Detection of Subgroup III Geminivirus Isolates in Leaf Extracts by Degenerate Primers and Polymerase Chain Reaction

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ABSTRACT

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The DNA of several monopartite and bipartite whitefly-transmitted (WFT) geminiviruses was amplified from a viral template present in infected leaves after either direct addition of clarified plant extracts to an otherwise complete polymerase chain reaction (PCR) mix or after immobilization of template to microfuge tubes. A degenerate primer pair was designed to specifically target the middle or 'core' region of the capsid protein gene of subgroup III geminivirus isolates and amplify a viral DNA fragment of approximately 550 bp. Using this method, a single PCR product of the expected size (550 bp), as estimated by agarose gel electrophoresis, was amplifiable from plants infected with a representative set of subgroup III geminivirus isolates with a broad biogeographic base. That the 550-bp PCR product had a geminiviral gene origin was demonstrated by direct sequencing of the 550-bp fragments (yielding approximately 470 to 490 bases of informative sequence) and was validated through comparison (alignment) of the sequences with the published DNA sequences of several well-characterized WFT geminiviruses. Analogous viral fragments were not detectable by PCR with the subgroup III core coat protein primers and extracts of plants infected with either subgroup I or II geminivirus isolates. The demonstrated exclusive specificity of the assay for subgroup III geminiviruses offers a highly simplified PCR-based assay that permits the detection of a geographically diverse collection of WFT geminiviruses infecting cultivated crops, ornamentals, and weed hosts with minimal sample preparation. This approach is highly useful for the amplification of subgroup III geminiviral DNA templates from total nucleic acid extracts from infected plants and partially purified virion preparations.

Whitefly-transmitted (WFT) geminiviruses, or subgroup III of the family Geminiviridae, cause serious diseases in vegetable and fiber crops and are emerging viral pathogens in tropical and subtropical regions (2,3). Identification of WFT geminiviruses by traditional virological methods has proven difficult. Many of the most economically important WFT geminiviruses are not readily mechanically transmitted; therefore, the use of definitive bioassay hosts for virus identification and evaluation of host range and other biological properties has been very difficult in many cases. Production of virus-specific antisera for subgroup III geminiviruses has proven difficult because (i) the physical and chemical properties of particles make them difficult to purify in a stable form; (ii) the virions appear to be poorly immunogenic; and (iii) the capsid proteins of WFT geminiviruses are not antigenically distinct with available polyclonal and most monoclonal antisera preparations (16,19,20,24,31,32,34).

DNA-DNA hybridization assays (1,4,13,23,24,33) and molecular cloning and DNA sequencing of viral genomes have been used for detection and identification of WFT geminiviruses and to predict or infer virus relationships at the quasispecies or strain levels (2,10,22,28,35). Polymerase chain reaction (PCR) and degenerate broad-spectrum oligonucleotide primers have been used for general detection and virus identification of subgroup III geminiviruses (5,8,14,26,29). Although the results of these studies are encouraging, the existing PCR-based diagnostic techniques for subgroup III geminiviruses still rely both on the procurement of purified viral DNA from plant tissues and the availability of geminivirus- or strain-specific PCR primers that must be empirically evaluated for each 'unknown.' Further, a negative result obtained because primers may not be targeted specifically to an uncharacterized geminivirus sequence, a typical scenario with the emergence of subgroup III viruses as important pathogens, does not accomplish the immediate goal of rapidly and accurately detecting geminiviruses, much less achieving virus identification. Thus, currently no universal PCR primers exist by which subgroup III geminiviruses can be detected and routinely identified with a high degree of accuracy and reliability.

Furthermore, a major caveat with current PCR-based detection systems that utilize suitably purified DNA from plant samples is the difficulty in obtaining high-quality purified DNA preparations from many field-collected samples and, in some cases, from greenhouse-grown plants of particular familial or generic affiliation. Among the most critical and troublesome problems are the presence of interfering secondary plant compounds, low or unpredictable virus titers, and degradation of samples due to a prolonged period of time between sample collection and DNA extraction.

