, St, Cr	M. Stela
TGV-MX2	*L. esculentum*	Solanaceae	Sinaloa, Mexico	MY, St, Cr	M. Stela
TGV-DR	*L. esculentum*	Solanaceae	Azua, Dominican Republic	MY, Cr	A. A. Villar
TGV-TX	*L. esculentum*	Solanaceae	Texas	Mo	J. Amador
TGV-TRD	*L. esculentum*	Solanaceae	Trinidad	Mo	A. A. St. Hill
ToMoV	*L. esculentum*	Solanaceae	Florida	G, Mo, St	E. Vallejos
TYLCV-EG1	*L. esculentum*	Solanaceae	Fayoum, Egypt	LC	M. K. Nakhla
BGMV-CR	*Phaseolus vulgaris*	Leguminosae	Alajuela, Costa Rica	G, Mo	Authors
BCMoV	*P. vulgaris*	Leguminosae	Sonora, Mexico	Mo	J. K. Brown
BCTV	*P. vulgaris*	Leguminosae	California	St, D, Cr	R. L. Gilbertson
SoyGMV	*Glycine max*	Leguminosae	Isabella, Puerto Rico	Mo, G	M. Blair
AbMV-CA	*Abutilon* sp.	Malvaceae	California	Mo	R. L. Gilbertson
CalGMV	*Calopogonium mucunoides*	Leguminosae	Quepos, Costa Rica	G, Mo	Authors
			Tilaran, Costa Rica	G, Mo	Authors
SidGMV	*Sida* sp.	Malvaceae	Alajuela, Costa Rica	G, Mo, St	Authors
MacGMV	*Macroptilium lathyroides*	Leguminosae	San Juan, Dominican Republic	G, Mo	A. Figueroa
LegGV	Legume weed	Leguminosae	Malawi	Mo	R. L. Gilbertson
ACMV-MAL	*Manihot esculenta*	Euphorbiaceae	Malawi	Mo	R. L. Gilbertson
ACMV-SAF	*M. esculenta*	Euphorbiaceae	South Africa	Mo	M. E. C. Rey
PGV-301	*Capsicum frutescens*	Solanaceae	Mexico	MY, Mo, D	M. Stela
PGV-406	*C. frutescens*	Solanaceae	Mexico	MY, Mo, D	M. Stela
RMV	*Rhynchosia minima*	Leguminosae	Puerto Rico	Cr, MY	J. Polston and J. Bird

[a]TGV-CR = tomato geminivirus, Costa Rica; TGV-MX1 and TGV-MX2 = tomato geminivirus, Mexico; TGV-DR = tomato geminivirus, Dominican Republic; TGV-TX = tomato geminivirus, Texas; TGV-TRD = tomato geminivirus, Trinidad; ToMoV = tomato mottle geminivirus, Florida; TYLCV-EG1 = tomato yellow leaf curl geminivirus, Egypt; BGMV-CR = bean golden mosaic geminivirus, Costa Rica; BCMoV = bean calico mosaic geminivirus; BCTV = beet curly top geminivirus; SoyGMV = soybean golden mosaic geminivirus; AbMV-CA = Abutilon mosaic geminivirus, California; CalGMV = Calopogonium golden mosaic geminivirus; SidGMV = Sida golden mosaic geminivirus; MacGMV = Macroptilium golden mosaic geminivirus; LegGV = legume geminivirus; ACMV-MAL = African cassava mosaic geminivirus, Malawi; ACMV-SAF = African cassava mosaic geminivirus, South Africa; PGV-301 = pepper geminivirus; PGV-406 = pepper geminivirus; RMV = Rhynchosia mosaic geminivirus.

[b]Cr = crumpling; D = distortion; G = golden mosaic; LC = leaf curling; Mo = mosaic; MY = mild yellowing; St = stunting.

fragments were electrophoresed in 0.7% agarose (FMC, Rockland, Maine) minigels in TBE buffer (Tris-borate EDTA) and visualized with ultraviolet light after staining in ethidium bromide.

Design of PCR-oligonucleotide primers. Degenerate primers for whitefly-transmitted geminiviruses (Table 2) were designed to anneal to highly conserved nucleotide sequence regions of the open reading frames (ORFs) or the common region of DNA-A and DNA-B. A degenerate primer is a mixture of molecules in which the nucleotides at one or more defined positions vary by design. The degenerate number for a primer is the product of all the numbers that designate how many nucleotides may occur at each position in that primer. The degenerate number for each primer was equal to or less than 16. Highly conserved regions were identified for primer design by aligning derived amino acid and/or nucleotide sequences of at least 10 of the characterized whitefly-transmitted geminiviruses (Table 3) and determining consensus sequences. The primers were synthesized as 17–20 mers, excluding the four nucleotides and a *Pst*I site, which were tailored into the 5′ end of some primers.

The name of a primer (P) includes its location in the genome (e.g., AL1 ORF), a designation of viral or complementary sense sequence (v or c), and a number that is the position of the nucleotide in the genome of BGMV-GA (GenBank accession nos. M91604 for DNA-A and M91605 for DNA-B) that corresponds to the 3′ nucleotide of the primer (e.g., PAL1v1978). Viral sense (v) refers to the sequence of the single-stranded DNA as it is found in the virion (42).

