Bean Golden Mosaic Geminivirus Type II Isolates from the Dominican Republic and Guatemala: Nucleotide Sequences, Infectious Pseudorecombinants, and Phylogenetic Relationships

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ABSTRACT

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Bean golden mosaic geminivirus (BGMV) causes major losses on beans (*Phaseolus vulgaris*) in the tropical and subtropical Americas and the Caribbean Basin. Infectious DNA-A and -B components of BGMV isolates from the Dominican Republic (BGMV-DR) and Guatemala (BGMV-GA) were sequenced, and their genome organizations were similar to those of other whitefly-transmitted geminiviruses from the Western Hemisphere. Whiteflies (*Bemisia tabaci*) transmitted BGMV from beans infected by particle-gun inoculation with clones of BGMV-DR or -GA. Infectious pseudorecombinants gave symptoms typical of wild-type infections when mixtures of BGMV-GA DNA-A and BGMV-DR DNA-B were used. Delayed and attenuated symptoms were obtained when mixtures of BGMV-DR DNA-A and BGMV-GA DNA-B were used. Phylogenetic analyses using the common region and four open reading frames placed 20 geminiviruses in eight clusters for the Western Hemisphere and at least five clusters for the Eastern Hemisphere viruses. BGMV-DR and -GA are in the BGMV type II cluster with the BGMV isolate from Puerto Rico. The BGMV type II cluster is clearly distinct from the BGMV type I cluster, which contains the BGMV isolate from Brazil. Bean dwarf mosaic geminivirus is in the Abutilon mosaic virus cluster, and bean calico mosaic geminivirus is in the squash leaf curl geminivirus cluster. Conserved nucleotide sequences of the common regions of members of these clusters were identified, and the presence of amino acid motifs with putative functions were identified in the derived amino acid sequences of the replication-associated proteins of BGMV-DR and -GA. The availability of sequenced, infectious clones of BGMV-DR and -GA provides opportunities for the study of genome functions and for the engineering of beans for resistance to these viruses.

Bean golden mosaic of common beans (*Phaseolus vulgaris* L.) was first described in Brazil by Costa (4) who found that the causal agent was transmitted by the whitefly (*Bemisia tabaci* Gennadius). Subsequently, a unique geminate viral particle was associated with beans showing golden mosaic symptoms, collected in Central America (12) and the Caribbean Basin (21). Goodman (20) demonstrated that the geminate particles of bean golden mosaic geminivirus (BGMV) from Puerto Rico (BGMV-PR) contain single-stranded DNA (ssDNA), and Haber et al (24) established that BGMV-PR is composed of two similar-sized molecules (each ~2.6 kb), now designated DNA-A and -B (36). The nucleotide sequence of BGMV-PR was subsequently published (29,42).

When first described, BGMV was a minor pathogen of beans (4); however, during the 1970s, fields with nearly 100% incidence became common in Brazil, Central America, and the Caribbean Basin (13,21). Even though moderately resistant bean cultivars are available (41), BGMV remains a major constraint to bean production in the tropical and subtropical Americas and the Caribbean Basin (3). BGMV is composed of at least two distinct types with major differences in DNA sequence and biological properties (e.g., sap-transmissibility, reaction of bean germ plasm, and host range). Type I is exemplified by the BGMV isolate from Brazil (BGMV-BZ) and type II by BGMV-PR (15). Other distinct bean-infecting geminiviruses are bean calico mosaic geminivirus (BCMoV; [39]) and bean dwarf mosaic geminivirus (BDMV; [27]), and bean can be experimentally infected with squash leaf curl geminivirus (SqLCV; [37]) and a tomato-infecting geminivirus from Mexico (46).

Because at least two types of BGMV exist, the present study was undertaken to molecularly characterize two additional, economically important BGMV isolates. One isolate, BGMV-GA, was collected near Monjas, Guatemala, from an experimental field jointly established by the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia, and the Instituto de Ciencias y Tecnologia Agricola, Guatemala. The mission of this collaborative research project is to select BGMV-resistant beans for tropical America. The other isolate, BGMV-DR, is from San Juan de la Maguana, the Dominican Republic, where BGMV causes severe losses. This isolate is thought to be more aggressive than the BGMV in Central America (F. J. Morales, *personal communication*). We had previously prepared infectious clones of these two isolates (16). Here we report the computer-assisted analysis of their nucleotide sequences, the relatedness of these isolates to other whitefly-transmitted geminiviruses, their whitefly trans-

missibility from beans infected with these clones, and the infectivity of pseudorecombinants made from heterologous mixtures of DNA-A and -B components of these two BGMV isolates.

MATERIALS AND METHODS

Viral clones. Full-length inserts (~2.6 kb) from recombinant plasmids containing DNA-A (pDRA1, cloned in the *Eco*RI site of pBluescript II KS+ [pBS]) and DNA-B (pDRB1, cloned in the *Hin*dIII site of pBS) from BGMV-DR isolate 87-1 from San Juan de la Maguana, the Dominican Republic (16), or DNA-A (pGAA1, cloned in the *Eco*RI site of pUC119) and DNA-B (pGAB1, cloned in the *Bam*HI site of pBS) from BGMV-GA isolate 0 from Monjas, Guatemala (41), were infectious in *P. vulgaris* inoculated by electric discharge particle acceleration (16).

Inoculation of beans with viral DNAs. Beans (*P. vulgaris* cv. Topcrop) were infected with the excised monomers of the DNA-A and -B components by electric discharge particle acceleration (16) at Agracetus, Inc., Middleton, WI, or with a particle gun (33) at CIAT, Cali, Colombia. At the University of Wisconsin, Madison, beans were grown in a growth chamber with a 26-C light period (14-h photoperiod, ~310 μE) and a 21-C dark period. At CIAT, beans were grown in growth chambers with a 23-C light period (13-h photoperiod 652 μE) and a 23-C dark period. Viral symptoms developed 6–10 days after inoculation under both sets of conditions.

Pseudorecombinants between BGMV-GA and -DR. To determine whether infectious pseudorecombinants could be made by exchanging the genome components of BGMV-DR and -GA, bean radicles were inoculated with mixtures of approximately equal molar concentrations of the linear monomers of viral DNA components as previously described (16).

***B. tabaci* transmission of BGMV from beans infected with cloned viral DNAs.** A colony of *B. tabaci* was initiated from adults collected on *Glycine max* (L.) Merr. at CIAT during 1990 and was maintained on bean plants in insect-proof cages in plant growth chambers. Adult whiteflies were determined to be nonviruliferous by the continued lack of symptoms on beans after they had been raised on beans for several generations. Nonviruliferous whiteflies were given acquisition access periods either on BGMV-infected beans inoculated with cloned viral DNAs or on uninoculated, symptomless beans. Groups of five nonviruliferous whiteflies in small cages were placed on the leaves of infected or uninfected beans. After a 2-h acquisition access period, each cage was placed on a primary leaf of a 10-day-old uninfected plant. After 24 h, the cages were removed and the whiteflies immediately destroyed. The beans were maintained in a whitefly-free plant growth room.