We report the design and testing of a degenerate PCR primer pair suitable for PCR-assisted amplification of viral DNA from a broad range of subgroup III geminiviruses. This PCR approach takes advantage of direct adsorption of a viral template from clarified extracts to polypropylene microcentrifuge tubes, which circumvents the usual requirement for extraction of total nucleic acids from plant samples prior to PCR amplification. There are several reasons for this work. First, there is a great demand for universal degenerate primers capable of rapidly and accurately detecting subgroup III geminiviruses in field- and greenhouse-collected plant samples. Using this method, leaf extracts may be readily prepared, and the template may be adsorbed to plastic tubes under even the least sophisticated conditions, thereby cir-

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cumventing the need to ship plant samples long distances, which often reduces sample quality, and limits the need to ship fresh plant samples. The ability to combine the development of 'core' region coat protein (CP) primers that exclusively target only the subgroup III geminivirus primers with existing PCR technology and automated DNA sequencing will permit both rapid detection of WFT geminiviruses and, ultimately, virus identification based on viral nucleotide sequences of this potentially informative region of the geminiviral genome.

MATERIALS AND METHODS

Primer design. Subgroup III geminivirus capsid protein gene sequences were obtained from GenBank and aligned by Align Plus software (Scientific and Educational Software, Durham, NC). Geminivirus DNA sequences used in this analysis were bean golden mosaic virus (BGMV) from Guatemala (BGMV-GA) (12), BGMV from Puerto Rico (BGMV-PR) (17), BGMV from Brazil (BGMV-BZ) (12), tomato golden mosaic virus (15), squash leaf curl virus strain E (SLCV-E), Abutilon mosaic virus (11), potato yellow mosaic virus (7), chino del tomate virus (J. K. Brown et al., *unpublished data*), African cassava mosaic virus (30), tomato yellow leaf curl virus from Israel (TYLCV-IS) (21), and from Thailand (TYLCV-TH) (25). Conserved regions in the capsid protein gene were identified (Fig. 1), and degenerate primers corresponding to highly conserved regions were designed by Primer Plus software (Scientific and Educational Software). Oligonucleotide primers were synthesized either by the Washington State University Bioanalytical Laboratory, Pullman, or the University of Arizona Biotechnology Laboratory, Tucson.

Direct PCR detection of geminiviruses in leaf extracts. Fresh or dried leaf tissue was ground in 50 mM Tris, pH 8.0, containing 10 mM EDTA (TE) (1 g/20 ml) with a mortar and pestle. Extracts were clarified by centrifugation (10,000 × *g*, 10 min, 4°C), and 50 µl of supernatant was transferred to an autoclaved, polypropylene microfuge tube. After a 30-min incubation at 4°C, the leaf extract was removed by pipetting, and the tubes were rinsed three times each with 200 µl of TE. In an alternate technique, 1 µl of leaf extract, prepared as before, was diluted 10-fold in TE and added directly to microfuge tubes, each containing 49 µl of complete PCR mix. PCR was carried out by the procedures described below.

PCR with nucleic acid extracts and purified virions. Total nucleic acids were purified from leaves by extraction in buffer (1 g/20 ml) by the method of Doyle and Doyle (9). Final total nucleic acid pellets were resuspended in cold TE. Geminivirus particles were partially purified from SLCV-infected pumpkin (*Cucurbita maxima*) leaves as previously described (4).

Either 1 µg of purified, total nucleic acid preparations or 1 µg of purified virions was added to each tube containing PCR mix and a

A. AR1-VIRAL STRAND (+) PRIMER SEQUENCE AV494

BGMV-PR	G	C	C	C	A	T	G	T	A	T	A	G	A	A	A	G	C	C	A	A	G
BGMV-GUAT.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BGMV-BR.	.	.	.	T	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
ABMV	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
PYMV	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
TGMV	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
SQLCV-EXT.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.	
ACMV	.	.	.	.	.	.	.	.	.	C	.	.	G	.	.	.	.	.	C	.	.
TYLCV-IS.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	.	.	C	.	.
TYLCV-TH.	A	.	.	T	G	C	A	.	.	C	.	.	.	.	.	.	.	.	C	.	.
DEGENERATE	G	C	C	C	A	T	G	T	A	T	A	G	A	A	A	G	C	C	A	A	G
PRIMER				T			A			C			G						C		