For DNA-A, primers PAL1v1978 and PAR1c496 were designed to anneal within the AL1 ORF, which codes for a replication-associated protein (10), and the AR1 ORF, which encodes for the coat protein (25), respectively (Fig. 1; Table 2). Primer PAL1v1978 was designed to anneal to the complementary sense strand of the replicative form of AL1 sequence encoding the derived amino acid sequence ThrGlyLysThrMetTrpAla, which is a conserved, putative NTP-binding site present in viral replication-associated proteins (17,20). Primer PAR1c496 was designed to anneal to the viral sense strand of the AR1 ORF sequence encoding for the conserved, derived amino acid sequence ProMetTyrArgLysProArg, which is located near the amino terminus of the coat protein (Fig. 1).

Four degenerate primers designed to anneal to the common region were determined from DNA-A common region nucleotide sequence alignments, which were adjusted to maximize nucleotide identities among eight geminiviruses for the Western Hemisphere (Fig. 2). Two of the primers were designed to anneal to the viral sense strand and two to the complementary sense strand (Fig. 2). Primers PCRv19 and PCRc1 (Table 2) were designed to anneal near the 5′ end of the common region, which contains the predicted start codon for AL1. Primers PCRc154 and PCRv181 were designed to anneal in the stem-loop region, which is conserved in all geminiviruses (27).

A degenerate primer for DNA-B, PBL1v2040 (Table 2), was designed to anneal to the complementary sense strand of the replicative form of the BL1 ORF sequence encoding the amino acid sequence CysMetLysIleAspHisCys, located in the amino terminus region of the predicted protein of the BL1 ORF. PBL1v2040 could be paired with either of the common region complementary sense primers (PCRc1 or PCRc154) to amplify a DNA-B fragment (Fig. 1).

Characterization of PCR-amplified fragments. Molecular biological tech-

Table 2. Sequences of the oligonucleotide primers

Primer[a]	Nucleotide sequence
PAL1v1978	5′ GCATCTGCAGGCCCACATYGTCTTYCCNGT 3′
PCRv19	5′ TGGCATWYTYGTAAATATG 3′
PCRc1	5′ CTAGCTGCAGCATATTTACRARWATGCCA 3′
PCRc154	5′ GGTAATATTATAHCGGATGG 3′
PCRv181	5′ TAATATTACCGGWTGGCC 3′
PAR1c496	5′ AATACTGCAGGGCTTYCTRTACATRGG 3′
PAR1c485	5′ CATGCTGCAGTACATYGGCCTYTTDACCC 3′
PBL1v2040	5′ GCCTCTGCAGCARTGRTCKATCTTCATACA 3′

[a]Primer nomenclature is coded as follows: P = primer; AR1 = open reading frame (ORF) for AR1, AL1 = ORF for AL1, BL1 = ORF for BL1, or CR = common region; v = viral sense primer (anneals to complementary sense strand of the replicative form and gives viral sense sequence) or c = complementary sense primer (anneals to viral sense strand and gives complementary sense sequence); number = nucleotide number for bean golden mosaic geminivirus from Guatemala for the 3′ nucleotide of the primer. Nucleotides added for the *Pst*I restriction site are underlined, and the four nucleotides 5′ of this restriction site were added to facilitate restriction endonuclease activity. Nucleotides at degenerate positions are represented by a single letter of the IUPAC ambiguity code: D = A,G,T; H = A,C,T; K = G,T; M =A,C; N = A,C,G,T; R = A,G; W = A,T; Y = C,T.

Table 3. Whitefly-transmitted geminiviruses that have been cloned and sequenced

Geminivirus[a]	Host	Geographic location	Reference
ACMV	*Manihot esculenta*	Kenya	42
TGMV-BZ	*Lycopersicon esculentum*	Brazil	19[b]
ToMoV	*L. esculentum*	Florida	15
BGMV-PR	*Phaseolus lunatus*	Puerto Rico	22[b]
BGMV-GA	*Phaseolus vulgaris*	Guatemala	J. C. Faria et al, *unpublished*
BGMV-DR	*P. vulgaris*	Dominican Republic	J. C. Faria et al, *unpublished*
BGMV-BZ	*P. vulgaris*	Brazil	R. L. Gilbertson et al, *unpublished*
BDMV	*P. vulgaris*	Colombia	S. H. Hidayat et al, *unpublished*
SqLCV	*Cucurbita pepo*	California	28
$AbMV_a$	*Abutilon sellovianum*	West Indies	11[c]
PYMV	*Solanum tuberosum*	Venezuela	7
TYLCV-ISR	*L. esculentum*	Israel	31

[a]ACMV = African cassava mosaic geminivirus; TGMV = tomato golden mosaic geminivirus; ToMoV = tomato mottle geminivirus; BGMV = bean golden mosaic geminivirus; BDMV = bean dwarf mosaic geminivirus; SqLCV = squash leaf curl geminivirus; $AbMV_a$ = Abutilon mosaic geminivirus; PYMV = potato yellow mosaic geminivirus; TYLCV = tomato yellow leaf curl geminivirus. Suffixes indicate place of origin.

