

Use of the asymmetric polymerase chain reaction and DNA sequencing to determine genetic variability of bean golden mosaic geminivirus in the Dominican Republic

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A combination of the polymerase chain reaction (PCR), asymmetric PCR (A-PCR) and DNA sequencing was used to determine the nucleotide sequence of a hypervariable region of the bipartite genome of bean golden mosaic geminivirus (BGMV). This region, which was part of the intergenic region of the DNA-B component, was amplified using primers designed from the nucleotide sequence of a DNA-B component clone (pDRB1) of an isolate of BGMV from the Dominican Republic (BGMV-DR). pDRB1 is infectious on beans when coinoculated with the DNA-A component of BGMV-DR (pDRA1), and typical bean golden mosaic symptoms are observed on infected plants. Bean leaf tissue infected with BGMV was collected at five separate field locations in the Dominican Republic and the hypervariable region was amplified by PCR, ssDNA was produced using A-PCR, and partial nucleotide sequences were determined. The

sequences of the hypervariable region from the field-collected samples ranged from 95% (one sample) to 98% (four samples) identical to the sequence of pDRB1. This contrasts with sequence identities of 86, 75 and 46% between the pDRB1 hypervariable region and the hypervariable regions of BGMV isolates from Guatemala, Puerto Rico and Brazil respectively, and 42% with bean dwarf mosaic geminivirus. These results indicate that Dominican Republic isolates of BGMV are very similar and should be considered isolates of the same virus (BGMV-DR), and that the infectious clones of BGMV-DR are representative of BGMV isolates in the Dominican Republic. The procedures described for DNA extraction from leaf tissue and for production of high quality ssDNA using PCR and A-PCR are rapid and efficient and could be applied to studies of variability and epidemiology of other viruses.

Geminiviruses are a unique group of plant viruses characterized by their twinned (geminate) icosahedral virions and ssDNA genomes. Diseases caused by geminiviruses are a major constraint on crop production, particularly in the tropics (Galvez & Morales, 1989). Two distinct subgroups of geminiviruses are recognized by the International Committee on Taxonomy of Viruses (ICTV); subgroup I geminiviruses possess a monopartite genome, are transmitted by various leafhopper species, and primarily infect monocotyledonous plants, whereas subgroup II geminiviruses possess a bipartite genome, are transmitted by the sweet potato whitefly (*Bemisia tabaci* Gennadius), and infect dicotyledonous plants (ICTV Group Descriptions, 1989). A number of subgroup II geminiviruses infect the common bean (*Phaseolus vulgaris* L.), and recent molecular studies on geminiviruses causing bean golden mosaic (BGM) and bean dwarf mosaic diseases have demonstrated that the genomes of these viruses are composed of two DNA

components, designated DNA-A and DNA-B (Howarth *et al.*, 1985; Gilbertson *et al.*, 1991*b*), and that considerable genetic diversity exists among these viruses (Gilbertson *et al.*, 1990). Four bean golden mosaic geminivirus (BGMV) isolates and one bean dwarf mosaic geminivirus (BDMV) isolate have been characterized. Three of the BGMV isolates from Puerto Rico (BGMV-PR; Howarth *et al.*, 1985), Guatemala (BGMV-GA; Morales & Niessen, 1988; Gilbertson *et al.*, 1991*a*) and the Dominican Republic (BGMV-DR; Gilbertson *et al.*, 1991*a*) are closely related. The fourth isolate, BGMV from Brazil (BGMV-BZ; Gilbertson *et al.*, 1988, 1991*a*), is highly divergent from the closely related BGMV isolates. The BDMV isolate from Colombia (Morales *et al.*, 1990) is divergent from all four BGMV isolates (Gilbertson *et al.*, 1990). Other subgroup II geminiviruses that infect the common bean include bean calico mosaic geminivirus (Brown *et al.*, 1990), squash leaf curl geminivirus (Cohen *et al.*, 1983), and the

recently isolated geminiviruses from tomatoes from Mexico (N. P. Paplomatas, E. Hidayat, D. P. Maxwell & R. L. Gilbertson, unpublished data) and Costa Rica (M. R. Rojas, N. P. Paplomatas, D. P. Maxwell & R. L. Gilbertson, unpublished data). Because of the diverse nature of geminiviruses that can infect the common bean, there is a need to develop rapid and specific methods for their detection and differentiation.