B. AR1-COMPLEMENTARY STRAND (-) PRIMER SEQUENCE AC1048

BGMV-PR.	G	G	A	T	T	A	G	A	G	G	C	A	T	G	T	G	T	A	C	A	T	G
BGMV-GUAT.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BGMV-BR.	G	.	G	A	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
ABMV	.	.	G	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.
PYMV	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
TGMV	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
SQLCV-EXT.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	
ACMV	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
TYLCV-IS.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.
TYLCV-TH.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
DEGENERATE	G	G	A	T	T	A	G	A	G	G	C	A	T	G	T	G	T	A	C	A	T	G
PRIMER			G			G			A						A							
						T									C							

Fig. 1. Alignment of conserved nucleotide sequences (from GenBank) of the 'core' region of the capsid protein gene for 10 subgroup III geminivirus isolates or viruses on which the design of degenerate core coat protein primers **A,** AV494 and **B,** AC1048 were based. BGMV = bean golden mosaic virus from Puerto Rico, Guatemala, and Brazil; ABMV = Abutilon mosaic virus; PYMV = potato yellow mosaic virus; TGMV = tomato golden mosaic virus; SQLCV = squash leaf curl virus; ACMV = African cassava mosaic virus; TYLCV = tomato yellow leaf curl virus from Israel and Thailand.

total reaction volume of 50 µl. The PCR master mix contained 150 µM dNTPs, 2.5 mM $MgCl_2$, 1.25 units of *Taq* polymerase, and 20 pmol of each primer. PCR was carried out in a thermocycler with 35 cycles, each consisting of 1 min at 92°C, 20 s at 60°C, and 30 s at 72°C. Reaction products were analyzed by electrophoresis on 1.5% agarose gels in Tris-acetate-EDTA buffer, pH 8.0. Experimental controls included a complete reaction mix to which was added either 1 µl of total nucleic acids purified from uninfected leaves, clarified extracts from uninfected leaves, or the resulting suspension purified from mock-inoculated pumpkin plants, using the procedure described for purifying SLCV virions. As an internal control, double-distilled water, substituted for viral DNA template, was added to complete reaction mixes.

All PCR reagents were purchased from GIBCO BRL (Gaithersburg, MD), and *Taq* polymerase was obtained from Perkin-Elmer/Cetus (Norwalk, CT). Geminivirus isolates were maintained in the greenhouse or laboratory, either as live cultures or as frozen (–70°C) or dried leaves, respectively, at the University of Arizona, Tucson. The clone of the full-length SLCV-E capsid gene was provided by S. G. Lazarowitz (University of Illinois, Urbana) and was used as a positive control in initial optimization of PCR conditions. Dried leaves infected with beet curly top virus (BCTV) were provided by D. M. Bisaro (The Ohio State University, Columbus), and tobacco yellow dwarf geminivirus (TYDV) (synonym bean summer death virus) was obtained from J. E. Thomas (Indooroopilly, Queensland, Australia).

RESULTS

Capsid protein gene degenerate PCR primers. Based on the alignment of capsid gene sequences of both bipartite and monopartite subgroup III geminiviruses, several highly conserved regions were located within a 700-base fragment, or 'core' region, of the capsid protein gene. A degenerate primer pair (Fig. 1), based on two conserved motifs that flank this core region of the CP, was used successfully to direct the amplification of geminiviral DNA fragments of the predicted molecular size (550 bp), as estimated by agarose gel electrophoresis. Using the published nucleotide sequence of BGMV-PR (17) as the prototype CP core region, these subgroup III-specific PCR primers were designated AV494 (viral strand) and AC1048 (complementary strand). When tested with leaf extracts or total nucleic acid purified from geminivirus-infected plants singly infected with geminivirus isolates comprising a representative set of isolates spanning a broad biogeographic base, the AV494/AC1048 primer pair successfully primed the amplification of a fragment of the expected molecular size, irrespective of geographic source or particular host plant species. We document the geminiviral CP gene fragments amplified from leaf extracts or DNA samples purified from the following viruses or isolates: Jatropha mosaic virus (JMV) in *Passiflora edulis*, serrano golden mosaic virus (SGMV) in tomato, SLCV from Arizona (SLCV-AZ) in pumpkin, and pepper mild tigré virus from Arizona (PepMTV-AZ) in pepper (Fig. 2).