[b]Corrected nucleotide sequences for the BL1 ORF of TGMV-BZ (44) and for AR1 ORF of BGMV-PR (A. Howarth, *personal communication*).

[c]The nucleotide sequences for the AL1 and AR1 ORFs of AbMV were assumed to be incorrect because of major differences in the derived amino acid sequences between those for AbMV and the other whitefly-transmitted geminiviruses. For the AR1 ORF, the A at nt 360 was assumed to be a G and this resulted in a start codon position similar to those for other whitefly-transmitted geminiviruses. For the AL1 ORF, two nucleotides (N) were inserted between nt 2010 and nt 2011 and one nucleotide (N) between nt 2093 and nt 2094. These changes in AL1 caused coding frame shifts and resulted in derived amino acid sequences which were consistent with the derived amino acids for the AL1 for other whitefly-transmitted geminiviruses from the Western Hemisphere (36).

niques were performed according to published protocols (29). For Southern hybridization analysis, DNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, New Hampshire), and Southern blots were hybridized with a radiolabeled general DNA probe for whitefly-transmissible geminiviruses (14). Denaturing and hybridization conditions were as described (14). For restriction fragment length polymorphism (RFLP) analysis or cloning, PCR-amplified DNA was precipitated with ethanol and dissolved in TE (Tris-EDTA) buffer. For RFLP analysis, the DNA was digested with selected restriction enzymes, electrophoresed in 0.7% agarose gels in TBE, and visualized with ultraviolet light after staining in ethidium bromide. For cloning PCR fragments, DNA was digested with *Pst*I restriction endonuclease, the site for which had been engineered into the 5′ ends of the PCR primers, and electrophoresed in 0.7% agarose in Tris-acetate-EDTA buffer. The PCR-amplified DNA band was excised from a gel stained with ethidium bromide, recovered with glass matrix (Gene Clean, BIO 101, La Jolla, California) according to the supplier's instructions, and ligated into pBluescript II KS+ (Stratagene, La Jolla, California) digested with the appropriate restriction enzyme. The DNA sequences were determined by the dideoxynucleotide chain termination method using Sequenase (United States Biochemical Corp., Cleveland, Ohio). DNA sequences were analyzed with the software of the Genetics Computer Group, University of Wisconsin-Madison (9).

RESULTS

Evaluation of primer pairs for amplification of geminivirus DNA. The most consistent amplification of a DNA-A fragment was obtained with the primer combination of PAL1v1978 and PAR1c496 (Table 4). As predicted from the annealing position of these primers with the DNA-A of BGMV-GA, PCR-amplified fragments of about 1.1 kb were obtained from DNA extracts of beans infected with the previously characterized geminiviruses, BGMV-GA (Fig. 3), BGMV-DR, BGMV-BZ, and BDMV. These fragments are predicted to include part of AL1 and AR1 and the entire common region. A 1.1-kb fragment was also amplified from beans infected with bean calico mosaic geminivirus (BCMoV) from Mexico (5). To determine if these primers could be used to amplify a similar-sized fragment from undescribed geminiviruses, leaf samples from bean, soybean, pepper, tomato, and weeds suspected to be infected with geminiviruses were collected in the field and tested for the presence of geminivirus nucleic acids by squash blot hybridization with a general geminivirus DNA probe (14). Samples positive for geminivirus were used for PCR, and a 1.1-kb fragment was amplified from these samples (Fig. 3), including tomato from Trinidad, the Dominican Republic, Costa Rica, Mexico, Texas, and Florida; bean from Costa Rica; soybean from Puerto Rico; pepper from Mexico; and three weeds, *Sida* sp. and *Calopogonium mucunoides* Desv. from Costa Rica, and *Macroptilium lathyroides* (L.) Urban from the Dominican Republic. The versatility of this primer pair was further evaluated using geminivirus-infected samples from Africa. A 1.4-kb fragment was obtained from cassava samples from South Africa (S. H. Hidayat et al, *personal communication*) and Malawi, from an unidentified legume weed from

