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Use of Cloned Viral DNAs in Characterization, Epidemiology, and Control of Bean Golden Mosaic Virus

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EXECUTIVE SUMMARY

TITLE: Use of Cloned Viral DNAs in Characterization, Epidemiology, and Control of Bean Golden Mosaic Virus

Bean-infecting geminiviruses are a major constraint to bean production in the tropical and subtropical regions of the Caribbean Basin, Central America, Mexico, and South America. Losses may reach 40% to 100%. Plant breeders have needed information on the diversity of bean-infecting geminiviruses so that appropriate breeding strategies could be developed; and also, the source of inoculum for these viruses has been unknown. Thus, a major effort was devoted to developing methods for the detection of these viruses in order to determine the diversity of bean-infecting geminiviruses and their presence in natural weed reservoirs. General geminiviral DNA probes and three probes specific for three different bean-infecting geminiviruses were developed for use with dot and squash blot hybridization methods. Additionally, general primers for whitefly-transmitted geminiviruses were designed for use with polymerase chain reaction (PCR) methods. These PCR primers were effectively used to amplify DNA fragments from undescribed geminiviruses from many countries in the Western Hemisphere, Africa, and Asia. Over 2,000 samples were processed by squash blot and PCR methods for scientists from these countries during the last 2 years. Four distinct bean-infecting geminiviruses were molecularly characterized and their distribution partially determined: bean golden mosaic geminivirus (BGMV) type I, BGMV type II, bean dwarf mosaic geminivirus, and bean calico mosaic geminivirus. These results indicate that plant breeders must consider the development of resistance for specific bean-infecting geminiviruses or develop bean cultivars with resistance to several geminiviruses. Geminiviruses were detected in many weeds, but the natural reservoir of BGMV was not found. It seems that weeds are not the major source of inoculum and that BGMV-infected beans were the inoculum. Thus, a bean-free growing period was initiated in the Dominican Republic and this greatly reduced losses during the January-April growing period. The detection methods devloped are being transferred to several developing countries (Costa Rica, Jamaica, and Egypt) and to international centers (AVDRC, CIAT). This project supported the training of one M.Sc. student from the Dominican Republic in an Integrated Pest Management program and provided partial support for M.Sc. students from Indonesia and Costa Rica.

RESEARCH OBJECTIVES

Overall aims and specific objectives:

This project used molecular biology approaches to improve control of bean-infecting geminiviruses, the major constraint to bean production, and thus protein production in tropical regions of South America, Central America and the Caribbean Basin. Our overall aims were to determine the full extent of pathogenic variability in bean-infecting geminiviruses, to identify the most prevalent bean golden mosaic geminivirus (BGMV) genotypes in Latin America and their geographic distribution, and to provide data and tools crucial for ongoing efforts to breed for BGMV-resistance in beans. Our specific goals were to:

1. Isolate and clone the DNA of bean-infecting geminiviruses from Latin America and characterize these clones by sequencing.

2. Develop improved methods for diagnosis and identification of bean-infecting geminiviruses, e.g., develop general and isolate-specific DNA probes for the identification of bean-infecting geminiviruses and develop general geminivirus primers for use with polymerase chain reaction methods.

3. Use these improved identification methods to define the relative importance of distinct BGMV genotypes in the Dominican Republic and selected regions in Latin America and to identify the plant reservoirs of these geminiviruses.

4. Assist in the evaluation of germplasm for resistance to various bean-infecting geminiviruses and to initiate studies on the inheritance of disease resistance factors.

This project was complemented by other research on bean-infecting geminiviruses in D. P. Maxwell's laboratory funded by the Bean/Cowpea CRSP Project and more recently by projects on tomato-infecting geminiviruses funded by OICD, AVDRC, and private seed companies.

Importance of project to international development:

The common bean (*Phaseolus vulagaris*)is one of the most important sources of protein and calories for the low-income populations of Latin America. One of the principal constraints on bean production is bean-infecting geminiviruses, particularly BGMV, which is the major viral disease of the crop in lowlands of tropical Latin America. Yield losses are substantial, ranging from 40% to 100%. The continued importance of this diseases is evident by the fact that BGMV was identified as the first priority for research in the September 1992 meeting of the PROFRIJOL project for Central America and the Caribbean Basin. BGMV has virtually precluded productive bean production throughout the year in some areas and during particular seasons in other areas.

When this project started, the most successful approach to controlling this viral disease was breeding for disease resistance. Because of the seriousness of crop losses to BGMV, extensive breeding efforts were initiated in several areas. One major site is the CIAT BGMV nursery in Guatemala which was started in the 1970's, and another was at CNPAF in Goiania, Brazil, which was started in the 1980's. During the course of this project, increased breeding efforts have occurred in Honduras and the Dominican Republic. One limiting factor for these breeding programs has been the lack of understanding of the number and variation of virus types causing bean golden mosaic as well as the occurrence and importance of other bean-infecting geminiviruses. Only by obtaining a better understanding of the overall complexity of bean golden mosaic etiology can breeding programs be planned to efficiently produce plants with the actual resistance characteristics required, such as resistance to the major BGMV type present in a particular region or to multiple BGMV types.

Our efforts were devoted to the molecular characterization of bean-infecting geminiviruses utilizing the most modern technology available. Initially, emphasis was placed on the development of DNA probes for geminivirus detection; but as the project developed, polymerase chain reaction methods became more important.

The relevance of this project extends beyond bean-infecting geminiviruses in Latin America as the techniques developed have applications to all geminiviruses. Major efforts have recently been devoted to assisting Egyptian scientists in the molecular characterization of the tomato yellow leaf curl virus infecting tomatoes (Nakhla et al., 1992) and working with scientists from AVDRC in the characterization of the diversity of geminiviruses infecting tomatoes in Asia. Also, we have aided United States-based seed companies in the characterization of geminiviruses in vegetables from the US, Mexico, and Central America. In these latter cases, the technology used was developed as part of this project and the Bean/Cowpea CRSP Project.

Background information:

Geminiviruses: Geminiviruses are a unique group of plant viruses that are characterized by their twin (geminate) isometric virions and single-stranded, circular DNA genome (reviewed by Harrison, 1985; Lazarowitz, 1987; Stanley, 1991). Geminiviruses that have been characterized can be classified into three major subgroups (Francki et al., 1991): those that possess a bipartite genome, are transmitted by whiteflies (Bemisia tabaci), and infect dicotyledonous plants; those that possess a monopartite genome, are transmitted by leafhoppers, and infect monocotyledonous hosts; and those that have a monopartite genome, are leafhopper-transmitted and infect dicotyledonous plants (Stanley et al., 1986). The genome of the whitefly-transmitted geminiviruses is usually divided between two DNA components, DNA-A and DNA-B (Fig. 1), both of which are required for infectivity (Hamilton et al., 1983; Morinaga et al., 1987; Stanley, Gay 1983; Gilbertson et al., 1991a). Recently, tomato yellow leaf curl geminivirus strains from Israel and Sardinia, which are whitefly-transmitted, were shown to have only a DNA-A component (Kheyr-Pour, et al., 1991; Navot et al., 1991).

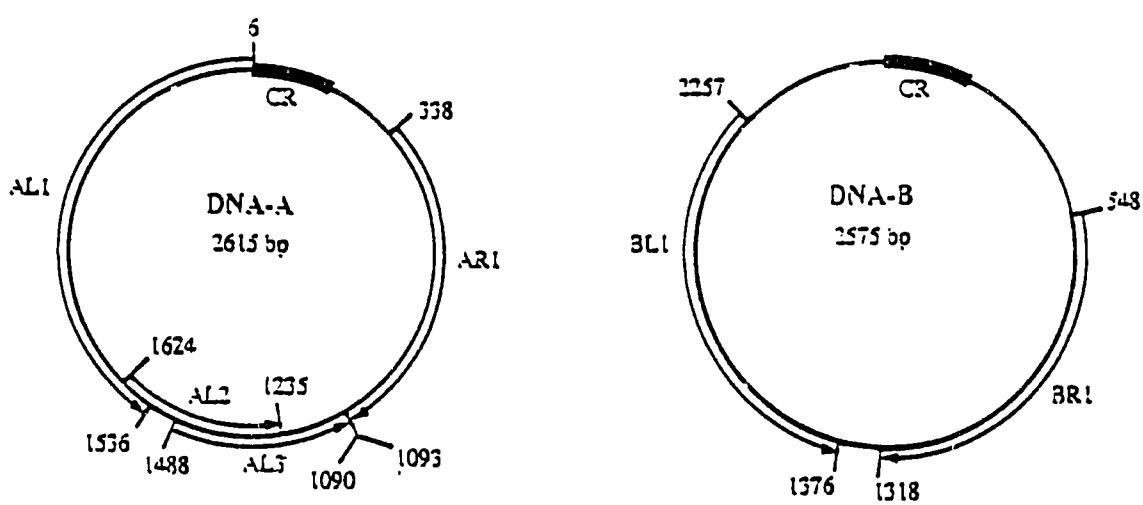

Fig. 1. Schematic maps of replicative forms of the genome of bean dwarf mosaic geminivirus. CR = Common Region; ORFs = AR1 (coat protein); AL1 (replication associated protein); AL2 (transactivation protein); AL3 (?, regulation of ssDNA levels); BL1 and BR1 (movement and/or symptoms).

The complete nucleotide sequences of the infectious cloned DNAs of African cassava mosaic geminivirus (ACMV; Stanley and Gay, 1983), tomato golden mosaic geminivirus (TGMV, Hamilton et al., 1984; corrected by von Arnim and Stanley,

1992), bean golden mosaic geminivirus from Puerto Rico (BGMV-PR; Howarth et al., 1985 and corrected by A. Howarth, personal communication; Morinaga et al., 1987), Abutilon mosaic geminivirus (AbMV; Frischmuth et al., 1990), squash leaf curl geminivirus (SqLCV, Lazarowitz and Lazdins, 1991), and potato yellow mosaic geminivirus (PYMV; Coutts et al., 1991) have been determined. The DNA-A and DNA-B components of each of these geminiviruses have little sequence identity except for approximately 200 nt of noncoding DNA, called the Common Region, which is almost identical between the two components of any single geminivirus, but is highly divergent among different geminiviruses.

Bean golden mosaic (BGM) disease of common bean (Phaseolus vulgaris) and certain other legumes is characterized by a brilliant golden mosaic pattern on infected leaves and stunted plant growth, and is a major constraint on dry bean production in Central and South America and the Caribbean Basin (Brown and Bird, 1992; Costa, 1975; Gálvez and Morales, 1989). BGMV-PR, which was characterized by Goodman and associates (Goodman et al., 1977; Howarth et al., 1985), was collected from lima bean (Phaseolus lunatus) in Puerto Rico and was readily sap-transmitted to P. vulgaris where it induces golden mosaic symptoms. In contrast, field isolates of BGMV from Brazil are not sap-transmissible (Costa, 1965; Costa, 1975; Figueira, 1980; Gilbertson et al., 1991b). Thus, it has been suggested, based on differences in sap-transmissibility, bean genotype reaction to BGMV isolates, and DNA hybridizations with specific DNA probes for bean-infecting geminiviruses, that genetic variation may exist among geminiviruses causing bean golden mosaic in the Americas and the Caribbean Basin (Costa, 1975; Gilbertson et al., 1991b; Morales and Niessen, 1988). Also, bean dwarf mosaic geminivirus (Morales et al., 1990) and bean calico mosaic geminivirus (Brown et al., 1990) have been reported but not molecularly characterized.

DNA probes: DNA probes have been used for detection of many plant viruses and viroids (Symons, 1984), including geminiviruses of tomatoes (Czosnek et al., 1988) and cucurbits (Polston et al., 1989). Dot blot and squash blot methods have been developed that allow for the efficient preparation of samples for hybridization with nucleic acid probes (Czosnek et al., 1988). It was expected that cloned DNA of the bean-infecting geminiviruses would be efficient DNA probes for their detection; and, thus, we devoted considerable effort at the beginning of this project to the cloning and sequencing of bean-infecting geminiviruses.

Polymerase chain reaction methods: The polymerase chain reaction (PCR) is an extremely sensitive and specific technique for the detection and identification of plant pathogens, and can be used to answer precise questions about pathogen populations and diversity (Gilbertson et al., 1991d; Robertson et al., 1991). PCR methods amplify a specific DNA sequence that lies between two regions of known nucleotide sequence (Kawasaki, 1990). The specificity of PCR is based on the use of oligonucleotide primers that are complementary to the regions flanking the DNA sequence to be amplified. Because PCR amplifies nucleic acids, it can overcome many of the problems associated with serological detection methods, such as low titer of antigen, cross-reaction of antibodies with heterologous antigens, and developmental or environmental regulation of antigen production. Furthermore, small amounts of sample tissues, which can be fresh, frozen, or even dried, are suitable for PCR. PCR methods have been used to study plant viruses, including luteoviruses (Robertson et al., 1991), potyviruses (Langeveld et al., 1991; Vunsh et al., 1990), leafhopper-transmitted geminiviruses infecting monocotyledonous plants (Rybicki and Hughes 1990), and BGMV in the Dominican Republic (BGMV-DR, Gilbertson et al., 1991d).

Geminiviruses are well suited for detection and identification by PCR methods because they replicate via a double-stranded, circular DNA intermediate, the replicative form (Stanley, 1991), which can serve as a template for amplification by PCR. The genome of most whitefly-transmitted geminiviruses is composed of two DNA components, designated DNA-A and DNA-B (Fig. 1). The complete nucleotide sequences of five bipartite, whitefly-transmitted geminiviruses from the Western Hemisphere and the more distantly related African cassava mosaic geminivirus (ACMV) from the Eastern Hemisphere have been published (Coutts et al., 1991; Frischmuth et al., 1990; Hamilton et al., 1984; Howarth et

al., 1985; Lazarowitz and Lazdins, 1991), and we have determined the complete nucleotide sequences for four bipartite bean-infecting geminivirus isolates from the Western Hemisphere (unpublished data). These sequences were aligned so that highly conserved regions could be identified for use in designing general PCR primers.

Innovative aspects of this project: This project was based on our observations that extensive genotypic variations exist among geminiviruses causing losses of beans. When initiated, this project was one of the first to apply modern molecular approaches to characterize types of diversity of geminiviruses, and polymerase chain technology was only beginning to emerge as a powerful tool for amplification of DNA. This project has produced extensive information i) on the complexity of bean-infecting geminiviruses in Latin America, ii) on the variability of field isolates of BGMV in Dominican Republic and Brazil, iii) on the existence of two types of BGMV, type I and type II, iv) on the weed reservoirs for BGMV in the Dominican Republic, and v) on the distribution of type II BGMV isolates in Central America and the Caribbean Basin. The development of PCR methods for the detection of undescribed geminiviruses using specially designed general primers for geminiviruses has applications well beyond this project and is being extensively used for diagnosis of geminiviruses in other crops, such as tomatoes, cucurbits, and cassava.

Support for this project by other grants and organizations: The Bean/Cowpea CRSP Project has provided complementary resources for the completion of part of these research objectives. The PSTC project provided the funds for training of one M. Sc. student in Integrated Pest Management from the Dominican Republic. The additional resources came from a Bean/Cowpea CRSP grant to D. P. Maxwell. Additional organizations that have supported this project include: i) CIAT, Cali, Colombia--Scientists provided the original bean-infecting geminivirus isolates from Colombia, Guatemala, and the Dominican Republic and constantly provided samples for analysis and participated in the planning of experiments; ii) CNPAF/EMBRAPA, Goiania, Brazil--Scientists provided samples for analysis and completed two short-term training programs at the University of Wisconsin during this project; iii) University of Arizona-Scientist furnished isolate of bean calico mosaic geminivirus and collaborated on its characterization; iv) OICD/USDA supported a project on geminiviruses on tomatoes as result of the technology developed on this project; and v) AVDRC, Taiwan supported short-term training for a technical person to characterize the tomato-infecting geminivirus from Taiwan. Agracetus, Inc., Middleton, WI sequenced the DNA-A component of BDMV and provided many primers for sequencing of the DNA-B. We have used our DNA probes and PCR technology to assist scientists in the identification of geminiviruses in various crops from the following areas: South Africa, Malawi, Syria, Sri Lanka, India, Nepal, Thailand, Indonesia, VietNam, Trinidad, Mexico, Jamaica, Nicaragua, Honduras, Costa Rica, Guatemala, Puerto Rico, Florida, and Texas. We have worked closely with four private companies in the U.S.A. on geminivirus diagnosis and was asked to assist a Dutch seed company with geminivirus research.

METHODS AND RESULTS

Cloning and Sequencing Bean-Infecting Geminiviruses:

Research reports:

Faria, J. C., R. L. Gilbertson, F. J. Morales, D. R. Russell, P. G. Ahlquist, S. F. Hanson, and D. P. Maxwell. 1990. Sequence of infectious clones of two mechanically transmissible isolates of bean golden mosaic geminiviruses. Phytopathology 80:983.

Hidayat, S. H., Gilbertson, R. L., Hanson, S. F., Morales, F. J., Ahlquist, P., Russell, D. R., and Maxwell, D. P. 19__. Complete nucleotide sequences of the infectious cloned DNAs of bean dwarf mosaic geminivirus. Phytopathology_____: ___-_____. (IN PRESS)

Loniello, A. O., Martinez, R. T., Pojas, M. R., Gilbertson, R. L., Brown, J. K.,

and Maxwell, D. P. 1992. Molecular characterization of bean calico mosaic geminivirus. (Abstr.) Phytopathology 82: (In Press)

Methods

Molecular cloning of viral dsDNA. Total viral DNA was isolated from bean calico mosaic geminivirus (BCMoV)-infected bean leaves by a procedure involving phenol/chloroform extraction and ethanol precipitation (Gilbertson et al., 1991a). Nucleic acids were fractionated by horizontal gel electrophoresis in a 0.7% agarose gel in 1x Tris-Acetate-EDTA buffer, and virus-specific dsDNA was then excised from the gel and recovered by binding to and elution from a silica matrix (Gene Clean, Bio 101, La Jolla, CA). Replicative form dsDNA was linearized by digestion with restriction endonucleases AccI (Promega Corp., Madison, WI) and ligated to a plasmid, pBluescript II KS (+) (Stratagene, La Jolla, CA) or pSP72 (Promega Corp., Madison, WI) previously linearized with the same enzyme. Recombinant plasmids were subsequently transformed into competent cells of *Escherichia coli* strain JM101. Transformants containing recombinant plasmids were selected on 1x YT plates containing ampicillin and IPTG/X-Gal and incubated at 37 C overnight. Standard DNA manipulation techniques were followed (Maniatis et al., 1982).

Characterization of cloned DNAs. Plasmids were extracted by the alkaline lysis procedure from *E. coli* grown in 2x YT broth cultures containing ampicillin. Insert sizes were determined by gel electrophoresis of plasmids digested with the appropriate restriction endonucleases. Clones having inserts of the expected size (2.6 kb) were characterized. Restriction endonuclease maps of the viral DNA inserts were prepared using a recombinant plasmid with each viral insert cloned in both orientations.

Nucleotide sequence determination. Different strategies were used to sequence DNA-A and DNA-B of bean dwarf mosaic geminivirus. Clones were available from our Bean/Cowpea CRSP project efforts (Gilbertson et al., 1991a). The DNA-A component was sequenced by subcloning of restriction fragments to make overlapping subclones in pBluescript SK (-) (Stratagene). Nucleotide sequences were determined using doublestranded, supercoiled plasmid DNA with flanking region primers or synthetic oligomers as primers. Potential full-length clones of the DNA-B component were sequenced by first creating libraries of nested deletions using exonuclease III (Erase-a-Base system, Promega Corp.). To complete the sequence of DNA-B, synthetic oligomers were used as primers (Biotechnology Center, University of Wisconsin-Madison, WI, and Agracetus Inc., Middleton, WI). Nucleotide sequence was determined by the dideoxy-nucleotide chain termination method using Sequenase (United States Biochemical Corp., Cleveland, OH) according to manufacturer's instructions. Both DNA strands of each component were sequenced completely to ensure accuracy. The total sequence of the viral DNA was assembled using database management software of the Genetics Computer Group, University of Wisconsin-Madison (Devereux et al., 1984). Relationships among bipartite geminiviruses were examined by comparing nucleotide sequences of different geminiviruses using the GAP program of this same software.

The nucleotide sequences of BDMV DNA-A (plasmid = pBDA1) and DNA-B (plasmid = pBDB1) have been deposited in GenBank as accession numbers M88179 and M88180, respectively.

Detection of viral nucleic acid by squash and dot blots. Viral nucleic acids were detected in infected plants by nucleic acid squash blot hybridization as described by Gilbertson et al. (1991b). Geminiviral DNA clones, radioisotope-labeled by nick translation with [α-^{32}P] dATP, were used as probes. A mixture of DNA-As and DNA-Bs of three different isolates of bean-infecting geminiviruses, BGMV from Brazil, BGMV from the Dominican Republic, and BDMV, served as a general probe; and the DNA-B's of each isolate were used as type-specific probes.