Nucleotide sequencing of BGMV-DR and -GA clones and sequence analyses. DNA manipulations were performed according to standard procedures (2,40). Recombinant plasmids were transformed into competent cells of *Escherichia coli* strains JM101 or DH5α. For DNA sequencing of each full-length insert, a set of overlapping subclones was created in both orientations with exonuclease III. Single-stranded DNA was generated with the helper phage M13K07, and nucleotide sequencing was performed on ssDNA templates by the dideoxy-nucleotide chain termination method with Sequenase (United States Biochemical Corp., Cleveland) according to the manufacturer's instructions. Sequence compressions were resolved by dITP or, in some cases, by sequencing with the Klenow fragment of DNA polymerase I. Both DNA strands of the insert in each recombinant plasmid were sequenced completely. Independent clones were sequenced across the cloning sites of the four full-length inserts to confirm that these inserts were full-length. Nucleotide sequences were assembled with the software of the Genetics Computer Group, University of Wisconsin-Madison (8). Relationships among geminiviruses were examined by comparing nucleotide sequences, using the GAP program of this software with a gap weight of 5 and a gap length weight of 0.3. Phylogenetic analyses were done with the computer program, Phylogenetic Analysis Using Parsimony, version 3.1 (PAUP), developed by D. L. Swofford (Illinois Natural History Survey, Champaign). Optimum trees were obtained with the Heuristic method with the branch-swapping option. One-hundred bootstrap replications were performed for each data set to assign branch strengths to the topological elements. Geminiviruses were assigned to a cluster when they occurred in the same branch >50% of the time in the bootstrap-replication analysis.

Detection of geminivirus DNA in plant extracts. Nucleic acids were extracted from 0.5–1 g of young bean leaves by a slight modification of the minipreparation method of Dellaporta et al (6). The extract was treated three times with phenol, the DNA was precipitated with ethanol, and the pellet was washed three times with 70% ethanol. The nucleic acid pellet was resuspended in 150 μl of Tris-EDTA (TE) buffer, and 7.5 μl of 100 mM spermidine was added. Fifteen microliters of this nucleic acid solution was digested with RNAse A, as well as with *Hin*dIII and *Sma*I restriction endonucleases, to give unique fragment sizes for each of the four components. The digestion mixtures were incubated with S1 nuclease to remove ssDNA, and the DNA fragments were precipitated with ethanol, resuspended in 40 μl of TE buffer, and separated on 0.7% agarose gels in 0.5× Tris-borate-EDTA buffer. Nucleic acids were transferred to nylon membranes, denatured (17), and hybridized in 50% formamide at 42 C with ^{32}P-labeled riboprobes prepared from pGAA1 or pGAB1 with T7 polymerase. Membranes were washed two times with 2× SSC/SDS (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0; SDS is sodium dodecyl sulfate), two times with 0.2× SSC/SDS at room temperature, two times with 0.2× SSC/SDS at 65 C, air-dried, and exposed to Kodak X-Omat AR X-ray film.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of the cloned viral DNAs. The BGMV-DR DNA-A insert in pDRA1 had 2,647 bp (GenBank L01635), and the DNA-B insert in pDRB1 had 2,608 bp (GenBank L01636). The BGMV-GA DNA-A insert in pGAA1 had 2,647 bp (GenBank M91604), and the DNA-B insert in pGAB1 had 2,596 bp (GenBank M91605). Comparison of the complete nucleotide DNA-A sequence of BGMV-GA with that of BGMV-DR, -PR, and -BZ gave identities of 97, 96, and 74%, respectively; for DNA-B, the identities were 92, 87, and 65%, respectively. These results clearly indicate that the BGMV-DR and -GA isolates are nearly identical to BGMV-PR but not to BGMV-BZ; thus, BGMV-DR and -GA are BGMV type II isolates (15).

Computer-assisted analysis of the BGMV-DR and -GA sequences for open reading frames (ORFs) coding for proteins larger than 10 kDa showed that each of the DNA-A components (Fig. 1) had four ORFs (AV1, AC1, AC2, and AC3) and each of the DNA-B components (Fig. 1) had two ORFs (BV1 and BC1). Nucleotide-sequence comparisons between homologous ORFs

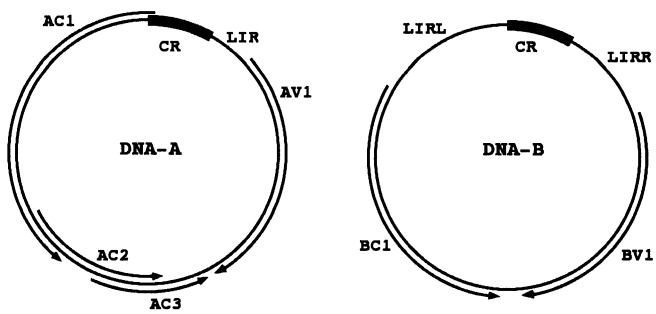

Fig. 1. Genome organization of bean golden mosaic geminivirus from Guatemala. Open reading frames (ORFs) are designated A or B (DNA-A or DNA-B component), V or C (viral or complementary sense polarity), and 1, 2, or 3 (position of ORF relative to the common region [CR]). The CR is represented by the wide bar. LIR = large intergenic region in DNA-A between the CR and the start of AV1. LIRR = large intergenic region between the CR and the start of BV1. LIRL = large intergenic region between the CR and BC1.

and noncoding regions (>200 nt) of BGMV-DR and -GA showed that the differences between the respective regions of the DNA-A components were less than those of the DNA-B components.

TABLE 1. Nucleotide (nt) differences between analogous regions of the genomes for bean golden mosaic geminivirus (BGMV) isolates from the Dominican Republic (BGMV-DR) and Guatemala (BGMV-GA)

Genome region[a]	Number of nt[b]	nt divergence[c] (%)
DNA-A		
Common region	204	4.9
LIR	135	4.4
AV1	786	2.7
AC1	1,062	2.6
AC2	519	2.1
AC3	399	2.3
DNA-B		
Common region	204	16.2
LIRR	299	11.4
LIRL	409	16.4
BV1	771	6.2
BC1	882	4.1

[a]Figure 1 shows the location of the open reading frames (AV1, AC1, AC2, AC3, BV1, and BC1). LIR = large intergenic region between the 3′ end of the common region and the start of AV1 (nucleotides 205–339 for BGMV-GA); LIRR = large intergenic region to the right of the common region (nucleotides 205–502 for DNA-B of BGMV-GA); LIRL = large intergenic region to the left of the common region (nucleotides 2,198–2,596 for DNA-B of BGMV-GA).
[b]Number of nucleotides in the region used for comparisons.
[c]A gap/insertion was counted as one change.

The percent differences between ORFs and noncoding regions of DNA-A were <3 and <5% (Table 1). The most variable region between the BGMV-GA and -DR genomes was the left intergenic region of DNA-B, which lies between the start codon for BC1 and the common region (hypervariable region; [19]); the nucleotide difference between these regions was 16.4%. The AC2 ORF had the most conserved nucleotide sequence (1.2% divergence), and the BV1 ORF was the most variable ORF (6.2%).

