Inoculation of Peppers with Infectious Clones of a New Geminivirus by a Biolistic Procedure

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ABSTRACT

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Several new viral diseases have appeared in almost all horticultural areas in México. Transmission of their causal agents by whiteflies and host-range data suggest the involvement of geminiviruses in most cases. One example of a severe case is the "rizado amarillo" disease affecting peppers in northern México (Tamaulipas State). The relationship of this disease to the tigré disease previously reported by Brown et al (7) is as yet unclear. Here we report the cloning and partial molecular characterization of the genome of a bipartite geminivirus associated with pepper rizado amarillo disease. The virus is tentatively named pepper huasteco virus (PHV). The cloned viral DNAs were infectious when inoculated by bombardment into pepper plants. The bombardment was accomplished using tungsten particles coated with DNA from both genomic components. The particles were delivered using a helium pressure-based apparatus (biolistic procedure). Replication of the viral DNA in plants was confirmed by Southern analysis and polymerase chain reaction (PCR) amplification of the coat-protein gene. Plants inoculated with either the A (plasmid pIGV22) or B (plasmid pIGV21) component alone did not develop any visible symptoms, and viral DNA was not detected by molecular hybridization. The advantages of this new inoculation procedure are discussed.

Previously unrecognized viral diseases have appeared in almost all agricultural production areas in México during the last 3–4 yr. Their etiologies remain unknown, although they share characteristics, such as transmission by whiteflies (*Bemisia tabaci* Genn.) (1,2). In 1989, two geminiviruses were reported infecting peppers (*Capsicum annuum* L.) affected with "tigré" disease in Tamaulipas, México (5). The first nonmechanically transmissible virus was tentatively called pepper mild tigré virus (PMTV). The second virus was the previously described chino del tomate virus (CdTV) (3,4). In an attempt to obtain more information about these new diseases, we characterized the causal agent(s) of a disease reported as "rizado amarillo" in peppers in the same geographical area (Tamaulipas, northern México). In a preliminary report, we suggested that the causal agent for rizado amarillo disease was a nonmechanically transmissible geminivirus (11), and more recently, we obtained evidence of the involvement of more than one geminivirus in rizado amarillo disease (J. A. Garzón and R. F. Rivera-Bustamante, *unpublished data*). The lack of mechanical transmission of the viruses associated with this complex eliminates the involvement of serrano golden mosaic virus (SGMV) (6) and Texas pepper geminivirus (TPGV) (22), both of which are mechanically transmitted. In addition, the involvement of CdTV, which infects beans, in rizado amarillo disease also is eliminated because no symptoms were observed in bean plants after inoculation with viruliferous whiteflies (4).

Geminiviruses are a group of plant viruses that contain single-stranded DNA (ssDNA) genomes. During infection, viral dsDNA (RF-DNA) is produced; this characteristic has been exploited to facilitate molecular characterization. Most geminiviruses can be classified in three subgroups, according to their host range, insect vector, and genome organization. The first group is composed of geminiviruses that infect monocots, are transmitted by leafhoppers, and possess only one ssDNA molecule (monopartite). The second group contains geminiviruses that infect dicots, are transmitted by leafhoppers, and are monopartite. The third group includes geminiviruses that are transmitted by whiteflies, infect dicots, and have a genome divided into two ssDNA molecules (bipartite) (8,17). An interesting exception is tomato yellow leaf curl geminivirus (TYLCV) from Israel and Sardinia, which is monopartite and is transmitted by whiteflies (15,18).

The lack of mechanical transmission of some geminiviruses is one of the major experimental constraints of investigating these plant pathogens. Inoculation of geminiviruses with *Agrobacterium tumefaciens* (agroinoculation) provides a useful tool for studying geminiviruses (10,13). This procedure, however, is cumbersome when several variants (or mutants) are tested. The two major disadvantages of agroinoculation can be the long period needed for the plant to recover and express symptoms after inoculation and more importantly, the time-consuming subcloning procedures needed to introduce each mutant into the vector, usually in the form of longer than unit-length constructs. Recently, the inoculation of four geminivirus isolates, including one nonmechanically transmissible virus, was reported using electric discharge particle acceleration. This procedure does not present the disadvantages inherent in the agroinoculation method and promises to facilitate geminivirus research (12).

Here we report the cloning and partial characterization of a geminivirus associated with rizado amarillo disease of peppers and the inoculation of infectious clones into plants by a biolistic procedure. Several results indicate that this virus differs from previously reported geminiviruses, and we have tentatively named it pepper huasteco virus (PHV) because it was isolated from the geographical area known as Las Huastecas in northern México. The biolistic procedure is more convenient than the agroinoculation method because the unit-length cloned DNA contained in the plasmids is infectious, eliminating the need for further modification. In addition, using this procedure, it is possible to observe symptom expression as early as 4–5 days after inoculation.

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MATERIALS AND METHODS

Virus isolation. Infected pepper tissue collected from the state of Tamaulipas, México, was used to transmit the geminivirus by grafting it onto pepper (*Capsicum annuum* L. 'Ancho San Luis') and tomato (*Lycopersicon esculentum* Mill. 'Hayslip') plants. The geminivirus-isolation strategy called for the use of whitefly (*Bemisia tabaci*) for disease transmission to eliminate some RNA viruses endemic in the area that are known to infect pepper (e.g., cucumber mosaic virus [CMV], tobacco mosaic virus [TMV], and tobacco etch virus [TEV]) and the use of indicator plants to determine the absence of some whitefly-transmitted RNA viruses (e.g., lettuce infectious yellows virus).

