Use of the Polymerase Chain Reaction to Amplify Tomato Yellow Leaf Curl Virus DNA from Infected Plants and Viruliferous Whiteflies

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ABSTRACT

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The genomic DNA molecule of an Israeli isolate of tomato yellow leaf curl virus (TYLCV), a whitefly-transmitted geminivirus, was amplified from total DNA extracts of TYLCV-infected plants (*Lycopersicon esculentum* 'M82') by the use of the polymerase chain reaction (PCR). Synthetic oligonucleotides complementary to different regions of the viral genome were used in different combinations to amplify the full-length as well as subgenomic fragments of the TYLCV DNA molecule. The procedure was also used to amplify TYLCV DNA from nucleic acid extracts of individual whiteflies (*Bemisia tabaci*) fed on TYLCV-infected tomato. TYLCV DNA was also amplified from whiteflies collected from naturally infected bean plants (*Phaseolus vulgaris* 'Tender Green') in the field.

Additional keyword: virus typing.

Tomato yellow leaf curl geminivirus (TYLCV) is transmitted by the tobacco whitefly (*Bemisia tabaci* Gennadius) (2–5), whose genome consists of a single molecule of covalently closed circular single-stranded DNA (11). It causes economic losses to tomato growers in the Middle East and other tropical and subtropical regions of the world (6,10). Nucleotide sequence analysis of TYLCV isolates collected in Israel (11), Italy (9), and Thailand (R. Beachy, *personal communication*) indicates that this virus is unusually heterogenous. The potential of the polymerase chain

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reaction (PCR) for amplification of the genomic length DNA molecule (14) could be of help in typing TYLCV isolates. PCR has been utilized, among many other applications, to detect and type related plant monopartite (13) and bipartite (7) geminiviruses, luteoviruses (8), and viroids (12).

We describe here the amplification of full-length TYLCV DNA as well as subgenomic fragments from total DNA extracts from infected plants and from natural and experimentally derived viruliferous whiteflies by the use of PCR.

MATERIALS AND METHODS

Virus, plants, and insects. The *Taq*I polymerase from Promega Corp. (Madison, WI) was routinely used; comparable results were achieved with *Taq*I polymerases from Boehringer Mannheim (Indianapolis, IN) and New England Biolabs (Beverly, MA). The DNA size marker was from Gibco-BRL (Gaithersburg, MD).

Whiteflies were reared on cotton plants (*Gossypium hirsutum* L. 'Akala') in wooden cages covered with porous unwoven propylene sheets, 17 g/m^2 (Agryl, Sodoca, France). Virus cultures originated from naturally infected tomato plants (*Lycopersicon esculentum* Mill. 'M82') from the Rehovot Experimental Field. They were maintained in tomato plants (M82) by whitefly-mediated inoculations performed in cages as previously described (17). Whiteflies were collected from these TYLCV-infected plants after a 24-h acquisition feeding period (17) and from field-infected bean (*Phaseolus vulgaris* L. 'Tender Green') grown at Yamma, in the Coastal Plain of Israel.

Extraction of plant and insect DNA. Total DNA was extracted from leaves of tomato plants (*L. esculentum* 'M82') according to the procedure of Taylor and Powell (16). Total DNA extracts prepared from whiteflies according to Zeidan and Czosnek (17) were purified by affinity chromatography, which eliminated detergents (Geneclean, Bio 101, La Jolla, CA).

Amplification of TYLCV DNA from plant and insect extracts. Reaction conditions were essentially those of Sambrook et al (15). The cycling protocol was initial annealing (0.5 μg of DNA from a TYLCV-infected plant with 0.2 mM of each primer) for 5 min at 65 C; extension for 5 min at 72 C and denaturation for 1 min at 92 C; subsequent cycles were 2 min at 55 C, 4 min at 72 C, and 1 min at 92 C. We performed 35 cycles by using a Techne PHC-2 thermocycler. Primer sequences were deduced from the nucleotide (nt) sequence of the TYLCV genome (11) and were purchased from Biotechnology General, Rehovot, Israel. They were (from 5′ to 3′) P1V, ATACTTGGA-CACCTAA-TGGC, nt 61–80; P2C, AAGTAAGACACCGATACACC, nt 41–60; P3C, AGCTAAGAGCTCAACAGA, nt 2,498–2,515; P4C, TGGACATCTAGACC-TAAG, nt 2,054-2,071; P5C, AGTCAC-GGGCCCTTACAA, nt 456–473. The sequence of primer P1V corresponds to the virion (+) strand, whereas primers P2C–P5C are complementary to the virion strand. Amplified samples were subjected to electrophoresis in a 1.5% agarose gel, stained with 0.5 μg/ml of ethidium bromide, and photographed.