In a previous study, we demonstrated that isolate-specific DNA probes, which consisted of the cloned DNA-B component of BGMV-DR, BGMV-BZ or BDMV used under high stringency hybridization conditions, differentiated these bean-infecting geminiviruses (Gilbertson *et al.*, 1991a). The cloned DNA-B of BGMV-DR was used as a probe to demonstrate that only viral isolates similar to BGMV-DR were prevalent in beans with BGM symptoms in different bean-growing regions of the Dominican Republic (Gilbertson *et al.*, 1991a). However, this probe does not differentiate closely related BGMV isolates, e.g. BGMV-DR and BGMV-GA, and there are differences among these isolates in symptomatology (D. P. Maxwell, R. L. Gilbertson & F. J. Morales, unpublished data), viral nucleic acid titre in infected plants (Gilbertson *et al.*, 1991a), and in symptoms when DNA components are exchanged (data for BGMV-DR and BGMV-GA; Faria *et al.*, 1990).

Because DNA sequence data are available for BGMV-PR (Howarth *et al.*, 1985), BGMV-BZ (R. L. Gilbertson, J. C. Faria, P. G. Ahlquist & D. P. Maxwell, unpublished data), BGMV-DR and -GA (J. C. Faria, R. L. Gilbertson, S. F. Hanson, F. J. Morales, P. G. Ahlquist & D. P. Maxwell, unpublished data) and BDMV (S. H. Hidayat, R. L. Gilbertson, P. G. Ahlquist, F. J. Morales, D. R. Russell, S. F. Hanson & D. P. Maxwell, unpublished data), we were able to determine the most variable region of the virus genome for these

five geminiviruses. This region, which we refer to as the hypervariable region, is part of the intergenic region of DNA-B (approx. 300 to 400 bp) which lies between the initiation codon of the open reading frame (BL-1) and the 5' end of the common region (Fig. 1). The percentage nucleotide sequence identities between the hypervariable regions of these geminiviruses are shown in Table 1.

Recently, we demonstrated that the cloned DNA-A and DNA-B components of BGMV-DR are infectious on beans (Gilbertson *et al.*, 1991b). We propose to use the

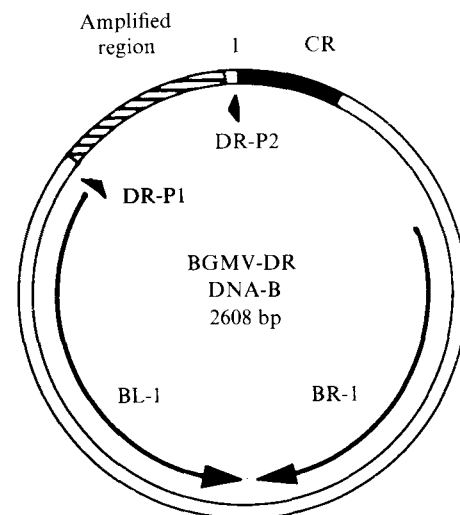


Fig. 1. Diagrammatic representation of the genomic organization of the DNA-B component of BGMV-DR showing the positions of the common region (CR), the two open reading frames (BR-1 and BL-1) and the region amplified for evaluation of genetic variability of BGMV-DR isolates [for the purposes of this figure, the circularized DNA-B insert of pDRB1 (Gilbertson *et al.*, 1991b) is shown, with nt 1 corresponding to the first nucleotide of the common region]. The locations at which amplification primers (DR-P1, viral-sense; DR-P2, complementary-sense) anneal to BGMV-DR DNA-B are shown by the arrowheads.

Table 1. Percentage nucleotide sequence identities between the hypervariable regions* of BGMV isolates and BDMV

Geminivirus isolate	Nucleotide sequence identity (%) [†]				
	BGMV-DR	BGMV-GA	BGMV-PR	BGMV-BZ	BDMV
BGMV-DR	—	86	75	46	42
BGMV-GA		—	70	44	44
BGMV-PR			—	52	50
BGMV-BZ				—	44
BDMV					—

* The hypervariable region is the intergenic region of component DNA-B that lies between the initiation codon of open reading frame BL-1 and the beginning of the common region.

[†] Numbers are the direct percentage nucleotide sequence identities rounded to the nearest whole number. All comparisons were made using the GCG program GAP.

DNA sequence of the hypervariable region to determine whether the BGMV-DR infectious clones are representative of BGMV isolates in the Dominican Republic and to assess variability among field isolates of BGMV. We report the use of polymerase chain reaction (PCR) (Saiki *et al.*, 1988) and asymmetric PCR (A-PCR; McCabe, 1990) to amplify DNA of this hypervariable region, and DNA sequencing to assess genetic variability. We demonstrate that the infectious clones of BGMV-DR are representative of BGMV in the Dominican Republic.