Viral DNA extraction. Viral RF-DNA was extracted by following the procedure described by Hamilton et al (14), which with the proper tissue produces a sample enriched for viral DNA with little host-DNA contamination. Briefly, pepper plants (cv. Ancho San Luis) were inoculated by stem grafting at the four to six true-leaf stage. Apical tissue was collected from plants exhibiting vein-yellowing symptoms typical of rizado amarillo disease. Fresh tissue (20 g) was frozen with liquid nitrogen and was ground in a mortar into a fine, white powder. The powder was resuspended 1:2 (w/v) with extraction buffer (0.5 M potassium phosphate buffer, pH 7, containing 0.75% sodium sulfite and 2.5% Triton X-100) and was gently agitated overnight at 4 C. The suspension was filtered through cheesecloth and clarified at 10,000 *g* for 10 min. The supernatant was centrifuged at 150,000 *g* for 3 h in a Beckman 70Ti rotor. The pellet was resuspended in buffer containing 40 mM Tris, 5 mM acetic acid, 10 mM Na_2 EDTA, pH 8.2, and 0.1% SDS (sodium dodecyl sulfate). This suspension was extracted twice with an equal volume of phenol-chloroform (1:1) and once with an equal volume of chloroform-isoamyl alcohol (14). Nucleic acids in the aqueous phase were precipitated with 2 volumes of cold ethanol and finally, were resuspended in distilled water and analyzed by agarose gel electrophoresis (20).

Cloning and sequencing of viral DNA. Viral RF-DNA extracted from infected pepper tissue was digested with the following restriction enzymes: *Bam*HI, *Pst*I, *Hin*dIII, *Eco*RI, *Xba*I, and *Kpn*I. Viral DNA restricted with *Hin*dIII was ligated into pBluescript SK+ (Stratagene, La Jolla, CA) and was used to transform *Escherichia coli* DH5α. Bacterial colonies with recombinant plasmids were selected in Luria plates with X-Gal and IPTG. Insert sizes were determined by agarose gel electrophoresis of plasmids digested with *Hin*dIII. Several colonies with inserts of approximately 2.6 kb were selected for further analysis. The analyses of the selected recombinant plasmids included restriction-endonuclease mapping performed by single and double digestions with *Bam*HI, *Cla*I, *Dra*I, *Pst*I, *Nru*I, *Hin*dIII, *Hin*cII, *Eco*RI, *Xba*I, *Kpn*I, *Ssp*I, and *Sty*I. Molecular hybridization (Southern blots) of recombinant plasmids against host-plant DNA confirmed the viral nature of the insert DNA. The insert DNAs were hybridized among themselves to evaluate insert relatedness. All standard procedures utilized during the cloning of viral DNA and characterization of the clones were used according to Sambrook et al (20). Hybridizations were conducted at 65 C using ^{32}P-labeled random, primed insert DNAs as probes. Autoradiographs were prepared by exposing Kodak X-Omat film at −70 C with an intensifying screen. Two plasmids, pIGV21 and pIGV22, containing different inserts, both approximately 2.6 kb, were selected for further analysis. The sequences of the extremes of both viral inserts were obtained using a Sequenase kit (version 2.0; United States Biochemicals, Cleveland, OH) according to the manufacturer's instructions.

Infectivity of the cloned viral DNA by a biolistic procedure. Pepper plants at the four-leaf stage were inoculated according to a biolistic procedure using a mixture of pIGV21 and pIGV22 plasmids. Plasmid DNA was deposited on the surface of tungsten microparticles (Sylvania No. 10) according to a previously described procedure (16,21). Microparticles (50 L) were mixed with 5 μl of a DNA solution (1 μg/L), 50 μl of 2.5 M $CaCl_2$, and 20 μl of 0.1 M spermidine. The suspension was vortexed and centrifuged at 10,000 *g* for 10 min, and the pellet containing the microparticles was washed once with cold 70% ethanol and once with absolute ethanol and was resuspended in ethanol for bombardment. The tungsten particles were accelerated by helium pressure at 800 or 1,200 psi using a Du Pont apparatus model (PDS-1000). The inoculated plants were transferred to a growth chamber maintained at 24–32 C for symptom expression.

Southern and PCR analyses. Viral replication in inoculated plants was verified by Southern blot analysis or PCR amplification of the coat-protein gene. Hybridization analyses of DNA extracted from inoculated plants were performed by following standard procedures (20). Samples were probed with labeled DNA excised from plasmid pIGV22 (component A) by *Hin*dIII. The viral insert was separated from plasmid DNA by agarose gel electrophoresis, extracted from the agarose matrix, and labeled by a random-priming procedure using ^{32}P dCTP. For Southern blot analysis, we used either total-plant DNA extracted by a modification of the method described by Dellaporta et al (9) or DNA extracts enriched for viral DNA, obtained as described by Hamilton et al (14). DNA extracts were fractionated by agarose gel electrophoresis and were transferred to nitrocellulose membrane by capillarity (20). PCR amplifications were performed with a GeneAmp kit from Perkin Elmer/Cetus (Norwalk, CT) according to the manufacturer's instructions. Briefly, nucleic acid extracts from inoculated or healthy pepper plants were mixed with the reaction mixture, which included *Taq* enzyme polymerase and oligonucleotides. The oligonucleotide sequences flank the open reading frame corresponding to the coat-protein gene (I. Torres-Pacheco and R. F. Rivera-Bustamante, *unpublished data*). The sequence of the 5′ oligonucleotide (viral) begins 14 nucleotides upstream from the start codon: 5′-ACCTTGAATTCAAAATGCCT-3′. The 3′ oligonucleotide (complementary) begins 22 nucleotides beyond the stop codon: 5′-AATGCTGTACTTGAGAATCA-3′. The samples were incubated in an Easy cycler from Ericomp, Inc. (San Diego, CA) or a thermal DNA cycler from Perkin Elmer for 25–30 cycles. Each cycle consisted of incubations at 95 C