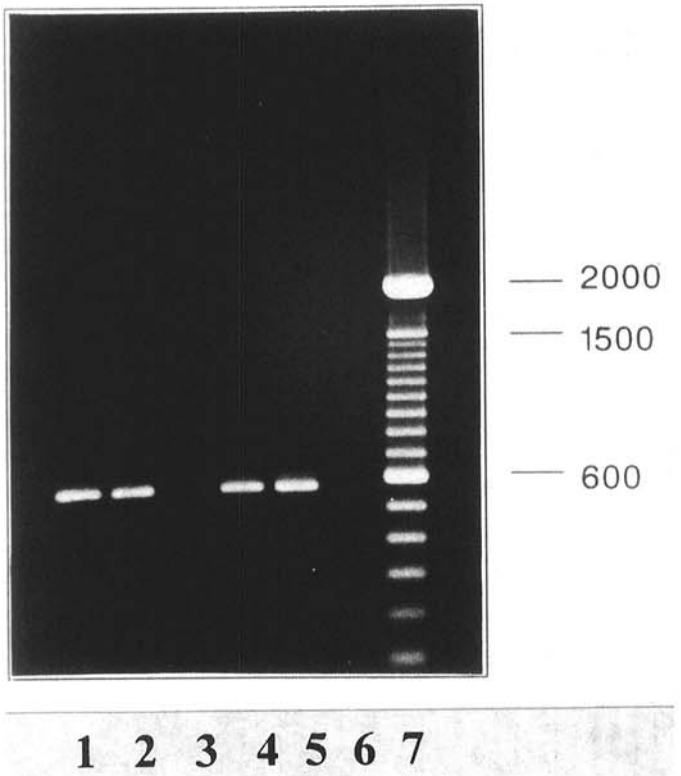

Fig. 2. Amplification of a geminivirus coat protein gene fragment with primer pair AV494/AC1048 and total leaf DNA samples. Samples are from: lane 1, Jatropha mosaic virus-infected *Passiflora edulis*; lane 2, serrano golden mosaic virus-infected tomato; lane 3, healthy pumpkin; lane 4, squash leaf curl virus-infected pumpkin; lane 5, pepper mild tigré virus-infected pepper; lane 6, water control; and lane 7, 100-bp standard.

The authenticity of the 550-bp PCR product as a DNA fragment amplified from the respective geminivirus CP gene was confirmed by direct sequencing of the products in both directions, using PCR primers as sequencing primers. The ends of the sequences were trimmed to remove sequencing errors close to the sequence primers. Figure 3 gives the alignment of 400-base sequences amplified from SLCV-AZ, JMV, SGMV, and PepMTV-AZ. The sequences obtained by direct sequencing of the PCR products aligned in the expected region of the CP gene of SLCV and BGMV-PR. The SLCV-AZ sequence fragment was different from the published sequence by only 4 bases. Even though the CP region was expected to be highly conserved, the six virus isolates are readily differentiated from each other based on CP core sequence comparisons. For example, the SLCV CP gene fragment at the nucleotide level is only approximately 80% homologous to BGMV-PR, 84% homologous to SGMV, and 82% homologous to PepMTV. The versatility and broad spectrum or universal capability of these PCR primers have been demonstrated in a preliminary report on the amplification of the 550-bp fragment and the respective DNA sequence of this conserved gene fragment from more than 80 geminivirus isolates (6). The suitability of the CP core sequence for subgroup III geminivirus identification is being investigated.

Optimization of PCR from aqueous extracts and relative detection limits. In preliminary experiments, we found that leaf extracts incubated in polypropylene microfuge tubes for 15 to 30 min resulted in absorption of sufficient template (either virions or free virus DNA), which remained despite exhaustive washing to permit PCR amplification of the targeted gene fragment. In a typical experiment, SLCV-infected leaf tissue was ground in TE buffer, pH 8.0 (1 g/10 ml), clarified by centrifugation, and 50 µl was added to microfuge tubes for a 30-min incubation on ice. Tubes were washed three times each with TE and completely drained between washes. Experiments in which PCR amplification was carried out, using a 10-fold dilution series of SLCV-infected or healthy pumpkin extracts, indicated that the core CP gene fragment was detectable in extracts diluted 10 to 10^4 (Fig. 4). No 550-bp fragment was detectable in reactions amplified from extracts of healthy (virus-free) pumpkin or in control reactions that contained water instead of plant extracts, as monitored by agarose gel electrophoresis.