The organization of the common regions of the BGMV-DR and -GA isolates is like that of BGMV-PR in that each common region has a nearly identical direct repeat of 11–15 nucleotides. The direct repeat motif, TGCGAGTGTCT, occurs in the DNA-A of BGMV-DR and in both DNAs of BGMV-GA; however, surprisingly, the DNA-B of BGMV-DR has a different direct repeat motif, GTGTCTCCATT(C/T)GA. These direct repeats are noticeably absent from 18 other geminiviruses, including BGMV-BZ, a type I isolate (Fig. 2; [47]). A conserved, potential stem-loop motif (36,47) present in the common regions of all geminiviruses is thought to be associated with the nicking of one DNA strand (38,54) during the rolling circle-type DNA replication of geminiviruses (36).

Infectivity of pseudorecombinants between BGMV-DR and -GA components. Pseudorecombinants between these type II isolates were formed by mixing the full-length, linearized cloned DNA components. The two heterologous mixtures were infectious (Table 2); however, symptom development depended on the components. The BGMV-GA DNA-A and BGMV-DR DNA-B pseudorecombinant gave symptoms similar to those of wild-type virus (16), whereas the BGMV-DR DNA-A and BGMV-GA DNA-B pseudorecombinant induced delayed and attenuated symptoms. Evidence for infection by the inoculated components

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          1                                                                                                  100
BGMV-GA   CATATTTGTAAA.TATGCGAGTGTCT....CCAAATGAGTTTGCGAGTGTCTCCAATTGAGGCTCCTCAAACTCTCGCTATGCAATTGGAGA..CTGGAG
BGMV-DR   CATATTTGTAAA.TATGCGAGTGTCTT...CCGAATGGGTTTGCGAGTGTCTCCAATTGAGGCTCCTCAAACTCTCGC.ATTCAATTGGAGA..CTGGAG
BGMV-PR   CATATTTGTAAA.TATGCGAGTGTCT....CCAAATGAGTTTGCGAGTGTCTCCAATTGAGGCTCCTCAAACTCTCGCTATGCAATTGGAGA..CTGGAG
  AbMV    CATTTATGTAA..TAAGAAGGGG..TACTCT.GGATGAGTTACT.......CCACTTGAGGCTCCTCAAAACTTGCTCATGTAATTGGAGT.ATTGGAG
  BDMV    CATTTTTGTAA..TAAGAGCTGG..TACT.CCAGTTGAGTTACT.......CCAATTCCCCCCTCTCAAAACTATCTCATTCTATTGGAGT.ATTGGAG
  ToMoV   CATTTTTGTAA..TAAGAAGGGG..TACT.CCAGATGAGTTACT.......CCAATTGAGCCTTCTCAAACTT.GCTCATTCAATTGGAGT.ATTAGAG
  PHV     CATATTTGTAA..TAAGAGAGGTG.TACACCGATTGGAG.....................CTCTTTAACCTGGGCTTATTGTATCGGTGT.ATTGGTA
BGMV-BZ   CATACTTGTAAA.TAAGAGGGTG..TACC.CCGATTGAG.....................CTCTCGTTCAAAAGTCTCT..ATGAATCGGTGT.AATGGTG
  TGMV    CATTTTTGTAAT.TAAGAGGCT...TACTACCAATTGAG.....................GAGGGGCTCCAAAAGT...TATATGAATTGGTAG.TAAGGTA
  PYMV    CATTTTTGTAAA.TATGAATGTTCT.....CCCAATATG......TTCCCCCTATTGCTCTGGCTCTCAAAACT...CTTATGAATTGGGGGAACTGGGG
SqLCV-E   CATATTTCGTAAATATGCATCGGGACACCAG.....GAG.....................GTGTCCTCTCAACTTTCTC..ATATTGCTGGTGTCC.TGGTG
  BCMoV   CATATTTGTAAA.TATGAGCCAGGACaCCAGGgGG.GAG.....................CTCTCTCCAAAACCT...ATTGTTTTTGGTGTCC.TGGTG
SqLCV-R   CATATTTGGTAAATATGAACCGGGACACCAGGGG..GAG.....................CTCTCTCTAAAACCT...ATTATTGCTGGTGTCC.TGGTG
  CalGMV  CATTTTTGTGAA.TATGAGCCAGGACACCAGGGG..GAG.....................CTCTCTCTAAAACTCT...ATTTTGCTGGTGTCC.TGGTG
  TPV     CATTTTTGCAAA.TAtgatATAGGACTCCA.....GCAG.....................atGTCCCTCAACTTCCTGTCATATATGTGGAGTCC.TGGAG
                                                                                                    ^^ ^     ^^ ^