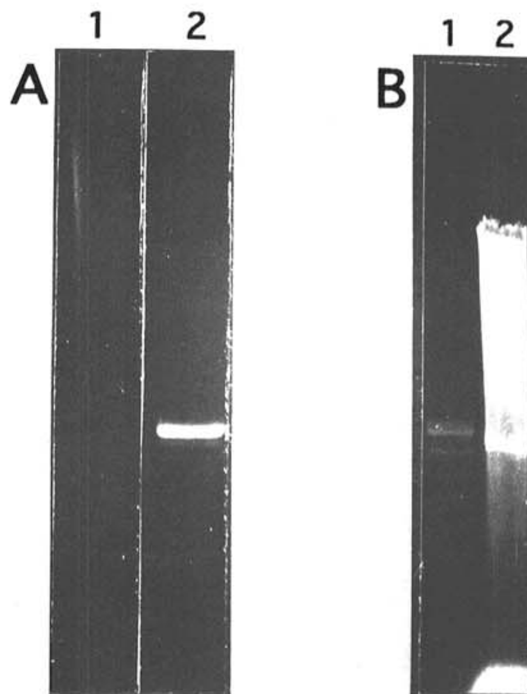

Fig. 1. Analysis of nucleic acids extracted from healthy and rizado amarillo-infected pepper plants. **A**, Lanes 1 and 2 show extracts from healthy and infected pepper tissue, respectively. Viral DNA bands are visible only in infected tissue extracts. **B**, Lanes 1 and 2 show extracts from young tissue at the onset of symptom expression, 12- to 14-days postinoculation (dpi), and from infected tissue, 25–30 dpi. The quality of the extracts changes with the course of the infection. Differences in the pattern are probably the result of changes in the ssDNA/dsDNA relationship.

for 1 min, 37 C for 1 min, and 70 C for 3 min. Amplified samples were analyzed directly by agarose gel electrophoresis and in some cases, were followed by Southern analysis.

Sequence analysis. PHV sequences were analyzed using the GeneWorks software package from Intelligenetics (Mountain View, CA). These geminivirus sequences (with respective accession numbers) were used for comparison and were obtained from the GenBank and EMBL databases: abutilon mosaic virus, (ABMV; AC: X15984 and X15983), tomato golden mosaic virus (TGMV; AC: K02030 and K02029), bean golden mosaic virus (BGMV; D00201 and D00200), and bean dwarf mosaic virus (BDMV; AC: X88180 and X88176).

RESULTS

Viral DNA extraction. As reported for other geminiviruses, the best tissue from which to extract viral DNA was young tissue from recently infected plants (8- to 10-days postinoculation [dpi]). Tissue from older plants or from plants grown for 20–25 dpi produced DNA extracts with heavy host-DNA contamination resulting from extended degradation. Although these preparations were not suitable for cloning purposes, their quality was sufficient for diagnosing field samples using Southern blot and PCR amplification analyses. Figure 1A shows an agarose gel electrophoresis analysis of DNA from healthy and infected pepper tissue (lane 2). Extracts from healthy plants did not contain bands (lane 1). Nuclease treatments demonstrated that both types of viral DNA, single-stranded and double-stranded molecules, were present in the extracts from infected plants (data not shown). Figure 1B shows a comparison of the quality of the DNA extracted at the onset of symptom expression, usually 10–12 dpi (stem grafting; lane 1), and DNA extracted 25–30 dpi (lane 2).

Cloning and characterization of the viral genome. Transmission by whiteflies and a host range restricted to dicots indicated that the geminivirus involved in rizado amarillo disease may be from the bipartite subgroup. Thus, we anticipated the cloning of two DNA fragments in the 2.6-kb range. Restriction of viral DNA extracted from infected tissue by *Hin*dIII appeared to produce linear dsDNA molecules ligated into the plasmid Bluescript, and the inserts were characterized by agarose gel electrophoresis. After restriction-enzyme mapping, the 2.6-kb inserts were easily classified into the two putative genomic components based on their restriction enzyme patterns with *Eco*RI and *Bam*HI. One plasmid from each group was selected and named pIGV21 and pIGV22, respectively. Figure 2A shows agarose gel electrophoresis analysis of plasmids pIGV21 and pIGV22 digested with several restriction enzymes. Viral inserts were first released with *Hin*dIII and then digested with a second enzyme. In some cases, the viral DNA did not have a specific restriction site for the second enzyme (e.g., pIGV22 with *Bam*HI). The restriction mapping confirmed that the viral DNA inserts in plasmids pIGV21 and pIGV22, although similar in size, are different. Figure 2B presents a more detailed restriction map for each insert. The dissimilarity of the inserts was corroborated by Southern blot hybridization using

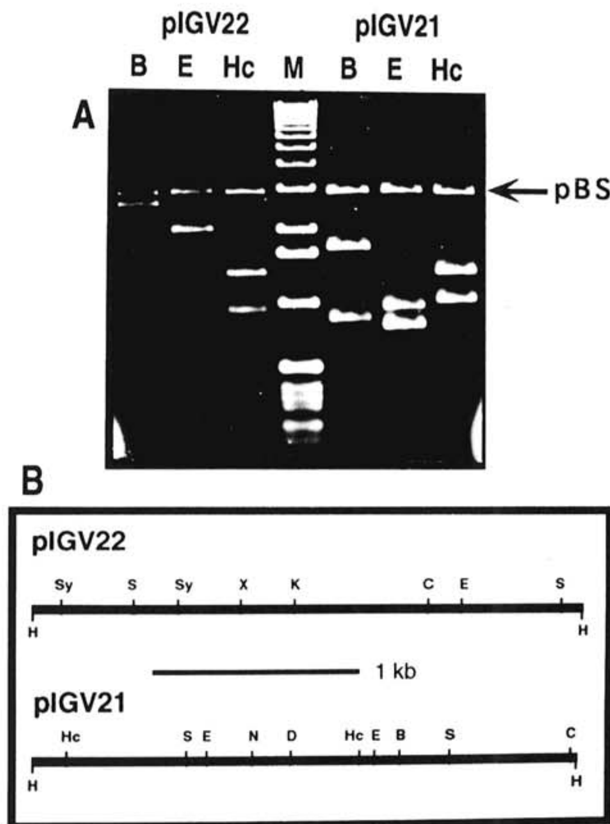

Fig. 2. Restriction maps of viral inserts in pIGV21 (PHV B) and pIGV22 (PHV A). **A,** DNA from plasmids pIGV22 and pIGV21 was digested first with *Hin*dIII to excise viral inserts and then was digested with different restriction enzymes. Lane M corresponds to molecular weight markers; the arrow indicates the position of the linearized pBluescript vector (2.9 kb) present in all lanes. Viral DNA in pIGV22 does not have a *Bam*HI site, so the insert remains intact (2.6 kb). In the other lanes, the enzymes digested the viral DNA producing smaller fragments. **B,** A restriction map constructed for both viral DNA inserts in plasmids pIGV22 and pIGV21. Both have unique *Hin*dIII sites. Three closely arranged *Hin*cII sites in pIGV22 were omitted. (H = *Hin*dIII, B = *Bam*HI, C = *Cla*I, D = *Dra*I, E = *Eco*RI, Hc = *Hin*cII, N = *Nru*I, Sy = *Sty*I, S = *Ssp*I, and X = *Xba*I).