Results

Molecular cloning and sequencing of viral DNA of BCMoV. The BCMoV isolate was supplied by Dr. J. K. Brown and was collected in Sonora, Mexico (Brown et al., 1990). The DNA extract from BCMoV-infected beans contained two predominantly viral DNA bands, which co-migrated with the linear dsDNA markers of 2.6 kb and 1.6 kb and were similar to the replicative forms of BGMV from Guatemala (Gilbertson et al., 1991a). DNA extracted from these bands was cleaved with AccI. After cloning, 48 recombinant plasmids were obtained, and the insert sizes were 0.4 kb, 1.1 kb, 1.6 kb, 1.9 kb, 2.2 kb, and 2.6 kb. Since the suspected size of full-length clones was 2.6 kb, the two clones with 2.6-kb inserts, pBCAcc911 and pBCAcc957, were characterized by restriction enzyme digestions and sequencing of the ends of the inserts. The sequence of one end of the insert in pBCAcc911 was 74% identical with the BL1 ORF of DNA-B of BGMV-GA and less than 42% identical to the DNA-A sequence of BGMV-GA. Similarly, the sequence of one end of the insert in pBCAcc957 was 61% identical with the BL1 ORF of DNA-B of BGMV-GA and only 45% identical to the DNA-A sequence of BGMV-GA. Thus, these inserts were both DNA-B components. The inserts in several other recombinant plasmids were sequenced and all were more similar to DNA-B than to DNA-A sequences of BGMV-GA.

Since no viral inserts of DNA-A were obtained by regular cloning methods, PCR methods (these will be discussed later in the report) were used to amplify a 1.2-kb fragment from DNA-A of BCMoV. This fragment was cloned and ends of the 1.2-kb insert in recombinant plasmid, pBCA1, were sequenced. These DNA sequences were 67% and 68% identical to the sequences of the AL1 ORF and AR1 ORF of BGMV-GA, respectively. To determine the relationship of BCMoV to other geminiviruses, the nt sequences of part of the AL1 and the Common Region were determined. Comparisons with seven other whitefly-transmitted geminiviruses from the Western Hemisphere indicated that BCMoV was most closely related to squash leaf curl geminivirus and not closely related to the other bean-infecting geminiviruses, such as BGMV type I and type II or BDMV.

Our data clearly showed that BCMoV is a bipartite, whitefly-transmitted geminivirus which is distinct from the known bean-infecting geminiviruses. Future efforts should define the geographical range of BCMoV so that it can be determined if this is the main bean-infecting geminivirus in Mexico. If it is, then breeding programs will need to consider this information.

Nucleotide sequence analysis of the cloned viral DNAs of BDMV. Nucleotide sequences of the terminal portions of the BDMV inserts were compared with the sequence of a BGMV isolate from Puerto Rico (BGMV-PR) (Howarth et al., 1985). These comparisons showed that the insert in pBDA1 had the greatest sequence identity with the DNA-A (83%) and the inserts in pBDB1 and pBDB21 with DNA-B (72% and 68%, respectively) of BGMV-PR. Thus restriction endonuclease maps and nucleotide sequence analysis of the cloned DNAs show that the genome of BDMV is bipartite. This is consistent with results for other whitefly-transmitted geminiviruses, including tomato golden mosaic geminivirus (TGMV) (Hamilton et al., 1984), squash leaf curl geminivirus (SqLCV) (Lazarowitz and Lazdins, 1991), BGMV-PR (Howarth et al., 1985), African cassava mosaic geminivirus (ACMV) (Stanley and Gay, 1983), tomato yellow leaf curl geminivirus isolate from Thailand (TYLCV-T) (Rochester et al., 1990), Abutilon mosaic geminivirus (AbMV) (Frischmuth et al., 1990), Texas pepper geminivirus (Rojas, 1992), and potato yellow mosaic geminivirus (PYMV) (Coutts et al., 1991). Recently, four bean-infecting geminiviruses from the Americas or the Caribbean Basin, including BDMV-CO were shown to require the two DNA components for infectivity (Gilbertson et al., 1991a). In contrast, two whitefly-transmitted strains of TYLCV from the Mediterranean region are monopartite (Kheyr-Pour et al., 1991; Navot et al., 1991).

The DNA-A component insert in pBDA1 had 2,615 bp and the two DNA-B component clones, pBDB1 and pBDB21, had BDMV inserts of 2,575 bp (GenBank Accession no. M88179) and 2,560 bp (GenBank Accession no. M88180), respectively. Computer-assisted sequence comparison of the viral inserts of pBDB1 and pBDB21

showed differences at 11 sites, including 10 single nucleotide mismatches and a 15-bp gap. This gap may have resulted from the loss of a 15-bp AccI fragment during cloning of pBDB21, since two AccI sites, which would result in a 15-bp fragment, are present in this region of the insert of pBDB1. The inserts in pBDA1 and pBDB1 were determined to be full-length by sequencing across the restriction sites used for cloning these inserts in independent BDMV DNA-A and DNA-B clones obtained with different restriction enzymes. These genome sizes for DNA-A and DNA-B are similar to those of other bipartite geminiviruses from the Western Hemisphere (Howarth et al., 1985; Lazarowitz and Lazdins, 1991) and are slightly smaller than the components for ACMV from Africa (Stanley and Gay, 1983) and TYLCV from the Mediterranean (Navot et al., 1991).

Relationship of BDMV to other whitefly-transmitted geminiviruses. Nucleotide sequence analysis of the DNA-A and DNA-B components of BDMV showed a genomic organization similar to that of other bipartite geminiviruses (Fig. 1). There were four ORFs on the DNA-A and two ORFs on the DNA-B, which are conserved with respect to size and relative location in the genomes of TGMV [Original sequence (Hamilton et al., 1984) was corrected by addition of two nt in BL1 (Von Arnim and Stanley, 1992).], BGMV (Howarth et al., 1985), and AbMV (Frischmuth et al., 1990). The BDMV DNA-A genome organization was similar to that for SqLCV DNA-A (Lazarowitz and Lazdins, 1991), but the position of ORFs in DNA-B in relation to the Common Region was different. The size of the intergenic region between BL1 and the Common Region is considerably smaller in SqLCV than in BGMV-PR, BDMV, AbMV, and TGMV.

Nucleotide sequence comparisons indicated that BDMV is distinct from other whitefly-transmitted geminiviruses including BGMV-PR, which is the other bean-infecting geminivirus that has been sequenced. Nucleotide sequence identities for the pairwise comparisons of the most conserved ORF of DNA-A (AR1) and DNA-B (BL1) among AbMV, BDMV, BGMV-PR, SqLCV, and TGMV indicated that BDMV is most similar to AbMV (86% and 87% identities for AR1 and BL1, respectively) and least similar to SqLCV (81% and 72% identities for AR1 and BL1, respectively). Comparisons of Common Region nucleotide sequences among these five geminiviruses further support the conclusion that BDMV is a distinct geminivirus and most similar to AbMV.

In each bipartite geminivirus the nucleotide sequences of the DNA-A and DNA-B components are different, except for a highly conserved ~200 nt sequence referred to as the Common Region (Lazarowitz, 1987). Since the Common Region sequence is a characteristic feature for each bipartite geminivirus (Rojas, 1992), Common Region comparisons may be indicators of the relatedness of bipartite geminiviruses. The BDMV Common Region, determined from comparison of DNA-A and DNA-B, is 187 nt long and is 96% identical. By contrast, the BDMV Common Region sequence showed 79% identity with that of AbMV and 68% identity with that of BGMV-PR. The BDMV Common Region contains a stretch of 33 nt that can form a stem-loop structure which consists of a stem of nine GC and two AT pairs and a loop of 11 nt including the invariant sequence TAATATTAC. This stem-loop region is a likely site for the origin of DNA replication (Lazarowitz et al., 1992). A direct repeat of TACTC is located at nt positions 25-29 and 39-43 in BDMV, and this same repeat is present in the closely related geminivirus, AbMV, at nt 28-32 and 42-46. The Common Region of BGMV-PR does not contain the direct repeat of TACTC, but does have a longer direct repeat of TGCGAGTGTCTCCAA at nt positions 18-32 and 40-54. Whether these direct repeats have functional significance remains to be investigated.

Pairwise comparisons of the derived amino acid sequences for the six ORFs in DNA-A and DNA-B of the two bean-infecting geminiviruses, BDMV and BGMV-PR, were made. The percent amino acid identities for these pairwise comparisons of AR1, AL1, AL2, AL3, BR1, and BL1 were 92%, 84%, 78%, 83%, 83%, and 92%, respectively. Thus, as with the nucleotide sequence comparisons, the most highly conserved amino acid sequences between BDMV and BGMV-PR were those of AR1 and BL1.

Since Howarth and Vandemark (Howarth and Vandemark, 1989) had used AR1

amino acid alignments to construct phylogenetic trees for monopartite and bipartite geminiviruses, the derived amino acid sequence of AR1 of BDMV was compared with those of the AR1 for AbMV, BGMV-PR, PYMV, SqLCV, and TGMV. All five identities were greater than 91%. The lowest identity (92%) was for BDMV and BGMV-PR. Again, this indicated that BDMV should be considered a different geminivirus from BGMV-PR. The high conservation of the derived amino acid sequence of AR1 among the whitefly-transmitted geminiviruses is consistent with the hypothesis that the coat protein is intimately associated with vector transmission. Briddon et al. (1990) showed that the coat protein of BCTV, a leafhopper-transmitted geminivirus, determines its vector specificity. This high conservation of the derived amino acid sequence of the coat protein among whitefly-transmitted geminiviruses explains why serological studies with polyclonal antisera (Roberts el al., 1984) showed that whitefly-transmitted geminiviruses are very closely related and distinct from leafhopper-transmitted geminiviruses, which have very similar amino acid sequences for their coat protein (Howarth and Vandemark, 1989).

From these nucleotide and amino acid comparisons, it is evident that BDMV is most closely related to the AbMV isolate from the West Indies. Nucleotide sequence identities for comparisons of the Common Region and ORFs were higher between BDMV and AbMV than among four other Western Hemisphere, whitefly-transmitted geminiviruses. The importance of understanding the relatedness of geminiviruses has recently been shown by the creation of infectious pseudorecombinants (heterologous mixtures of DNA-A and DNA-B) from strains of BGMV (Faria et al., 1990). Gilbertson et al. (1991c) showed that BDMV-CO is closely related to tomato mottle geminivirus from Florida, and subsequently, that a pseudorecombinant between these two distinct geminiviruses would infect the common host, *N. benthamiana* (Hou et al., 1992). Pseudorecombinants between distinct geminiviruses may be an important way in which new geminiviruses evolve.

Development of DNA Probes for Detection of Bean-Infecting Geminiviruses

Research reports:

Gilbertson, R. L., Hidayat, S. H., Martinez, R. T., Leong, S. A., Faria, J. C., Morales, F., and Maxwell, D. P. 1991. Differentiation of bean-infecting geminiviruses by nucleic acid hybridization probes and aspects of bean golden mosaic in Brazil. Plant Dis. 75:336-342.

Hidayat, S. H. 1990. The construction and application of DNA probes for the detection of bean dwarf mosaic geminivirus. M. Sc. Thesis, University of Wisconsin-Madison, pp. 53.

Methods

Detection of geminiviral nucleic acids in leaves by squash and dot blot hybridization. Leaf disks were excised with a flame-sterilized No. 4 cork borer. For the squash blot procedure, leaf disks were squashed on Zetabind nylon membrane (CUNO, Ind. Meriden, CT) with a flame-sterilized round-bottomed glass rod. Two dot blot procedures were used, one in which one or three leaf disks were ground in 0.5 ml of distilled water or TE buffer (10 mM Tris, pH 7.5, 1mM EDTA) in 1.5-ml Eppendorf tubes with a sterilized plastic pestle, and 10-μl samples were spotted onto Zetabind. The other procedure involved grinding a single leaf disk in 50-μl of TE, making twofold serial dilutions of the resulting plant sap in microtiter plates (Becton Dickinson Co., Lincoln Park, NJ), and spotting 5-μl samples of each dilution on Zetabind (dilution method). Similar dilution series were prepared from sap of mock-inoculated plants and from a solution containing 2 ng/μl of cloned BGMV-GA components A and B, and 5 μl samples were spotted on Zetabind.

Membranes were placed on filter paper, which had been saturated with 0.2 N NaOH, for 5 min to lyse cells and denature DNA, and then washed for 5 min in

1 M Tris, pH 7.5, then 2X SSC (Maniatis et al., 1982), followed by 95% ethanol. Membranes were air-dried and stored or used immediately for hybridization. Prehybridization (10 min) and hybridization (16-24) hr) were carried out in a solution of 30 (low stringency) to 50% (high stringency) formamide-0.125 M $Na2HPO_4$-7% SDS-1mM EDTA (pH 8.0) at 42 C. Membranes were washed twice at 42 (low stringency) formamide-0.125 M Na_2HPO_4-2% SDS-1 mM EDTA (pH 8.0) (Amasino, 1986). Membranes were blotted dry and exposed at -70 C to Kodak X-Omat AR or OG-1 X-ray film with Cronex Lightening Plus or Lanex intensifying screens, respectively. Exposure time varied from 4 to 24 hr.

DNA Probes. Full-length DNA clones of BGMV-BZ, BGMV-GA, BGMV-DR, and BDMV-CO components A and B (Gilbertson et al. 1991a) were used as DNA probes. Probe DNA was radioisotope-labeled with [^{32}P]-dATP by nick translation with kits from Bethesda Research Laboratories (Gaithersburg, MD) or Promega BioTech (Madison, WI).

Results

Detection of geminiviral DNA in bean and *N. benthamiana* leaves by nucleic acid squash and/or dot blot hybridization with general and specific DNA probes. Dot and squash blots of leaves infected with BGMV-GA, BGMV-DR, BGMV-BZ, and BDMV were hybridized under low-stringency conditions with a general probe, which was a mixture containing cloned DNA components A and B of BGMV-BZ, BDMV, and BGMV-GA or BGMV-DR, or hybridized under high-stringency conditions with specific probes, which were the individual component B clones of these virus isolates. For BGMV-BZ, leaves from beans infected via whiteflies were collected at CNPAF, frozen or dried, and brought to Madison. Subsequently, we successfully infected beans with a mixture of the cloned DNA components A and B of BGMV-BZ (Gilbertson et al., 1991a); and in some cases, leaves from these infected plants were used for detection of BGMV-BZ. Before preparation of dot and squash blots, dried leaf samples were rehydrated with sterile distilled water for 10-15 min, whereas frozen leaf samples were thawed for 1-5 min.

With the general probe, geminiviral nucleic acids were detected in squash and dot blots of bean leaves infected with BGMV-BZ, BFMV-GA, BGMV-DR, and BDMV and in *N. benthamiana* plants inoculated with BDMV (Fig. 2), but not in mock-inoculated plants (Fig. 2) or *N. benthamiana* inoculated with BGMV-GA or BGMV-DR (*data not shown*). When dot and squash blots of these infected leaves were probed with the specific probes, the BGMV-BZ component B probe hybridized only with tissue infected with BDMV; and the BGMV-GA and BGMV-DR component B probes each hybridized only with tissue infected tissues could be visualized after 4 hr of exposure, with very strong signals observed after 12-16 hr. These experiments were repeated three times, with similar results obtained for each experiment.

We now routinely use squash blot hybridization to confirm results of sap-transmission experiments, results of experiments involving inoculation of plants with cloned geminiviral DNAs, and to detect geminiviral nucleic acids in bean samples from Central America, South America, and the Caribbean. In all cases (more than 300 samples have been tested), hybridization signals have been observed in squash blots from beans with typical golden mosaic symptoms (no false negatives), and no signals have been observed in squash blots from beans without symptoms (no false positives).

The stability of squash and dot blotted samples on membranes before lysis was evaluated by preparing membranes with squash and dot blot samples from uninfected bean trifoliolate leaves and leaves infected with BGMV-GA. One membrane was exposed to lysis buffer immediately, one after a 2-wk storage, and the other after a 2-mo storage at 22 C. Membranes were probed with BGMV-GA component A 24-48 hr after exposure to lysis buffer. This experiment was conducted twice. We observed no reduction in hybridization signal 2 mo after samples had been applied to membranes. After exposure to lysis buffer, geminiviral nucleic acids can be detected on membranes for at least 1 yr and probably longer.

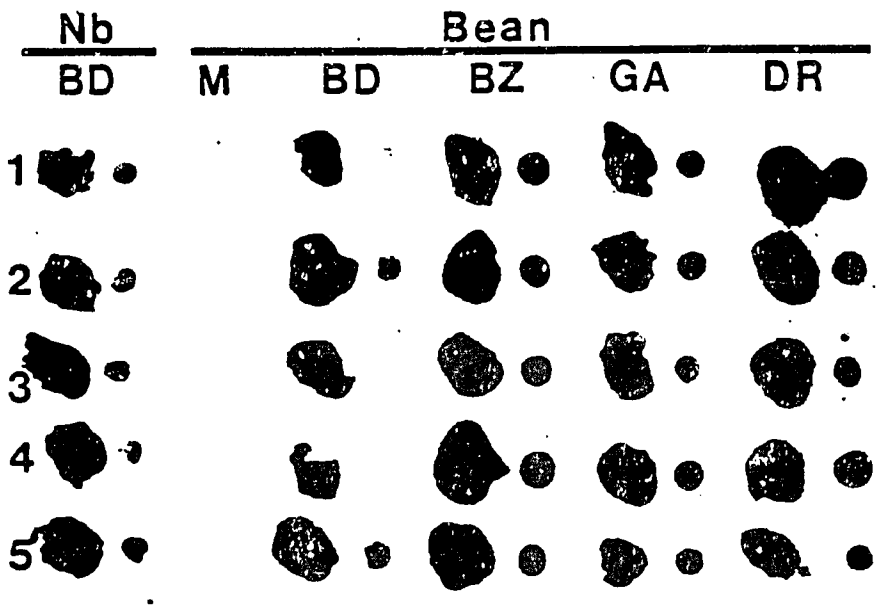

Fig. 2. Comparison of squash (samples on left in each column) and dot (samples on right in each column) blot hybridization for detection of bean-infecting geminiviruses in *Nicotiana benthamiana* (Nb) and bean (*Phaseolus vulgaris*) leaf tissue. Samples are mock-inoculated beans (M), plants infected with bean dwarf mosaic geminivirus (BD), or beans infected with a bean golden mosaic geminivirus (BGMV) isolate from Brazil (BZ), Guatemala (GA), or the Dominican Republic (DR). Samples were taken from each of five plants (1-5) for each treatment. Samples were hybridized with a general probe, which was a mixture of the cloned DNA components A and B of BGMV-BZ, BGMV-GA, and BDMV, using low-stringency conditions.

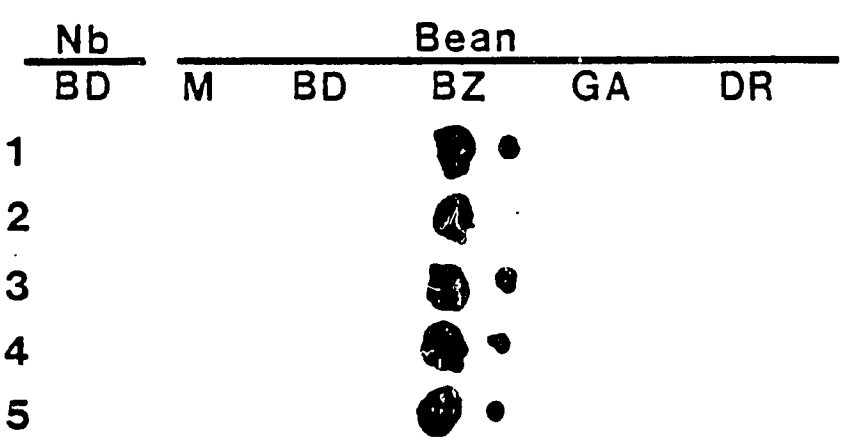

Fig. 3. Specific detection of a bean golden mosaic geminiviral isolate from Brazil (BGMV-BZ) in infected bean leaves by nucleic acid squash (samples on left) and dot blot (samples on right) hybridization. Samples are mock-inoculated leaves (M), *Nicotiana benthamiana* (Nb), or beans (*Phaseolus vulgaris*) infected with bean dwarf mosaic geminivirus (BD), or beans infected with BGMV-BZ or BGMV isolates from Guatemala (GA) or the Dominican Republic (DR). Samples were taken from each of five plants (1-5) for each treatment and were hybridized with the cloned DNA component B of BGMV-BZ using high-stringency conditions.

To determine if we could detect differences in viral nucleic acid titer in bean leaves by dot blot hybridization, beans (cv. Topcrop) were inoculated with BGMV-GA or BDMV on three separate dates (2-3 wk apart) so that infected trifoliolate leaves at different stages of development could be sampled. Infected leaves were collected from plants at three stages of development: V2 growth stage, young, partially expanded (7-10 days after inoculation; R1 growth stage, medium-aged, fully expanded (21-28 days after inoculation; and R6 growth stage, old, fully expanded, and beginning to senesce (49-56 days after inoculation). In a separate experiment, young, medium-aged, and old trifoliolate leaves infected with BGMV-GA or BDMV were collected from each of three bean plants 42 days after inoculation (R2 growth stage). Leaf disks were cut from leaves, dot blotted (dilution method), and membranes were probed with BGMV-GA or BDMV DNA components A and B under high-stringency conditions. These experiments were repeated three times. For both BGMV-GA and BDMV, the strongest hybridization signals were always observed from young partially expanded trifoliolate leaves were collected from plants 10 to 42 days after inoculation. Less intense signals were observed from fully expanded trifoliolates, and the weakest signals were from older fully expanded trifoliolates that were beginning to senesce (*data not shown*).