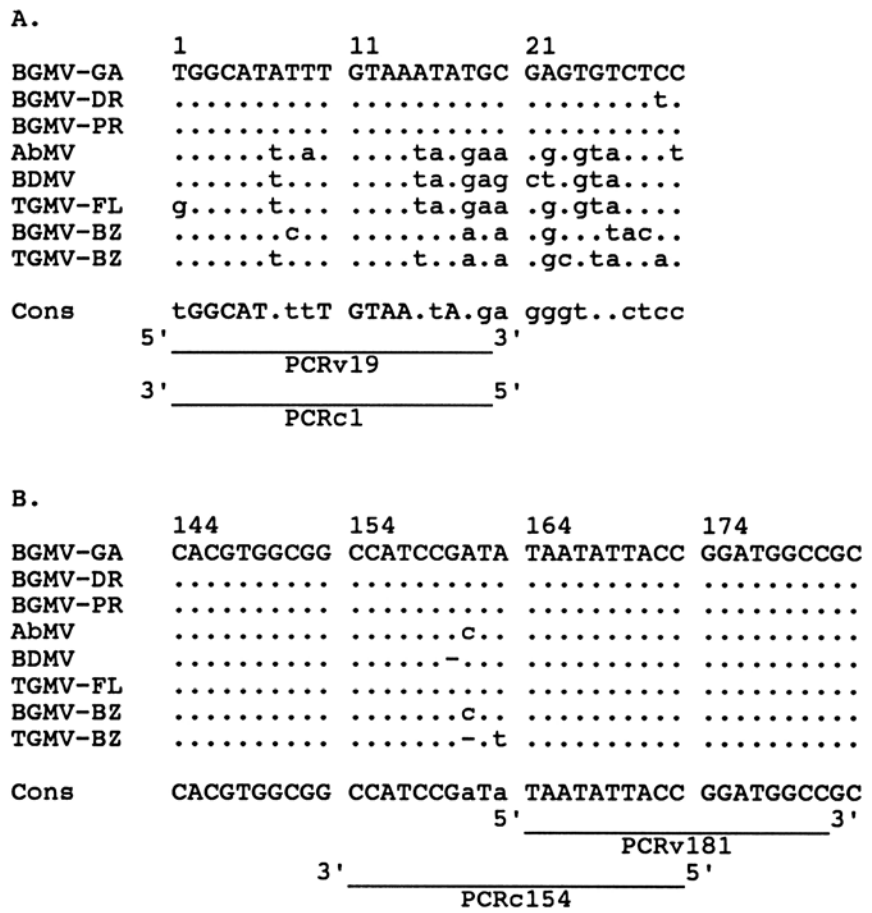

Fig. 2. Alignment of the DNA sequences of the common region for eight bipartite, whitefly-transmitted geminiviruses. Nucleotide sequences differing from that of the bean golden mosaic geminivirus (BGMV-GA) are shown as lower-case letters, gaps are indicated as a dash, and the identical nucleotides as a period. **(A)** Sequences used to design primers PCRv19 and PCRc1. **(B)** Sequence in the stem-loop region used to design primers PCRv181 and PCRc154. Nucleotide sequences used to design primers are underlined. Abbreviations for geminiviruses are given in Table 3. Cons = consensus sequence; lower-case letters represent the nucleotides for the majority of geminiviruses; upper-case letters indicate that all geminiviruses have the same nucleotide; a period indicates no consensus.

Table 4. Fragment size (kb) obtained with different primer pairs in the polymerase chain reactions from five field samples infected with uncharacterized geminiviruses and beans infected with BGMV

	Primer Pairs				
Geminivirus[a]	PAL1v1978 PAR1c496	PAL1v1978 PCRc1	PAL1v1978 PAR1c485	PAL1v1978 PCRc154	PCRv181 PAR1c485
SoyGMV	1.1	0.7	1.1, 0.4	0.8	0.35
BGMV-GA	1.1	0.7	1.1	0.8	0.35
TGV-MX1	1.1	0.7	0.8	0.8	0.35
BCTV	...[b]	0.7	...	0.8	...
TYLCV-EG1	1.4	0.8	1.4	...	...
LegGV	1.4	0.8	0.5	...	...

[a] SoyGMV = soybean golden mosaic geminivirus; BGMV-GA = bean golden mosaic geminivirus, Guatemala; TGV-MX = tomato geminivirus, Mexico; BCTV = beet curly top geminivirus; TYLCV-EG1 = tomato yellow leaf curl geminivirus, Egypt; LegGV = legume weed geminivirus, Malawi.
[b] Not tested.

Malawi, and from tomatoes infected with TYLCV from Egypt (30). In most, a single DNA fragment of the expected size of 1.1 or 1.4 kb was amplified (Fig. 3). In some samples, however, a second fragment of ≅2.5 kb at a lower concentration was observed. This 2.5-kb fragment did not hybridize with the general geminivirus probe (14), and therefore it was not considered to be a virus fragment. No PCR-DNA fragments were amplified from extracts from healthy bean or tomato with any primer pair.

Because the putative NTP-binding site and the stem-loop regions of whitefly-transmitted geminiviruses are also present in leafhopper-transmitted, monopartite geminiviruses (20,27,43), the primer pairs that anneal to the AL1 ORF (PAL1v1978) and the common region (PCRc1 or PCRc154) were tested with an extract from a field sample of beans from California with beet curly top symptoms. Fragments of 0.7 kb and 0.8 kb were obtained (Table 4). Squash blots of the infected bean tissue and Southern blots of the amplified fragments hybridized with a specific probe for beet curly top geminivirus (BCTV) under conditions of high stringency.

PCR-amplified DNA-B fragments of the predicted ≅0.6 kb were obtained from the three BGMV isolates and BDMV (Fig. 4, Table 4) with the primer pair PBL1v2040 and PCRc1. These primers were then used to amplify DNA-B fragments from uncharacterized geminiviruses, including Rhynchosia mosaic geminivirus (RMV), and tomato-infecting geminiviruses from Mexico (33) and Florida (tomato mottle geminivirus, ToMoV; 1,15) (Fig. 4). In some, different sizes of DNA-B fragments were amplified (Fig. 4); for example, the fragment amplified from BGMV-DR was larger than those from BDMV and the tomato-infecting geminiviruses (Fig. 4). To confirm the identity of the putative DNA-B fragments, Southern hybridization analyses were carried out under low stringency conditions with a probe consisting of DNA-B clones of BGMV-GA, BGMV-BZ, and BDMV. Because these primers are expected to amplify only a portion of DNA-B, including part of BL1 and the hypervariable region (16) but not the common region, any hybridization would be specific for DNA-B. All but one of the amplified DNA-B fragments hybridized strongly with the DNA-B probe (Fig. 4). The DNA-B fragment from TGV-MX1 hybridized only weakly; therefore, this portion of its nucleotide sequence may be different from the corresponding sequences of the geminiviruses used in the probe. The strong hybridization of the probe with ToMoV from Florida was due to the high level of sequence similarity between ToMoV and BDMV (15).