The original field isolate of BGMV-DR was collected by A. Figueroa (Gilbertson *et al.*, 1991a) in San Juan de la Maguana, Dominican Republic in 1987. The isolate was passed through *P. vulgaris* by sap transmission at least 10 times before DNA extracts were made from which full-length infectious clones of DNA-A and DNA-B were obtained (Gilbertson *et al.*, 1991b). The recombinant plasmid pDRB1 contains the full-length BGMV-DR DNA-B cloned at a unique *Hind*III site (Gilbertson *et al.*, 1991b).

Bean leaf tissue with typical BGM symptoms was collected in February 1990 from five separate locations in the southwestern region of the Dominican Republic: Azua, San Juan de la Maguana (fields 1 and 2), Niebo and Lake Enriquillo. From each location, a single half-expanded trifoliate leaflet was collected and allowed to dry at ambient temperature between sheets of paper. The samples were stored at room temperature for approximately 4 months.

DNA was extracted from an approximately 25 mm² piece of dried leaf tissue. The tissue was placed in a 1.5 ml microfuge tube, 500 µl of extraction buffer was added [50 mM-EDTA, 500 mM-NaCl, 10 mM-2-mercaptoethanol (Dellaporta *et al.*, 1983)] and the tissue was ground thoroughly with a pestle (Kontes). Immediately after the tissue had been ground, 33 µl of 20% SDS was added, the tube was vigorously agitated in a vortex mixer, and was then incubated at 65 °C for 10 min. After incubation, 160 µl of 5 M-potassium acetate (pH 4.5) was added, the tube was vortexed and centrifuged for 10 min at about 10000 *g*. The supernatant (450 µl) was removed and placed in a new tube (carefully avoiding plant debris), 225 µl of isopropanol (0.5 volume) was added, and the tube was vortexed and centrifuged for 10 min at 10000 *g*. The supernatant was removed, the pellet was washed with 500 µl of 70% ethanol, and the tube was centrifuged for 5 min at 10000 *g*. The pellet was dried for 5 min under partial vacuum and resuspended in 500 µl of sterile distilled water.

From the nucleotide sequence of pDRB-1, two primers were designed that would amplify the hypervariable region of BGMV-DR DNA-B. DR-P1 [nucleotides (nt) 2220 to 2242, Fig. 1] is a virus-sense sequence and is located upstream from the start of open reading frame

BL-1. DR-P2 (nt 2583 to 2603, Fig. 1) is a complementary-sense sequence and is located adjacent to the start of the common region. The oligonucleotide sequences for these primers are: DR-P1, 5' CTGAAGCGCAAAGATAGAGAAGC 3' (23 nt), DR-P2, 5' CTCAAAACGATATCGTTTTGC 3' (21 nt).

To produce a ssDNA template for sequencing, two separate PCRs were carried out: a standard PCR to amplify the hypervariable region of BGMV-DR and an A-PCR to produce ssDNA. For the standard PCR, 20 µl of the DNA minipreparation was used. PCR was carried out according to Saiki *et al.* (1988) using Taq polymerase (Promega) according to manufacturer's recommendations in a Perkin-Elmer Cetus DNA Thermal Cycler. The final reaction volume was 100 µl with final dNTP and primer concentrations of 200 µM and 0.4 µM, respectively. Samples were amplified by 30 cycles of PCR with melting, annealing and polymerizing conditions of 60 s at 96 °C, 45 s at 55 °C and 60 s at 72 °C, respectively. Amplified DNA was precipitated with ethanol, resuspended in 25 µl of TE buffer (10 mM-Tris-HCl 1 mM-EDTA), electrophoresed in 1% Seaplaque low-melting point agarose (FMC), the gel was stained with ethidium bromide, and DNA bands were excised. The low-melting point agarose containing the DNA was melted by heating at 65 °C for 10 min and 5 µl of this solution was used for the A-PCR reaction. The A-PCR reactions were identical to those used for the standard PCR except the primer DR-P2:DR-P1 ratios were 50:1 or 100:1. After A-PCR, the amplified DNA was extracted with chloroform, adjusted to 2.5 M-ammonium acetate, and precipitated with two volumes of ethanol. The DNA was resuspended in 30 µl of TE buffer and 7 µl (approx. 1 µg) was used per sequencing reaction. Primer DR-P1 (approx. 0.5 pmol) was used as the sequencing primer. DNA was sequenced using the dideoxynucleotide chain termination method with Sequenase (U.S. Biochemical) according to manufacturer's specifications.