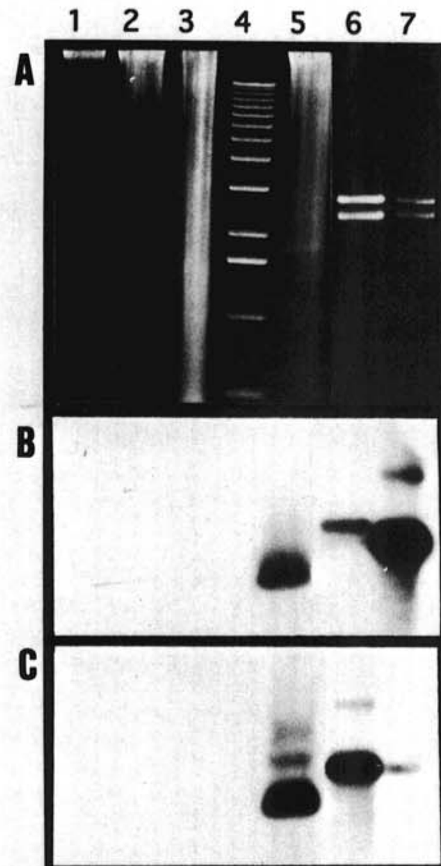

Fig. 3. Analysis of pepper plants inoculated by biolistic procedure. **A,** Agarose gel stained with ethidium bromide. DNA extracts from plants inoculated with: lane 1, pBluescript DNA (control); lane 2, pIGV21 DNA (component B); lane 3, pIGV22 DNA (component A); lane 4, molecular weight markers; lane 5, pIGV22 + pIGV21 DNA (components A + B); as reference, lanes 6 and 7 contain *Hin*dIII-digested DNA from pIGV21 (PHV B) and pIGV22 (PHV A), respectively. Top bands correspond to linear pBluescript and bottom bands correspond to released insert. **B,** Nucleic acids in **A** transferred to nitrocellulose membrane and hybridized with ^{32}P-labeled pIGV21 insert DNA (component B). **C,** As in **B** but hybridized with ^{32}P-labeled pIGV22 insert DNA (component A).

A

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TAATGGCATATTTGTAATAAGAGAGGTGTACACCGATTGGAGCTCTTTAACCTGGGCTTATTGTATCGGTGTATT    75

GGTAGCCAATATATAGTATATGGGAGTTATCTAGGATCTTCGTACACGTGAGGGCCATCCGTTATAATATTACCG    150

GATGGCCGACCGCTTACCTTATCTATCCGTACTGCTTTATTTGAATTAAAGATGTTACTTTTATGCTATCCAATG    225
         HindIII
            ▼
AAGCGTAGCGTCTGGGAAGCTTAGTTATCAGTTCCAGACGTGGGGACCAAGTAGTGTATGACCACTTTATTGACT    300

GTCAGCTTTATAAATTGAAATTAAAACATAAGTGGTCCATGTACCTTTAATTCAAAATGCCTAAGCGTGATGCTC    375
                                                          M  P  K  R  D  A  P
CTTGGCGATTAACGGCGGGGACCGCCAAGATTAGCCGAACTGGCAATAATTCACGGGCTCTTATCATGGGCCCGA    450
 W  R  L  T  A  G  T  A  K  I  S  R  T  G  N  N  S  R  A  L  I  M  G  P  S
GTACTAGCAGGGCCTCAGCTTGGGTTAATCGCCCAATGTACAGGAAGCCCCGGATTTATCGTATGTACAGAACTC    525
 T  S  R  A  S  A  W  V  N  R  P  M  Y  R  K  P  R  I  Y  R  M  Y  R  T  P
CGGATGTGCCGAAAGGTTGTGAAGGTCCCTGTAAGGTTCAATCGTTTGAACAACGACATGACGTCTCTCATGTTG    600
 D  V  P  K  G  C  E  G  P  C  K  V  Q  S  F  E  Q  R  H  D  V  S  H  V  G
GTAAGGTTATTTGTATATCCGACGTAACTCGTGGTAATGGTATTACCCATCGTGTTGGCAAACGATTCTGCGTTA    675
 K  V  I  C  I  S  D  V  T  R  G  N  G  I  T  H  R  V  G  K  R  F  C  V
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B

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PHV  ORF  ----------  MPKRDAPWRL  TAGTAKISRT  GNNSRALIMG  P-S-TSRASA   38
ABMV AR1  ----------  ----------  MPGTSKTSRN  ANYSPRARIG  P-R-VDKASE   28
BDMV AR1  ----------  MPKRDAPWRS  MAGTTKVSRN  ANYSPRGGIG  P-K-MTRAAE   38
BGMV AR1  MYAHSMCKSR  MPKRDAPWRH  MAGTSKVSRS  GNYSPSGGMG  S-K-SNKANA   48
TGMV AR1  ----------  MPKRDAPWRL  MAGTSKVSRS  ANYSPRGSLP  K-R-D----A   34