          101                                                                                        188
BGMV-GA   .TACAATATATACTAGTACCCTCAATCTCGTGAATTATCAGATTCAC.ACACGTGGCGGCCATCCGATATAATATTACCGGATGGCCG
BGMV-DR   .TACAATATATACTAGAACCCTCAATCTCGTGAATTACGAGATTCAC.ACACGTGGCGGCCATCCGATATAATATTACCGGATGGCCG
BGMV-PR   .TACAATATATACTAGAACCCTCAATCTCTTGAATTATCACATCCAT.ACACGTGGCGGCCATCCGATATAATATTACCGGATGGCCG
  AbMV    GTCT.TTATATACTAGAACTCTCATTAACGGATTTGCA.....ACACGTGGCGGCCATCCGCTATAATATTACCGGATGGCCG
  BDMV    TTAC.TTATATACTAGAACCCTCAATCTGGTTTCGGA.....ACACGTGGCGGCCATCCG.TATAATATTACCGGATGGCCG
  ToMoV   TAAC.TTATATATAAGAACCCTCTATAGAATTATTAATCTGGTTCAT.ACACGTGGCGGCCATCCGATATAATATTACCGGATGGCCG
  PHV     GCC.AATATATAGTATATGGGAGTTATCTAGGATCTTCGT.....ACACGTGAGGGCCATCCGTTATAATATTACCGGATGGCCG
BGMV-BZ   CC..AATATATAGTAAGAAGTTCTTTAAGGATCTGTAG.....ACACGTGGCGGCCATCCGCTATAATATTACCGGATGGCCG
  TGMV    GCTC.TTATATATTA.GAAGTTCCTAAGGGG.....CACGTGGCGGCCATCCG.TTTAATATTACCGGATGGCCG
  PYMV    GAAC.TTATATAGTA.GAAGTTCCTAAAGGCAGATCA.....ACACGTG.CGGCCATCCGTTATAATATTACCGGATGGCCG
SqLCV-E   TCC.TATATATACCTCAAGACACATAAAGCCTCTAGGGGACACCAAGGGGCAAAATCGGCCATCCGCAATAATATTACCGGATGGCCG
  BCMoV   TCCC.ATATATACTACAAGTCTCTTTAGGCCTCTTTAGGACACCAT.GGGCAAAATCGgCCATCCGAAATAATATTACCGGATGGCCG
SqLCV-R   TCCC.ATTTATA...CAACTCTCT.....GGGGAGGACACCAGGGGCAAAATCGGCCATCCGCAATAATATTACCGGATGGCCG
  CalGMV  TCCC.ATTTATACTAAAACCCTCT.....GGGGGACACCATGGGCAAAATCGGCCATCCGTAATAATATTACCGGATGGCCG
  TPV     TCCC.TTATATACATAAAGCC.CTCTT.....GGGGACTCCAAGGGCAAAAGCGGCCATCC..TATAATATTACTGGATGGCCG
            ****                                                                  ----------
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Fig. 2. Alignment of nucleotide sequences for viral sense polarity of common regions for DNA-A of 15 whitefly-transmitted geminiviruses from the Western Hemisphere. Sequences start at the initiation codon of the AC1 open reading frame (CAT) and terminate at the 3′ nucleotide of the stem-loop motif (dashed line). Sequences were aligned by hand, and gaps are indicated by periods (.). The direct repeats for the bean golden mosaic geminivirus (BGMV) type II isolates are underlined, the TATA box is marked with an asterisk (*), and the G's in direct repeats, immediately 5′ of the TATA box (toward nucleotide 1 of the common region), are marked with carets (△). BGMV from Guatemala (-GA), from the Dominican Republic (-DR), and from Puerto Rico (-PR, Howarth et al [29] as corrected in Hidayat et al [28]), Abutilon mosaic virus from the West Indies (AbMV; [11]), bean dwarf mosaic virus from Colombia (BDMV; [28]), tomato mottle virus from Florida (ToMoV; [1,18]), pepper huasteco virus from Mexico (PHV, GenBank no. X70418 [DNA-A] and X70719 [DNA-B]), BGMV from Brazil (-BZ; [15]), tomato golden mosaic virus from Brazil (TGMV; [25,52,56]), potato yellow mosaic virus from Venezuela (PYMV; [5]), squash leaf curl virus from California (SqLCV-E, extended host range isolate [37]), bean calico mosaic virus from Mexico (BCMoV; [39]), SqLCV-R (restricted host range isolate [35]), Calopogonium golden mosaic virus from Costa Rica (CalGMV; [47]), and Texas pepper virus (TPV, A. O. Loniello, and D. P. Maxwell, *unpublished data*).

was confirmed by detection of appropriate fragment sizes with Southern hybridization analyses (data not shown). These results support previous observations that geminiviruses with similar common regions can form infectious pseudorecombinants (e.g., two isolates of African cassava mosaic virus [ACMV], [51]; two strains of tomato golden mosaic virus [TGMV], [56]; and BDMV and tomato mottle geminivirus [ToMoV], [18]). Conversely, mixtures of the components of geminiviruses with different common regions (>25% nucleotide differences) are not infectious (e.g., BGMV-BZ and -GA, [15]; TGMV and SqLCV, [38]; ACMV from Kenya and from India, in Stanley [48]). Thus, the infectious pseudorecombinants between BGMV-DR and -GA further support the classification of these isolates as BGMV type II (15).

Whitefly-transmission of BGMV from beans infected with viral clones. Even though infectious clones of 17 bipartite, whitefly-transmitted geminiviruses have been reported, only the progeny of the cloned components of Texas pepper virus (TPV) have been whitefly-transmitted (53). Whiteflies fed on beans infected separately with the monomers of BGMV-DR or -GA were able to transmit each of these isolates to beans (all three test plants for each isolate were infected), and the successful transmission was confirmed by serologically specific electron microscopy ([7]; data not shown). Control plants exposed to whiteflies that had fed on symptomless beans did not develop symptoms.

Relationship of BGMV-DR and -GA to other whitefly-transmitted geminiviruses. Nucleotide- and derived amino acid-sequence comparisons indicated that the BGMV-DR and -GA isolates are closely related to BGMV-PR, that these three isolates are all BGMV type II, and that they are distinctly different from other whitefly-transmitted geminiviruses, including the BGMV-BZ type I (15). These differences are particularly evident from the alignment of the common regions for 15 geminivirus isolates from the Western Hemisphere (Fig. 2). This alignment was used to construct a cladogram for these viruses (Fig. 3). Fourteen of the 15 isolates were placed into the four previously proposed cluster groups of Western Hemisphere geminiviruses (47): the BGMV type II cluster (BGMV-DR, -GA, and -PR), the BGMV type I cluster (BGMV-BZ, TGMV, and potato yellow mosaic virus [PYMV]), the Abutilon mosaic virus (AbMV) cluster (AbMV, BDMV, and ToMoV), and the SqLCV cluster (BCMoV, TPV, two strains of SqLCV, and Calopogonium golden mosaic virus [CalGMV]). Interestingly, bean-infecting viruses are present in all four of these clusters. Pepper huasteco virus (PHV) formed a new cluster. Distinguishing common-region characteristics are clearly evident for three of the clusters. Members of the BGMV type II cluster have the previously described direct repeat in the 5′ end of the common region, whereas the members of the AbMV cluster have a different direct repeat (TACT) in this area. Members of the SqLCV cluster have a GGGCAAAA motif immediately 5′ of the stem-loop motif. Members of the BGMV type I cluster are more divergent than the members within the other clusters and may represent distantly related geminiviruses that could be subdivided.

Derived amino acid sequences of the AC1 ORF were used by Howarth and Vandemark (30) to study the phylogenetic relationships of eight whitefly-transmitted and seven leafhopper-transmitted geminiviruses. These geminiviruses were separated into two groups according to their insect vectors. Among the whitefly-transmitted geminiviruses, the five Western Hemisphere viruses were differentiated from the three Eastern Hemisphere viruses. In our study, the nucleotide sequences of the AC1 ORFs of 17 whitefly-transmitted and three leafhopper-transmitted geminiviruses were aligned, and a cladogram was constructed (Fig. 4). One cluster, which included two monopartite, leafhopper-transmitted geminiviruses from the Eastern Hemisphere, was far removed from the other 18 geminiviruses. The two geminiviruses, maize streak virus (MSV; [44]) and tobacco yellow mosaic virus (TobYMV; [43]), have an intron within the AC1 ORF, unlike the other geminiviruses, including the only other monopartite, leafhopper-transmitted geminivirus, beet curly top virus (BTCV). The SqLCV cluster is most closely related to the MSV and TobYMV group; and, among the whitefly-transmitted geminiviruses, the AC1 ORF for SqLCV has a distinct derived amino acid sequence that includes a unique gap of four amino acids (Fig. 5). This gap is only found in members of this cluster of the whitefly-transmitted geminiviruses (e.g., BCMoV; [39] and CalGMV) (M. R. Rojas, J. Karkashian, and D. P. Maxwell, *personal communication*). Additional clusters included the BGMV type II cluster (BGMV-DR, -GA, and -PR), the AbMV cluster (AbMV, BDMV, and ToMoV), the PYMV cluster, the TGMV cluster, and the BGMV type I cluster (BGMV-BZ). Curiously, BCTV, which is a monopartite, leafhopper-transmitted geminivirus, and the bipartite, whitefly-transmitted PHV (14) from Mexico are situated in the phylogenetic tree between the

TABLE 2. Infection of *Phaseolus vulgaris* with cloned DNAs of bean golden mosaic virus (BGMV) isolates from the Dominican Republic (DR) and Guatemala (GA) and pseudorecombinants between the components of these two geminivirus isolates

Pseudorecombinant[a] (DNA-A & DNA-B)	No. of plants with symptoms/ no. inoculated[b]		
	Exp. 1	Exp. 2	Exp. 3
GP	0/3	0/3	0/3
GA & GA	3/3	3/3	2/3
DR & DR	3/3	1/3	6/6
GA & DR	2/6	3/3	6/6
DR & GA	1/6	1/3	2/5

[a]GP = Gold particles only.