DNA sequences were assembled and analysed using programs from the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984). Identities among nucleotide sequences were determined using the BESTFIT and GAP programs.

Amplified DNA fragments of the predicted size (approx. 300 to 400 bp) were detected in the five bean leaf samples with BGM symptoms collected in the Dominican Republic (DR samples), which indicates that these plants were infected with BGMV-DR, and from pDRB1 (included as a positive control). No fragments of the predicted size were detected in uninfected beans or a sterile water control. Clear unambiguous DNA sequence data (approx. 200 nt) was obtained from the ssDNA templates generated by A-PCR (similar results were

obtained for both primer ratios) for the five DR samples and pDRB1. The alignment of these nucleotide sequences with the previously determined nucleotide sequence of the hypervariable region of BGMV-DR is shown in Fig. 2. The sequence of the fragment amplified from pDRB1 was identical to that previously determined for pDRB1. When the sequences from the DR field samples were compared with the pDRB1 sequence, identities ranged from 95% (one sample) to 98% (the other four samples) (Fig. 2). Because the hypervariable region is the most variable region in the genome of the bipartite geminiviruses (including the common region), the high level of sequence identity (low level of genetic variability) in the hypervariable regions of these BGMV isolates suggests that these are isolates of the same virus (BGMV-DR). More variability exists between the hypervariable region of BGMV-DR and the hypervariable regions of BGMV-GA and BGMV-PR (86 and 75%, respectively; Table 1), which represent the known diversity of closely related BGMV isolates. The overall sequence identity among the DNA-A and DNA-B components of BGMV-DR, BGMV-GA and BGMV-PR is approximately 95 to 98% (J. C. Faria, R. L. Gilbertson, S. F. Hanson, F. J. Morales, P. G. Ahlquist & D. P. Maxwell, unpublished data). The BGMV-DR hypervariable region is highly divergent from those of the distantly related BGMV-BZ and BDMV (46 and 42% similar, respectively; Table 1).

These data support our hypothesis that the full-length infectious DNA-A and DNA-B clones of BGMV-DR are representative of BGMV isolates in the Dominican Republic. The BGMV-DR infectious clones or progeny virus derived from these clones could be used in future efforts to screen bean germplasm for resistance to BGMV-DR and to study the epidemiology of BGM in the Dominican Republic. It appears that passage of BGMV-DR through beans by sap transmission has not resulted in the selection of a variant virus, and that there has not been a major change in the viral population in the Dominican Republic since the original BGMV-DR isolate was collected in 1987. The demonstration that BGMV-DR causes BGM in the Dominican Republic extends our previous results obtained using BGMV-DR DNA-B as an isolate-specific DNA probe (Gilbertson *et al.*, 1991a), which indicated that one or more of the closely related BGMV isolates BGMV-DR, BGMV-GA and/or BGMV-PR, but not BGMV-BZ or BDMV, was causing BGM in the Dominican Republic. However, these data cannot rule out the possibility that our samples contained mixed infections of BGMV-DR isolates with closely related BGMV isolates like BGMV-GA, because primers DR-P1 and/or DR-P2 are not complementary to appropriate sequences of BGMV-GA and BGMV-PR, and the primers did not amplify the BGMV-GA