RFLP analysis of PCR-amplified fragments. PCR fragments generated with the same primer pair from different plant samples were digested with restriction enzymes to detect polymorphisms. The 1.1-kb DNA-A fragments amplified with PAL1v1978 and PAR1c496 from extracts of plants infected with ToMoV, TGV-MX1, a tomato-infecting geminivirus from Costa Rica (TGV-CR), a pepper-infecting geminivirus from Mexico (MX-P2), BDMV, or BGMV-DR were separately digested with the restriction endonucleases, *Bgl*II, *Hin*cII, *Sal*I, and *Rsa*I. Only the BGMV-DR fragment was cleaved with *Sal*I. *Bgl*II digestions resulted in three RFLP patterns (*unpublished*). One RFLP pattern (0.3- and 0.8-kb fragments) was found for BDMV, ToMoV, and BGMV-DR and another for MX-P2 (0.5- and 0.6-kb fragments), whereas *Bgl*II did not digest fragments from TGV-MX1 and TGV-CR. RFLP patterns resulting from *Hin*cII digestions distinguished TGV-MX1 and TGV-CR (Fig. 5), and different *Hin*cII RFLP patterns were found among the PCR fragments for BDMV, BGMV-DR, ToMoV, and MX-P2 (Fig.

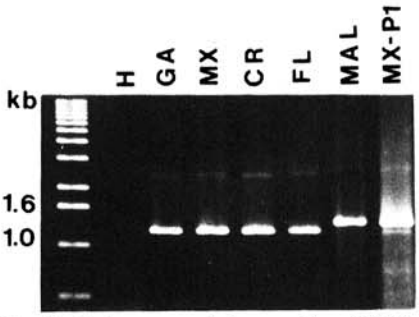

Fig. 3. Polymerase chain reaction (PCR) amplification of a ≅1.1-kb DNA fragment with the PCR primer pair for DNA-A (PAL1v1978 and PAR1c496) from seven different plant samples. A 2-μl aliquot of the 100-μl reaction mixture was analyzed in a 0.7% agarose gel. Plant samples: H = healthy bean; GA = bean infected with bean golden mosaic geminivirus from Guatemala; MX = tomato infected with a geminivirus from Mexico (TGV-MX1); CR = tomato infected with a geminivirus from Costa Rica; FL = tomato infected with tomato mottle geminivirus isolate FL-B1 from Florida; MAL = cassava infected with a geminivirus from Malawi; and MX-P1 = pepper infected with a pepper geminivirus isolate P1 from Mexico. Left lane = 1 kb ladder size marker.

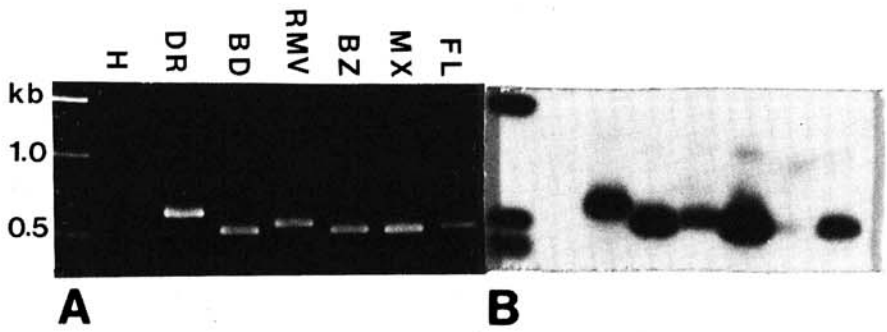

Fig. 4. Polymerase chain reaction (PCR)-amplified products for **(A)** DNA-B of six geminiviruses and **(B)** Southern blot DNA hybridizations. **(A)** Amplification of 0.5- to 0.65-kb fragments with the primer pair PBL1v2040 and PCRc1. A 2-μl aliquot of each PCR reaction mixture was analyzed in a 0.7% agarose gel. Left lane = 1 kb ladder size marker. Plant samples are: H = healthy bean; DR = bean infected with bean golden mosaic geminivirus (BGMV) from the Dominican Republic; BD = bean infected with bean dwarf mosaic geminivirus (BDMV); RMV = bean infected with Rhynchosia mosaic geminivirus; BZ = bean infected with BGMV from Brazil; MX = tomato infected with a geminivirus from Mexico (TGV-MX1); and FL = tomato infected with tomato mottle geminivirus isolate FL-B1 from Florida. **(B)** Southern blot hybridization of the agarose gel in **(A)** (lanes in the same order). The blot was probed with a mixture of DNA-B recombinant plasmids of BGMV from Guatemala and Brazil and BDMV at low stringency.