[b]Plant radicles were inoculated by electric discharge particle acceleration. Symptoms were recorded 7–8 and 13–14 days after inoculation. Numbers of plants with symptoms 13–14 days after inoculation are presented. Symptoms always developed within 7 days, except after inoculation with BGMV-DR DNA-A and BGMV-GA DNA-B, in which symptoms were delayed and attenuated. Exp. = experiment.

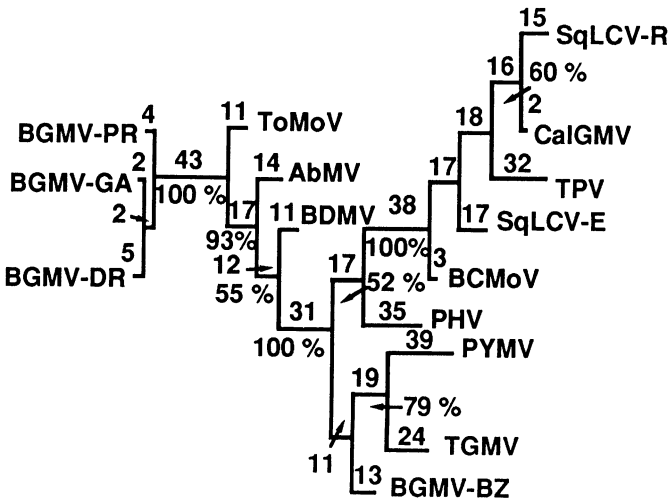

Fig. 3. Cladogram showing the relationships among 15 whitefly-transmitted, Western Hemisphere geminiviruses based on alignment of nucleotide sequences for the common region (Fig. 2). Sequences were analyzed by the Phylogenetic Analysis Using Parsimony, version 3.1, developed by D. L. Swofford (Illinois Natural History Survey, Champaign), using Heuristic tree construction, and branch strength was tested by constructing 100 trees by bootstrap with branch swapping. The numbers of nucleotide changes are noted on top of the horizontal lines, and the percentage of trees with a given branch are below the horizontal lines. Vertical distances are arbitrary, and horizontal distances are in proportion to the number of nucleotide differences between branch nodes. Bean golden mosaic virus (BGMV) from Puerto Rico (-PR), from Guatemala (-GA), and from the Dominican Republic (-DR), tomato mottle virus (ToMoV), Abutilon mosaic virus (AbMV), bean dwarf mosaic virus (BDMV;), squash leaf curl virus (SqLCV-R), Calopogonium golden mosaic virus (CalGMV), Texas pepper virus (TPV), SqLCV-E, bean calico mosaic virus (BCMoV), pepper huasteco virus (PHV), potato yellow mosaic virus (PYMV), tomato golden mosaic virus (TGMV), and BGMV from Brazil (-BZ). Figure 2 provides more complete information on the viruses.

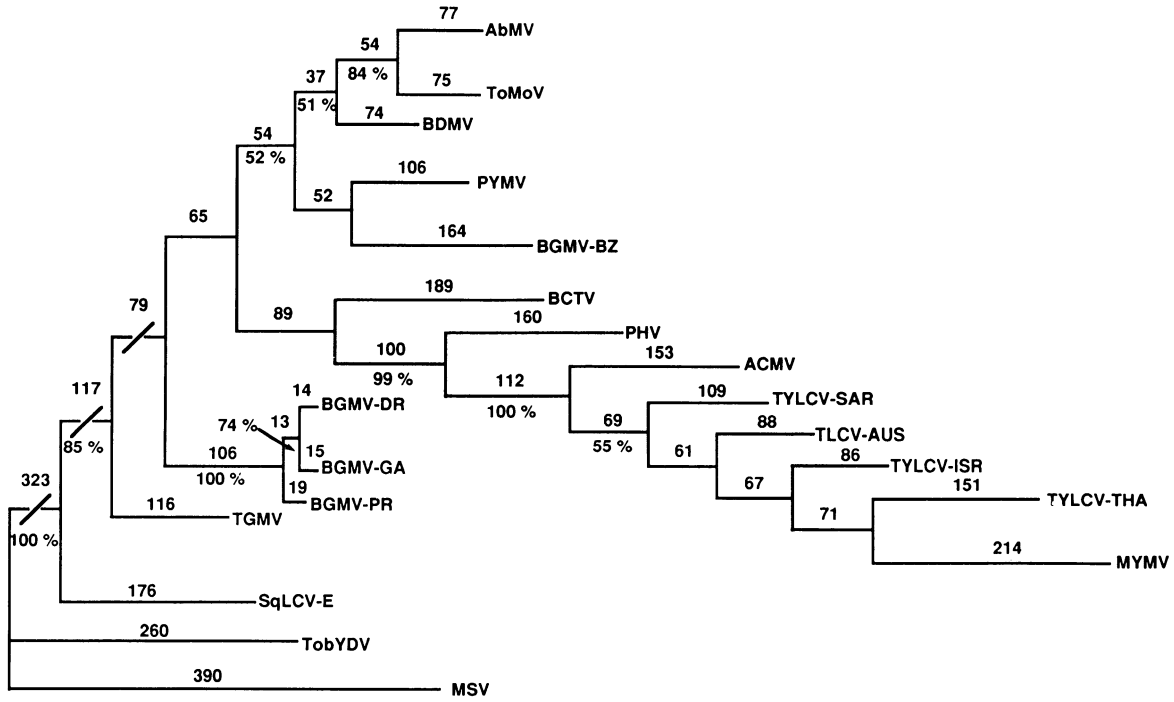

Fig. 4. Cladogram showing relationships among three leafhopper-transmitted geminiviruses (beet curly top virus [BCTV], [50]; maize streak virus [MSV], [44]; and tobacco yellow dwarf virus [TobYDV], [43]) and 17 whitefly-transmitted geminiviruses, based on the total nucleotide sequences of open reading frame (ORF) AC1: bean golden mosaic virus (BGMV) from the Dominican Republic (-DR), from Guatemala (-GA), and from Puerto Rico (-PR), tomato golden mosaic virus (TGMV), squash leaf curl virus (SqLCV-E), Abutilon mosaic virus (AbMV), tomato mottle virus (ToMoV), bean dwarf mosaic virus (BDMV), potato yellow mosaic virus (PYMV), BGMV from Brazil (-BZ), pepper huasteco virus (PHV), African cassava mosaic virus (ACMV), tomato yellow leaf curl virus (TYLCV) from Sardinia (-SAR; [32]), tomato leaf curl virus from Australia (TLCV-AUS), TYLCV from Thailand (-THA; S. Attathom, *personal communication*), and mung bean yellow mosaic virus from Thailand (MYMV; T. Morinaga, M. Ikegami, and K.-I. Miura, *personal communication*). Figure 2 provides more complete information on the viruses. The nucleotide sequence for the AC1 ORF of AbMV was adjusted as discussed previously (28).