RESULTS

Amplification of the full-length TYLCV genome from a TYLCV-infected plant. Two 20-mer primers P1V and P2C from the intergenic region of the viral genome were used to amplify the full-length (2,787 nt) TYLCV genomic molecule. Three TYLCV subgenomic fragments were amplified with P1V in combination with the three other 18-mer primers complementary to the virion strand (P3C, P4C, and P5C). Figure 1 shows the location of the primers in the circular viral genome.

A DNA band of approximately 2.8 kbp, the size of the TYLCV genome, was amplified from plant extracts with primers P1V and P2C (Fig. 2A, P2C). Restriction digests of this amplified DNA with *Sac*I and *Bcl*I yielded fragments of the size predicted from the restriction map of the cloned TYLCV genome (Fig. 2B). The sizes of the amplified fragments obtained with the other

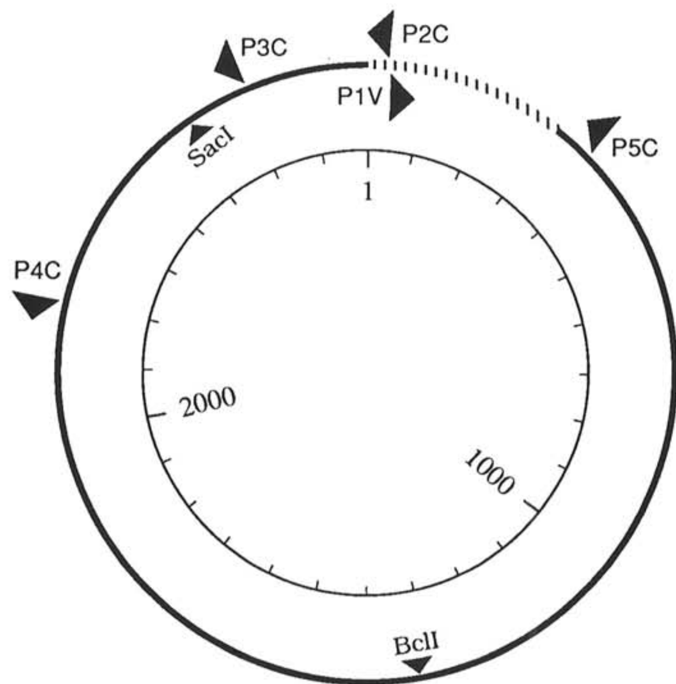

Fig. 1. Location of the primers used for amplification of the complete tomato yellow leaf curl virus (TYLCV) genome (P1V and P2C) and of subgenomic fragments (P1V and P3C; P1V and P4C; P1V and P5C). The cross-hatched region of the outer circle (nucleotides 1–314) denotes the intergenic region of the viral genome. Arrows indicate the direction of DNA synthesis from each primer.

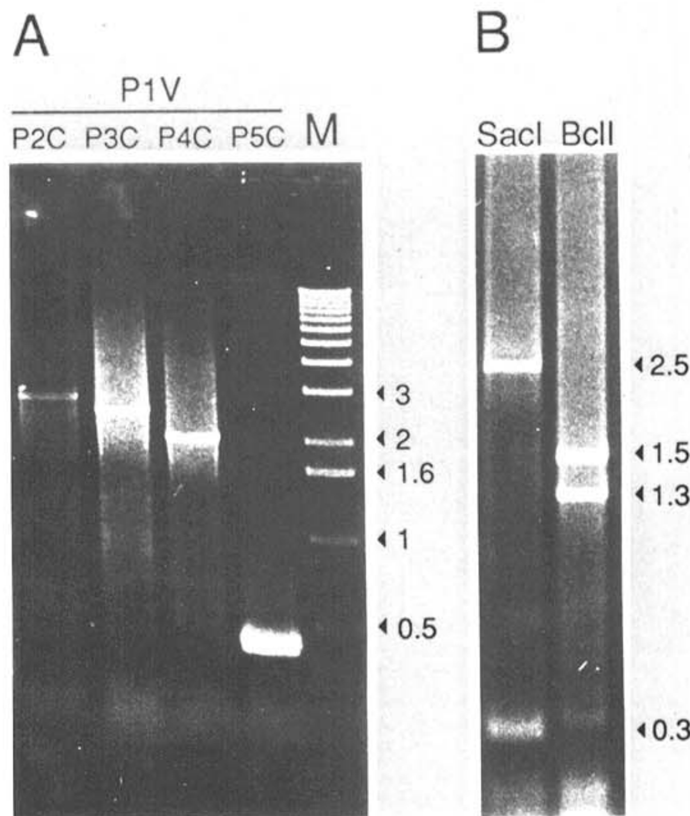

Fig. 2. A, Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified tomato yellow leaf curl virus (TYLCV) DNA from infected tomato. Primers were (from left to right) P1V and P2C; P1V and P3C; P1V and P4C; P1V and P5C. Lane M, DNA size marker (bp). **B,** Restriction endonuclease analysis of the PCR-amplified DNA from TYLCV-infected tomato produced with primers P1V and P2C. One-fifth of the reaction mix was treated with *Sac*I or *Bcl*I. Sizes (in kbp) of the restriction fragments are indicated.