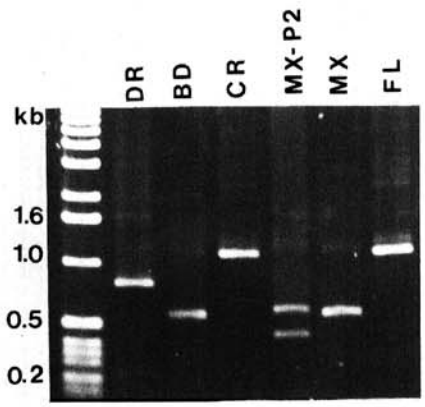

Fig. 5. Restriction fragment length polymorphism patterns of the 1.1-kb DNA-A fragments amplified by polymerase chain reaction with the primer pair PAL1v1978 and PAR1c496 for six geminiviruses. DNA fragments were digested with the *Hin*cII restriction endonuclease and electrophoresed in a 0.7% agarose gel. Plant samples: DR = bean infected with bean golden mosaic geminivirus from the Dominican Republic; BD = bean infected with bean dwarf mosaic geminivirus; CR = tomato infected with a geminivirus from Costa Rica; MX-P2 = pepper infected with pepper geminivirus isolate P2 from Mexico; MX = tomato infected with a geminivirus from Mexico (TGV-MX1); FL = tomato infected with tomato mottle geminivirus isolate FL-B1 from Florida. Left lane = 1 kb ladder size marker.

5). PCR fragments from TGV-CR and ToMoV were not digested with *Hin*cII. Also, the *Hin*cII digestions of the PCR fragments from BDMV and TGV-MX1 gave the same size fragment (≅0.5 kb). *Rsa*I cleaved the DNA-A fragments from all six geminiviruses, resulting in a different RFLP pattern for each geminivirus (Fig. 6).

Detection of mixed geminivirus infections. To determine if a mixed infection could be detected by restriction enzyme analysis of DNA-A fragments amplified with the primer pair PAL1v1978 and PAR1c496, beans (cv. Topcrop) were coinoculated with two distinct geminiviruses, BDMV and BGMV-DR. For control treatments, beans were mock-inoculated or inoculated with either BDMV or BGMV-DR. Seven days after inoculation, typical leaf symptoms developed on beans inoculated with BDMV (stunting, distortion, and downcupping) or BGMV-DR (golden mosaic), whereas beans inoculated with both BDMV and BGMV-DR developed both golden mosaic and downcupping symptoms. Two-week-old, infected trifoliolate leaves were collected, DNA extracted, and PCRs were carried out. Because only BGMV-DR has a *Sal*I site within the 1.1-kb DNA-A fragment, *Sal*I digests were used to distinguish BGMV-DR from BDMV. The 1.1-kb DNA-A fragments amplified from beans infected with BGMV-DR were cleaved into the expected 0.3- and 0.8-kb fragments, whereas DNA-A fragments from BDMV-infected plants were not cleaved (Fig. 7). When the DNA-A fragments from beans inoculated with BGMV-DR and BDMV were digested with *Sal*I, three DNA bands of 1.1, 0.8, and 0.3 kb were observed. The sum of these three bands equaled 2.2 kb; therefore a mixed infection of BDMV and BGMV-DR was confirmed (Fig. 7). If only one geminivirus had been present, the expected sum of the bands would have been 1.1 kb.

Using the same primers and *Pst*I digestions, a mixed geminivirus infection was detected in a field sample of tomato from Mexico (TGV-MX2, Table 1). When the 1.1-kb DNA-A fragments amplified from this sample were digested with *Pst*I, three fragments of 1.1, 0.6, and 0.5 kb were observed (Fig. 8). To eliminate the possibility of an incomplete *Pst*I digestion and to further characterize these fragments, each fragment was excised from an agarose gel, purified, cloned, and partially sequenced. The nucleotide sequence comparison of the common regions and part of the intergenic region of these fragments showed only 82% identity (Fig. 9), so this was probably a mixed infection.

DISCUSSION

Degenerate PCR primers for amplification of portions of the DNA-A or DNA-B components of whitefly-transmitted geminiviruses were designed from highly conserved regions of the viral genome identified from nucleotide and/or amino acid sequence alignments. Some of these conserved regions may have functional significance (e.g., NTP-binding site of AL1 and the stem-loop of the common region); therefore, these primers should have general application for the amplification of DNA-A or DNA-B fragments from a wide range of whitefly-transmitted geminiviruses. This was shown by amplification of fragments from numerous samples from the Americas and Caribbean Basin as well as from four samples from Africa. When the general primer pair for the AL1 ORF (PAL1v1978) and the AR1 ORF (PAR1c496) was used, 1.1- and 1.4-kb fragments were obtained from geminivirus-infected samples from the Western Hemisphere and Africa, respectively. The larger PCR fragments from the African geminiviruses are consistent with the larger DNA-A for these geminiviruses from the Eastern Hemisphere (31,42). Furthermore, one primer pair (PAL1v1978 and PCRc1) was effectively used to amplify fragments from the highly divergent, monopartite, leafhopper-transmitted BCTV (23) and, thus, might also serve as general primers to amplify fragments of monopartite, leafhopper-transmitted geminiviruses infecting monocotyledonous plants.