PHV  ORF  WVNRPMYRKP  RIYRMYRTPD  VPKGCEGPCK  VQSFEQRHDV  SHVGKVICIS   88
ABMV AR1  WVHRPMYRKP  RIYRTLRTAD  MPRGCEGPCK  VQSYEQRHDI  SHVGKVMCIS   78
BDMV AR1  WVNRPMYRKP  RIYRTLRTRD  MPRGCEGPCK  VQSYEQRHDI  SHVGKVMCIS   88
BGMV AR1  WVNRPMYRKP  RIYRMYKSPD  VPKGCEGPCK  VQSYEQRHDI  SHVGKVMCIS   98
TGMV AR1  WVNRPMYRKP  RIYRSLRGPD  VPKGCEGPCK  VQSYEQRHDI  SLVGKVMCIS   84
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C

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TAATGGCATATT-TGTAATAAGAGAGGTGTACACCGATTGGAGCTCTTTAACCTGGGCTTATTGTATCGGTGTATT   75
CAGTGGCAT-TTGTGTAATATG-GAGGGGTACACCGATTGGAGCTCTTTAACCTGGGCTTATTGTATCGGTGTATT   74

GGTAGCCAATATATAGTATATGGGAGTTATCTAGGATCTTCGTACACGTG-AGGGCCATCCGTTATAATATTACCG   150
GGTAGCCAATATATAGTATATGGGAGTTATCTAGGATCTTCGTACACGTGGA-GGCCATCCGTTATAATATTACCG   149

GATGGCCGACCGCTTAC-CTTATCTATCCGT          PHV A                                  180
GATGGCCGACCGCTTCCACTC-TCTTTCCTT          PHV B                                  179
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Fig. 4. Partial sequence of viral insert in pIGV22. The sequences of both extremes of the viral *Hind*III fragment inserted in pIGV22 were determined. **A,** An incomplete ORF (nucleotides [nt] 357–675) coding for the putative PHV coat protein was identified downstream from the *Hind*III site The amino acid sequence of the putative ORF is shown below the nucleotide sequence. The common region for PHV was identified upstream from the *Hind*III site (nt 1–180). The underlined region corresponds to the 30-bp consensus region found in all bipartite geminiviruses able to form a stem-loop structure. **B,** Comparison of the first 88 amino acids of the putative PHV coat protein with the N-terminals of several bipartite geminivirus coat proteins. **C,** Alignment of the homologous regions (92%) found in PHV A (top) and PHV B (bottom). The 30-bp consensus for bipartite geminiviruses is underlined.

the two clones in reciprocal tests.

To confirm that the inserts in plasmids pIGV21 and pIGV22 were part of the viral genome, the inserts were labeled and used as probes in molecular hybridization experiments against DNA extracts from healthy and infected pepper plants. Only infected plants produced the expected positive signal. An example of the results is shown in Figure 3, in which DNA extracts from an uninoculated plant or plants inoculated with only one component did not hybridize against viral probes.

The inserts in plasmids pIGV22 and pIGV21 were identified as being like components A and B of a typical bipartite geminivirus. The first evidence came from infectivity experiments in which only a combination of both plasmids (or inserts) were infectious. This identification was confirmed after the viral inserts were partially sequenced, and the data compared with geminivirus sequences were submitted to GenBank and EMBL databases. Figure 4 shows a partial sequence of the insert in pIGV22, in which a putative coat-protein gene was identified through comparison with the coat protein of several geminiviruses (Fig. 4B). The first 180 bp correspond to the common region of PHV because a similar region with 92% homology was found in pIGV21 (Fig. 4C). This common region contains the 30-bp consensus found in all bipartite geminiviruses able to form a stem-loop structure. Figure 5 shows a partial nucleotide sequence of the insert in pIGV21. An ORF with high homology to the N-terminals of BL1 gene products from several bipartite geminiviruses was identified. Figure 5B shows the alignment of PHV-B ORF to the first 120 amino acids of the predicted products of BL1 ORFs of abutilon mosaic virus (ABMV), tomato golden mosaic virus (TGMV), bean golden mosaic virus (BGMV), and bean dwarf mosaic virus (BDMV).

Biolistic inoculation. Attempts to mechanically transmit disease and infect test plants using infected plant sap or cloned viral DNAs were unsuccessful. However, a biolistic procedure (21) used to inoculate plants at the four true-leaves stage using tungsten microparticles coated with full-length viral DNAs excised from their respective plasmid vectors resulted in 60–70% infection efficiency. Two different pressures (800 and 1,200 psi) were used for the inoculation, but no detectable difference in efficiency was observed, except that at the higher pressure some plants were decapitated.

Plants were also inoculated using intact plasmids containing the viral DNA inserts (pIGV21 + pIGV22) and *Hin*dIII-digested pIGV21 and pIGV22, which releases the full-length inserts from the plasmids. The efficiency, measured as the proportion of inoculated plants with symptoms, did not vary. However, the plants

A

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ATGGATTCATGGTTGGCGAATCCTCCTAGCGCATTCAATTATATAGAGTCACATAGAGATGAATATCAGCTCTCTCATGACTTAACGGAG
 M  D  S  W  L  A  N  P  P  S  A  F  N  Y  I  E  S  H  R  D  E  Y  Q  L  S  H  D  L  T  E

ATAATACTTCAATTTCCGTCAACGGCGTCGCAGTACGCTGCCAGACTTAGTCGTAGCTGTATGAAAATTGACCATTGCGTTATCGAGTAT
 I  I  L  Q  F  P  S  T  A  S  Q  Y  A  A  R  L  S  R  S  C  M  K  I  D  H  C  V  I  E  Y
                                                                                     HindIII

AGACAGCAAGTTCCGATAAACGCCACTGGGTCGGTCATAGTGGAAATCCATGACAAACGAATGACAGACAATGAATCATTACAAGCTTCT
 R  Q  Q  V  P  I  N  A  T  G  S  V  I  V  E  I  H  D  K  R  M  T  D  N  E  S  L  Q  A  S

TGGACATTTCCACTAAGATGTAACATCGATCTCCATTATTTCTCAGCATCCTTCTTCTCCTTGAAGGACCCCATACCCTGGAAGCTATAT
 W  T  F  P  L  R  C  N  I  D  L  H  Y  F  S  A  S  F  F  S  L  K  D  P  I  P  W  K  L  Y