Results obtained from additional dilution-series experiments indicated that relative amounts of 550-bp product obtained from amplification of leaf extracts were inconsistent with a proportional decrease in product per dilution. Possible inhibition of PCR by tissue extract components was suggested by the results of some dilution-series experiments. The lowest extract dilution often yielded less amplification product than the higher dilutions. Repeated washing (20 times) of tubes with TE after standard incubation of ex-

tracts in tubes did not affect fragment intensity (data not shown), suggesting that at the highest viral DNA template/sap constituent concentrations amplification was inhibited, possibly as a result of interfering host constituents.

Specificity of PCR tissue assays. The utility of this PCR assay was demonstrated in an experiment with leaf extracts from infected tissues representing 14 subgroup III geminivirus isolates and numerous host plant species (Fig. 5). The targeted core region of the CP gene was successfully amplified from all geminivirus isolates and host species tested after sufficient dilution of the extract, indicating that the ability to detect geminivirus in infected plant tissues was not notably affected, or inhibited, by any particular plant species tested. For example, the diagnostic 550-bp viral DNA fragment was detected in greenhouse-grown, cultivated plant species: cv. Topcrop bean, cv. DP 70 cotton, cv. Anaheim peppers, cv. Big Max pumpkin, and cv. Pole Boy tomato; and in extracts of field-infected weed or cultivated plants: *Sida glabrous*, *Malva parviflora*, *Abutilon striatum* var. *thompsonii*, *P. edulis*, and *Datura stramonium*. The direct-binding technique described here was used to successfully detect WFT geminiviruses in more than 200 field- or greenhouse-collected samples tested during an 18-month period.

When extracts of leaves infected with TYDV and BCTV, two leafhopper-transmitted, monopartite geminiviruses (subgroups I and

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                    160        170        180        190        200        210
                     *          *          *          *          *          *
BGMV-PR (17)    GATGTGAAGGACCTTGCAAGGTCCAATCATATGAACAACGCCATGATATATCTCATGTTG
JMV-PR.SEQ      .G.............T.....T..G..T..C..G..GA.A......G.T........C.
SQLC-EXT (18)   .......G..G..A........T..G..T..C..G..G..G........C......C...
SQLC-AZ.SEQ     .......G..G..A........T.....T..C..G..G..G........C......C...
SGMV.SEQ        .T........G.....T........G....T...G..G..G........C..........
PMTV.SEQ        .T........G.....T........G....TC..G..G..G........C..G.......

                    220        230        240        250        260        270
                     *          *          *          *          *          *
BGMV-PR         GTAAGGTTATGTGTATATCCGATATCACACGTGGTAATGGTATTACTCATCGTGTTGGTA
JMV-PR.SEQ      .G..........C..C..T.....T..C........C........C..C..C.......
SQLC-EXT        .C.....A........T..T..CG.G..G..........C........C..C..C..C.
SQLC-AZ.SEQ     CC.....A........T..T..CG.G..G..........C........C..C..C..C.
SGMV.SEQ        .C.....C.....C..T..G..CG.G.............................A..G.
PMTV.SEQ        .C.....C.....C..T..G..CG.G...........C...........C.....A..C.

                    280        290        300        310        320        330
                     *          *          *          *          *          *
BGMV-PR         AACGTTTTTGTGTGAAGTCTGTGTATATTTTAGGTAAGATATGGATGGATGAAAACATCA
JMV-PR.SEQ      .G..........T.....C.............................C.....T....
SQLC-EXT        .G..C........T........A...........C.............C.....T....
SQLC-AZ.SEQ     .G..C........T........A...........C.............C.....T....
SGMV.SEQ        .G.....C.....C.......CA...G....G.................C..G..T....
PMTV.SEQ        .G.....C..C..........CA..CG....G.................C..G..T....