	112 132
BGMV-GA	EWGQFQVDGRSARGGQQSAND
BGMV-DR	EWGQFQVDGRSARGGQQTAND
BGMV-PR	EWGQFQVDGRSARGGQQSAND
AbMV	EWGEFQIDGRSARGGQQTAND
BDMV	EWGVFQIDGRSARGGQQSAND
ToMoV	EWGDFQIDGRSARGGQQSAND
PHV	EWGEFQIDGRSARGGQQSAND
BGMV-BZ	EWGHFQVDGRSARGGQQTAND
TGMV	VWGEFQVDGRSARGGCQTSND
PYMV	EWGLFQIDGRSARGGQQTVND
SqLCV-E	ESGQYKVSGGS....KSNKDD
BCTV	EWGEFQIDGRSARGGQQTAND
ACMV	EWGQFQIDGRSARGGQQSAND
TYLCV-ISR	DFGVSQIDGRSARGGQQSAND
TLCV-AUS	EWGEFQIDGRSARGGQQSAND
TYLCV-THA	DHGIFQIDGRSARGGCQSAND
TYLCV-SAR	EWGTFQIDGRSARGGQQTAND
MYMV	DHGSFQVDGRSARGGKQSAND

Fig. 5. Derived amino acid-sequence alignment for open reading frame (ORF) AC1 of 18 geminiviruses showing the proposed gap for squash leaf curl virus (SqLCV). Bean golden mosaic virus (BGMV) from Guatemala (-GA), from the Dominican Republic (-DR), and from Puerto Rico (-PR), and Abutilon mosaic virus (AbMV), bean dwarf mosaic virus (BDMV), tomato mottle virus (ToMoV), pepper huasteco virus (PHV), BGMV from Brazil (-BZ), tomato golden mosaic virus (TGMV), potato yellow mosaic virus (PYMV), SqLCV-E (extended host range isolate), beet curly top virus (BCTV), African cassava mosaic virus (ACMV), tomato yellow leaf curl virus (TYLCV) from Israel (-ISR), tomato leaf curl virus from Australia (TLCV-AUS), TYLCV from Thailand (-THA), TYLCV from Sardinia (-SAR), and mung bean yellow mosaic virus (MYMV). Figures 2 and 4 provide more complete information on the viruses. The nucleotide sequence for the AC1 ORF of AbMV was adjusted as discussed previously (28). Numbers correspond to the amino acid positions for the derived amino acid sequence of AC1 protein of BGMV-GA.

Western and Eastern Hemisphere viruses. The six Eastern Hemisphere viruses were placed in a distinct group that can be divided into at least the ACMV cluster, the tomato yellow leaf curl virus (TYLCV) cluster (TYLCV from Isreal [-ISR] and from Sardinia [-SAR] and tomato leaf curl virus from Australia, each with a monopartite genome), and the TYLCV from Thailand (-THA) cluster (TYLCV-THA and mung bean yellow mosaic virus, each with a bipartite genome). It is very likely that these clusters will be subdivided in the future.

When the phylogenetic cladograms generated from the common-region sequences (Fig. 3) and AC1 ORF (Fig. 4) are considered, similar clusters of the whitefly-transmitted geminiviruses emerge. This might be expected since the replication-associated AC1 protein interacts with specific nucleotides in the common region (10). The importance of these relationships also is shown by the finding that infectious pseudorecombinants can only be made between the components of members within the same cluster (e.g., BGMV-DR and -GA; two isolates of TGMV, [57]; SqLCV strains, [35]; ACMV isolates, [51]; and ToMoV and BDMV, [18]). No infectious pseudorecombinants have been made between components of geminiviruses from different clusters (e.g., BGMV-GA and -BZ, [15]; SqLCV and TGMV, [37]; ACMV and TGMV [K. W. Buck, R. H. A. Coutts, I. T. Petty, and J. Stanley, *unpublished data* in von Arnim and Stanley (57)]). It will be valuable to determine the minimal similarities in common-region nucleotides and in AC1-derived amino acid sequences that allow the formation of an infectious combination, because these mixtures are likely to have a major role in the evolution of geminiviruses (18).

The phylogenetic analysis of the nucleotide sequences of the AV1 ORFs for the 17 whitefly-transmitted geminiviruses (Fig. 6A) indicates that they can be divided into the Western and Eastern Hemisphere groups and that PHV is again intermediate between these two groups. In general, this phylogenetic cladogram shows

a relationship among these viruses similar to that shown by the cladograms for the common region (Fig. 3) and AC1 ORF (Fig. 4). The BGMV type II isolates were placed in a distinct cluster, BDMV was part of the AbMV cluster, and BGMV-BZ type I was not closely related to other members of the Western Hemisphere group. Alignment of the derived amino acids for the AV1 ORFs supports the placement of BGMV type II isolates into a distinct phylogenetic cluster because there are three potential start codons in this ORF unique to BGMV type II isolates (Fig. 7). The second start codon is 30-nt from the 3′ end of the first start codon, and this second start codon appears to be the preferred initiation codon because it is present in the majority of gemini-