Dot blot hybridization was also used to detect differences in BGMV-GA and BGMV-DR nucleic acid titers in a susceptible cultivar (Topcrop) and in two cultivars that are moderately resistant or tolerant (HMX 7958 and Pinto 114). HMX 7958 is an experimental cultivar that has been reported to be moderately resistant (reduced symptoms) to BGM in Mexico and was kindly provided by the Harris Moran Seed Co. (San Juan Bautista, CA). Pinto 114 was reported to develop only moderate golden mosaic symptoms under field conditions, and when sap-inoculated with a BGMV-GA isolate, some plants did not develop symptoms. These cultivars were planted in a controlled environment chamber, and 7 days later, when beans had one-half expanded primary leaves, plants were inoculated with BGMV-GA or BGMV-DR (only Pinto 114). Ten to 14 days later, the first trifoliolate leaves with symptoms were collected from five infected plants of each cultivar and disks cut from these leaves were analyzed by dot blotting (dilution method). Membranes were probed with BGMV-GA components A and B under high stringency conditions. The experiment with HMX 7958 was repeated three times, and the experiment with Pinto 114 was conducted twice. HMX 7958 was repeated three times, and the experiment with Pinto 114 was conducted twice. HMX 7958 plants developed mild golden mosaic symptoms after inoculation with BGMV-GA but not the brilliant golden mosaic, leaf distortion, and poor pod set that was observed with susceptible cv. Topcrop. Similarly, Pinto 114 developed golden mosaic symptoms after inoculation with BGMV-GA or BGMV-DR that were less severe than those observed on Topcrop. Greater hybridization signals were observed from dot blots of the susceptible Topcrop that from cvs. HMX 7958 (data not shown) or Pinto 114 (Fig. 4). Stronger hybridization signals also were observed from Topcrop plants infected with BGMV-DR that from those infected with BGMV-GA (Fig. 4). By making serial dilutions of plant sap and dotting samples of each dilution, the differences in viral nucleic acid titer could be clearly seen; in both HMX 7958 and Pinto 114, viral nucleic acids were not detected after two to three dilutions. These results clearly demonstrate that, at least for the infected first trifoliate leaves, HMX 7958 and Pinto 114 had lower viral nucleic acid titers that Topcrop.

Survey of beans with golden mosaic symptoms in the Dominican Republic. A trip was taken to the Dominican Republic in February 1990 to prepare squash blots of beans and weeds with golden mosaic symptoms for hybridization with the general or specific geminiviral DNA probes. Beans with golden mosaic, stunting, leaf distortion and/or green mosaic symptoms; various crops with or without symptoms; or weeds with or without golden mosaic symptoms were collected from 24 locations in the Central and Western Dominican Republic. Each membrane had a sample from a bean plant with no symptoms (negative control) and a bean plant with typical BGM symptoms (positive control). The samples were squash blotted on four replicate membranes for hybridization with the general and specific probes. Before samples were lysed and hybridized in Madison, WI, samples from plants infected with BDMV and BGMV-BZ were squash blotted onto each of the replicate

membranes to provide positive controls for the specific probes.

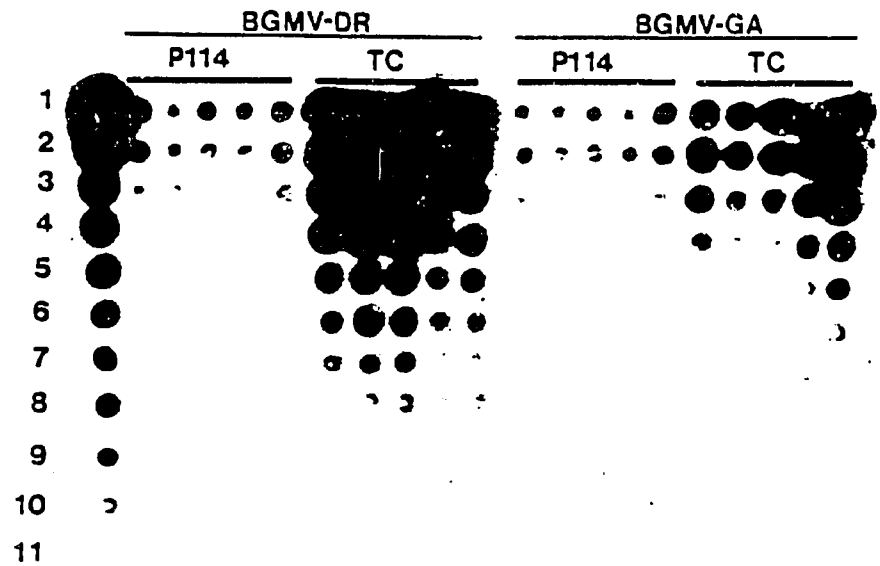

Fig. 4. Detection of differences in bean golden mosaic geminiviral nucleic acid titer in a moderately resistant bean cultivar, Pinto 114 (P114), and a susceptible cultivar, Topcrop (TC), by nucleic acid dot blot hybridization. Samples were prepared from infected first trifoliolate leaves from each of five individual plants of each cultivar inoculated with a BGMV isolate from Guatemala (BGMV-GA) or the Dominican Republic (BGMV-DR). Column one contains twofold dilutions (1-11) of cloned DNA components A and B of BGMV-GA (10 ng, 5 ng, 2.5 ng, 1.3 ng, 625 pg, 313 pg, 156 pg, 78 pg, 39 pg, 20 pg, 10 pg). Samples were hybridized with the cloned DNA components A and B of BGMV-GA using high-stringency conditions.

When the general probe was used, hybridization signals were not observed from crop samples (tomato, cassava, green pepper, or tobacco), weeds without golden mosaic symptoms, or beans without symptoms; hybridization signals were observed from all beans with golden mosaic symptoms, six of seven beans with stunting and leaf distortion, and one of four beans with green mosaic (similar to bean common mosaic symptoms). Weeds with golden mosaic symptoms that gave weak to strong hybridization signals with the general probe included: *Croton lobatus* L., *Jatropha* spp., *Sida* spp., *Urena lobata* L., *Bastardia bivalvis* (Cav.) Kunth., *Rhynchosia minima* (L.) Dc., and *Euphorbia heterophylla* L. No hybridization signals were observed from five of nine *Sida* spp. and two of four *E. heterophylla* plants with golden mosaic symptoms.

With the BGMV-DR-specific probe at high stringency conditions, hybridization signals were observed from beans with golden mosaic symptoms from all locations, whereas no signals were observed with the BDMV or BGMV-BZ specific probes (*data not shown*). Three bean plants with stunting and/or crinkling symptoms that hybridized with the general probe also hybridized with the specific probes; two of these did not hybridize with any of the specific probes, whereas one hybridized with the BDMV-specific probe. The only weed that hybridized with the specific probes was *R. minima*, which gave strong hybridization signals with all three specific probes.

Development of PCR Methods for Detection of Bean-Infecting Geminiviruses

Research reports:

Rojas, M. R. 1992. Detection and characterization of whitefly-transmitted geminiviruses by the use of polymerase chain reaction. M. S. thesis. University

of Wisconsin-Madison at Madison. 92 pp.

Rojas, M. R., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. 1993. Use of Polymerase Chain Reaction with General Primers in the Detection and Identification of Whitefly-Transmitted Geminiviruses. Plant Dis. 76:xxx-xxx. (Submitted August 1992)

Methods

Extraction of viral DNA. Two methods were used for extraction of DNA from plant tissues. In the first method (D. R. Russell and J. Cooley, personal communication), young leaf tissue (5-30 mg) was ground with 300 µl of extraction buffer (100 µM EDTA, 2.5 M NH_4Ac, 100 Mm Tris buffer, pH 8) in a 1.5-ml microfuge tube, and then centrifuged for 10 min at ca 10,000 g. The supernatant fluid (ca 250 µl) was transferred to a clean microfuge tube and 500 µl of isopropanol added. The tube was vortexed and centrifuged for 10 min at 10,000 g, and the supernatant fluid removed. The pellet was washed with 200 µl of 70% ethanol, the tube centrifuged for 3 min at 10,000 g, the fluid removed, and the pellet dried for 5 min in a Speed-Vac dryer prior to resuspension in 100 to 300 µl of distilled water. The second DNA extraction method was developed by Dellaporta et al. (1983) and used as previously described by Gilbertson et al. (1991d) for extraction from BGMV-infected bean samples. Twenty to 40 µl of the DNA extract were used per 100 µl PCR reaction mixture.

For some plant samples, an additional purification step was added to obtain suitable viral DNA. The DNA extracted by the Dellaporta et al. (1983) method was suspended in 500 µl Milli-Q water, extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and centrifuged at 10,000 g for 5 min. The aqueous layer was removed and placed in a clean microfuge tube. One ml of 100% ethanol was added, the sample centrifuged for 5 min at 10,000 g, the liquid phase removed, and the precipitated nucleic acid pellet washed twice with 500 µl of 70% ethanol. The pellet was dried under a partial vacuum and resuspended in 50 µl of distilled water. Twenty µl of this DNA extract were used in 100 µl PCR reaction mixtures.

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

Polymerase chain reaction. Primers were synthesized by the University of Wisconsin-Madison Biotechnology Center and were adjusted to 10 µM with Milli-Q water. PCR reaction mixtures of 100 µl contained 20-40 µl of sample DNA solution, and dNTP and primer concentrations of 25 µM for each nucleotide and 0.2 µM for each primer, respectively. The Mg^{++} concentration was between 0.5 and 2.5 mM, and this concentration could greatly influence the success of the viral DNA amplification. Taq polymerase (Promega, Corp., Madison, WI) was used according to manufacturer's instructions. Each reaction mixture was covered with 50 µl of mineral oil to prevent evaporation. Presumed viral DNA was amplified in a Coy Thermal Cycler by 30 cycles of melting, annealing and DNA extension conditions of 1 min at 94 C, 1 min at 50 C, and 3 min at 72 C, or 1 min 94 C, 2 min 55 C, and 2 min 72 C. For the last cycle, the extension time was increased to 3 min, and then the temperature was decreased to 4 C until the reaction mixtures were removed. Amplified DNA fragments were electrophoresed in 0.7% agarose (FMC, Rockland, Maine) minigels in TBE buffer (Tris-borate EDTA) and visualized with UV light after staining in ethidium bromide.

Design of PCR-oligonucleotide primers. Degenerate primers for whitefly-transmitted geminiviruses (Table 1) were designed to anneal to highly conserved nucleotide sequence regions of the open reading frames (ORFs) or the Common Region of DNA-A and DNA-B. These regions were identified by aligning derived amino acid and/or nucleotide sequences of at least ten of the characterized whitefly-transmitted geminiviruses, and consensus sequences were produced from these alignments. The primers were synthesized as 17-20 mers, excluding the four

nucleotides and a PstI site which were tailored into the 5' end of some primers. A degenerate primer is a mixture of molecules in which the nucleotides at one or more defined positions vary by design. The degenerate number for a primer is the product of all the numbers which designate how many nucleotides may occur at each position in that primer. The degenerate number for each primer was equal to or less than 16. The sequences of all primers were compared with sequences of DNA-A or DNA-B of the ten geminiviruses to confirm that they would anneal at the predicted locations. The name of a primer (P) includes its location in the genome (e.g., AL1 ORF), a designation of viral or complementary sense sequence (v or c), and a number which is the position of the nucleotide in the genome of BGMV-GA (GenBank accession No. M91604 and M91605 for DNA-A and DNA-B, respectively) that corresponds to the 3' nucleotide of the primer (e.g., PAL1v1978). Viral sense (v) refers to the sequence of the single-stranded DNA as it is found in the virion (Stanley and Gay, 1983).

Table 1. Sequences of the oligonucleotide primers

Primer[a]	Nucleotide sequence
PAL1v1978	5' GCATCTGCAGGCCCACATYGTCTTYCCNGT 3'
PAL1v2589	5' GAGCAMTGWGGATAWGTTGAGGAAAT 3'
PCRc1	5' CTAGCTGCAGCATATTTACRARWATGCCA 3'
PCRc154	5' GGTAATATTATAHCGGATGG 3'
PCRv168	5' TAATATTACCGGWTGGCC 3'
PAR1c496	5' AATACTGCAGGGCTTYCTRTACATRGG 3'
PAR1c503	5' CATGCTGCAGTACATYGGCCTYTTDACCC 3'
PBL1v2042	5' GCTCTGCAGCARTGRTCKATCTTCATACA 3'

[a]P = primer; AR1 = ORF for AR1, AL1 = ORF for AL1, BL1 = ORF for BL1, or CR = Common Region; V = viral sense primer (anneals to complementary sense and gives viral sense sequence) or C = complementary sense primer; number = nt number for bean golden mosaic geminivirus from Guatemala for the 3' nt of the primer. Nucleotides added for the PstI restriction site are underlined, and the four nt 5' of this restriction site were added to facilitate restriction endonuclease activity. Nucleotides at degenerate positions are represented by a single letter of the IUPAC ambiguity code: D = A,G,T; H = A,C,T; M = A,C; N = A,C,G,T; R = A,G; W = A,T; Y = C,T.

For DNA-A, primers PAL1v1978 and PAR1c496 were designed to anneal within the AL1 ORF, which codes for a replication-associated protein (Elmer et al., 1988), and AR1 ORF, which encodes for the coat protein (Kallender et al., 1988), respectively (Fig. 1). Primer PAL1v1978 was designed to anneal to the complementary sense strand of the replicative form of AL1 sequence encoding the derived amino acid sequence ThrGlyLysThrMetTrp, which is a conserved, putative NTP-binding site present in viral replication-associated protein (Gorbalenya and Koonin, 1989; Hanson et al., 1991). Primer PAR1c496 was designed to anneal to the viral sense strand of the AR1 ORF sequence encoding for the conserved, derived amino acid sequence ProMetTyrArgLysProArg, which is located near the amino terminus of the coat protein (Fig. 1).

Four degenerate primers designed to anneal to the Common Region were determined from DNA-A Common Region nucleotide sequence alignments, which were adjusted to maximize nucleotide identities among the eight geminiviruses for the Western Hemisphere. Two of the primers were designed to anneal to the viral sense strand and two to the complementary sense strand. Primers PCRv1 and PCRc1 were designed to anneal near the 5' end of the Common Region, which contains the

predicted start codon for AL1. Primers PCRc154 and PCRv168 were designed to anneal in the stem-loop region, which is conserved in all geminiviruses (Lazarowitz, 1987).

A degenerate primer for DNA-B, PBL1v2042 (Table 1), was designed to anneal to the complementary sense strand of the replicative form of the BL1 ORF sequence encoding the amino acid sequence GlnTrpSerPheMetGln, located in the amino terminus region of the predicted protein of the BL1 ORF. PBL1v2042 could be paired with either of the Common Region complementary sense primers (PCRc1 or PCRc154) to amplify a DNA-B fragment.

Characterization of PCR-amplified fragments. Molecular biological techniques were performed according to accepted protocols (Maniatis et al., 1982). For Southern hybridization analysis, DNA was transferred to Nytran membranes (Schleicher & Schuell Inc., Keene, NH), and Southern blots were hybridized with a radiolabeled general DNA probe for whitefly-transmissible geminiviruses (Gilbertson et al., 1991b). Denaturing and hybridization conditions were as described in Gilbertson et al.(1991b). For restriction fragment length polymorphism (RFLP) analysis or cloning, PCR-amplified DNA was precipitated with ethanol and dissolved in TE buffer. For RFLP analysis, the DNA was digested with selected restriction enzymes, electrophoresed in 0.7% agarose gels in TBE, and visualized with UV light after staining in ethidium bromide. For cloning PCR fragments, DNA was digested with PstI restriction endonuclease, the site for which had been engineered into the 5' ends of the PCR primers, and electrophoresed in 0.7% agarose in Tris-acetate-EDTA buffer. The PCR-amplified DNA band was excised from an ethidium bromide-stained gel, recovered with glass matrix (Gene Clean, BIO 101, La Jolla, CA) according to the supplier's instructions, and ligated into pBluescript II KS+ (Stratagene, La Jolla, CA) digested with the appropriate restriction enzyme. The DNA sequence was determined by the dideoxynucleotide chain termination method using Sequenase (United States Biochemical Corp., Cleveland, OH). DNA sequences were analyzed with the software of the Genetics Computer Group, University of Wisconsin-Madison (Devereux et al., 1984).

Results

Evaluation of primer pairs for amplification of geminivirus DNA. The most consistent amplification of a DNA-A fragment was obtained with the primer combination of PAL1v1978 and PAR1c496 (Table 2). As predicted from the annealing position of these primers with the DNA-A of BGMV-GA, PCR-amplified fragments of about 1.1 kb were obtained from DNA extracts of beans infected with the previously characterized geminiviruses, BGMV-GA, BGMV-DR, BGMV-BZ, and BDMV. These fragments are predicted to include part of AL1 and AR1, and the entire Common Region. A 1.1-kb fragment was also amplified from beans infected with bean calico mosaic geminivirus (BCMoV) from Mexico (Brown et al., 1990). To determine if these primers could be used to amplify a similar-sized fragment from undescribed geminiviruses, leaf samples from beans, soybeans, peppers, tomatoes and weeds suspected to be infected with geminiviruses were collected in the field and tested for the presence of geminiviral nucleic acids by squash blot hybridization with a general geminiviral DNA probe (Gilbertson et al., 1991b). Samples positive for geminiviruses were used for PCR, and a 1.1-kb fragment was amplified from these samples, including tomatoes from Trinidad, the Dominican Republic, Costa Rica, Mexico, Texas (U.S.A.) and Florida (U.S.A.), beans from Costa Rica, soybeans from Puerto Rico, peppers from Mexico, and three weeds, *Sida* sp. and *Calopogonium* sp. from Costa Rica and *Macroptilium lathyroides* (L.) Urb. from the Dominican Republic. The versatility of this primer pair was further evaluated using geminivirus-infected samples from Africa. A 1.4-kb fragment was

obtained from cassava samples from South Africa (S. H. Hidayat and associates, personal communication) and Malawi, from an unidentified legume weed from Malawi, and from tomatoes infected with TYLCV from Egypt (Nakhla et al., 1992). In most cases, a single DNA fragment of the expected size of 1.1 or 1.4 kb was amplified. In some cases, however, a second fragment of ≈2.5-kb at a lower concentration was sometimes observed. This 2.5 kb fragment did not hybridize with the general geminiviral probe (Gilbertson et al., 1991b); and therefore, it was not likely to be a fragment from a geminivirus. No PCR-DNA fragments were amplified from extracts from healthy beans or tomatoes with any primer pair.

Table 2. Fragment size (kb) obtained with different primer pairs in the polymerase chain reactions with five undescribed geminivirus-infected samples and bean golden mosaic geminivirus-infected beans

	PRIMER PAIRS				
	PALc1978	PAL1c1978	PAL1v1978	PAL1v1978	PCRv168
Geminivirus	PAR1c496	PCRc1	PAR1c503	CRc154	PARc503
SoyGMV	1.1	0.70	1.1, 0.40	0.80	0.35
BGMV-GA	1.1	0.70	1.1	0.80	0.35
TGV-MX	1.1	0.70	0.80	0.80	0.35
BCTV		0.70			
TYLCV-EG1	1.4	0.80	1.4		
LegGV	1.4	0.80	0.50		

[a]SoyGMV = soybean golden mosaic geminivirus; BGMV-GA = bean golden mosaic geminivirus, Guatemala; TGV-MX = tomato geminivirus, Mexico; BCTV = beet curly top geminivirus; TYLCV-EG1 = tomato yellow leaf curl geminivirus from Egypt; LegGV = legume weed geminivirus from Malawi.

Because the putative NTP-binding site and the stem-loop regions of whitefly-transmitted geminiviruses are also present in leafhopper-transmitted, monopartite geminiviruses (Hanson et al., 1991; Lazarowitz, 1987, Stanley et al., 1986), the primer pairs that anneal to the AL1 ORF (PAL1v1978) and the Common Region (PCRc1 or PCRc154) were tested with an extract from a field sample of beans with beet curly top symptoms from California. Fragments of 0.7 kb and 0.8 kb were obtained (Table 2). Squash blots of the infected bean tissue and Southern blots of the amplified fragments hybridized with a specific probe for beet curly top geminivirus under conditions of high stringency (unpublished data).

PCR-amplified DNA-B fragments of the predicted ≈0.6 kb were obtained from the three BGMV isolates and BDMV with the primer pair PBL1v2042 and PCRc1. These primers were then used to amplify DNA-B fragments from uncharacterized geminiviruses, including Rhynchosia mosaic geminivirus (RMV), and tomato-infecting geminiviruses from Mexico (TGV-MX1; Paplomatas et al., 1991) and

Florida (tomato mottle geminivirus, ToMoV; Gilbertson et al., 1991c). In some cases, different sized DNA-B fragments were amplified (Fig. 4); for example, the fragment amplified from BGMV-DR was larger than those from BDMV and the tomato-infecting geminiviruses. To confirm the identity of the putative DNA-B fragments, Southern hybridization analyses were carried out under low stringency conditions with a probe consisting of DNA-B clones of BGMV-GA, BGMV-BZ, and BDMV. Because these primers are expected to amplify only a portion of DNA-B including part of BL1 and the hypervariable region (Gilbertson et al., 1991d) but not the Common Region, any hybridization would be specific for DNA-B. All but one of the amplified DNA-B fragments hybridized strongly with the DNA-B probe. The DNA-B fragment from TGV-MX1 hybridized only weakly, therefore, this portion of its nucleotide sequence is highly divergent from the corresponding sequences of the geminiviruses used in the probe. The strong hybridization of the probe with ToMoV from Florida was due to the high level of sequence similarity between ToMoV and BDMV (Gilbertson et al., 1991c).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified fragments. PCR fragments generated with the same primer pair from different plant samples were digested with restriction enzymes to detect polymorphisms. The 1.1-kb DNA-A fragments amplified with PAL1v1978 and PAR1c496 from extracts of plants infected with ToMoV, TGV-MX1, a tomato-infecting geminivirus from Costa Rica (TGV-CR), pepper-infecting geminivirus from Mexico (MX-P2), BDMV, or BGMV-DR were separately digested with the restriction endonucleases, BglII, HincII, SalI, and RsaI. Only the BGMV-DR fragment was cleaved with SalI. BglII digestions resulted in three RFLP patterns (data not shown). One RFLP pattern (0.3- and 0.8-kb fragments) was found for BDMV, ToMoV, and BGMV-DR, one for MX-P2 (0.5- and 0.6-kb fragments), whereas fragments from TGV-MX and TGV-CR were not digested with BglII. RFLP patterns resulting from HincII digestions distinguished TGV-MX1 and TGV-CR; and different HincII RFLP patterns were found among the PCR fragments for BDMV, BGMV-DR, ToMoV, and a pepper-infecting geminivirus (MX-P2). PCR fragments from TGV-CR and ToMoV were not digested with HincII. Also, the HincII digestions of the PCR fragments from BDMV and TGV-MX1 gave the same size fragment (≈0.5 kb). RsaI cleaved the DNA-A fragments from all six geminiviruses, resulting in a different RFLP pattern for each geminivirus.