                    340        350        360        370        380        390
                     *          *          *          *          *          *
BGMV-PR         TGCTTAAGAACCATACCAATAGTGTCATTTTTTGGTTGGTTCGTGACCGTAGACCATATG
JMV-PR.SEQ      A.T.A........C..G..C........G..C.........A.A..T..............
SQLC-EXT        A.T.G.....T..C..G..C.....G..G........A.....A................
SQLC-AZ.SEQ     A.T.G.....T..C..G..C.....G..G........A.....A................
SGMV.SEQ        A...C..A.....C.....C..C.....G..C...C.T..G..A..TA.G...........
PMTV.SEQ        A...C........C........C.....G..C...C.C..GA.A..TA.G...........

                    400        410        420        430        440        450
                     *          *          *          *          *          *
BGMV-PR         GAACCCCTATGGATTTTGGTCAAGTTTTTAACATGTTTGACAATGAACCTAGTACTGCTA
JMV-PR.SEQ      .T..T...C.T........C.....G..C..............C................
SQLC-EXT        .C..T...........C..C..G..G..C..T.....C.....C..G..C.....G..A.
SQLC-AZ.SEQ     .C..T...........C..C..G..G..C..T.....C.....C..G..C.....G..A.
SGMV.SEQ        .T..............C..G..G..G..C.......A......C..G........A..C.
PMTV.SEQ        .T.....................G..G..C.......A......C..G.....C..A.GC.

                    460        470        480        490        500        510
                     *          *          *          *          *          *
BGMV-PR         CGGTCAAGAACGATTTTCGTGATCGTTATCAAGTTATGCATAGGTTCAATGCAAAGGTTT
JMV-PR.SEQ      .T..T.....T.....G..........T......C........AA..TT...GT......A
SQLC-EXT        .TA...........C.A..................C.....C......T.C..G.....CA
SQLC-AZ.SEQ     .AA...........C.A..................C.....C......T.C..G.....CA
SGMV.SEQ        .T..G.........C.G............C...........C......TCA..T.....CA
PMTV.SEQ        .C..G.........C.G..........TC.....CC....C......TCC..T.....CA

                    520        530        540        550
                     *          *          *          *
BGMV-PR         CTGGTGGTCAATATGCAAGCAACGATCAAGCCTTGGTAAG
JMV-PR.SEQ      .A.....A........C........A.....AC....G..
SQLC-EXT        ..........G.....G........A...........T..
SQLC-AZ.SEQ     ..........G.....G........G...........T..
SGMV.SEQ        .A.....G..G.....C........G..G..G.....G..
PMTV.SEQ        .A.....A..G.T............G..G..G.....T..
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Fig. 3. Nucleotide sequence alignment of polymerase chain reaction-amplified coat protein gene fragments of squash leaf curl virus (SQLC-AZ), Jatropha mosaic virus (JMV-PR), serrano golden mosaic virus (SGMV), and pepper mild tigré virus (PMTV). The fragments were amplified and sequenced with primer pair AV494/AC1048. Bean golden mosaic virus (BGMV-PR) and SQLCV-EXT published sequences (18) were included for comparison.

II, respectively), were assayed with the subgroup III-specific primers under the stated conditions, no 550-bp product was detectable, as judged by visualization on agarose gels (data not shown).

Stability of the viral template. The stability of the polypropylene microfuge tube-bound viral template is an important consideration with respect to detection and, ultimately, the accuracy of a diagnostic result. In preliminary experiments, leaf extracts were incubated in tubes, washed as described before, and air-dried. Tubes were placed in plastic bags and held at room temperature for 1 to 2 weeks in each location (Arizona or Washington) prior to transporting to the collaborating laboratories using the U.S. postal service. PCR reactions were carried out either immediately or 2 months after the arrival of treated tubes. In these experiments, the 550-bp fragment was readily detectable in extracts from subgroup III geminivirus-infected plants but was not detectable in the accompanying control tube. These results suggest the viral template, adsorbed in the manner described, is stable for at least moderate lengths of time.