TACAGAGTCTCCGATACTAACGTACATCAGAACACCCATTTTGCCAAGTTCAAAGGGAAATTGAAGTTGTCCACAGCTAAACACTCCGTG
 Y  R  V  S  D  T  N  V  H  Q  N  T  H  F  A  K  F  K  G  K  L  K  L  S  T  A  K  H  S  V

GATATACCTTTCCGGGCTCCGACGGTGAAGATTTTATCGAAACAGTTCACC-----
 D  I  P  F  R  A  P  T  V  K  I  L  S  K  Q  F  T  -----
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B

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PHV  ORF  MDSWLANPPS AFNYIESHRD EYQLSHDLTE IILQFPSTAS QYAARLSRSC MKIDHCVIEY   60
ABMV BL1  MDSQLVNPPN AFNYIESHRD EYQLSHDLTE IILQFPSTAA QLTARLSRSC MKIDHCVIEY   60
TGMV BL1  MDSQLACPPN VFNYIESNRD EYQLSHDLTE IILQFPSTAS QLSARLSRSC MKIDHCVIEF   60
BGMV BL1  MDSQLANPPN AFNYIESHRD EYQLSHDLTE IILQFPSTAS QLSARFSRSC MKIDHCVIEY   60
BDMV BL1  MDSQLVNPPN AFNYIESHRD EYQLSHDLTE IILQFPSTAS QLTARLSRSC MKIDHCVIEY   60

PHV  ORF  RQQVPINATG SVIVEIHDKR MTDNESLQAS WTFPLRCNID LHYFSASFFS LKDPIPWKLY  120
ABMV BL1  RQQVPINATG SVIVEIHDKR MTDNESLQAS WTFPIRCNID LHYFSASFFS LKDPIPWKLY  120
TGMV BL1  RQQVPINATG SVVVEIHDKR MTDNESLQAS WTFPVRCNID LHYFSSSFFS LKDPIPWKLY  120
BGMV BL1  RQQVPINATG SVVVEIHDKR MTDNESLQAS WTFPIRCNID LHYFSSSFFS LKDPIPWKLY  120
BDMV BL1  RQQVPINATG SVIVEIHDKR MTDNESLQAS WTFPIRCNID LHYFSASFFS LKDPIPWKLY  120
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Fig. 5. Partial sequence of viral insert in pIGV21. The sequences of both extremes of the viral *Hin*dIII fragment inserted in pIGV21 were determined. **A,** *Hin*dIII restriction site in pIGV21 was found inside a possible ORF. The predicted amino acid sequence is shown below the nucleotide sequence. **B,** Alignment of the predicted amino acid sequence of ORF in **A** with the first 120 amino acids from the BL1 gene products of several bipartite geminiviruses. The arrow indicates the location of the *Hin*dIII site in relation to the aa sequence. No discontinuity is observed in PHV-B ORF, suggesting the clone for PHV B is full length.

inoculated with the undigested clones developed symptoms 4–5 days earlier than did the plants inoculated with *Hin*dIII-digested viral DNA (Table 1). Plants inoculated with either component alone or with Bluescript DNA by itself did not develop any symptoms.

Symptoms developed in the leaves that emerged after inoculation. The main characteristic was an initial vein yellowing, as well as curling and some rugocity of the leaves (Fig. 6). After several days, the disease syndrome went into remission; however, when the plants were trimmed, new growth exhibited severe symptoms. When tissue from plants inoculated by the biolistic procedure was used for graft inoculation the symptoms observed in the graft-inoculated plants were usually as severe as the symptoms observed in the trimmed plants.

Analysis of inoculated plants. We verified the presence of viral DNA in the infected plants using two approaches. First, we analyzed DNA extracts from biolisticly inoculated plants with Southern blot analysis. DNA was extracted from newly developed leaves that were not present when the plants were inoculated. We used labeled DNAs from either pIGV22 (component A) or pIGV21 (component B) plasmids as probes. Only plants inoculated with both components produced a positive hybridization with both probes, indicating the presence of the whole viral genome (Fig. 3, lane 5). In contrast, viral DNA was not detected in plants inoculated with only one component (either A [Fig. 3, lane 3] or B [lane 2]) or with Bluescript DNA (Fig. 3, lane 1). The same DNA pattern was detected in plants inoculated by the biolistic procedure using either digested or undigested plasmid DNA observed in plants inoculated by whiteflies or by grafting (data not shown).

The second approach detected the presence of the viral DNA by PCR amplification of the viral coat-protein gene from total DNA isolated from plant tissue. Figure 7 shows that a fragment of approximately 800 bp could be amplified from different DNA extracts from inoculated tissue as well as from cloned viral DNA (lanes 2–4 and 6–8). As expected, no amplification product was obtained when DNA from uninoculated plants was used in the PCR procedure (lane 1). As a positive control, we used DNA extracts from graft-inoculated plants and cloned viral DNA (pIGV22) that produced the expected amplified fragment (lanes 2 and 8). In every case, an indistinguishable amplification product was observed. This procedure allowed us to analyze the inoculated plants at an early stage because less plant material was needed. As verification, the products of PCR amplification were positively identified using hybridization against labeled viral DNA (Fig. 7B). In all cases, the PCR products hybridized with a similar intensity.

TABLE 1. Infection of *Capsicum annuum* plants with cloned viral DNA by biolistic inoculation

Inoculum[a]	Number of experiments	Plants with symptoms/plants inoculated[b]	%	Incubation period[c]
Water	3	0/12	0	...[d]
pBluescript DNA	2	0/12	0	...
pIGV22 (A)	2	0/6	0	...
pIGV21 (B)	2	0/6	0	...
PHV (A + B) excised	3	11/16	68	10–12
PHV (A + B) undigested	3	9/13	76	5–6

[a] Excised indicates circular plasmids were digested with *Hin*dIII to release viral DNA as a single fragment; undigested indicates circular plasmids were used as inoculum.
[b] Number of plants showing symptoms at 20 days postinoculation.
[c] Number indicates first day after inoculation when symptoms were observed.
[d] No symptoms.