The present methods for molecular characterization of whitefly-transmitted geminiviruses involve laborious DNA extraction and cloning procedures (22). The PCR methods presented here will facilitate molecular characterization of this diverse group. In this study, geminiviral DNA fragments were amplified from a number of crop plants and weeds. The *Pst*I sites engineered into the 5′ ends of the primers make PCR-amplified fragments readily available for cloning and sequencing. This has been recently done for 11 uncharacterized gemini-

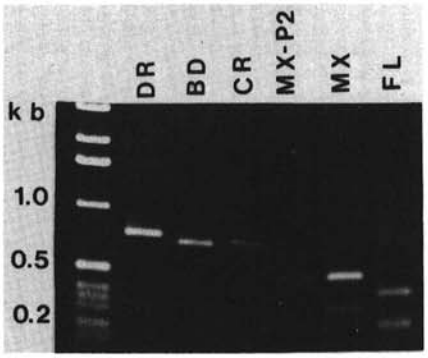

Fig. 6. Restriction fragment length polymorphism patterns of the 1.1-kb DNA-A fragment amplified by polymerase chain reaction with the primer pair PAL1v1978 and PAR1c496 for six geminiviruses. DNA fragments were digested with *Rsa*I restriction endonuclease and electrophoresed in a 0.7% agarose gel. Plant samples: DR = bean infected with bean golden mosaic geminivirus from the Dominican Republic; BD = bean infected with bean dwarf mosaic geminivirus; CR = tomato infected with a geminivirus from Costa Rica; MX-P2 = pepper infected with pepper geminivirus isolate P2 from Mexico; MX = tomato infected with a geminivirus from Mexico (TGV-MX1); FL = tomato infected with tomato mottle geminivirus isolate FL-B1 from Florida. Left lane = 1 kb ladder size marker.

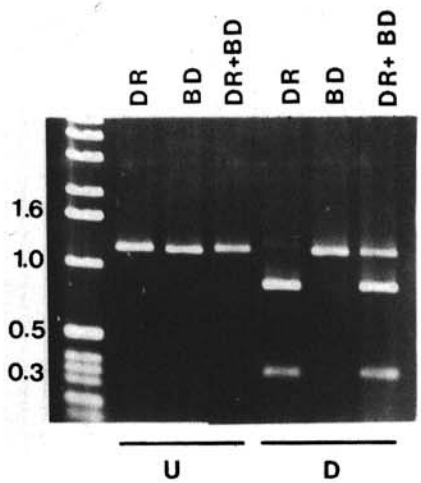

Fig. 7. Detection of a mixed geminivirus infection of beans by restriction enzyme digestion of the 1.1-kb DNA-A fragment amplified using the polymerase chain reaction (PCR) and the primer pair PAL1v1978 and PAR1c496. PCR-amplified fragments from infected plant samples: DR = bean golden mosaic geminivirus from the Dominican Republic, BD = bean dwarf mosaic geminivirus, DR + BD = mixed infection of both geminiviruses. U = PCR-amplified fragments not digested with a restriction enzyme. D = PCR-amplified fragments digested with *Sal*I. From sequence data (Table 3), it was predicted that a *Sal*I site was present in the PCR fragment from DR and not in that from BD. Left lane = 1 kb ladder size marker.

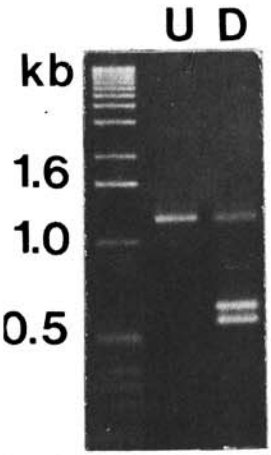

Fig. 8. Detection of a mixed geminivirus infection in field-collected tomato from Mexico (TGV-MX2, Table 1) by restriction enzyme digestion of the 1.1-kb DNA-A fragment amplified using the polymerase chain reaction and the primer pair PAL1v1978 and PAR1c496. The polymerase chain reaction-amplified fragments were digested (D) with the *Pst*I restriction endonuclease or not digested (U). Left lane = 1 kb ladder size marker.

viruses from weeds and crop plants from Central America, the Caribbean Basin, and Africa (36). The cloned PCR fragments can also be used as DNA probes. Application of these PCR techniques alone or in combination with other methods such as DNA probes, RFLP analyses, sequencing of PCR fragments, and serological methods will greatly enhance our understanding of geminivirus epidemiology and etiology.