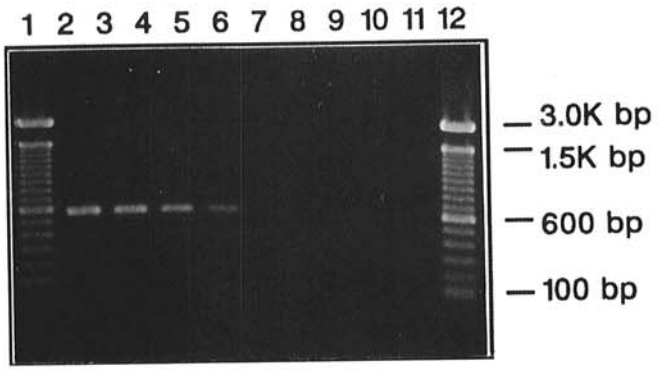

Fig. 4. Polymerase chain reaction analysis of 10-fold serial dilutions of leaf tissue extracts for the presence of a squash leaf curl geminivirus coat protein gene fragment with primer pair AV494/AC1048. Lanes 2 through 10, infected tissue; lane 11, noninfected tissue; and lanes 1 and 12, 100-bp standard ladder.

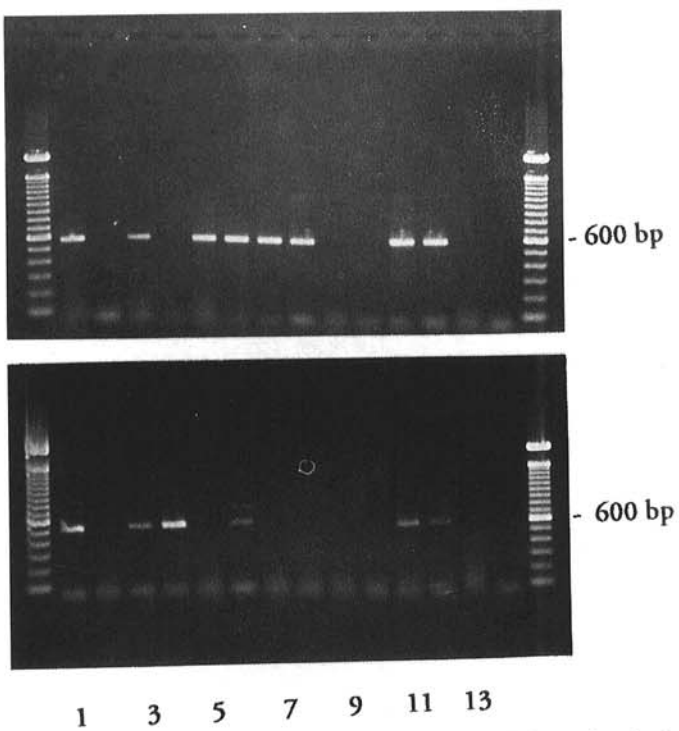

Fig. 5. Leaf tissue extracts were assayed by polymerase chain reaction for the presence of a geminivirus coat protein gene fragment with primer pair AV494/AC10. Top gel: lane 1, squash leaf curl virus in pumpkin; lane 2, pumpkin control; lane 3, cotton leaf curl virus in cotton; lane 4, cotton control; lane 5, tomato yellow leaf curl virus from Thailand in tomato; lane 6, tomato golden mosaic virus in tomato; lane 7, tomato mottle virus FL3132 in tomato; lane 8, chino del tomate virus in tomato; lane 9, beet curly top virus (BCTV) in tomato; lane 10, tomato control; lane 11, serrano golden mosaic virus in pepper; lane 12, Texas pepper virus in pepper; lane 13, pepper control; and lane 14, water control. Bottom gel: lane 1, *Sida glabrous* geminivirus from Puerto Rico; lane 2, *S. glabrous* control; lane 3, Abutilon mosaic virus (AbMV) in *Malva parviflora*; lane 4, AbMV in *Abutilon theophrastii*; lane 5, *M. parviflora* control; lane 6, Passiflora virus in *Passiflora edulis*; lane 7, *P. edulis* control; lane 8, tobacco yellow dwarf virus in *Datura stramonium*; lane 9, *D. stramonium* control; lane 10, bean summer death virus 1 in bean; lane 11, bean golden mosaic virus from Puerto Rico in bean; lane 12 BCMV in bean; lane 13, bean control; and lane 14, water control.