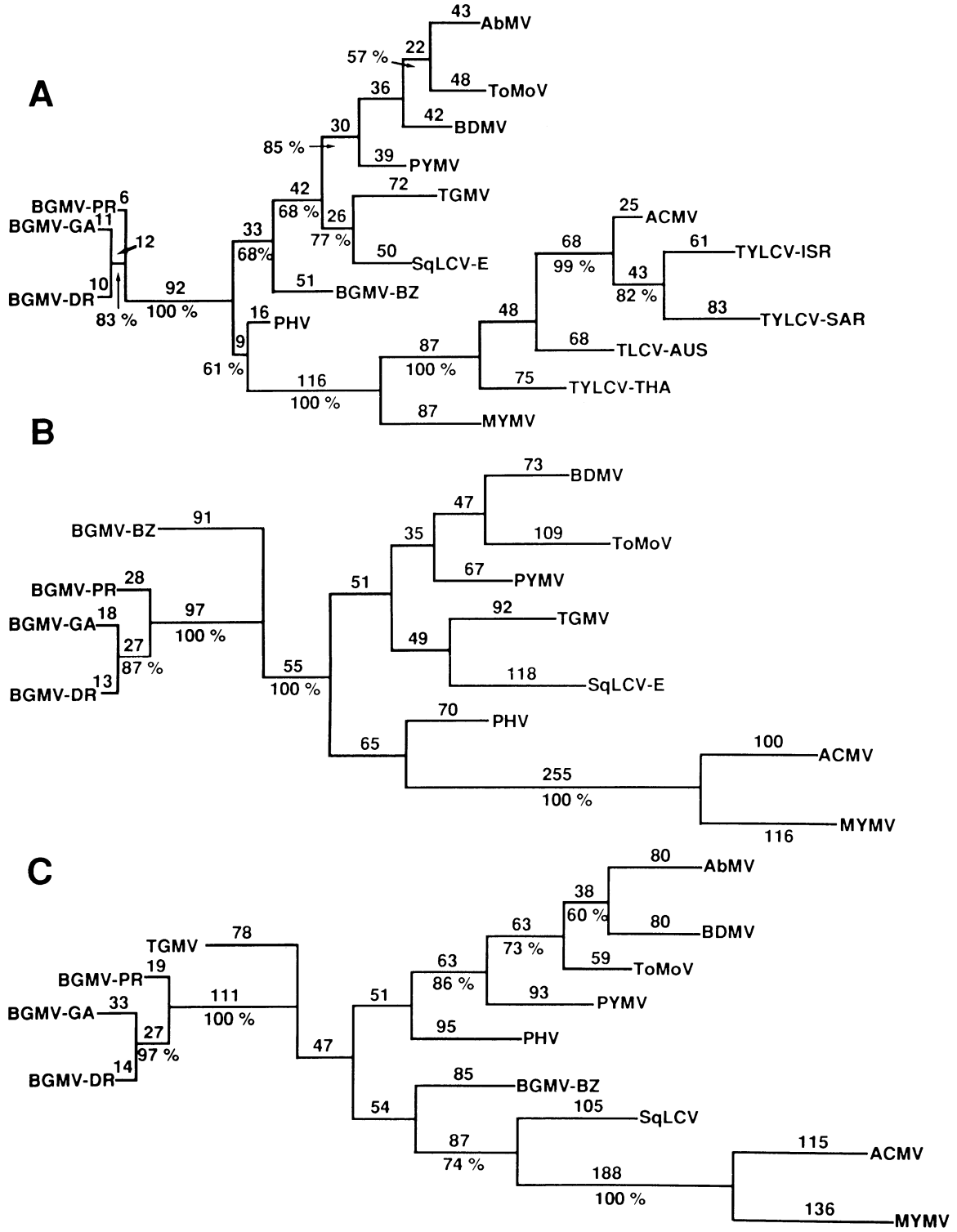

Fig. 6. Cladogram showing relationships among the whitefly-transmitted geminiviruses based on total nucleotides for open reading frames (ORFs) **A,** AV1, **B,** BC1, and **C,** BV1. Bean golden mosaic virus (BGMV) from Puerto Rico (-PR), from Guatemala (-GA), and from the Dominican Republic (-DR), Abutilon mosaic virus (AbMV), tomato mottle virus (ToMoV), bean dwarf mosaic virus (BDMV), potato yellow mosaic virus (PYMV), tomato golden mosaic virus (TGMV), squash leaf curl virus (SqLCV-E, extended host range isolate), BGMV from Brazil (-BZ), pepper huasteco virus (PHV), African cassava mosaic virus (ACMV), tomato yellow leaf curl virus (TYLCV) from Israel (-ISR), TYLCV from Sardinia (-SAR), tomato leaf curl virus from Australia (TLCV-AUS), TYLCV from Thailand (-THA), and mung bean yellow mosaic virus (MYMV). Figures 2 and 4 provide more complete information on the viruses. The BC1 ORF of AbMV is not included because of major differences in the derived amino acids for the C-terminal region. The sequence of the BV1 ORF of AbMV was modified as discussed previously (18).

viruses and the transcriptional initiation for the AV1 ORF of TGMV is consistent with this ATG initiation codon (55). A similar situation exists for the start of the AC2 ORF (data not shown). Because the amino terminal end shows considerable variation among the geminiviruses, this region may function as different epitopes for interaction with monoclonal antibodies (MAbs) (27). MAbs produced against coat protein of ACMV were used to distinguish BGMV-GA and -DR from -BZ (B. D. Harrison, J. C. Faria, and D. P. Maxwell, *personal communication*).

Phylogenetic analyses also were completed with the two DNA-B ORFs, BC1 (12 viruses) and BV1 (13 viruses; Fig. 6). It was thought that a different clustering of geminiviruses might be obtained with an analysis of these ORFs because of the role of the BC1 and BV1 proteins in virus movement and, perhaps, host range. The general clustering of viruses was similar in cladograms derived from DNA-A (Figs. 3, 4, and 6A) and DNA-B (Fig. 6B and C) sequences. Thus, BGMV type II isolates always form a cluster, and BDMV associates more closely with another cluster that contains AbMV and ToMoV. The phylogenetic analyses based on BC1 and BV1 do not indicate a strong relationship between the nucleotide sequences of these ORFs and the host range of the viruses.

Phylogenetic analysis of the common region and the four ORFs of the Western Hemisphere bipartite geminiviruses clearly define three clusters: BGMV type II, AbMV, and SqLCV; it is likely that BGMV-BZ type I, PYMV, TGMV, and PHV represent members of four additional clusters. Thus, seven cluster groups are proposed for these Western Hemisphere whitefly-transmitted geminiviruses.

Conservation of possible functional regions of the common region and AC1 protein. Recently, Fontes et al (10) showed that the AC1 protein of TGMV specifically binds to the DNA region between nucleotide 53 and 105 of the common region of DNA-A. This region includes the transcription initiation site (nucleotide 62) of the AC1 transcript (10). In the alignment of the common regions of 15 geminiviruses (Fig. 2), the region homologous to the nucleotide 53–105 region of TGMV is contained within nucleotide 67–125. A direct repeat of 4–8 nt containing at least three G's ($GGXGX_nGGXG$, X = any nucleotide, n = 3 or 4 nucleotides) is 4–6 nt immediately 5′ (toward the start of the common region) of a conserved TATA box. One exception is ToMoV, which has only two G's in the second motif. The similarities of these direct repeats are evident within the members of the phylogenetic clusters (Fig. 3). For example, the three BGMV type II isolates have a TGGAG repeat sequence; four of the five members of the SqLCV cluster have TGGTGTC and one member has TGGAGTC; and the three members of the AbMV cluster have ATT(G/A)GAG. The separation of BGMV type I, TGMV, and PYMV into three clusters is supported by differences in their direct repeats, i.e., GGTG for BGMV-BZ, GGTAG for TGMV, and TGGGGGAA for PYMV. PHV has an imperfect repeat of T(C/T)GGT, and we suggest that it forms a separate cluster. In all cases, the two sets of GG were separated by 5–6 nt. These highly conserved direct repeats are likely targets for the binding of the homologous AC1 protein. Interestingly, infectious pseudorecombinants have been made only between geminiviruses that have similar direct repeats (e.g., BGMV-DR and -GA, SqLCV-E and -R [extended and restricted host ranges, respectively (35)], and BDMV [repeat = ATTGGAGT] and ToMoV [repeat = ATTAGAGT] [18]). Attempts to make infectious pseudorecombinants with components of geminiviruses that have different direct repeats (e.g., BGMV-GA and -BZ, [15], and TGMV and SqLCV, [38]) have failed. Additional common region sequence similarities exist within phylogenetic clusters and also may interact specifically with AC1 proteins (e.g., the direct repeats of the BGMV type II isolates or the GGGCAAAA motif that is immediately 5′ of the stem-loop region in the members of the SqLCV cluster).