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

have been 1.1 kb.

Using the same primers and PstI digestions, a mixed geminivirus infection was detected in a field sample of tomatoes from Mexico. When the 1.1-kb DNA-A fragments amplified from this sample were digested with PstI, three fragments of 1.1, 0.6, and 0.5 kb were observed. This indicated a possible mixed infection. To eliminate the possibility of an incomplete PstI digestion and to further characterize these fragments, each fragment was excised from an agarose gel, purified, cloned, and partially sequenced. The nucleotide sequence comparison of the Common Regions and part of the intergenic region of these fragments showed only 82% identity. This confirmed a mixed infection.

Diversity of bean-infecting geminiviruses:

Research reports:

Faria, J. C., Gilbertson, R. L., Hanson, S. F., Morales, F. J., Ahlquist, P., and Maxwell, D. P. 1993. Bean golden mosaic geminivirus type II isolates form the Dominican Republic and Guatemala: Nucleotide sequence of the infectious clones and pseudorecombinants between these clones. Phytopathology 83:___-____. (In preparation)

Gilbertson, R. L., Rojas, M. R., Russell, D., and Maxwell, D. P. 1991d. The use of the asymmetric polymerase chain reaction and DNA sequencing to determine genetic variability among isolates of bean golden mosaic geminiviruses. J. Gen. Virol. 72:2843-2848.

Hidayat, S. H., Gilbertson, R. L., Hanson, S. F., Morales, F. J., Ahlquist, P., Russell, D. R., and Maxwell, D. P. 1993. Complete nucleotide sequences of the infectious cloned DNAs of bean dwarf mosaic geminivirus. Phytopathology 83:___-_____. (IN PRESS)

Loniello, A. O., Martinez, R. T., Rojas, M. R., Gilbertson, R. L., Brown, J. K., and Maxwell, D. P. 1992. Molecular characterization of bean calico mosaic geminivirus. (Abstr.) Phytopathology 82:1992.

Methods

Asymmetric polymerase chain reaction. 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 mm^2 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 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 those primers are: DR-P1, 5' CTGAAGCGCAAAGATAGAGAAGC 3' (23nt), 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 μ/g) 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.

Sequence analysis. DNA sequences were assembled and analyzed using programs from the Univeristy of Wisconsin Genetics Computer Group (Devereux et al., 1984). DNA relatedness (phylogenetic trees) were constructed with the nucleic acid sequences of the AL1 ORF and the Common Region using the Phylogenetic Analysis Using Parsimony (PAUP) program.

Results

Genetic variability of BGMV in the Dominican Republic. Amplified DNA fragments of the predicted size (approx. 300 to 400 bp) were detected in the five bean leaf samples with BGM symptom 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. 5. 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 region of BGMV-GA and BGMV-PR (86 and 75%, respectively), 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).

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., 1991b), 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 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 (Pybicki & 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 (e.g. 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

```
            1
pDRB1       TCATTTGTTG ACACTCGCAT ACAACTGATA AACTGACCAA CCAAGAGATT
DR-1        .......t.. .......... .......... ......c... ..........
DR-2        .......... .......... .......... ...aa.c... ..........
DR-3        .......... .......... .......... ......c... ..........
DR-4        .......... .......... .......... ......c... ..........
DR-5       A...-..at.. .....g.... .......... ...a..c... ..........
pDRB-PCR    .......... .......... .......... .......... ..........

            51
pDRB1       ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
DR-1        .......... .......... .......... .......... ..........
DR-2        .......... .......... .......... .......... ..........
DR-3        .......... .......... .......... .......... ..........
DR-4        .......... .......... .......... .......... ..........
DR-5        .......... .......... ....t..... .......... ..........
pDRB-PCR    .......... .......... .......... .......... ..........

            101
pDRB1       CAGTTGTATG CCTTTATATC GCCTTTTGAA AAAGAACATA TTATTTAGTT
DR-1        .......... .......... ........t. .......... ..........
DR-2        .......... .......... .......... .......... ..........
DR-3        .......... .......... ..t....... ...a...... ..........
DR-4        .......... .......... ........t. .......... ..........
DR-5        .......... .......... .......... .......... .......a..
pDRB-PCR    .......... .......... .......... .......... ..........

            150
pDRB1       AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC GGATATATAT
DR-1        .......... .......... .......... .......... ..........
DR-2        .......... ......a... .......... .......... ..........
DR-3        .......... .......... .......... .......... ..........
DR-4        .......... .......... .......... .......... c.........
DR-5        .........t ...c...... .......... .......... ..........
pDRB-PCR    .......... .......... .......... .......... ..........

            200
pDRB1       ATT
DR-1        ...    (98%)
DR-2        ...    (98%)
DR-3        ...    (98%)
DR-4        ..a    (98%)
DR-5        ...    (95%)
pDRB-PCR    ...   (100%)
```

Fig. 5. 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 sequenced of the BGMV-DR component DNA-B (nt 2279 to 2481 of pDRB1). Variations in nucleotide sequence are shown as lower-case letters, the same nt as a period (.), and gaps as a dash (-). The percentage sequence identities ofthe amplified fragment with the pDRB1 sequence are shown in parentheses after the sequence of each fragment.

it has been reported that ssDNA templates suitable for sequencing can be produced by direct A-PCR amplification, 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 & Fwok, 1990), human T cell lymphotrophic 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 & Sanger, 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 & Sanger, 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.

Distribution of type II isolates of bean golden mosaic geminivirus in the Caribbean Basin and Central America. Our research group has sequenced the infectious clones of BGMV from Guatemala and the Dominican Repulbic (unpublished results) and found that these isolates had sequence idenities of > 90% with each other and with the isolate from Puerto Rico (Howarth et al., 1985). In contrast, they were not closely related to an isolate of BGMV from Brazil (Gilbertson et al., 1991b; unpublished results). We have proposed that the two distinct types of BGMV be designated type I and type II. Type I isolates would be similar to BGMV from Brazil and type II isolates similar to the ones from Guatemala, the Dominican Republic, and Puerto Rico. Type I isolates are not sap transmissible whereas those of type II are. Additionally, it is expected that bean germ plasm will respond differently to these two types of BGMV.

The genetic diversity of BGMV isolates was determined by cloning and sequencing PCR amplified fragments of BGMV from bean samples collected in Central America and Jamaica as well as Brazil. The nt sequence of the Common Region, AL1, and AR1 for isolates Jamaica, Nicaragua, Puerto Rico, and Costa Rica were found to be >90% identical to those of type II isolates and not closely related to type I. Thus, it appears that the type II isolates are most prevalent in the Caribbean Basin and Central America. It is not clear where type II isolates shift to bean calico mosaic geminivirus in Mexico. So far, BGMV type II isolates have not been detected in Mexico. For five samples from Brazil (three from Goiás, one each from Paraná and São Paulo), nt sequences for AL1 and AR1 were 5-

obtained and these were > 90% identical to the type II isoaltes.

Our results clearly document the occurrence of four distinct geminiviruses infecting beans. This includes the type I and type II isolates of BGMV, BDMV and BCMoV (Fig. 6). Plant breeders need to be aware of these different geminiviruses as strategies are designed to breed beans for resistance to geminiviruses. Before our research was started, only BDMV and BGMV were known and they were thought to be closely related.

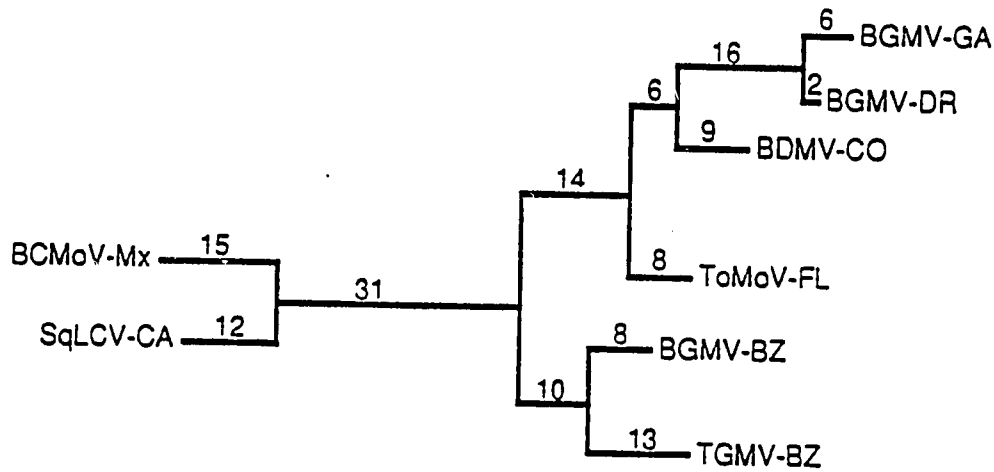

Fig. 6. A phylogenetic tree was constructed using the PAUP program to compare the Common Regions of eight whitefly-transmitted, bipartite geminiviruses. The number of nucleotide differences between the different geminiviruses is shown above each branch of the tree.

Weed reservoirs of geminiviruses:

Pulbished reports:

Gilbertson, R. L., Hidayat, S. H., Martinez, R. T., Leong, S. A., Faria, J. C., Morales, F., and Maxwell, D. P. 1991. Differentiation of bean-infecting geminiviruses by nucleic acid hybridization probes and aspects of bean golden mosaic in Brazil. Plant Dis. 75:336-342.

Rojas, M. R. 1992. Detection and characterization of whitefly-transmitted geminiviruses by the use of polymerase chain reaction. M. S. thesis. University of Wisconsin-Madison at Madison. 92 pp.

Rojas, M. R., J. P. Karkashian, and D. P. Maxwell. Characterization of Geminiviruses Infecting Bean and *Calopogonium* sp. in Costa Rica. Ann. Reptr. Bean Improv. Coop. 35:150-151.

Martinez, R. T., M. R. Rojas, A. Figueroa, and D.P. Maxwell. *Macroptilium lathyroides* Infected by a Geminivirus Distinct From Bean Golden Mosaic Geminivirus in the Dominican Republic. Ann. Reptr. Bean Improv. Coop. 35:152-153.

Methods

DNA hybridizations with geminiviral probes and PCR methods as described above were used to study the diversity of geminiviruses in weeds in the Dominican Republic and Costa Rica. These methods would allow us to determine if weeds were a common reservoir of bean-infecting geminiviruses.

Results

Use of the DNA probes for detection of geminiviruses in weeds in the Dominican Republic. When the general probe was used, hybridization signals were not observed from crop samples (tomato, cassava, green pepper, or tobacco), or weeds without golden mosaic symptoms. Weeds with golden mosaic symptoms that gave weak to strong hybridization signals with the general probe included: *Croton lobatus* L., *Jatropha* spp., Sida spp., *Urena lobata* L., *Bastardia bivalvis* (Cav.) Kunth., *Rhynchosia minima* (L.) Dc., and *Euphorbia heterophylla* L. No hybridization signals were observed from five of nine *Sida* spp. and two of four *E. heterophylla* plants with golden mosaic symptoms.

When the isolate-specific probes for BGMV-BZ, BGMV-DR, and BDMV were used at high stringency, the only weed sample which reacted with any of these three probes was *R. minima* and surprisingly it gave strong hybridization signals with all three specific probes. This means that bean-infecting geminiviruses were not present in the other six weeds and that *R. minima* may contain a mixture of geminiviruses and/or may have a geminivirus which has sequences similar to the three isolate specific-probes.

Use of PCR methods to detect geminiviruses in weeds in the Dominican Republic. *Macroptilium lathyroides* is a common legume weed and it has been described as the natural source of inoculum for BGMV in Puerto Rico, the Dominican Republic, and Jamaica (Bird et al., 1975; Abreu and Gálvez, 1979; Pierre, 1975). In the Dominican Republic, large areas of *M. lathyroides* with striking golden mosaic symptoms have been observed by us in areas close to bean fields infected with BGMV. Samples from these plants gave weak hybridization signals with the general geminivirus probe; and thus, we could not rule out the possibility that these plants have a very low titer of a bean-infecting geminivirus. Plant samples showing typical golden mosaic symptoms were collected in the Southwest zone (San Juan de la Maguana) and the Central zone (Azua). PCR reaction methods with general primers for geminiviruses were used to amplify a 1.1-kb fragment and sequence was obtained for the AL1 ORF, Common Region, and AR1 ORF. The nucleotide sequence identity of the AL1 region for the Macroptilium geminivirus from Azua was compared with that of the isolate from San Juan de la Maguana and BGMV-DR and the nt identities were 100% and 83%, respectively.

From the sequence comparison data, we concluded that *M. lathyroides* was infected by a new geminivirus which is distinct from BGMV-DR. Thus, it seems to be that *M. lathyroides* is not the source of inoculum for BGMV in the Dominican Republic. There is no variability between the two geminivirus isolates from *M. lathyroides* from the two locations. This new geminivirus is designated Macroptilium golden mosaic geminivirus (MacGMV).

PCR methods were used to show that MacGMV is a bipartite geminivirus, which is typical of the whitefly-transmitted geminiviruses in the Western Hemisphere.

A DNA-B component was detected when the PCR primer pair for the BL1 ORF and Common Region (Rojas, 1992) were used to amplify a 600-bp fragment. Nucleotide sequence of the end of this fragment in the intergenic region confirmed that it was a DNA-B component.

Besides *M. lathyroides*, *C. lobatus* was commonly found adjacent to bean fields and many plants had golden symptoms typical of a geminivirus. The same PCR methods as described above were used to obtain DNA-A and DNA-B fragments from plant samples with symptoms. Nucleotide sequence of the ends of these fragments indicated that these plants contained a new whitefly-transmitted, bipartite geminivirus which was not closely related to any of the bean-infecting geminviruses. Nucleotide sequence is not yet available for the Common Region, and this will be completed in the near future.

In Costa Rica, a legume weed *Calopogonium* sp. has been reported as a host for BGMV (Gálvez and Morales, 1989) and we used the PCR methods to amplify a 1.1-kb fragment from DNA-A from two samples of *Calopogonium* sp. collected from diverse areas. Nucleotide sequence of the AL1 ORF for these the fragments from these two samples were 97% identical with each other and 75% identical BGMV from Costa Rica. Thus, this weed is not the weed reservoir for BGMV in Costa Rica. This is the first report of sequence data from a geminivirus infecting *Calopogonium* sp. and the name Calopogonium golden mosaic geminivirus (CalGMV) is proposed for this new virus.

In summary, we have been unable to locate a weed which could serve as the source of inoculum in the Dominican Republic or Costa Rica. Based on this, we have proposed that beans are the major source of BGMV in the Dominican Republic and that the severity of infection could be reduced by requiring a bean-free growing period just previous to the main bean planting in the San Juan area. This was implemented by the government and as predicted the losses to BGMV have been greatly reduced during the January to April growing season. Other regions may also consider using this approach.

Germplasm evaluation for reaction to BGMV and BDMV:

Results reported:

Hidayat, S. H. 1990. The construction and application of DNA probes for the detection of bean dwarf mosaic geminivirus. M. Sc. Thesis, University of Wisconsin-Madison, pp. 53.

Results

One of our objectives was to assist with the evaluation of germplasm using standard methods in the plant growth chambers. Because of the need to devote our resources to the characterization and development of detection methods, limited effort was devoted to this aspect of the grant.

Thirty-four bean cultivars/bean genotypes were tested for their reaction to BDMV and BGMV-DR (Table 3). Several of the bean lines were immune to BDMV, but none of them were immune to BGMV-DR. Generally, the meso-american lines were immune to BDMV and some of these lines had reduced symptoms with BGMV-DR. The two lines, Pompadour J and Pompadour G, which were thought to be resistant to

BGMV-DR, due to field test results in the Dominican Republic, had dwarf symptoms, rather than the typical golden mosaic symptoms, when inoculated with BGMV-DR. Once this was known, it was possible to find these dwarfed plants in the field plots in the Dominican Republic.

Table 3. Reaction of bean genotypes to bean dwarf mosaic virus (BDMV) and a bean golden mosaic virus isolate from the Dominican Republic (BGMV-DR).

	Reaction score to[a]	
Cultivar/bean genotypes	BGMV-DR	BDMV
Topcrop	++++	++++
Pinto 114[b]	++++	-
DOR 303	nt	-
California Dark Red	nt	++++
A429	nt	++++
Improved Tendergreen	nt	++++
Mountcalm	nt	++++
Black Turtle Soup	nt	-
Topaz	nt	-
Sierra[b]	++++	-
ISB 82-354	nt	-
ISB 84-114	nt	-
Pindak	nt	-
Nodak	nt	-
BillZ	nt	-
Othello	nt	-
Olathe	nt	-
Red Mexican	nt	-
Mayflower[b]	++++	nt
Seafarer[b]	++++	nt
Desarrual	++++	-
Pompadour K	++++	-
Pompadour J	++++[c]	-
Pompadour G	++++[c]	-
9056-9[d]	++++	-
9056-10[d]	++++	+
Xan 174	++++	+
Belneb	++++	-
9056-48[d]	++++	-
Roma	+++	+++
PC-50	++++	++++

Cultivar/bean genotypes	Reaction score to[a]	
	BGMV-DR	BDMV
P86225[e]	++++	+
P86291[e]	++++	-
P86297[e]	++++	-

[a] ++++ = 100% of inoculated plants are infected
++ = 75% of inoculated plants are infected
+ = 50% of inoculated plants are infected
- = immume plants
nt = not tested

[b] infected plants showing delayed symptoms
[c] infected plants showing dwarf-like symptoms
[d] supplied by Dr. J. Beaver, University of Puerto Rico
[e] supplied by Dr. J. Kelly, Michigan State University

PROJECT IMPACT, RELEVANCE, AND TECHNOLOGY TRANSFER

Usefulness of project findings for developing countries. The impact of the findings can be divided into four areas:

Diversity of bean-infecting geminiviruses: When this project was initiated, there was suggestive evidence based on symptoms that two different geminiviruses infected beans, bean golden mosaic geminivirus (BGMV) and bean dwarf mosaic geminivirus (BDMV). We have provided conclusive evidence that at least four distinct geminiviruses infect beans: BGMV type I from Brazil, BGMV type II from the Caribbean Basin and Central America, bean calico mosaic geminivirus from Mexico, and bean dwarf mosaic geminivirus from Colombia. These results clearly emphasize the need for breeding programs for resistance to geminiviruses to recognize these major differences. Thus, beans with resistance to one virus may not be resistant to other geminiviruses.

Development of detection methods for geminiviruses: A general geminivirus probe was developed which effectively detected whitefly-transmitted geminviruses from the Western Hemisphere, but was not very effective for detection of geminiviruses from the Eastern Hemisphere. Also, three specific probes for BGMV type I, BGMV type II, and BDMV isolates were developed and tested. It is likely that the cloned DNA-B component of bean calico mosaic geminivirus would be a specific probe for this virus. These technologies have been useful for the groups at the University of Wisconsin and CIAT, but the general availability to developing countries is limited because of the use of radioisotopes.

General primers for geminiviruses were designed for use with polymerase chain reaction methods and these methods allowed the amplification of geminiviral fragments from a wide range of undescribed geminiviruses from the Western and Eastern Hemisphere. This technology has had a tremendous impact on this project and the methods have been transferred to Costa Rica, and is currently being transferred to Egypt, Jamaica, South Africa, CIAT, and AVDRC.

Our laboratory processed over 2,000 samples in the last 3 years using these DNA hybridization and PCR methods. Samples have been obtained from scientists in seven countries in Asia, four countries in Africa, two countries in the Middle East, five countries in Central America plus Mexico, four islands in the Caribbean Basin, and five countries in South America. For the U.S.A., our efforts have resulted in the detection of geminiviruses in ornamentals in Florida and in tomatoes in Florida and Texas.

Detection of geminiviruses in weed hosts: No weed host was found to be the main source of inoculum for BGMV in the Dominican Republic, and the previously proposed legume weed host for BGMV was shown to have a distinct geminivirus. These observations led to major changes in the culture of beans in the Dominican Republic and a bean-free period is now imposed by the government before the major growing season for beans in January to April. This has resulted in an increase in bean yields during this season. Other countries may follow this practice.