The physical nature or topology of the bound template is either viral single-stranded genomic DNA encapsidated within bound virions (virion +ssDNA), nonencapsidated genomic plus strand ssDNA (free + ssDNA), or free double-stranded replicative form (RF) DNA. Experiments were designed to evaluate this question, in which the addition of either SLCV dsDNA or purified SLCV virions to healthy pumpkin extracts to an otherwise complete PCR reaction mix resulted in amplification of the expected 550-bp fragment. Further investigation of the structural nature of the template (virion +ssDNA, free +ssDNA, and RF DNA) proved inconclusive. Treatment of microcentrifuge tubes charged with extracts of SLCV-infected pumpkin with mungbean nuclease (ssDNA specific) to remove free +ssDNA, treatment of charged tubes with *Hin*cII endonuclease (specific for a RF site within the CP gene), or simultaneous incubation of charged tubes with both enzymes diminished the detectable amount of amplified 550-bp PCR product but did not entirely eliminate the ability to obtain a detectable product (data not shown).

DISCUSSION

The objective of this study was to develop and optimize a universal assay that permits sensitive and accurate detection of biogeographically diverse subgroup III geminiviruses. We reasoned that PCR primers that anneal to two highly conserved sequences within the most highly conserved gene of the WFT geminivirus subgroup would be useful for broad-spectrum PCR-based virus detection. Although there are numerous highly conserved DNA motifs within the capsid gene of subgroup III geminiviruses that are potentially suitable as degenerate priming sites, the motifs targeted by the AV494/AC1048 primer pair are ideal because of the anticipated conservation and, hence, their apparently universal nature. In addition, these primers permit accurate amplification of a fragment of DNA sequence, yielding a PCR product that may be directly sequenced in a single run, without prior molecular cloning of the viral gene fragment.

Although the AV494 and AC1048 primers are degenerate, nucleotide heterogeneity occurs primarily in the third position of most codons, leading to their observed broad-spectrum capability. Not all base differences were accounted for in the design of the AV494 primer, and five alternate bases were omitted because they occurred only rarely. Omission of the respective mismatched base did not interfere with the amplification of the expected 550-bp fragment from any putative geminivirus isolate tested thus far.

The PCR technique described here allows rapid, sensitive, and accurate detection of a diverse array of WFT geminiviruses in cultivated and weed hosts, with minimal sample preparation. This assay also is highly effective with purified nucleic acid extracts or partially purified virion preparations, either treated or untreated with a ssDNA-specific nuclease. Although the evidence is equivocal, the results suggest that some of the virion +ssDNA that serves as a template for PCR is derived from virions bound to plastic tubes. If this is the case, populations of virions may remain stable, whereas others swell or are disrupted over time, exposing the virion +ssDNA during the incubation or the PCR reaction, making the virion +ssDNA available for priming and amplification. A similar observation has been made for certain RNA viruses and cDNA

synthesis (27,36). Removal of the capsid was not required for reverse transcription of the genomes of many types of RNA viruses. Working with woody tissues as a source of several RNA viruses, Rowhani et al. (27) were able to directly bind virions, reverse transcribe, and PCR amplify specific genomic fragments. Whether binding of virions to polypropylene tubes destabilizes virions and preserves a viral template for subsequent PCR amplification after prolonged storage of charged tubes remains to be assessed. Nevertheless, the fact that the template can be stored or shipped at room temperature provides obvious advantages for virus detection and identification purposes.

The versatility of this method makes it the simplest, most broad-spectrum PCR-based detection system available to date for detection of subgroup III WFT geminiviruses. DNA sequences of the 550-bp core capsid gene fragment amplified by this technique from numerous WFT geminiviruses have been accumulated and are maintained in a geminivirus subgroup III database in the University of Arizona laboratory, Tucson. In combination with computer-aided, automated DNA-sequencing systems, the rapid, PCR-based amplification technique described here will facilitate geminivirus detection and identification based on conserved and intervening nucleotide sequences of the capsid gene. Direct comparisons of subgroup III core capsid protein sequences may permit an evaluation of relationships and geographic origins of subgroup III members based on the sequence similarity of this highly conserved capsid gene fragment among subgroup III geminiviruses (6). This strategy may prove analogous to serological comparisons employed for other plant viruses—an approach that is not currently feasible for the geminiviruses.

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