DISCUSSION

The initial strategy used for PHV isolation allowed us to eliminate most RNA viruses known to be endemic in the area. This was confirmed by ELISA (enzyme-linked immunosorbent assay). Nevertheless, our procedures could not rule out the possible presence of a mixture of similar, nonmechanically transmitted geminiviruses. As discussed below, we believe that at least two geminiviruses are involved in rizado amarillo disease. The first

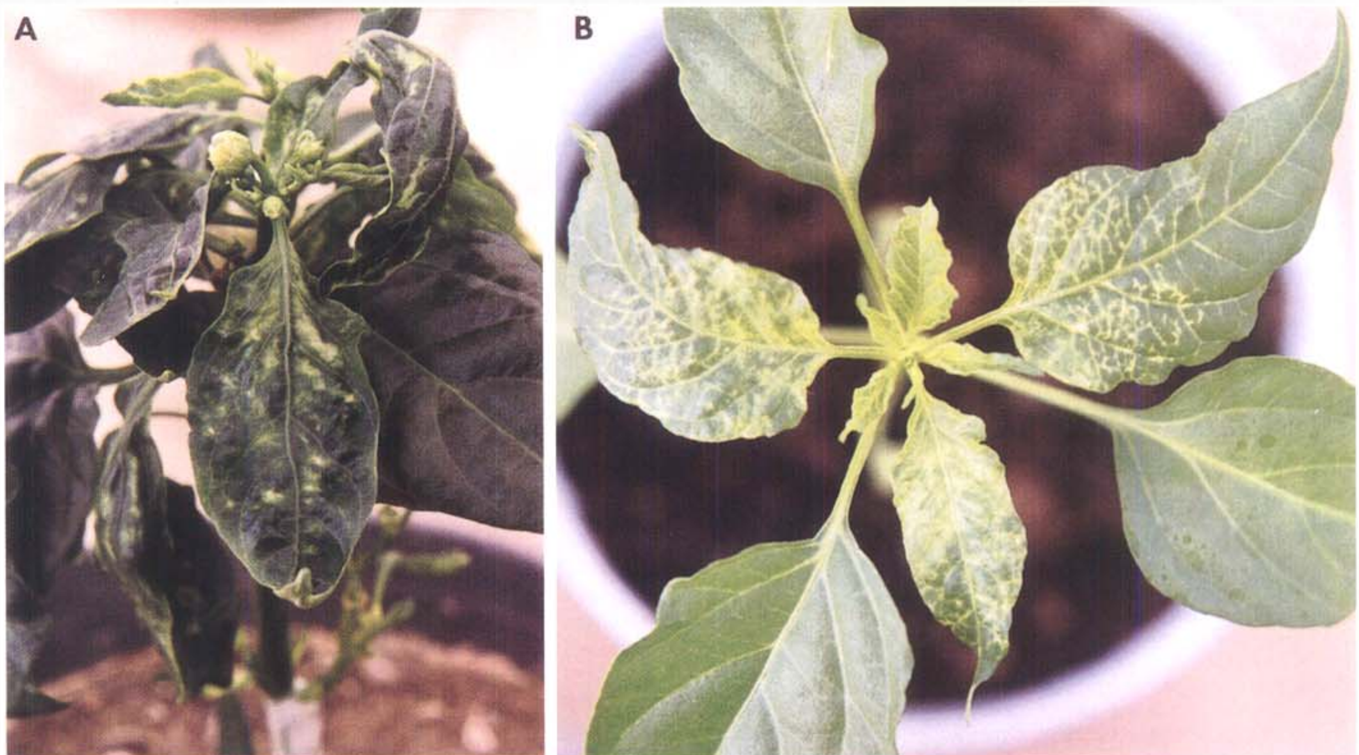

Fig. 6. Symptoms in pepper infected with rizado amarillo disease (A) and inoculated with PHV (B). **A,** Leaf distortion as well as yellow mosaic. **B,** Yellowing of the veins is a main characteristic of the symptoms caused by PHV

geminivirus is reported here as pepper huasteco virus (PHV). The second geminivirus also is being characterized in our laboratory. However, we do not, as yet, have convincing evidence to conclude whether the second virus is a new geminivirus or one of those previously reported in northern México.

Our decision to name this virus pepper huasteco virus (PHV) instead of using the disease name (rizado amarillo) is based on the differences in the symptoms obtained in both cases, and our belief that there are other viruses involved in the disease. The distribution of PHV in México and its involvement in other complex diseases (e.g. pepper tigré disease) is currently being studied.

PHV is considered to be different from other geminiviruses previously reported. First, the lack of mechanical transmission eliminates SGMV and TPGV, which are mechanically transmitted (6,22). In addition, the restriction maps of PHV differ from the maps for TPGV (22). Second, the severity of the symptoms induced by PHV excludes CdTV and PMTV, which produce mild symptoms in pepper (4,7). A definitive answer will be obtained when nucleotide sequences for these geminiviruses are available. The partial nucleotide sequence obtained for PHV allows us to eliminate geminiviruses whose nucleotide sequences have been reported (e.g., ABMV, ACMV, BGMV, TGMV, SqLCV, TYLCV-S, and TYLCV-IS).

The cloning of several fragments in the 2.6-kb range facilitated the selection of two viral clones that in combination were infectious. The infectivity of the clones when inoculated in combination indicates they both represent the complete genome of a bipartite geminivirus. In addition to the infectivity results, the identity of the components was confirmed by an analysis of their partial nucleotide sequences. Both components share a common region (180 bp) that had 92% homology. As expected, this common region contained a 30-bp consensus sequence found in all bipartite geminiviruses (Fig. 4A and 4C).