Evaluation of germplasm for resistance to bean-infecting geminiviruses: Our results have confirmed and extended those of other researchers. In general, the meso-american lines have a higher level of resistance to geminiviruses than the Andean lines and many are immune to BDMV. Unexpectedly, we found that BGMV

infection of two Andean lines from the Dominican Republic caused extreme dwarf symptoms and no golden mosaic symptoms. These genotypes were originally thought to be immune to BGMV.

Impact on the Dominican Republic, other developing countries and International Research Centers. This project was organized so that most of the funds went towards the support of a person from the Dominican Republic to receive training in virus diagnosis and disease control. R. Teresa Martinez from the Dominican Republic completed three years of training on this project and finished an M. Sc. Degree in Integrated Pest Management in August, 1992. She will return to the Dominican Republic in September 1992 and start a position with the Secretaria de Estado de Agricultura. This position will be funded initially by the Bean/Cowpea CRSP project. She will have continued responsibilities for research on management practices to control bean golden mosaic virus on beans. Ms. Martinez learned the techniques associated with nucleic acid based methods for the detection of geminiviruses.

As indicated above, several developing countries and two international centers are implementing the use of the diagnostic methods developed during this grant. Also, close association has been maintained with Brazilian scientists and they are using the PCR technologies to assist them in their efforts to control BGMV in Brazil.

PROJECT ACTIVITIES/OUTPUTS:

Meetings attended:

The annual meeting of the American Phytopathological Society was attended each of the three years of the project by D. P. Maxwell and two years by R. T. Martinez.

Two Bean Improvement Cooperative meetings were attended by D. P. Maxwell and one by Freddy Saladin.

Two Caribbean Divisonal meetings of the American Phytopathological Society were attended by D. P. Maxwell and six invited papers presented.

Two meeting organized for the PSTC host country and U.S.A. scientists in Canada (Sept. 1991) and Mexico (Dec. 1992) were attended by O. Azzam and R. T. Martinez.

Fourth conference of the International Plant Biotechnology Network, January 14-18, 1991, San Jose, Costa Rica was attended by D. P. Maxwell.

International Bonference on Bean Golden Mosaic, Guatemala City, Nov. 1992. Talks were presented by D. P. Maxwell (3), A. O. Loniello (1), W. McLaughlin (1), J. C. Faria (1), and J. Karkashian (2).

Training:

R. T. Martinez from the Dominican Republic received training for three years at the University of Wisconsin and earned a M. Sc. Degree in August 1992 in Integrated Pest Management.

Dr. Josais C. Faria from Brazil received three months training in molecular techniques used for virus characterization.

Two undergraduate students completed research projects assocaited with the objectives of this project.

S. H. Hidayat from Indonesia completed a M. Sc. Degree in Plant Pathology in January 1990. She developed the specific probe for BDMV and evaluated the bean germplasm.

M. R. Rojas from Costa Rica completed a M. Sc. Degree in Plant Pathology in January 1992. She completed the design of general primers for geminiviruses for PCR.

Publications:

Refereed

Gilbertson, R. L., M. R. Rojas, D. R. Russell, and D. P. Maxwell. 1991. The use of asymmetric polymerase chain reaction and DNA sequencing to determine genetic variability of bean golden mosaic geminivirus in the Dominican Republic. J. Gen. Virol. 72:2843-2848.

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Hidayat, S. H., R. L. Gilbertson, S. F. Hanson, F. J. Morales, P. Ahlquist, D. R. Russell, and D. P. Maxwell. 1992. Complete nucleotide sequences of the infectious cloned DNAs of bean dwarf mosaic geminivirus. Phytopathology (IN PRESS)

Rojas, M. R., R. L. Gilbertson, D. R. Russell, and D. P. Maxwell. 1992. Design of general primers and use of polymerase chain reaction for the detection and identification of whitefly-transmitted geminiviruses. Plant Dis. (IN PRESS)

Abstracts

Faria, J. C., R. L. Gilbertson, F. J. Morales, D. R. Russell, P. G. Ahlquist, S. F. Hanson, and D. P. Maxwell. 1990. Sequence of infectious clones of two mechanically transmissible isolates of bean golden mosaic geminiviruses. Phytopathology 80:983.

Gilbertson, R. L., J. C. Faria, S. F. Hanson, S. H. Hidayat, M. Rojas, F. J. Morales, P. G. Ahlquist, D. R. Russell, and D. P. Maxwell. 1991. Molecular characterization of bean-infecting geminiviruses. Fourth conference of the International Plant Biotechnology Network, January 14-18, 1991, San Jose, Costa Rica.

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Non-Refereed

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PROJECT PRODUCTIVITY:

The major goals of the project were completed. DNA probe and PCR methods were developed for the detection of geminivirues in a wide-range of plant species. These techniques allowed our group to test over 2,000 plants in the last three years. The bean-infecting geminivirus in the Dominican Republic has been cloned and sequenced and found to represent the Type II BGMV isolates. BDMV from Colombia was also cloned and sequenced. Progress was made towards determining the potential weed reservoirs, but this is a much bigger task than expected. One species of legume weed, *Rhynchosia minima*, may be a reservoir, but it was not possible to use PCR methods to amplify viral DNA fragments from infected tissue. Interfering compounds were present in this plant. Attempts to use whiteflies to transfer a geminivirus from infected plants into beans in the Dominican Republic met with technical problems. Much more needs to be learned of the epidemiology of BGMV.

FUTURE WORK:

The techniques developed in this project are now available for use in extensive epidemiological studies on the spread of geminiviruses between natural

hosts and crop plants. These methods can be used to determine if strains of whiteflies exist which are more efficient vectors of certain geminiviruses. Extensive characterization of the many geminiviruses found in natural/weed plants is now possible and studies to understand the evolution of geminiviruses from natural plants to economic plants can be initiated. Additionally, the geminiviruses infecting economically important crops such as tomatoes, peppers, and melons throughout the world can be studied using polymerase chain reaction methods and the general primers for whitefly-transmitted geminiviruses. For example, these methods could assist in the understanding of the geminiviruses causing losses in tomatoes and mung beans in Asia.

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Differentiation of Bean-Infecting Geminiviruses by Nucleic Acid Hybridization Probes and Aspects of Bean Golden Mosaic in Brazil

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ABSTRACT

Gilbertson, R. L., Hidayat, S. H., Martinez, R. T., Leong, S. A., Faria, J. C., Morales, F., and Maxwell, D. P. 1991. Differentiation of bean-infecting geminiviruses by nucleic acid hybridization probes and aspects of bean golden mosaic in Brazil. Plant Dis. 75:336-342.

A bean golden mosaic geminiviral isolate from Goiania, Goiás, Brazil (BGMV-BZ), was determined to be transmitted by whiteflies and induced golden mosaic symptoms and diagnostic geminiviral inclusion bodies and ultrastructural abnormalities in infected bean leaves. In contrast to BGMV isolates from Central America and the Caribbean, BGMV-BZ could not be sap-transmitted to beans. Cloned geminiviral DNA components were used as DNA probes for the rapid and specific detection of BGMV-BZ and three sap-transmissible bean-infecting geminiviral isolates—BGMV from Guatemala (BGMV-GA) and the Dominican Republic (BGMV-DR) and bean dwarf mosaic geminivirus from Colombia (BDMV-CO). A general DNA probe detected all four viral isolates, whereas specific probes detected BGMV-BZ, BDMV, or BGMV-GA and BGMV-DR. Nucleic acid dot and squash blot methods were used to prepare samples for hybridization, and the dot blot method was used to determine relative differences in viral nucleic acid titers in infected bean leaves. The general and specific probes were employed to study the variability of BGMV isolates and potential weed reservoirs of geminiviruses in the Dominican Republic.

Bean golden mosaic geminivirus (BGMV) is a virus transmitted by whiteflies (*Bemisia tabaci* Gennadius) that posseses a single-stranded DNA genome, infects common bean (*Phaseolus vulgaris* L.) and certain other legumes, and occurs throughout Central and South America and the Caribbean (13). Bean golden mosaic (BGM) is characterized by a striking yellow-green mosaic pattern on infected leaves and by stunted and distorted growth. This disease can cause up to 100% yield loss and seriously diminishes the quality of surviving harvestable seed (13). The first report of BGM was by A. S. Costa in 1960 in the state of São Paulo, Brazil (7). Though first considered a disease of minor importance, BGM has spread rapidly and has now been reported to cause significant losses in at least 12 Latin American countries (13). In Brazil, BGM is the most important disease of beans during the dry season and has eliminated commercial bean production in some traditional bean-growing regions, such as areas in the states of Minas Gerais and Paraná (10). Commercial bean production in the dry season in the western region of the Dominican Republic is also threatened by BGM (F. Saladin, *personal communication*).

Recent evidence indicates that genetic variation exists among geminiviruses causing BGM (15,16) and that this variation has been, and will continue to be, a major complication in ongoing attempts to control BGM by classical plant breeding methods. Two bean lines developed by scientists from the Centro Internacional Agricultura Tropical (CIAT), A429 and DOR303, which are moderately resistant (moderate symptoms and acceptable yield) to BGM in Guatemala (25), developed severe BGM symptoms in Brazil (J. C. Faria, *unpublished*). Moreover, BGMV isolates from Central America, Puerto Rico, and the Caribbean are transmissible via plant sap (13,25), whereas isolates from Brazil are not (7,11). Additional genetic variation in bean-infecting geminiviruses is demonstrated by the recent report of bean dwarf mosaic geminivirus (26).

As part of a study on the genetic diversity of bean-infecting geminiviruses throughout South America, Central America, and the Caribbean, experiments were conducted at the Centro Nacional de Pesquisa Arroz-Feijão (CNPAF), Goiânia, Goiás, Brazil, to determine the transmission of the geminivirus(es) associated with BGM. Light and electron microscopy were used to determine the cytopathology of infected leaves and to diagnose viruses. Results indicated that BGM is induced by a geminivirus transmitted by whiteflies that is not sap-transmissible. It also became apparent that methods for rapid detection and differentiation of bean-infecting geminiviruses are needed to adequately investigate genetic diversity among these viruses.

DNA probes have been used for detection of many plant viruses and viroids (2,24,28,32), including geminiviruses (8,18, 27,29,30). Dot blot and squash blot methods have been developed that allow for the efficient preparation of samples for hybridization with nucleic acid probes. Dot blot hybridization has been used to detect viral nucleic acids in animal and plant tissues (2,4,24,28–30) and was quantitative for viral nucleic acids of cauliflower mosaic caulimovirus in plant sap (24). Squash blot hybridization was first used to detect DNA sequences in root tip segments (19) and in Drosophila flies (33) and has more recently been adapted to detect the mycoplasmalike organism that causes Western X-disease (21), the plant pathogenic bacterium *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye (17), and tomato yellow leaf curl geminivirus (27).

In this report, we describe the results of studies conducted with a BGMV isolate from Brazil (BGMV-BZ) and the detection of BGMV-BZ and other bean-infecting geminiviruses by nucleic acid dot and squash blot hybridization methods with cloned geminiviral DNA as probes. These methods were used to detect differences in viral nucleic acid titer in bean cultivars infected with geminiviruses, to study the potential genetic diversity of BGMV in the Domin-

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ican Republic, and to determine weed reserviors of geminiviruses in the Dominican Republic.

MATERIALS AND METHODS

Virus isolates. The three BGMV isolates used—BGMV-Guatemala (BGMV-GA isolate O) and BGMV-Dominican Republic (BGMV-DR)—were mechanically transmitted via plant sap from bean plants with golden mosaic symptoms collected from Monjas, Guatemala, and San Juan de la Maguana, the Dominican Republic, respectively, and maintained in beans (cv. Topcrop) by sap transmission (25). BGMV-BZ was transmitted by whiteflies from bean plants with golden mosaic symptoms collected at CNPAF and maintained in Topcrop beans by whitefly transmission. Bean dwarf mosaic geminivirus-Colombia (BDMV-CO) was mechanically transmitted via plant sap from infected beans collected near Cali, Colombia, and was maintained in beans by sap transmission (26). Geminivirus-infected plant tissue was imported under APHIS permits PPQ 549 and 573.

Sap transmission. Young infected trifoliolate leaves and leaf buds were collected, frozen in liquid nitrogen in a mortar, and ground into a fine powder with a pestle. The mortar was placed on ice and, for experiments in Brazil, a sap inoculum was produced by adding a 1% suspension of magnesium trisilicate in 0.1 M potassium phosphate buffer (pH 8.0) (1:4 w/v) and a small amount of 600-mesh Carborundum and grinding thoroughly with a pestle. For experiments conducted in Madison, WI, only the potassium phosphate buffer was used. The sap suspension was immediately inoculated onto the entire adaxial leaf surface of 7- to 10-day-old bean seedlings (cv. Topcrop) that had been dusted with 600-mesh Carborundum, by dipping the base of the pestle into the sap and rubbing leaves in a circular motion. The youngest three leaves of *Nicotiana benthamiana* Domin plants at the five- to seven-leaf stage were similarly inoculated. Control treatments were plants inoculated with buffer and Carborundum and, for experiments conducted in Brazil, plants were exposed to viruliferous whiteflies. Plants were maintained in a growth chamber (28 C, 16-hr photoperiod) in Brazil and Madison or in a whitefly-free greenhouse in Brazil, except for plants exposed to viruliferous whiteflies, which were in a separate growth chamber or greenhouse. Results were recorded 17 days after inoculation.

Light and electron microscopy of bean leaves. Leaves were prepared for staining in azure-A, which stains nucleic acids, by removing the cuticle and epidermal tissues by gently rubbing leaves with 600-mesh sandpaper (5). Areas of abraded leaves (3–5 mm^2) were excised and placed in 2-methoxyethanol for 15–30 min to dissolve chlorophyll and then placed in 0.1% azure-A in 0.01 M Na_2HPO_4 for 15–30 min. The tissue pieces were destained in 95% ethanol for 5–15 min, followed by 2-methoxyethyl acetate for 15–30 min, blotted dry, placed on a drop of Euparol (Carolina Biological Supply, Burlington, NC) on a glass slide, and covered with a coverslip. Stained tissue pieces were viewed with a light microscope at various magnifications.

Bean leaves infected with the virus, which had abundant viral inclusion bodies, and uninfected leaves were selected for examination by electron microscopy. Tissue pieces (3 mm^2) were fixed in 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4, and postfixed in 2% osmium tetroxide in phosphate buffer (9). The fixed tissue was dehydrated in a graded series of acetone to 70%, then treated with a saturated solution of uranyl acetate in 70% acetone for 24 hr at 4 C. Dehydration was continued in 100% acetone and tissues were infiltrated, under vacuum, with epoxy resin. Embedded tissue was sectioned on a Reichert OMU3 ultramicrotome, stained with lead citrate, and examined in a JEM 7 electron microscope.

Detection of geminiviral nucleic acids in leaves by squash and dot blot hybridization. Leaf disks were excised with a flame-sterilized No. 4 cork borer. For the squash blot procedure, leaf disks were squashed on Zetabind nylon membrane (CUNO, Inc., Meriden, CT) with a flame-sterilized round-bottomed glass rod. Two dot blot procedures were used, one in which one or three leaf disks were ground in 0.5 ml of distilled water or TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) in 1.5-ml Eppendorf tubes with a sterilized plastic pestle, and 10-μl samples were spotted onto Zetabind. The other procedure involved grinding a single leaf disk in 50 μl of TE, making twofold serial dilutions of the resulting plant sap in microtiter plates (Becton Dickinson Co., Lincoln Park, NJ), and spotting 5-μl samples of each dilution on Zetabind (dilution method). Similar dilution series were prepared from sap of mock-inoculated plants and from a solution containing 2 ng/μl of cloned BGMV-GA components A and B, and 5-μl samples were spotted on Zetabind.

Membranes were placed on filter paper, which had been saturated with 0.2 N NaOH, for 5 min to lyse cells and denature DNA, and then washed for 5 min in 1 M Tris, pH 7.5, then 2× SSC (23), followed by 95% ethanol. Membranes were air-dried and stored or used immediately for hybridization. Prehybridization (10 min) and hybridization (16–24 hr) were carried out in a solution of 30 (low stringency) to 50% (high stringency) formamide-0.125 M Na_2HPO_4-7% SDS-1 mM EDTA (pH 8.0) at 42 C. Membranes were washed twice at 42 (low stringency) or 65 C (high stringency) for 20 min with 0.125 M Na_2HPO_4-2% SDS-1 mM EDTA (pH 8.0), and once at the same temperature for 20 min in 20 mM Na_2HPO_4-1% SDS-1 mM EDTA (pH 8.0) (1). Membranes were blotted dry and exposed at −70 C to Kodak X-Omat AR or OG-1 X-ray film with Cronex Lightning Plus or Lanex intensifying screens, respectively. Exposure time varied from 4 to 24 hr.

DNA probes. Full-length DNA clones of BGMV-BZ, BGMV-GA, BGMV-DR, and BDMV-CO components A and B (15,16; R. L. Gilbertson et al, *unpublished data*) were used as DNA probes. Probe DNA was radioisotope-labeled with [^{32}P]-dATP by nick translation (23) with kits from Bethesda Research Laboratories (Gaithersburg, MD) or Promega BioTech (Madison, WI).

RESULTS

Sap transmission. Three separate experiments were conducted in Brazil to determine if BGMV-BZ could be sap-transmitted. Inoculum was collected from beans with golden mosaic symptoms in the field at CNPAF (field inoculum) or from beans infected with

Table 1. Results of experiments on the sap transmission of a Brazilian isolate of bean golden mosaic (BGMV-BZ) geminivirus

Treatment	Plants with symptoms/total plants[a]	
	Greenhouse	Growth chamber
Field inoculum[b]	0/30	0/48
Greenhouse inoculum[c]	0/127	0/200
Control-buffer plus Carborundum	0/74	0/85
Control exposed to viruliferous whiteflies	60/60[d]	...

[a] Plants with any symptoms of viral infection were counted. Numbers represent totals of three separate experiments.

[b] Sap prepared from leaves collected from bean plants with golden mosaic symptoms in the field.

[c] Sap prepared from bean leaves infected with BGMV-BZ by whiteflies in the greenhouse and included separate inoculations with sap from leaves collected 10, 17, or 21 days after exposure to viruliferous whiteflies.

[d] All 60 plants had golden mosaic symptoms. Large nuclear inclusion bodies were observed in phloem parenchyma cells of leaves from 10 randomly selected plants. Strong hybridization signals were observed from squash and dot blots of leaves from 10 plants after being probed with ^{32}P-labeled BGMV-BZ DNA.

BGMV in a greenhouse at 10, 17, or 21 days after exposure to viruliferous whiteflies (greenhouse inoculum). We were unable to transmit BGMV via plant sap, regardless of the inoculum source, whereas beans exposed to viruliferous whiteflies developed golden mosaic symptoms 7–10 days after exposure (Table 1).

Light and electron microscopy of bean leaves having viral symptoms in Brazil. Trifoliolate leaves from beans with golden mosaic symptoms were collected from the field and greenhouse (plants infected via whiteflies) at three stages of growth: very young, just beginning to expand; one-half to three-quarter expanded and showing mild (vein clearing) or severe symptoms (brilliant golden mosaic); or fully expanded leaves with golden mosaic symptoms. Leaves were also collected from healthy plants.

After azure-A treatment, blue-stained nuclear inclusions were observed in phloem parenchyma cells of bean leaves with BGM (Fig. 1A) but not in healthy leaves. Inclusions were most readily observed in one-half to three-quarter expanded trifoliolate leaves that showed vein clearing and were just beginning to develop golden mosaic symptoms. These leaves were usually collected from plants at the V3–R3 stages of growth (plants with first trifoliolate leaves to plants just after flowering) (22). Inclusions were not readily observed in very young leaves (less than one-half expanded), because of difficulties in visualizing nuclei, or in older fully expanded leaves where there was a scarcity of inclusions and nuclei were collapsed. Other nuclear abnormalities were observed only in leaves with BGM symptoms such as hypertrophy of nuclei and nucleoli and nucleolar elongation and vacuolization (Fig. 1A). Ultrastructural studies of leaves with BGM revealed hypertrophy of nuclei and nucleoli, nucleolar segregation, and the presence of fibrillar rings in nuclei of phloem parenchyma cells (Fig. 1B). These ultrastructural changes were not observed in nuclei of phloem parenchyma cells of healthy leaves.

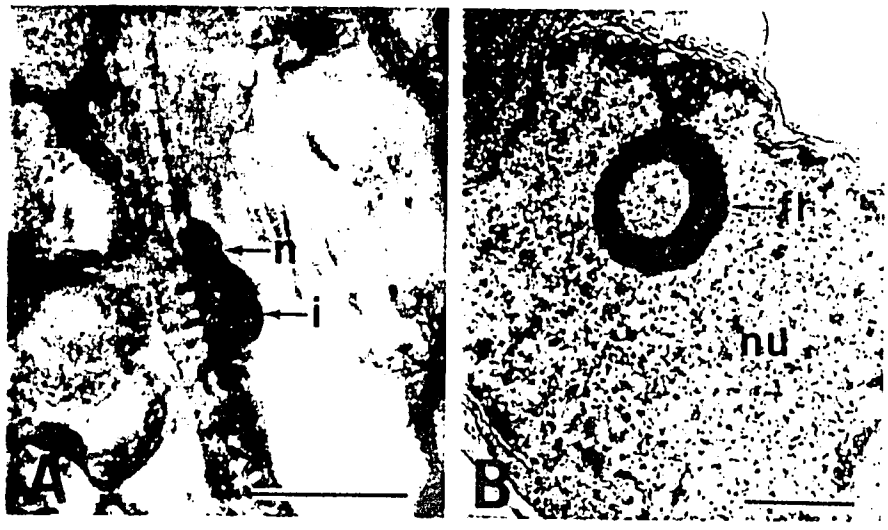

Fig. 1. (A) Light micrograph of infected bean leaf tissue stained with azure-A showing a nuclear inclusion body and nucleolar hypertrophy and vacuolization associated with infection by a Brazilian isolate of bean golden mosaic geminivirus. i = inclusion body, n = nucleolus. Bar = 10 μm. (B) Electron micrograph of a fibrillar ring observed in the nucleus of bean leaf tissue infected with a Brazilian isolate of bean golden mosaic geminivirus. fr = fibrillar ring, n = nucleus. Bar = 1 μm.