The AC1 protein is the only viral protein required for replication (36), and several functional motifs consistent with this role in replication have been identified within the AC1-derived amino acid sequence. It is proposed that the AC1 protein functions by specifically binding to common-region sequences (10) and thereby initiates the generation of ssDNA from the double-stranded DNA (dsDNA) intermediate via a rolling circle mechanism (54). This process probably involves the nicking of one strand of the dsDNA

```
          1                                30
BGMV-GA   MYAHFTCKSR MPKRDAPWRH MAGTSKVSRS
BGMV-DR   MHAHSTCKSR MPKRDAPWRN NAGTYKVSRS
BGMV-PR   MYAHSTCKSR MPKRDAPWRH MAGTSKVSRS
AbMV      .......... IPKRDLPWRS MPGTSKTSRN
BDMV      .......... MPKRDAPWRS MAGTTKVSRN
ToMoV     .......... MPKRDLPWRS MAGTSKVSRN
PHV       .......... MPKRDAPWRL TAGTAKISRT
BGMV-BZ   .......... MPKRDAQWRH MGGTSKISRS
TGMV      .......... MPKRDAPWRL MAGTSKVSRS
PYMV      .......... MPKRDAPWRS MAGTSKVSRN
SqLCV-E   .......... MVKRDAPWRL MAGTSKVSRS
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Fig. 7. Derived amino acid sequence alignment for the N-terminal region of open reading frame (ORF) AV1 showing the potential variation in the initiation codon between bean golden mosaic virus (BGMV) type II isolates and eight other whitefly-transmitted geminiviruses. BGMV from Guatemala (-GA), from the Dominican Republic (-DR), and from Puerto Rico (-PR), Abutilon mosaic virus (AbMV), bean dwarf mosaic virus (BDMV), tomato mottle virus (ToMoV), pepper huasteco virus (PHV), BGMV from Brazil (-BZ), tomato golden mosaic virus (TGMV), potato yellow mosaic virus (PYMV), and squash leaf curl virus (SqLCV-E, extended host range isolate). Figures 2 and 4 provide more complete information on the viruses. Numbers correspond to the amino acid positions for the derived amino acid sequence of AV1 protein of BGMV-GA.

```
          10                               30
BGMV-GA   QSKNYFLTYPRCPIPKEEVLS
BGMV-DR   QSKNYFLTYPHCSIPKEEALS
BGMV-PR   QSKNYFLTYPRCTIPKEEALS
BDMV      QSRNYFLTYPQCSLTKEEALS
BGMV-BZ   NAKNYFLTYPQCSITKESAIE
SqLCV-E   TARNIFLTYPRCDVPKEEVLE
TYLCV-ISR YAKNYFLTYPNCSLSKEEALS
               ----  -
               FLTY  C
          50                               70
BGMV-GA   RHENGEPHLHALIQFEGKFVC
BGMV-DR   RHENGEPHLHALIQFEGKFVC
BGMV-PR   RHDNGEPHLHALIQFEGKFIC
BDMV      LHEDGEPHLHVLIQFEGKYQC
BGMV-BZ   IHENGEPHLHALIQFEGKFQC
SqLCV-E   EHSDGSPHLHCLIQLSGKSNI
TYLCV-ISR LHENGEPHLHVLIQFEGKYQC
                - -   -
                H H   Q
          90                              110
BGMV-GA   NIQGAKSSSDVKAYIDKDGVT
BGMV-DR   NIQGAKSSSDVKAYIDKDGDT
BGMV-PR   NIQGAKSSSDVKEYIDKDGVT
BDMV      NIQGAKSSSDVKSYIDKDGDT
BGMV-BZ   NIQSAKSSSDVKSYIEKDGDY
SqLCV-E   NIQAAKDTNAVKNYITKEGDY
TYLCV-ISR NIQAAKSSTDVKTYVEKDGNF
                       *  -
                       Y  K
```

Fig. 8. Putative DNA-nicking motif (31,34) for the derived amino acids of the N-terminal region of open reading frame (ORF) AC1 of bean golden mosaic virus (BGMV) from Guatemala (-GA), the Dominican Republic (-DR), and Puerto Rico (-PR), bean dwarf mosaic virus (BDMV), BGMV from Brazil (-BZ), squash leaf curl virus from California (SqLCV-E, extended host range), and tomato yellow leaf curl virus from Israel (TYLCV-ISR). The three potentially important regions for DNA-nicking activity are marked with a dash, and the putatively active Tyr (Y) is indicated by an asterisk (*). The numbers above the derived amino acid sequence correspond to the amino acid positions for BGMV-GA.

intermediate as in the replication of other circular ssDNA viruses and plasmids (31). Recently, Koonin and Ilyina (34) reported the conservation in monopartite and bipartite geminiviruses of three amino acid motifs involved in nicking dsDNA. As expected, these motifs are also conserved in the AC1-derived amino acid sequences of BGMV-DR and -GA (Fig. 8). Additionally, a purine NTP-binding motif has been identified within the putative AC1 protein of geminiviruses (22,26) and has the following characteristics for BGMV-DR and -GA: (β-strand) Glu Asp XXXX Gly Lys Thr (α-helix) X_{32} (β-strand) Asp Asp X_{43} Asn (Asn is preceded by several hydrophobic amino acids; X = any amino acid residue). Because this motif is found in helicases of several viruses, the AC1 protein may have helicase activity (23). Hanson et al (26) reported that codon changes for conserved amino acids in this motif abolished infectivity of BGMV-GA; and they suggested that a trans-dominant interference scheme for virus-derived resistance might be considered using these mutants. Also, codon changes in the nicking motif may be attractive candidates for engineering plants with resistance to geminiviruses. By analogy to the large T antigen of the animal DNA virus, simian virus 40 (9), other putative amino acid domains associated with AC1 protein would include nuclear location signals, common region-binding domains, and phosphorylation sites.

In summary, our observations on genome organization (Figs. 1, 2, and 7), phylogenetic relationships (Figs. 3, 4, and 6), and the formation of infectious pseudorecombinants (Table 2) clearly establish BGMV-DR and -GA as BGMV type II (15). These isolates are distinct from three other characterized bean-infecting geminiviruses, BGMV type I (15), BDMV (28), and BCMoV (39). This diversity must be considered when implementing programs to develop beans resistant to geminiviruses. The sequenced, infectious clones of bean-infecting geminiviruses can be used for research on genome function and for development of antiviral schemes for producing resistant plants.

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