Whereas DNA sequencing of PCR-amplified fragments is the most precise method for identifying geminiviruses, other approaches can be used to characterize these fragments. Fragment size can be different, as was demonstrated for DNA-A fragments of Eastern versus Western Hemisphere geminiviruses, and for the DNA-B fragment of BGMV-DR versus those of the tomato-infecting geminiviruses. Southern hybridization analysis of PCR-amplified fragments with cloned geminiviral DNA components as probes is another method of characterization. For example, the DNA-B fragment amplified from TGV-MX1 hybridized weakly with the cloned DNA-B components of bean-infecting geminiviruses, whereas the fragment from ToMoV hybridized strongly with the same probe. This indicates that the nucleotide sequence of TGV-MX1 is highly divergent from those of the other geminiviruses. An easier method for characterizing PCR-amplified fragments was RFLP analysis. By digesting PCR-amplified fragments with one or more restriction endonucleases, known geminiviruses were distinguished from uncharacterized geminiviruses. The clearest differences were obtained with enzymes that have multiple recognition sites, e.g., *Hin*cII or endonucleases that recognize four-nucleotide sequences (*Rsa*I). RFLP analysis could be combined with DNA-DNA hybridizations with nonradioactive geminiviral probes as an effective diagnostic tool. A general probe for whitefly-transmitted geminiviruses and/or isolate-specific probes could be used (14,34). Haber et al (18) showed that RFLP and Southern blot analyses were useful techniques for differentiating geminiviruses infecting several plants collected in Puerto Rico.

One important application of the PCR method and RFLP analysis was the demonstration of mixed geminivirus infection. The presence of two geminivirus strains in a single tomato plant from Mexico was shown in this study. DNA sequence analysis of the cloned PCR-amplified fragments clearly demonstrated that two geminivirus strains, TGV-MX1 and TGV-MX2, were present in this plant (36; E. J. Paplomatas et al, *personal communication*). A mixed geminivirus infection was previously detected in infected squash plants using strain-specific probes for two squash leaf curl geminivirus strains (34). Mixed infections may prove to be common in field-infected plants; thus, their detection and characterization will be one of the important applications of the PCR techniques.

Added in galley: PAR1c715 (5′ GATTTCTGCAGTTDATRTTYTCRTCCATCCA 3′) was designed to anneal to the viral sense strand of AR1 ORF and was used effectively with PAL1v1978 to amplify ~1.6-kb DNA-A fragments from geminivirus-infected tomatoes from India and Taiwan (B.-T. Chiange et al, *personal communication*). PCRc2 (deletion of the 3′ A from PCRc1) was used with PBL1v2040 to amplify DNA-B fragments from geminivirus-infected tomatoes, beans, and melons from Central America (M. K. Nakhla et al, *personal communication*).

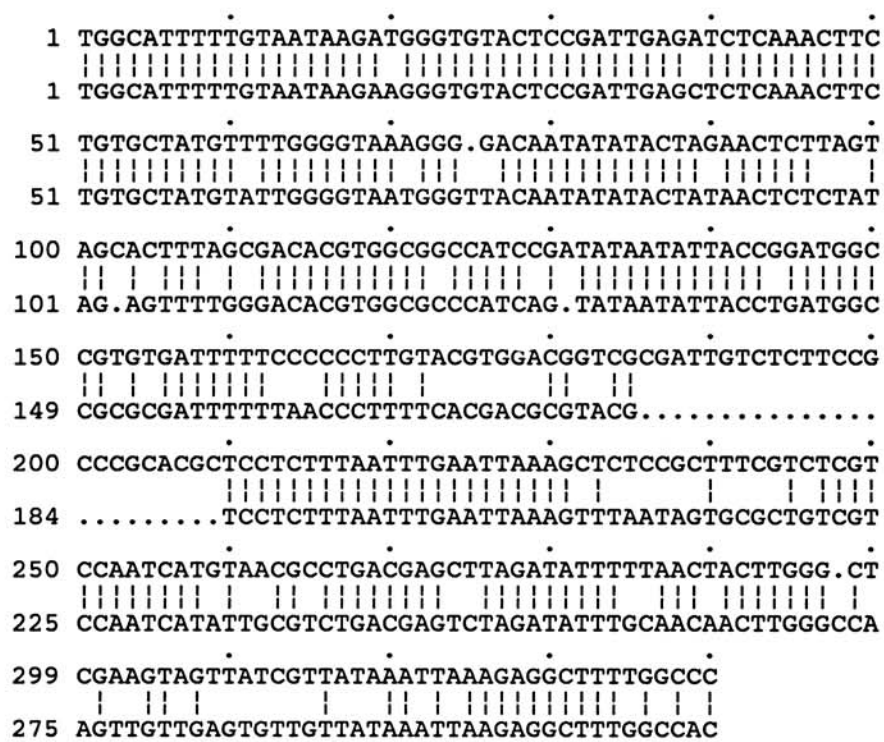

Fig. 9. Nucleotide sequence alignments of the common region and part of the intergenic region (Fig. 1) for the cloned geminiviral DNA fragments amplified from a field-collected tomato sample from Mexico (Fig. 8) using polymerase chain reaction and the primer pair PAL1v1978 and PAR1c496. First row in pair: TGV-MX1, tomato geminivirus isolate 1 recombinant plasmid, pTMX1. Second row: TGV-MX2, tomato geminivirus isolate 2 recombinant plasmid, pTMX2. Nucleotide 1 represents the presumed start of the common region. The conserved stem-loop region is from nt 120 to nt 152 for TGV-MX1. The end of the common region and the start of the intergenic region is estimated to be near nt 160 for TGV-MX1. Note the large gap (25 nt) in the intergenic region for TGV-MX2 starting at nt 185 for TGV-MX1.

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