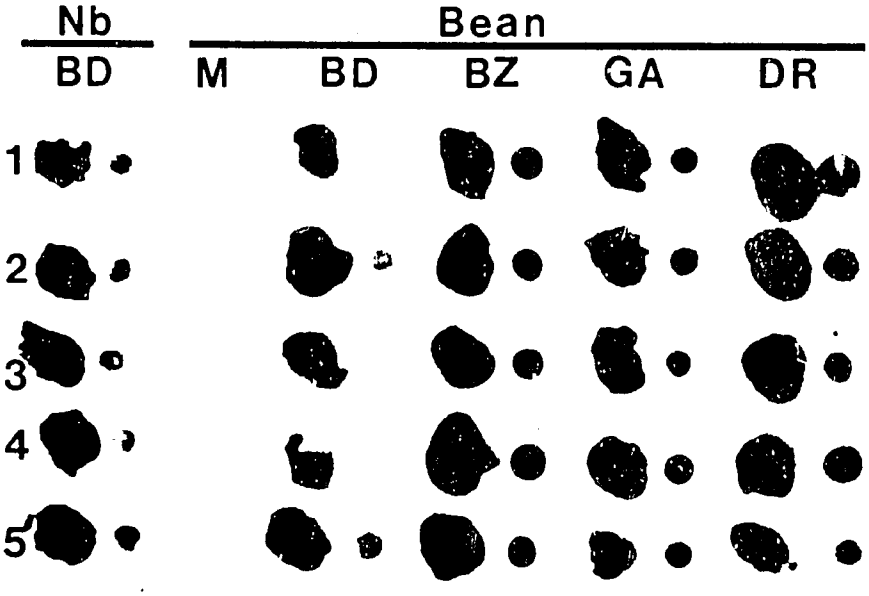

Fig. 2. Comparison of squash (samples on left in each column) and dot (samples on right in each column) blot hybridization for detection of bean-infecting geminiviruses in *Nicotiana benthamiana* (Nb) and bean (*Phaseolus vulgaris*) leaf tissue. Samples are mock-inoculated beans (M), plants infected with bean dwarf mosaic geminivirus (BD), or beans infected with a bean golden mosaic geminivirus (BGMV) isolate from Brazil (BZ), Guatemala (GA), or the Dominican Republic (DR). Samples were taken from each of five plants (1–5) for each treatment. Samples were hybridized with a general probe, which was a mixture of the cloned DNA components A and B of BGMV-BZ, BGMV-GA, and BDMV, using low-stringency conditions.

Sap transmission of BGMV-GA, BGMV-DR, and BDMV to beans and *N. benthamiana*. Bean (cv. Topcrop) and *N. benthamiana* plants were inoculated with BGMV-GA, BGMV-DR, and BDMV in Madison, WI. Five plants were inoculated with each isolate and the experiment was repeated three times. All of the bean plants inoculated with BGMV-GA and BGMV-DR developed golden mosaic symptoms and 14 of 15 bean plants inoculated with BDMV were dwarfed. *N. benthamiana* plants inoculated with BGMV-GA and BGMV-DR did not develop symptoms, whereas 13 of 15 plants inoculated with BDMV were stunted and leaves were cupped, distorted, and had mosaic symptoms. These results confirm the sap transmission of these isolates to beans (25,26) and indicate that BDMV can infect *N. benthamiana*.

Detection of geminiviral DNA in bean and *N. benthamiana* leaves by nucleic acid squash and/or dot blot hybridization with general and specific DNA probes. Dot and squash blots of leaves infected with BGMV-GA, BGMV-DR, BGMV-BZ, and BDMV were hybridized under low-stringency conditions with a general probe, which was a mixture containing cloned DNA components A and B of BGMV-BZ, BDMV, and BGMV-GA or BGMV-DR, or hybridized under high-stringency conditions with specific probes, which were the individual component B clones of these virus isolates. For BGMV-BZ, leaves from beans infected via whiteflies were collected at CNPAF, frozen or dried, and brought to Madison. Subsequently, we successfully infected beans with a mixture of the cloned DNA components A and B of BGMV-BZ (14); and in some cases, leaves from these infected plants were used for detection of BGMV-BZ. Before preparation of dot and squash blots,

dried leaf samples were rehydrated with sterile distilled water for 10–15 min, whereas frozen leaf samples were thawed for 1–5 min.

With the general probe, geminiviral nucleic acids were detected in squash and dot blots of bean leaves infected with BGMV-BZ, BGMV-GA, BGMV-DR, and BDMV and in *N. benthamiana* plants inoculated with BDMV (Fig. 2), but not in mock-inoculated plants (Fig. 2) or *N. benthamiana* inoculated with BGMV-GA or BGMV-DR (*data not shown*). When dot and squash blots of these infected leaves were probed with the specific probes, the BGMV-BZ component B probe hybridized only with tissue infected with BGMV-BZ (Fig. 3), the BDMV component B probe hybridized only with tissue infected with BDMV, and the BGMV-GA and BGMV-DR component B probes each hybridized only with tissue infected with BGMV-GA-and BGMV-DR (*data not shown*). For the general and specific probes, signals from infected tissues could be visualized after 4 hr of exposure, with very strong signals observed after 12–16 hr. These experiments were repeated three times, with similar results obtained for each experiment.

We now routinely use squash blot hybridization to confirm results of sap-transmission experiments, results of experiments involving inoculation of plants with cloned geminiviral DNAs, and to detect geminiviral nucleic acids in bean samples from Central America, South America, and the Caribbean. In all cases (more than 300 samples have been tested), hybridization signals have been observed in squash blots from beans with typical golden mosaic symptoms (no false negatives), and no signals have been observed in squash blots from beans without symptoms (no false positives).

The stability of squash and dot blotted samples on membranes before lysis was evaluated by preparing membranes with squash and dot blot samples from uninfected bean trifoliolate leaves and leaves infected with BGMV-GA. One membrane was exposed to lysis buffer immediately, one after a 2-wk storage, and the other after a 2-mo storage at 22 C. Membranes were probed with BGMV-GA component A 24–48 hr after exposure to lysis buffer. This experiment was conducted twice. We observed no reduction in hybridization signal 2 mo after samples had been applied to membranes. After exposure to lysis buffer, geminiviral nucleic acids can be detected on membranes for at least 1 yr and probably longer.

To determine if we could detect differences in viral nucleic acid titer in bean leaves by dot blot hybridization, beans (cv. Topcrop) were inoculated with BGMV-GA or BDMV on three separate dates (2–3 wk apart) so that infected trifoliolate leaves at different stages of development could be sampled. Infected leaves were collected from plants at three stages of development: V2 growth stage (22), young, partially expanded (7–10 days after inoculation); R1 growth stage, medium-aged, fully expanded (21–28 days after inoculation); and R6 growth stage, old, fully expanded, and beginning to senesce (49–56 days after inoculation). In a separate experiment, young, medium-aged, and old trifoliolate leaves infected with BGMV-GA or BDMV were collected from each of three bean plants 42 days after inoculation (R2 growth stage). Leaf disks were cut from leaves, dot blotted (dilution method), and membranes were probed with BGMV-GA or BDMV DNA components A and B under high-stringency conditions. These experiments were repeated three times. For both BGMV-GA and BDMV, the strongest hybridization signals were always observed from young partially expanded trifoliolate leaves, regardless of whether the leaves were collected from plants 10 or 42 days after inoculation. Less intense signals were observed from fully expanded trifoliolates, and the weakest signals were from older fully expanded trifoliolates that were beginning to senesce (*data not shown*).

Dot blot hybridization was also used

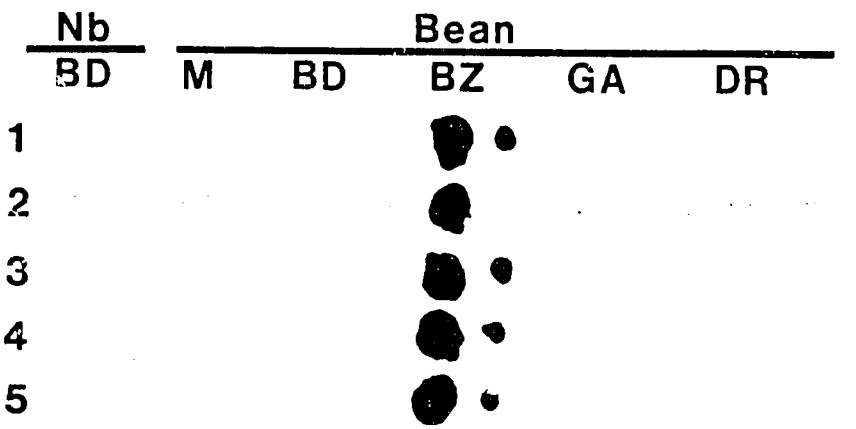

Fig. 3. Specific detection of a bean golden mosaic geminiviral isolate from Brazil (BGMV-BZ) in infected bean leaves by nucleic acid squash (samples on left) and dot blot (samples on right) hybridization. Samples are mock-inoculated leaves (M), *Nicotiana benthamiana* (Nb), or beans (*Phaseolus vulgaris*) infected with bean dwarf mosaic geminivirus (BD), or beans infected with BGMV-BZ or BGMV isolates from Guatemala (GA) or the Dominican Republic (DR). Samples were taken from each of five plants (1–5) for each treatment and were hybridized with the cloned DNA component B of BGMV-BZ using high-stringency conditions.

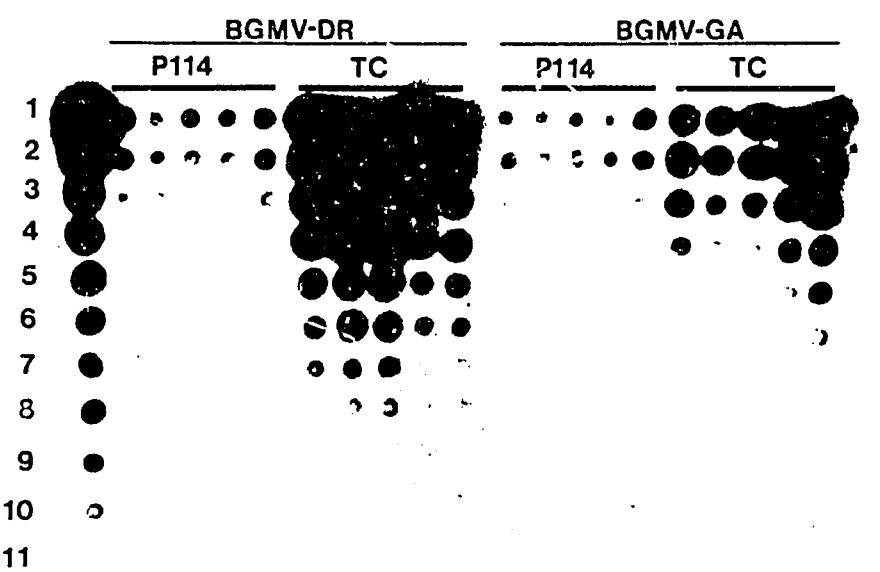

Fig. 4. Detection of differences in bean golden mosaic geminiviral nucleic acid titer in a moderately resistant bean cultivar, Pinto 114 (P114), and a susceptible cultivar, Topcrop (TC), by nucleic acid dot blot hybridization. Samples were prepared from infected first trifoliolate leaves from each of five individual plants of each cultivar inoculated with a BGMV isolate from Guatemala (BGMV-GA) or the Dominican Republic (BGMV-DR). Column one contains twofold dilutions (1–11) of cloned DNA components A and B of BGMV-GA (10 ng, 5 ng, 2.5 ng, 1.3 ng, 625 pg, 313 pg, 156 pg, 78 pg, 39 pg, 20 pg, 10 pg). Samples were hybridized with the cloned DNA components A and B of BGMV-GA using high-stringency conditions.

to detect differences in BGMV-GA and BGMV-DR nucleic acid titers in a susceptible cultivar (Topcrop) and in two cultivars that are moderately resistant or tolerant (HMX 7958 and Pinto 114). HMX 7958 is an experimental cultivar that has been reported to be moderately resistant (reduced symptoms) to BGM in Mexico and was kindly provided by the Harris Moran Seed Co. (San Juan Bautista, CA). Pinto 114 was reported to develop only moderate golden mosaic symptoms under field conditions, and when sap-inoculated with a BGMV-GA isolate, some plants did not develop symptoms (25). These cultivars were planted in a controlled environment chamber, and 7 days later, when beans had one-half expanded primary leaves, plants were inoculated with BGMV-GA or BGMV-DR (only Pinto 114). Ten to 14 days later, the first trifoliolate leaves with symptoms were collected from five infected plants of each cultivar and disks cut from these leaves were analyzed by dot blotting (dilution method). Membranes were probed with BGMV-GA components A and B under high-stringency conditions. The experiment with HMX 7958 was repeated three times, and the experiment with Pinto 114 was conducted twice. HMX 7958 plants developed mild golden mosaic symptoms after inoculation with BGMV-GA but not the brilliant golden mosaic, leaf distortion, and poor pod set that was observed with susceptible cv. Topcrop. Similarly, Pinto 114 developed golden mosaic symptoms after inoculation with BGMV-GA or BGMV-DR that were less severe than those observed on Topcrop. Greater hybridization signals were observed from dot blots of the susceptible Topcrop than from cvs. HMX 7958 (*data not shown*) or Pinto 114 (Fig. 4). Stronger hybridization signals also were observed from Topcrop plants infected with BGMV-DR than from those infected with BGMV-GA (Fig. 4). By making serial dilutions of plant sap and dotting samples of each dilution, the differences in viral nucleic acid titer could be clearly seen; in both HMX 7958 and Pinto 114, viral nucleic acids were not detected after two to three dilutions, whereas viral nucleic acids were detected in Topcrop after five to eight dilutions. These results clearly demonstrate that, at least for the infected first trifoliolate leaves, HMX 7958 and Pinto 114 had lower viral nucleic acid titers than Topcrop.

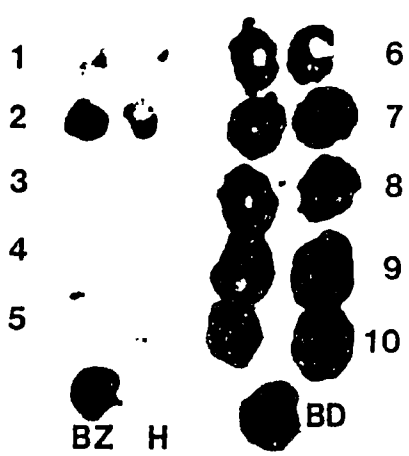

Fig. 5. Detection of geminiviral nucleic acids in bean and weed samples collected in the Dominican Republic by nucleic acid squash blot hybridization. Samples are (**1,2**, *Rhynchosia minima* with golden mosaic symptoms, (**3,4**) *Sida* spp. with golden mosaic symptoms, (**5**) *Jatropha* spp. with golden mosaic symptoms, and (**6–10**) beans with golden mosaic symptoms. H = bean plant with no symptoms, BZ = bean plant infected with a bean golden mosaic geminiviral isolate (BGMV) from Brazil, BD = bean plant infected with a bean dwarf mosaic geminiviral (BDMV) isolate from Colombia. Samples 1–10 were applied as duplicate samples. Samples were hybridized with a mixture of cloned DNA components A and B of BGMV isolates from Brazil and the Dominican Republic and BDMV.

Survey of beans and weeds with golden mosaic symptoms in the Dominican Republic. A trip was taken to the Dominican Republic in February 1990 to prepare squash blots of beans and weeds with golden mosaic symptoms for hybridization with the general or specific geminiviral DNA probes. Beans with golden mosaic, stunting, leaf distortion, and/or green mosaic symptoms; various crops with or without viral symptoms; or weeds with or without golden mosaic symptoms were collected from 24 locations in the Central and Western Dominican Republic. Each membrane had a sample from a bean plant with no symptoms (negative control) and a bean plant with typical BGM symptoms (positive control). The samples were squash blotted on four replicate membranes for hybridization with the general and specific probes. Before samples were lysed and hybridized in Madison, WI, samples from plants infected with BDMV and BGMV-BZ were squash blotted onto each of the replicate membranes to provide positive controls for the specific probes.

When the general probe was used, hybridization signals were not observed from crop samples (tomato, cassava, green pepper, or tobacco), weeds without golden mosaic symptoms, or beans without symptoms (Fig. 5); hybridization signals were observed from all beans with golden mosaic symptoms (Fig. 5), six of seven beans with stunting and leaf distortion, and one of four beans with green mosaic (similar to bean common mosaic symptoms). Weeds with golden mosaic symptoms that gave weak to strong hybridization signals with the general probe included: *Croton lobatus* L., *Jatropha* spp. (Fig. 5), *Sida* spp., *Urena lobata* L., *Bastardia bivalvis* (Cav.) Kunth., *Rhynchosia minima* (L.) Dc. (Fig. 5), and *Euphorbia heterophylla* L. No hybridization signals were observed from five of nine *Sida* spp. (see Fig. 5 for an example of *Sida* spp. without signal) and two of four *E. heterophylla* plants with golden mosaic symptoms.

With the BGMV-DR-specific probe at high-stringency conditions, hybridization signals were observed from beans with golden mosaic symptoms from all locations, whereas no signals were observed with the BDMV or BGMV-BZ specific probes (*data not shown*). Three bean plants with stunting and/or crinkling symptoms that hybridized with the general probe also hybridized with the specific probes; two of these did not hybridize with any of the specific probes, whereas one hybridized with the BDMV-specific probe. The only weed that hybridized with the specific probes was *R. minima*, which gave strong hybridization signals with all three specific probes.

DISCUSSION

Whitefly transmission, symptomatology, and light and electron microscopy were used to confirm that a geminivirus (BGMV-BZ) was associated with bean golden mosaic at CNPAF. In contrast to BGMV isolates from Central America and the Caribbean, BGMV-BZ could not be sap-transmitted to beans despite the use of a procedure successfully used for transmission of BGMV isolates from Florida and Puerto Rico (E. Hiebert, *personal communication*). Moreover, BGMV-GA, BGMV-DR, and BDMV-CO were easily transmitted with sap prepared by simply grinding infected leaves in 0.1 M potassium phosphate buffer. The apparent lack of sap transmissibility of BGMV-BZ is consistent with results of other researchers (7,11). Attempts to sap transmit BGMV from Argentina have also been unsuccessful (F. Morales, *unpublished data*). These results indicate that isolates of BGMV from Brazil and those from Central America and the Caribbean may be genetically different.

Thus, there is a need to determine the distribution of various bean-infecting geminiviruses throughout Latin America and to develop techniques for their rapid identification in beans and other plants, particularly weeds. Detection of inclusion bodies by light microscopy confirmed that a geminivirus was associated with BGM in Brazil, and the cytopathology of these leaves was similar to that for other geminiviral infections (6,12,20). However, using viral inclusion bodies for diagnosis is time consuming, technically difficult, and is generally diagnostic only for a viral group and not for virus strains (5,6).

We have demonstrated that DNA probes can rapidly detect and differentiate geminiviruses that infect bean. Squash blot hybridization with cloned geminiviral DNA was a rapid, efficient,

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and reliable method for detecting all four geminiviral isolates used in this study. For example, we used squash blots to confirm that BDMV infected *N. benthamiana* and that BGMV-GA and BGMV-DR did not infect this host.

By using excised leaf disks, many samples can be squash blotted on a single membrane. The procedure is very flexible—fresh, frozen, or dried tissue can be used, and once tissues are squashed on membranes, hybridizations can be done months later. Thus, samples can be squashed at different locations and times, and lysis and hybridizations can be conducted at a later date at a central location. Similar results were reported when squash blot hybridization was used to detect tomato yellow leaf curl geminivirus (27).

The dot blot method was used to detect geminiviral nucleic acids in plant tissues and differences in geminiviral nucleic acid titers. The highest geminiviral nucleic acid titers were always detected in young expanding trifoliolate leaves. This observation agrees with results of an earlier study using physical measurements of geminiviral concentrations (31). Thus, young trifoliolate leaves should be collected for detection of geminiviral nucleic acids rather than fully expanded leaves.

It was also possible to detect differences in viral nucleic acid titers among bean cultivars with dot blot hybridization. It has been suggested that resistant cultivars may have lower viral titers than susceptible cultivars (3,13), and the dot blot assay showed that the viral nucleic acid titers in two moderately resistant cultivars (HMX 7958 and Pinto 114) were considerably lower than the titer in the susceptible cv. Topcrop. These results indicate that some viral function (e.g., replication or spread) may be inhibited in HMX 7958 and Pinto 114, and they support previous observations that these cultivars are moderately resistant to BGMV (S. Magneson, *personal communication*, 25). Dot blot hybridization can provide only an initial indication as to whether a cultivar may be resistant, and field evaluations must follow.