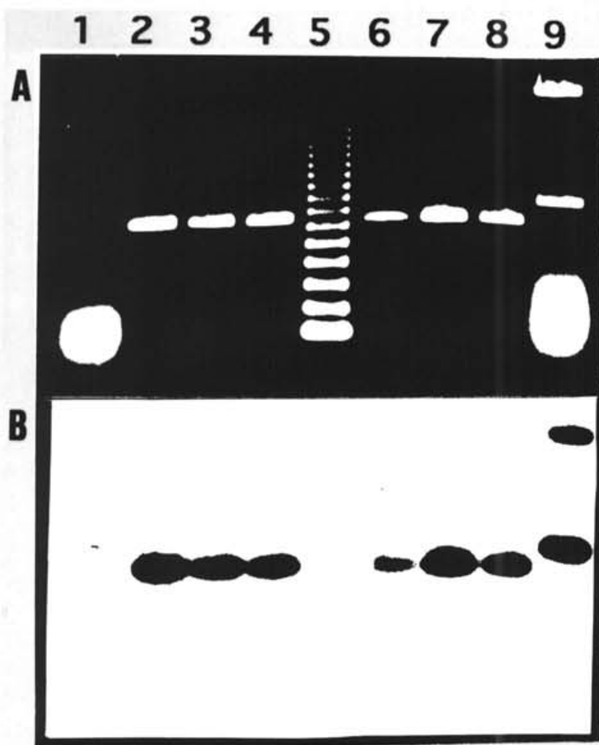

Fig. 7. PCR (polymerase chain reaction) amplification of PHV coat-protein gene. PHV coat-protein gene was amplified by PCR from different DNA extracts: lane 1, uninoculated plant; lane 2, plant with rizado amarillo disease; lanes 3 and 7, plants inoculated with *Hin*dIII-digested pIGV21 + pIGV22 DNA (PHV); lanes 4 and 6, plants inoculated with undigested pIGV21 + pIGV22 DNA; lane 8, pIGV22 DNA (positive control for PCR). Lane 5 corresponds to 123-bp ladder molecular weight marker; lane 9 contains undigested DNA from plasmid pIGV24 (positive control for hybridization). **A,** Agarose gel electrophoresis analysis of PCR products. The amplified fragments migrate between bands 6 (728 bp) and 7 (861 bp) of the 123-bp ladder corresponding to the expected 810 bp. **B,** Nucleic acids in gel shown in **A** were transferred to a nitrocellulose membrane and hybridized against pIGV24 DNA. pIGV24 consists of pBluescript with a 982-bp insert from pIGV22 (*HindIII-Xba*I fragment) that contains the entire PHV coat-protein gene.

Clones pIGV22 and pIGV21 were identified as being components A and B, respectively. This identification was obtained after analysis of the partial nucleotide sequences of both inserts. In pIGV21, the *Hin*dIII site lies inside a typical BL1 ORF (Fig. 5). Computer alignment of PHV-B ORF with the BL1 products from other geminiviruses also confirmed that the insert in pIGV21 is a full-length clone for component B because there is a perfect continuity in the amino acid sequence of the ORF (Fig. 5B). In pIGV22, the *Hin*dIII site lies between the common region and the coat-protein gene (Fig. 4). In this case, it was not possible to verify the continuity of the sequence. However, because the *Hin*dIII site lies in an important regulatory region, it is unlikely that a deletion in this area will render an infectious clone.

Inoculation with PHV A by itself did not cause any visible symptoms. This suggests component A alone is either not infectious, or it cannot move from the inoculation site to the rest of the plant. Although this is the general rule for dicot-infecting geminiviruses, it important to verify it because recent reports showed the infectivity of component A and/or the lack of component B in some isolates of tomato yellow leaf curl virus (TYLCV) (15,18,19).

The inoculation procedure used here to confirm the infectivity of the viral DNA clones presents several advantages over previously described methods. First, the symptoms develop in a few days, and the plants do not go through a stressful treatment, such as the one reported in some cases of agroinoculation in which the recovery could take up to 3 wk. During the procedure we used, the plants are not severely damaged, and they recover in a few hours. Second, we can use intact plants as long as they fit into the chamber. Also, by covering parts of the plants, we can inoculate specific organs or tissues. This allows the inoculation of plants either at different developmental stages or in different tissues to study virus movement and replication. Finally, this procedure allows a fast, simple analysis of a large number of constructions. All cloning procedures can be accomplished in *E. coli* vectors (e.g., pBluescript, pUC series) that are easy to handle and ideal for procedures such as sequencing and analysis of point mutations.

The reason undigested plasmid DNA produced symptoms earlier than did the *Hin*dIII-digested plasmid remains unexplained. Perhaps the linear DNA inoculum is more sensitive to nuclease degradation after being introduced into the plant cells. The uncut or circular DNA, on the other hand, could be more resistant because it is a closed molecule. This, however, does not explain how the viral DNA is excised from the harboring vector to begin replication. Analysis of the viral DNA recovered from inoculated plants showed that pBluescript DNA is lost between inoculation and the time when the first symptoms are expressed (the earliest time we analyzed our plants). We have been unable to detect any DNA rearrangements because the same viral DNA pattern is observed whether the plant is inoculated by the biolistic procedure with cloned DNA or by whiteflies or grafting. We have attempted to detect pBluescript DNA in the inoculated plants by Southern blot analysis and have failed. A more sensitive method, such as PCR amplification, combined with a more detailed kinetic analysis beginning just after inoculation could provide insight into the fate of pBluescript DNA.

Agroinoculation has been an important procedure in circumventing the problem presented by nonmechanically transmitted geminiviruses. However, to our knowledge, all reports indicate the requirement of longer than unit-length constructions to obtain infectivity. Gilbertson et al (12) reported the successful inoculation of monomeric forms of some nonmechanically transmitted variants of BGMV by electric discharge particle acceleration. In contrast to our results, however, they obtained infectivity only

when the viral DNA was excised from the vectors. We do not know if our results are the result of a particular characteristic of our virus (PHV) or of the specific constructions we are using. We are in the process of making several monomeric and dimeric constructs to investigate the process of excision and circularization that cloned DNA suffers in vivo. We also are interested in increasing the efficiency of inoculation using our biolistic procedure.

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