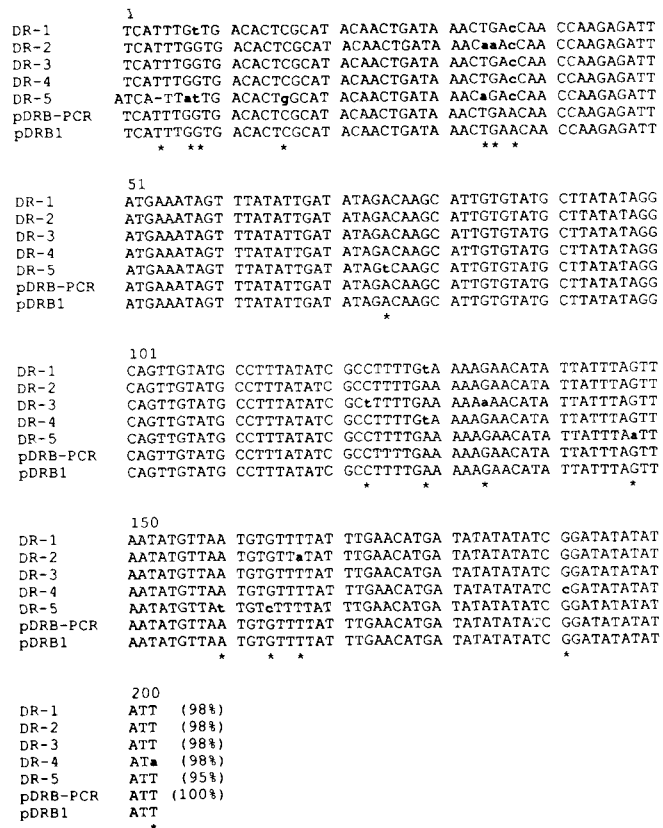


Fig. 2. Alignment of the DNA sequences of the amplified hypervariable region of five BGMV-infected bean field samples from the Dominican Republic and from the cloned BGMV-DR DNA-B (pDRB-PCR), with the previously determined hypervariable region sequence of the BGMV-DR component DNA-B (nt 2279 to 2481 of pDRB1). Variations in nucleotide sequence of the amplified fragments from the pDRB1 sequence are shown as bold lower-case letters, and the positions of these variations with respect to pDRB1 are indicated by an asterisk below the pDRB1 sequence. The percentage sequence identities of the amplified fragment with the pDRB1 sequence are shown in parentheses after the sequence of each fragment.

hypervariable region (BGMV-PR was not tested). Thus, these primers can be used to identify BGMV-DR specifically.

A similar PCR-based approach can be developed to determine whether mixed geminiviral infections occur in the field and to detect and identify geminiviruses in general. PCR primers could be designed that would anneal to highly conserved geminiviral consensus sequences, theoretically allowing amplification and DNA sequence analysis of a specific region of the genome of any bipartite geminivirus. This would allow the rapid characterization of the apparently wide range of whitefly-transmitted geminiviruses that infect weeds and crop plants in Latin America and the southern United States. In some cases, this approach could provide an alternative to the more laborious technique of

cloning and sequencing viral DNA. Recently, the feasibility of such an approach has been demonstrated for the detection and typing of monopartite geminiviruses that infect grasses (Rybicki & Hughes, 1990).

The procedure used for isolation of DNA for PCR amplification is very simple and can be done on a large number of samples in a short period of time. Furthermore, the method can be applied to samples in various conditions (eg. fresh, dry or frozen), and in this study, we used air-dried tissue which had been stored for approximately 4 months. This allows considerable flexibility in how, and where, samples are collected, and at what time they are processed. Although it has been reported that ssDNA templates suitable for sequencing can be produced by direct A-PCR amplification (McCabe, 1990), we found that an important step in the production of a high quality template by A-PCR was the initial amplification of the target fragment by a standard PCR followed by agarose gel isolation of the fragment. This provides more target DNA for the A-PCR and eliminates background DNA and contaminants that may interfere with the A-PCR and/or the sequencing reactions (D. R. Russell, unpublished data).

PCR-based assays have been used to detect and type a number of important viral pathogens of humans and animals, including human immunodeficiency virus (Kellogg & Kwok, 1990), human T cell lymphotropic virus (Ehrlich *et al.*, 1990), hepatitis B virus (Larzul *et al.*, 1988; Kaneko *et al.*, 1989), cytomegalovirus (Demmler *et al.*, 1988) and human papillomavirus (Manos *et al.*, 1989). More recently, PCR methods have been used to detect and characterize viroids (Puchta & Sanger, 1989), monopartite geminiviruses infecting grasses (Rybicki & Hughes, 1990) and luteoviruses (Robertson *et al.*, 1991).

We expect that the use of PCR and A-PCR will provide new and detailed insight into the population structure of plant viruses and viral evolution. The method described in this report can be used to generate the precise nucleotide sequence of a given region of the viral genome, which enables the specific identification of a given virus isolate. Clearly, this approach is more definitive than other methods of virus detection, and can greatly reduce the possible misidentification of a virus. Although we used this method to study a virus with a DNA genome, it could readily be applied to viruses or viroids with RNA genomes by including a reverse transcription reaction before PCR amplification (Puchta & Sanger, 1989; Robertson *et al.*, 1991). Thus, this method may be a useful tool for the detection of viruses and other pathogens in seed certification programs.

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