By using individual DNA components of these geminiviruses or mixtures of these components and varying the hybridization stringency, we developed a general probe that detected all four geminiviruses and specific probes that detected either BGMV-BZ, BDMV, or BGMV-GA and BGMV-DR. General and specific DNA probes have been used for detection and differentiation of barley yellow dwarf luteovirus serotypes (34). Polston et al (29) used a mixture of cloned DNA components as a general probe to detect two strains of squash leaf curl geminivirus and unique restriction fragments of the components of these strains as specific probes to differentiate the strains. For the general probe used in this study, a mixture of DNA-A and DNA-B components of BGMV-BZ, BDMV, and BGMV-GA or BGMV-DR was used under low-stringency conditions. The DNA-A component was included because of the presence of the highly conserved coat protein gene of the geminiviruses transmitted by whiteflies (27). This general probe should detect other geminiviruses transmitted by whiteflies. For example, this general probe detected geminiviral nucleic acids in tomatoes from Florida that showed symptoms of a geminivirus infection in November 1989 (R. L. Gilbertson et al, *unpublished data*). Conversely, because of the significant level of DNA sequence divergence that exists among the DNA-B components of BGMV-BZ, BDMV, and the closely related geminivirus isolates, BGMV-GA and BGMV-DR (15,16, R. L. Gilbertson et al, *unpublished data*), the cloned DNA-B components of these isolates can be used as specific DNA probes when used under high-stringency conditions.

We used the squash blot hybridization method and the general and specific probes to conduct a survey of potential weed hosts of BGMV and to evaluate the genetic diversity among BGMV isolates in the Dominican Republic. Our results clearly showed that the predominant BGMV isolate in the Dominican Rebublic was similar to BGMV-DR and that viruses related to BGMV-BZ and BDMV were not present in beans with golden mosaic symptoms. Thus, BGMV-DR could be used for screening bean lines for BGM resistance in the Dominican Republic. One stunted bean with crinkled leaves was possibly infected with BDMV or a related geminivirus, whereas two other plants with these symptoms were infected with a different and unknown geminivirus. Because beans with these symptoms were infrequently encountered, these other geminiviruses do not appear to be important in the Dominican Republic. Although numerous weeds had golden mosaic symptoms and geminiviral nucleic acids were detected in some of these, it appears that most of these weeds were not infected with BGMV-DR, BGMV-GA, BDMV, or BGMV-BZ, because they hybridized only with the general probe. The leguminous weed *R. minima*, which hybridized with all three specific probes, may be infected with more than one geminivirus or with a geminivirus that is closely related to those used in this study.

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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.

Germiniviruses 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

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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.*, 1991*a*). 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.*, 1991*a*). 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.*, 1991*a*), 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.*, 1991*b*). We propose to use the

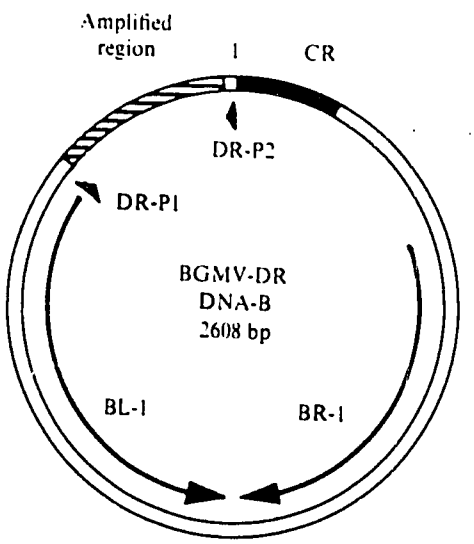

Fig. 1. Diagrammatic representation of the genomic organization of the DNA-B component of BGMV-DR showing the positions of the common region (CR), the two open reading frames (BR-1 and BL-1) and the region amplified for evaluation of genetic variability of BGMV-DR isolates [for the purposes of this figure, the circularized DNA-B insert of pDRB1 (Gilbertson *et al.*, 1991*b*) 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.

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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.*, 1991*a*) in San Juan de la Maguana, Dominican Republic in 1987. The isolate was passaged 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.*, 1991*b*). The recombinant plasmid pDRB1 contains the full-length BGMV-DR DNA-B cloned at a unique *Hin*dIII site (Gilbertson *et al.*, 1991*b*).

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 mm^2 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 issue 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

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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.*, 1991*a*), 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 hypervariable region (BGMV-PR was not tested). Thus, these primers can be used to identify BGMV-DR specifically.

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            1
DR-1        TCATTTGtTG ACACTCGCAT ACAACTGATA AACTGAcCAA CCAAGAGATT
DR-2        TCATTTGGTG ACACTCGCAT ACAACTGATA AACaaAcCAA CCAAGAGATT
DR-3        TCATTTGGTG ACACTCGCAT ACAACTGATA AACTGAcCAA CCAAGAGATT
DR-4        TCATTTGGTG ACACTCGCAT ACAACTGATA AACTGAcCAA CCAAGAGATT
DR-5       ATCA-TTatTG ACACTgGCAT ACAACTGATA AACaGAcCAA CCAAGAGATT
pDRB-PCR    TCATTTGGTG ACACTCGCAT ACAACTGATA AACTGAACAA CCAAGAGATT
pDRB1       TCATTTGGTG ACACTCGCAT ACAACTGATA AACTGAACAA CCAAGAGATT
              *  **         *                          **  *

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DR-1        ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
DR-2        ATGAaATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
DR-3        ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
DR-4        ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
DR-5        ATGAAATAGT TTATATTGAT ATAGtCAAGC ATTGTGTATG CTTATATAGG
pDRB-PCR    ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
pDRB1       ATGAAATAGT TTATATTGAT ATAGACAAGC ATTGTGTATG CTTATATAGG
                                            *

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DR-1        CAGTTGTATG CCTTTATATC GCCTTTTGtA AAAGAACATA TTATTTAGTT
DR-2        CAGTTGTATG CCTTTATATC GCCTTTTGAA AAAGAACATA TTATTTAGTT
DR-3        CAGTTGTATG CCTTTATATC GCtTTTTGAA AAAaAACATA TTATTTAGTT
DR-4        CAGTTGTATG CCTTTATATC GCCTTTTGtA AAAGAACATA TTATTTAGTT
DR-5        CAGTTGTATG CCTTTATATC GCCTTTTGAA AAAGAACATA TTATTTAaTT
pDRB-PCR    CAGTTGTATG CCTTTATATC GCCTTTTGAA AAAGAACATA TTATTTAGTT
pDRB1       CAGTTGTATG CCTTTATATC GCCTTTTGAA AAAGAACATA TTATTTAGTT
                                     *     *     *           *

            150
DR-1        AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC GGATATATAT
DR-2        AATATGTTAA TGTGTTaTAT TTGAACATGA TATATATATC GGATATATAT
DR-3        AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC GGATATATAT
DR-4        AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC gGATATATAT
DR-5        AATATGTTAt TGTcTTTTAT TTGAACATGA TATATATATC GGATATATAT
pDRB-PCR    AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC GGATATATAT
pDRB1       AATATGTTAA TGTGTTTTAT TTGAACATGA TATATATATC GGATATATAT
                     *     *  *                                *

            200
DR-1        ATT  (98%)
DR-2        ATT  (98%)
DR-3        ATT  (98%)
DR-4        ATa  (98%)
DR-5        ATT  (95%)
pDRB-PCR    ATT (100%)
pDRB1       ATT
            *
```

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.

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

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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 lymphotrophic 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 & Sänger, 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 & Sänger, 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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(Prepared by F. J. Morales)

Bean Golden Mosaic

Bean golden mosaic, or bean golden yellow mosaic, caused by bean golden mosaic virus (BGMV), occurs in most tropical and subtropical areas of the New World where beans are grown. Disease incidence has been increasing in the Dominican Republic, Puerto Rico, and Central America, where crops such as tomato and potato have been grown near beans. The disease was first reported from Brazil in 1965. A similar golden mosaic disease of lima bean was reported in 1948 from India, but it is not known if the two diseases are identical. If infection occurs at a very early stage, yield losses may be as high as 100% because flowers fail to develop. The extent of losses in the field is related to the timing and efficiency of whitefly transmission. If infection occurs within 3 weeks of planting, losses may be 50% or higher. Losses from infection 5 weeks after planting may be less than 25%.

Symptoms

Symptoms of BGMV infection of bean seedlings begin with fine, vein-limited lines of bright yellow chlorosis, usually on the first trifoliolate leaves emerging after inoculation. These fine chlorotic lines typically appear first on only half of the leaf and near the leaf tips. Within 3-5 days, veinal chlorosis spreads to cover one third or more of the leaf area, giving a characteristic netlike appearance, with bright yellow veins contrasting with dark green interveinal areas (Plate 121). Veinal chlorosis later expands into a bright golden mosaic that appears striking in the field (Plate 122). Younger trifoliolate leaves emerging after the first leaves develop symptoms frequently become curled downwards within a week after the initial appearance of reticulate chlorosis. Curling leaves fail to expand properly, and their surfaces become stiff and leathery (Plate 123). Leaves with chlorosis and distortion may become necrotic. Seed setting is very poor if plants are infected at a very early stage.

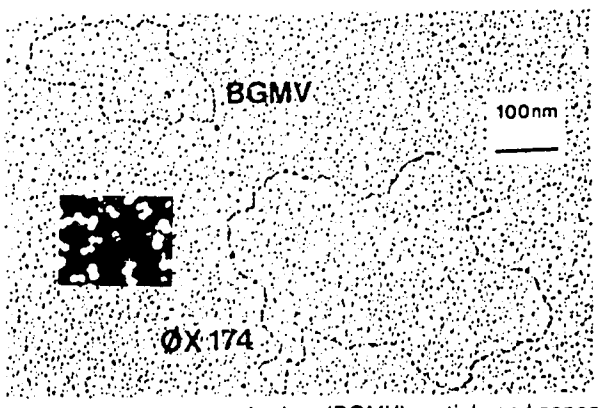

Fig. 23. Bean golden mosaic virus (BGMV) particle and genome. Inset electron micrograph shows BGMV particles negatively stained with phosphotungstate. Circular, single-stranded DNA molecules from BGMV and bacteriophage OX 174 are at the same scale as the virus particles. (Courtesy S. Haber)

All of more than 4,000 lines of common bean germ plasm tested were susceptible to BGMV infection. No bean varieties or lines of related legume species respond consistently to BGMV infection with local lesions. Resistance has been found only in *Phaseolus* spp. of Asiatic origin that do not cross with common bean. There are minor differences in symptomatology among cultivars, but Top Crop is the cultivar that has been used in most studies involving mechanical inoculation of BGMV to beans.

BGMV has a narrow host range. The virus infects *Calopogonium mucunoides* Desv., *Macroptilium lathyroides* (L.) Urb., *M. longepedunculatum* (Benth.) Urb., *Phaseolus lunatus* L., *P. vulgaris* L., and *Vigna unguiculata* (L.) Walp. but not *Cajanus cajun* (L.) Huth, *Cassia occidentalis* L., or *Rhynchosia minima* (L.) DC. The malvaceous weed *Malvastrum coromandelianum* (L.) Garcke may be a host for BGMV.

Causal Agent

BGMV is a member of the whitefly-transmitted subgroup of the geminiviruses. Geminiviruses are named for their unique twin-icosahedral (18 × 30 nm) particle morphology. They are also the only plant viruses with genomes of circular, single-stranded (ss) DNA. Each geminate particle encapsidates only one ssDNA molecule (Fig. 23). Among whitefly- and leafhopper-transmitted geminiviruses that have been characterized to date, members of the former subgroup have genomes consisting of two different DNA molecules of about 2,600 nucleotides, whereas genomes of members of the latter subgroup contain only one DNA molecule of 2,600-2,700 nucleotides.

Mechanical transmissibility of BGMV isolates depends on their geographic origin. Isolates from Central America, the Caribbean, and Colombia can be mechanically transmitted, whereas isolates from Argentina and Brazil cannot. BGMV is not transmitted through seed and has never been recovered from seeds harvested from infected plants.

Most studies on the physicochemical properties and molecular biology of BGMV have been conducted on an isolate originally obtained from *Macroptilium lathyroides* in Puerto Rico. A Venezuelan isolate of BGMV behaves similarly to the type Puerto Rican isolate in host range, transmission, symptomatology, and cytopathology, and its DNA is more than 90% homologous with DNA from the Puerto Rican isolate. However, the two isolates have different sets of hybridizing restriction fragments of DNA, indicating that the isolates are closely related but distinct variants. Analysis of the complete DNA sequences of BGMV isolates from Brazil, Guatemala, and the Dominican Republic also indicates genetic diversity among geminiviruses causing bean golden mosaic.

Disease Cycle and Epidemiology

Bean golden mosaic virus is transmitted by the aleyrodid whitefly *Bemisia tabaci* (Plate 124). Adult *B. tabaci* may acquire the virus in as little as 6 min from infected plants, but efficient transmission requires longer feeding periods. Whiteflies can transmit the virus for periods ranging from a few days to several weeks and probably through the last molt. Transmission efficiency of an individual insect is often intermittent and erratic. Male and female whiteflies transmit with equal efficiency. No evidence has been found that BGMV can be transmitted through the ovaries.

Bean golden mosaic is a serious constraint to bean production in areas where the seedling stage of the plant coincides with the presence of large numbers of the vector. High temperatures (higher than 25°C) hasten development of *B. tabaci*, leading to higher vector population densities. Disease spread also depends on vector migration and suitable reservoirs

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of inoculum. Spread of bean golden mosaic in southern Brazil, for example, is greater in crops planted in the cooler, dry season than in those planted in the warmer, wet season, possibly because of mass migration of whiteflies from soybean and other summer crops maturing at the end of the wet season. Natural enemies of *B. tabaci* are also more prevalent in the wet season.

After inoculation of expanding, unifoliolate primary leaves (9-12 days after seeding) of plants of the cultivar Top Crop maintained at 24-30°C, symptoms appear 5-6 days later on the first trifoliolate leaves. Usually, no symptoms are seen on the inoculated primary leaves, although yellow spots may develop occasionally. Virus is limited to phloem and phloem-associated cells 6 days after inoculation but spreads to mesophyll cells by 20 days after inoculation. The titer of infectious BGMV in leaf tissue peaks 10-12 days after inoculation and declines over the next 10 days. Only tissue collected at the peak of titer can be used with consistent success as inoculum for sap transmission of BGMV. Although symptoms continue to appear on subsequently emerging trifoliolate leaves and are most striking on those that develop 3-6 weeks after inoculation, leaf tissue collected later than 10-14 days after inoculation is not consistently infective.

Symptom development depends on temperature. Foliar symptoms appear earlier and are more pronounced at 30°C than at 25°C in bean seedlings inoculated mechanically. Similarly, in bean seedlings inoculated by the whitefly vector, symptoms appear 1 week after inoculation when plants are held at 27°C or above and 2-3 weeks after inoculation when maintained at 21-24°C. Races of the whitefly vector may be distinguished on the basis of their ability to transmit agents of different golden yellow mosaic or rugose mosaic diseases. Race *jatrophae* transmits the agent of Jatropha mosaic, and race *sidae* transmits BGMV and the geminivirus agents of Rhynchosia mosaic, Euphorbia mosaic, and Abutilon mosaic.

Control

Planting time should be adjusted so that the young seedling stage will not coincide with periods of high whitefly populations. Weed reservoirs of inoculum, such as *M. lathyroides*, should be controlled. Measures to control whitefly vectors have met with varying success. Insecticidal treatments have generally not proved feasible, but early treatment with a systemic insecticide followed by foliar sprays for the first 35 days can reduce the incidence of whitefly and BGMV. Use of colored mulches to reduce whitefly populations appears promising. Moderately resistant cultivars such as ICTA-Quetzal and ICTA-Jutiapan are available in black-seeded beans adapted to Central America. The breeding line LM 30630 from Brazil shows great promise of moderate resistance to the Brazilian isolate of BGMV.

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(Prepared by S. Haber, D. P. Maxwell, and R. L. Gilbertson)

Bean Mild Mosaic

Bean mild mosaic is caused by bean mild mosaic virus (BMMV), a highly contagious virus that infects many varieties of dry and snap beans. It has been isolated from bean plants in El Salvador and Colombia. Its occurrence in other countries is not known. It causes mild or no symptoms in beans by itself but in mixed infections with other viruses acts synergistically to increase symptom severity. The virus was originally discovered in El Salvador mixed with bean curly dwarf mosaic virus in bean plants that were showing severe disease symptoms. Its effects on yield are not known.

Symptoms

BMMV infects several legumes, including common bean, scarlet runner bean, soybean, tepary bean, *Canavalia ensiformis* (L.) DC., *C. gladiata* (Jacq.) DC., *Lablab purpureus* (L.) Sweet, *Macroptilium atropurpureum* (Moc & Sessé ex DC.) Urb., *M. lathyroides* (L.) Urb., *Rhynchosia minima* (L.) DC., and *Sesbania exaltata* (Raf.) Cory. Infection has not been observed following inoculation of the legumes *Arachis hypogaea* L., *Cicer arietinum* L., *Crotalaria juncea* L., *Lathyrus sativus* L., *Lens culinaris* Medikus, *Phaseolus lunatus* L., *Pisum sativum* L., *Vicia faba* L., *Vigna radiata* (L.) R. Wilcz., and *V. unguiculata* (L.) Walp.

The nonlegumes *Chenopodium quinoa* Willd. and *Gomphrena globosa* L. are susceptible to the Central American isolate of BMMV but not to the Colombian isolate. No infection has been observed following inoculation of any of 40 other nonleguminous genera, including grasses, solanaceous crops, crucifers, cucurbits, and several annual ornamental crops.

In greenhouse experiments conducted in the United States, all of 25 snap and dry bean cultivars became infected with the virus, but reactions ranged from symptomless infection to barely discernible mild mosaic. The virus became fully systemic and sometimes incited slight veinbanding or roughening of the leaf surface (Fig. 24). Mechanically inoculated plants of some varieties developed chlorotic local lesions on primary leaves.

The virus incited a diagnostic mosaic leaf deformation and stunting in red tepary bean, hyacinth bean (*L. purpureus*), and in guar (*Cyamopsis tetragonoloba* L. Taubert). BMMV systemically infected other legume crops, including *C. ensiformis*, *M. lathyroides*, and *S. macrocarpa* Muhlenb., but incited no symptoms. Soybean cultivars Kanrich and Scott reacted with a mild mottle symptom.

Causal Agent

BMMV is easily purified. Particles are isometric, are 28 nm in diameter, and contain single-stranded RNA of molecular weight 1.27×10^6, which composes 20% of the particle weight. Particles sediment as a single component with an $s_{20,w}$ of about 127 S. In sap from Top Crop bean plants, the dilution end point is 10^{-8}, the thermal inactivation point is 84°C, and infectivity is retained for at least 6 weeks at 22°C.

This virus is serologically unrelated to 35 other viruses with spherical particles, including 10 usually associated with legumes and 11 other viruses with single sedimenting components. In symptomatology and host range, BMMV could be confused with bean pod mottle virus, even though they are serologically unrelated.

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CONSTRUCTION OF DNA PROBES FOR THE DETECTION OF BEAN DWARF MOSAIC GEMINIVIRUS

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Bean dwarf mosaic (BDM) was first described in Brazil as a minor disease of Phaseolus vulgaris L. The etiology of BDM has been investigated by various workers. In 1975, Costa suggested that BDM was caused by a strain of the Abutilon mosaic virus that adapted to beans (Costa, 1975). More recently, Morales et al. (1990) demonstrated that the virus is a member of the geminivirus group which is whitefly-transmitted and possesses a circular, single-stranded DNA genome. This disease is widely distributed in Latin America and is closely associated with the presence of the whitefly and malvaceous weeds, mainly Sida spp. Beans infected at the seedling stage are generally dwarfed and tend to become bunchy or rosseted (CIAT, 1987; Costa, 1975).

Geminiviruses occur mostly in phloem and phloem-associated parenchyma cells, and thus they are present in low-to-moderate concentrations. This can make detection of these viruses more difficult than non-phloem limited viruses. Besides that, the coat proteins of whitefly-transmitted geminiviruses share extensive amino acid similarities; and polyclonal antibodies do not distinguish between different geminiviruses (Roberts et al., 1984; Sequeira and Harrison, 1982). Since no serological or other methods were available to detect bean dwarf mosaic virus (BDMV), this study was initiated to develop viral DNA probes which could be used to identify BDMV.

The BDMV isolate was collected from infected beans at Cali, Colombia by F. J. Morales. The viral double-stranded DNA replicative form was cloned and recombinant plasmids were characterized. Restriction endonuclease analysis of the recombinant plasmids demonstrated two different restriction endonuclease patterns. Sequence comparison of the terminal portion of the insert DNAs of these plasmids to the sequence of bean golden mosaic virus (BGMV) isolate from Guatemala (BGMV-GA), also, indicated that two distinct classes of DNA, known as DNA-A and DNA-B, had been cloned. Since DNA sequence homologies are lowest among DNA-B's of whitefly-transmitted geminiviruses, a DNA-B clone was chosen for designing BDMV-isolate-specific probes.

A potential full-length clone of DNA-B, designated as pBDB21, was sequenced by the dideoxy nucleotide chain-termination method. Both strands of the clone were completely sequenced as well as another clone which showed that pBDB21 is missing 15 nt at the cloning site.

Sequence comparison between pBDB21 and BGMV-GA DNA-B was done for the non-coding regions and coding regions. Sequence homology between coding regions are 70% and 76% for BR1 and BL1, respectively. Non-coding regions gave comparisons of 50%, 70%, and 41% sequence homology for the nucleotides at positions 2257-2600, 1-200, and 201-548, respectively. This sequence similarity data indicate the presence of conserved DNA sequences as well as divergent sequences in DNA-B of BDMV. This character allows the design of isolate-specific DNA probes, which can be used for detection and identification of BDMV in beans and other plants. There is little information about genetic variation for BDMV. Since there are isolates of BDMV from

Argentina which are not mechanically transmissible (Morales, unpublished data), in contrast to the isolate from Colombia, it is expected that there will be some sequence differences between these two groups of BDMV. Thus, the strategy for developing BDMV-specific probes must address the issue of potential sequence variability. The specific probes were constructed by subcloning the BL1 region and the non-transcribed region between BL1 and BR1 including the Common Region to represent the highly conserved and the highly variable regions, respectively.

Geminiviral nucleic acids were detected in dot blots of bean leaves infected with BGMV-GA, BGMV isolate from Dominican Republic (BGMV-DR), BGMV isolate from Brazil (BGMV-BZ), and BDMV. Leaf disks from infected plants were macerated in TE buffer and spotted on membranes. Dilution series from these suspensions were prepared to test the sensitivity of the DNA probes. After lysis in sodium hydroxide and washing, membranes were probed under low stringency conditions with the general probe, a mixture containing cloned DNA components A and B of BGMV-GA, BGMV-BZ, and BDMV; or under high stringency conditions with the specific probes derived from full-length BDMV DNA-B, BL1 fragment, and non-coding fragment. With the general probe, hybridization signals were observed from all virus-infected bean samples. When the specific probes were used, only bean samples infected with BDMV hybridized to them. No significant hybridization differences were observed with the three different BDMV-specific probes, and each probe could detect at least 20 pg of viral DNA. To test the specificity of the specific probes, more samples are needed. When a given sample hybridizes with a probe derived from the highly conserved region of the DNA-B, this indicates that the virus is closely related to BDMV. When the other probe from the most divergent region of the DNA-B hybridizes with a given sample, this indicates that the sequence similarity is greater than 90% for the other parts of the viral genome, and it can be concluded that the given virus is an isolate of BDMV.

Geminiviral nucleic acid probes have been used to evaluate the distribution and incidence of various bean-infecting geminiviruses and to evaluate bean germplasm for its reaction to geminivirus infection.

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LACK OF SEED TRANSMISSION BY BEAN GOLDEN MOSAIC GEMINIVIRUS FROM THE DOMINICAN REPUBLIC AND PUERTO RICO

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Beans (Phaseolus vulgaris) are one of the principle sources of protein in developing countries including Dominican Republic in which the majority of the population have a low income. Bean golden mosaic geminivirus (BGMV) is economically important in commercially grown beans throughout the Caribbean, Central America, and South America. This virus is transmitted by the sweetpotato whitefly Bemisia tabaci.

Experiments have been conducted to determine if BGMV from Dominican Republic and Puerto Rico are seed transmission. Twenty three different germplasm lines of P. vulgaris from Puerto Rico and three from Dominican Republic were used. Seeds were collected from field grown plants showing typical BGMV symptoms. Six seeds from each germplasm line were planted. Topcrop, a susceptible bean cultivar, was inoculated with a BGMV isolate from the Dominican Republic, and non-inoculated Topcrop plants served as the control. All plants were grown in a plant growth chamber with 26 C for the 14 hr photoperiod and 21 C for the dark period. Typical bean golden mosaic symptoms develop on susceptible cultivars in about 10-14 days when plants are grown in this plant growth chamber.

Golden mosaic symptoms were recored at 15 and 21 days after planting. Inoculated Topcrop showed typical symptoms in 7 - 10 days and the non-inoculated Topcrop plants did not develop symptoms. No symptoms were observed on any of the plants from the seeds collected from infected beans in the Dominican Republic or Puerto Rico. It should be recognized that the seed numbers were very small and thus only a high rate of seed transmission would have been detected.

Our results agree with those of previous researchers (Costa, 1975; Pierre, 1975) who did not obtain seed transmission. Thus, insect transmission is the most important means of transmission in the field, and it is important to determine the natural reservoir of bean-infecting geminiviruses in the Dominican Republic and Puerto Rico.

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Molecular Characterization of Bean Calico Mosaic Geminivirus

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Whitefly-transmitted geminiviruses have a ssDNA genome generally divided into two circular components, DNA-A and DNA-B (Howarth et al., 1985). The two components have different nucleotide sequences with the exception of a 200 nucleotide region which is nearly identical between both components of each geminivirus and different among different geminiviruses. This region is designated the Common Region. Both components are necessary for infection for geminiviruses from the Western Hemisphere (Gilbertson et al., 1991). Geminiviruses are found throughout the Caribbean Basin, Central America, South America, Africa, the Middle East, and Asia. They are a serious constraint on vegetable production in those areas (Bock, 1982; Gálvez and Morales, 1989).

Bean calico mosaic geminivirus (BCMoV) was described by Brown et al. (1990) as a new whitefly-transmitted geminivirus from Sonora, Mexico. Brown observed a 60% occurrence of BCMoV in infected bean fields (Phaseolus vulgaris). Characteristic particles of the geminivirus were observed in infected bean plants (Brown et al., 1990).

The symptoms of BCMoV include bright yellow calico and mosaic patterns on the leaves and stunting of the beans. These symptoms are similar to those caused by bean golden mosaic geminivirus (BGMV). Thus, it is necessary to determine if BCMoV is a new virus, or if it is a strain of a previously discovered geminivirus, e.g., BGMV from Guatemala (Gilbertson et al., 1991).

The double-stranded replicative form of BCMoV was extracted from infected beans (Gilbertson et al., 1991) and used for cloning. Three putative full-length DNA-B clones (pBCB1, pBCB2 and pBCB3) were obtained. Endonuclease restriction maps showed that these BCMoV DNA-B clones were different than those for other bean-infecting geminiviruses characterized in our laboratory (unpublished); i.e., BGMV from Brazil, Guatemala and the Dominican Republic and bean dwarf mosaic geminivirus (BDMV). The nucleotide sequence of the presumed Common Region of pBCB1 has the characteristically conserved stem-loop (Fig. 1).

Nucleotide sequence identities of the Common Region of BCMoV with those of DNA-Bs' for BGMV from Guatemala, BGMV from Brazil, and BDMV were 45%, 60% and 61%, respectively. The Common Region of BCMoV had the greatest nucleotide sequence identity (86%) to the restricted host range isolate of squash leaf curl geminivirus (SqLCV) from California (Fig. 1; Lazarowitz, 1991).

The polymerase chain reaction technique (Rojas, 1992) was used to amplify a portion of the BCMoV DNA-A containing a portion of the AL1 gene (the putative replicative protein gene), the Common Region (the geminivirus origin of replication), an intergenic region and part of the AR1 gene (the coat protein gene). Part of the AL1 gene and the AR1 gene were sequenced and compared to sequences from other geminiviruses. The nucleotide sequence of the AL1 region of BCMoV was compared with respective regions for other bean-infecting geminiviruses and identities were

always less than 80%. Identities of greater than 90% are indicative of strains of the same geminivirus (unpublished data).

In conclusion, BCMoV is a distinct bean-infecting geminivirus and is not closely related to BGMV isolates from Central America, the Caribbean Basin or Brazil or BDMV; but appears to be more closely associated with the squash leaf curl geminivirus types (Rojas, 1992).

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        1                                                  50
BCMoV   ATTAGGG.TT CAGTGGCAT. TGTCGTAAAT AAGACCCGGG ACACCAGGGG
SqLCR   GTTAGGGTTT CAGTGGCATA TTTGGTAAAT ATGAACCGGG ACACCAGGGG
 CONS   .TTAGGG.TT CAGTGGCAT. T.T.GTAAAT A.GA.CCGGG ACACCAGGGG

        51                                                100
BCMoV   GGAGCTCCTC TCAAAAACCC TATTGTTGTT GGTGTCCTGG TGTCCCATAT
SqLCR   .GAGCTC.TC TCTAAAACC. TATTATTGCT GGTGTCCTGG TGTCCCATTT
 CONS   .GAGCTC.TC TC.AAAACC. TATT.TTG.T GGTGTCCTGG TGTCCCAT.T

        101                                               150
BCMoV   ATACTACAAG TCTCTTTAGG CCCTTTTAGG ACACCATGGG CAAAATCGGC
SqLCV   A...TACAAC TCTCTGGGG. .......AGG ACACCAGGGG CAAAATCGGC
 CONS   A...TACAA. TCTCT...G. .......AGG ACACCA.GGG CAAAATCGGC

        151                                           194
BCMoV   CATCCGAAAT AATATTACCG GATGGCCG.A AATTTTTTGG TGTCC
SqLCV   CATCCGCAAT AATATTACCG GATGGCCGCA AATTTTTTGG TGTCC
 CONS   CATCCG.AAT AATATTACCG GATGGCCG.A AATTTTTTGG TGTCC
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Fig. 1. Common Region of BCMoV DNA-B compared to the Common Region of the restricted host range isolate of SqLCV DNA-B. The stem-loop region, which is conserved in all geminiviruses, is underlined. CONS = consensus sequence. A period (.) indicates no consensus.

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CHARACTERIZATION OF GEMINIVIRUSES INFECTING BEAN AND *Calopogonium* sp. IN COSTA RICA

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Bean golden mosaic is a major constraint to bean (*Phaseolus vulgaris*) production in Latin America and the Caribbean. The disease is caused by bean golden mosaic geminivirus (BGMV) and causes yield losses of 40 to 100% depending on the age of the plants at the time of infection, cultivar differences and strains of the virus (Morales and Niessen, 1988).

The genome of BGMV is composed of two circular, single-stranded DNAs (termed DNA-A and DNA-B), which are encapsidated in twinned icosahedral particles (Goodman, 1977). The host range of BGMV has been reported to include several species in the genera *Phaseolus* and *Vigna*, as well as weed legumes such as *Macroptilium lathyroides* and *Calopogonium* sp. (Gálvez and Morales, 1989). This indicates the possibility that these weeds function as reservoirs of BGMV in the field.

This study presents an initial characterization of the geminiviruses infecting bean and *Calopogonium* sp. in Costa Rica. Bean and *Calopogonium* sp. samples with typical golden mosaic symptoms were collected in Costa Rica and viral DNA was amplified from infected tissue by polymerase chain reaction (PCR). The procedure yielded a 1.1 kb DNA fragment from the DNA-A, which was cloned and sequenced.

The sequence obtained from the bean sample showed 100% identity to that of BGMV-GA, a well characterized isolate from Guatemala (Gilbertson et al., 1991). Thus, the same virus is infecting beans in Guatemala and Costa Rica. This means that the bean germplasm selected for resistance to BGMV in Guatemala at the CIAT nursery should be useful in Costa Rica. Comparison of two isolates from *Calopogonium* sp. presented 97% sequence identity. Despite the fact that these samples were collected at two distant and ecologically different places in Costa Rica, they appear to be infected with the same geminivirus. Comparison of these isolates with BGMV-CR presented only 75% sequence identity. This result suggests that *Calopogonium* sp. is not the source of inoculum for beans in Costa Rica. This is the first report of sequence data from a geminivirus infecting *Calopogonium* sp. and the name Calopogonium golden mosaic geminivirus (CalGMV) is proposed to designate this new virus.

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          1                                                  50
 BGMV-CR  TTGAAAGAAG AACAACCGAA AGATTACGTC CTTCAACATC ACAACATCCG
 BGMV-GA  TTGAAGGAAG AACAACCGAA AGATTACGTC CTTCAACATC ACAACATCCG
CalGMV-Q  ATCAAGGTCG GTGATCCACG AACGTTCTTA GTTAGTTATC ATCATGCCAA
CalGMV-T  ATCAAGGTCG GTGATCCACT AACGTTCTTA GTTAGTTATC ATCATGTCAA
    CONS  .T.AAgG..G ...A.CC..a A...T.C.T. .TT....ATC A..A..tC..

          51                                                100
 BGMV-CR  TTCTAATCTC GAACGGATCT TCGTCAAAGT GcCgGAACCA TGGGTTCCTC
 BGMV-GA  TTCTAATCTC GAACGGATCT TCGTCAAAGT GCCGGAACCA TGGGTTCCTC
CalGMV-Q  GGCTAACATC GAGCGCCTCT TTCATAAGGC TCCGGAGCCA TGGACTCCTC
CalGMV-T  GGCTAACATC GAGCGCCTCT TTCATAAGGC TCCGGAGCCA TGGACTCCTC
    CONS  ..CTAA..TC GA.CG..TCT T....AA.G. .CCGGA.CCA TGG..TCCTC

          101                                               150
 BGMV-CR  CATTTCCGTT GTCATCATTC ATCAATGTTC CGGTTGTTAT GCAAGAATGG
 BGMV-GA  CATTTCCGTT GTCATCATTC ATCAATGTTC CGGTTGTTAT GCAAGAATGG
CalGMV-Q  CGTTTCAACT CTCCACATTC ACTAACGTCC CCGTGGAGAT GCAAAGGTGG
CalGMV-T  CGTTTCAACT CTCCACATTC ACTAACGTCC CCGTGGAGAT GCAAAGGTGG
    CONS  C.TTTC...T .TC..CATTC A..AA.GT.C C.GT.G..AT GCAA...TGG

          151                                               200
 BGMV-CR  GTTGACGACT ATTTCGGAAG GGGTTCCGCT GCGCGGCCGG AAAGACCTAT
 BGMV-GA  GTTGACGACT ATTTCGGAAG GGGTTCCGCT GCGCGGCCGG AAAGACCTAT
CalGMV-Q  GCTGATGACT ATTTCGGGAG AGATGCCGCA GCGCGGCCTG AGAGACCTAT
CalGMV-T  GCTGATGACT ATTTCGGGAG AGATGCGTxx GCGCGGCCGG AGAGACCTAT
    CONS  G.TGA.GACT ATTTCGG.AG .G.T.Ccgct GCGCGGCCgG A.AGACCTAT

          201                     225
 BGMV-CR  TAGTATCATC GTCGAAGGTG ATTCA
 BGMV-GA  TAGTATCATC GTCGAAGGTG ATTCA
CalGMV-Q  TAGCATAATC ATTGAGGGTG ATTCA
CalGMV-T  TAGCATAATC ATTGAGGGTG ATTCA
    CONS  TAG.AT.ATC .T.GA.GGTG ATTCA
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Fig. 1. Alignment of the complementary sense nucleotide sequences for the AL1 region of four whitefly-transmitted geminiviruses from Central America. One sequence is for the characterized geminivirus isolate BGMV-GA (Bean golden mosaic geminivirus, Guatemala), and three sequences are for the polymerase chain reaction (PCR) fragments of DNA-A from the uncharacterized geminiviruses, BGMV-CR = Bean golden mosaic geminivirus from Costa Rica; CalGMV-Q = Calopogonium golden mosaic geminivirus from Quepos, Costa Rica; CalGMV-T = Calopogonium golden mosaic geminivirus from Tilaran, Costa Rica. CONS = consensus sequence. Upper-case letters indicate that all geminiviruses have the same nt. Lower-case letters represent the nt for the majority of the geminiviruses. A period (.) indicates no consensus.

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Macroptilium lathyroides INFECTED BY A GEMINIVIRUS DISTINCT FROM BEAN GOLDEN MOSAIC GEMINIVIRUS IN THE DOMINICAN REPUBLIC

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Beans are very important component in the diet of all Dominicans. There are several constraints to bean production in the Dominican Republic and one of these constraints is bean golden mosaic geminivirus (BGMV) which occurs mainly in the South and West.

The percentage of incidence of the viral disease infecting beans has increased in the last few years and recently a geminivirus has also been detected infecting tomatoes production (Rojas, 1992).

Macroptilium lathyroides is a common legume weed and it has been described as natural source of inoculum for BGMV in Puerto Rico, the Dominican Republic, and Jamaica (Bird et al., 1975; Abreu and Gálvez, 1979; Pierre, 1975).

M. lathyroides samples showing typical golden mosaic symptoms were collected in the Dominican Republic in the Southwest zone (San Juan de la Maguana) and the Central zone (Azua), which are major areas of bean production. These samples were dried and brought to our laboratory at the University of Wisconsin-Madison, to determine if M. lathyroides is a reservoir of BGMV.

The squash blot hybridization and polymerase chain reaction (PCR) techniques were used to detect geminiviruses. The PCR technique was used to amplify viral DNA from the dried plant samples. The universal primer pair PAR1C503 and PAL1V1979 was used to amplify part of AL1, the Common Region, and part of AR1 (Rojas, 1992). A PCR fragment of 1.1 kb was digested with PstI restriction endonuclease and subsequently cloned into Bluescript plasmid (pBS). The insert sizes were 1.1 kb for both samples of Macroptilium. These clones were partially sequenced and the data compared with BGMV from the Dominican Republic and other characterized geminiviruses. The nucleotide sequence identity of the AL1 region for the Macroptilium geminivirus from Azua was compared with that of the isolate from San Juan de la Maguana, BGMV-DR (Dominican Republic) and BGMV-GA (Guatemala) and the nucleotide sequence identities were 100, 83, and 84%, respectively (Fig. 1).

From the sequence comparison data we concluded that M. lathyroides was infected by a new geminivirus which is distinct from BGMV-DR. Thus, it seems to be that M. lathyroides is not the source of inoculum for BGMV in the Dominican Republic. There is no variability between the two geminivirus isolates from M. lathyroides from the two locations. This new geminivirus is designed Macroptilium golden mosaic geminivirus (MacGMV).

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           1                                                  50
MacGMV-A   ATATTGAAGG AGGAGCAGCC GAAAGATTAC GTCCTTCATT ATCACAACAT
MacGMV-SJ                    CAGCC GAAAGATTAC GTCCTTCATT ATCACAACAT
BGMV-DR    ATATTAAAGG AAGAACAACC GAAAGATTAC GTCCTTCAAA ATCACAACAT
BGMV-GA    ATATTGAAGG AAGAACAACC GAAAGATTAC GTCCTTCAAC ATCACAACAT
CONS       ATATTgAAGG AaGAaCA.CC GAAAGATTAC GTCCTTCA.t ATCACAACAT

           51                                                 100
MacGMV-A   CCGTTCTAAC CTCGAACGGA TCTTCGTCAA GGTTCCGGAA CCGTGGGTTC
MacGMV-SJ  CCGTTCTAAC CTCGAACGGA TCTTCGTCAA GGTTXCXGAA CCGTGGGTTC
BGMV-DR    CCGTTCTAAT CTCGAACGGA TCTTCTTCAA AGTGCCGGAA CCATGGGTTC
BGMV-GA    CCGTTCTAAT CTCGAACGGA TCTTCGTCAA AGTGCCGGAA CCATGGGTTC
CONS       CCGTTCTAA. CTCGAACGGA TCTTCgTCAA .GT.cCgGAA CC.TGGGTTC

           101                                                150
MacGMV-A   CTCCATTTCA TCTCTCCACC TTCACGAACG TTCCGGCTGC GATGCAAGAC
MacGMV-SJ  CTCCATTTCA TCTCTCCACC TTCACGAACG TTCCGGCTGC GATGCAAGAC
BGMV-DR    CTCCATTTCC GTTGTCATCA TTCATCAATG TTCCGGTTGT TATGCAAGAA
BGMV-GA    CTCCATTTCC GTTGTCATCA TTCATCAATG TTCCGGTTGT TATGCAAGAA
CONS       CTCCATTTC. ..T.TC..C. TTCA..AA.G TTCCGG.TG. .ATGCAAGA.

           151                                                200
MacGMV-A   TGGGTGAATC GTTATTTTGG GGTCAGtxcc ggTGCGCGGC CGGAGAGACC
MacGMV-SJ  TGGGTGAATC GTTATTTTGG GGTCAGTGCC GCTGCGCGGC CGGAGAGACC
BGMV-DR    TGGGTTGACG ACTATTTCGG AAGGGGTTCC GCTGCGCGGC CGGAGAGACC
BGMV-GA    TGGGTTGACG ACTATTTCGG AAGGGGTTCC GCTGCGCGGC CGGAAAGACC
CONS       TGGGT..A.. ..TATTT.GG .....GTtCC GcTGCGCGGC CGGAgAGACC

           201                                 231
MacGMV-A   TATTAGTATC ATCGTCGAGG GTGATTCAAG A
MacGMV-SJ  TATTAGTATC ATCGTCGAGG GTGATTCA
BGMV-DR    TATTAGTATC ATCGTCGAAG GTGATTCACG A
BGMV-GA    TATTAGTATC ATCGTCGAAG GTGATTCACG A
CONS       TATTAGTATC ATCGTCGA.G GTGATTCAcG A
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Fig. 1. Alignment of the complementary sense nucleotide sequences for the AL1 region of four whitefly-transmitted geminiviruses. Two sequences are for the characterized geminivirus isolates of bean golden mosaic geminivirus from Guatemala (BGMV-GA) and from the Dominican Republic (BGMV-DR), and two sequences are for fragments of DNA-A from the polymerase chain reaction (PCR) for the uncharacterized geminiviruses from Macroptilium lathyroides collected at Azua (MacGMV-A) and San Juan de la Maguana (MacGMV-SJ). CONS = consensus sequence. Upper-case letters indicate that all geminiviruses have the same nt. Lower-case letters represent the nt for the majority of the geminiviruses. A period (.) indicates no consensus.

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