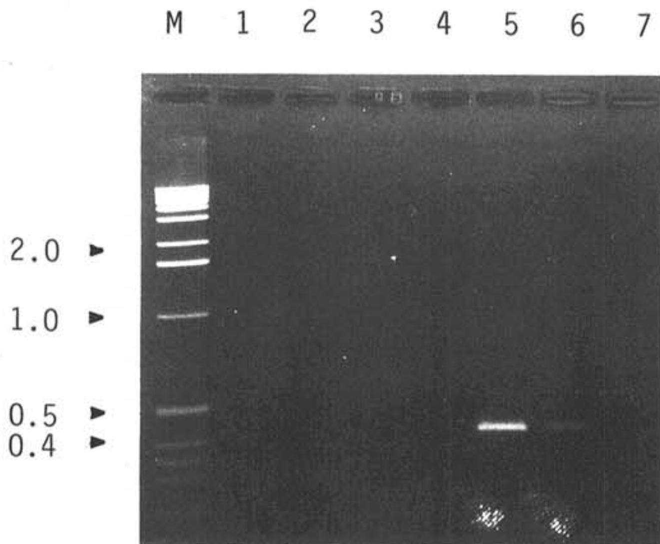

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified tomato yellow leaf curl virus (TYLCV) DNA from viruliferous whiteflies. Primers were P1V and P5C. Lane M, DNA size marker (bp). Lanes 1–7 contain PCR products from a single whitefly: whitefly reared on cotton plants (lanes 1 and 2); whitefly fed on uninfected tomato (lane 3) and on uninfected bean (lane 4); whitefly collected from field-infected bean (lane 5); whitefly after a 24-h acquisition feeding period on TYLCV-infected tomato, PCR with (lane 6) and without primers (lane 7).

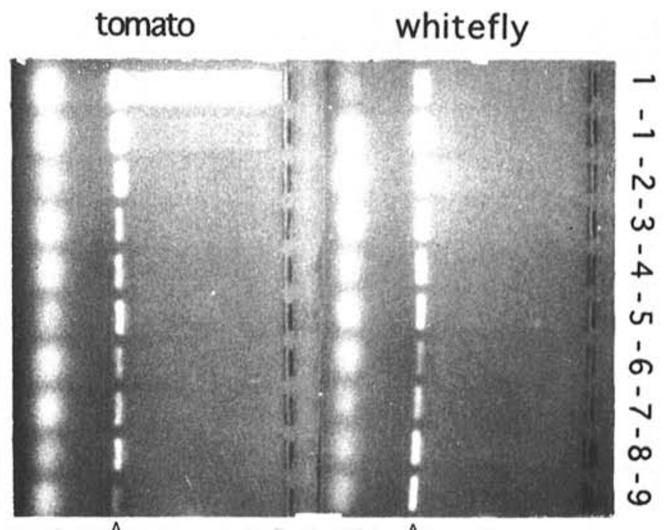

Fig. 4. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified tomato yellow leaf curl virus (TYLCV) DNA from a single whitefly and from infected tomato after serial 10-fold DNA dilutions from 10^{-1} (−1) to 10^{-9} (−9). Primers were P1V and P5C. Upper panel, whitefly after a 24-h acquisition feeding period on infected tomato (1); lower panel, 4.5 μg of DNA from an infected tomato (1). The amplified DNA fragment of about 450 bp is indicated by an arrowhead at the bottom.

three primers (Fig. 2A, lanes P3C, P4C, P5C) were their expected lengths: about 2,150 nt, 1,650 nt, and 440 nt, respectively.

Amplification of TYLCV DNA from individual viruliferous whiteflies. We amplified TYLCV DNA from total nucleic acid extracts of individual whiteflies (about 50 ng of DNA per insect [17]) given 24-h acquisition periods on a TYLCV-infected tomato plant by using primers P1V and P5C (Fig. 3). A fragment of about 440 bp was amplified; the size was as expected from the location of the primers on the TYLCV genome (Fig. 1).

We tested the applicability of the protocol for epidemiological studies by assaying whiteflies from fields of bean cultivars in the Israel Coastal Plain suspected of containing TYLCV. TYLCV DNA was amplified from a total nucleic acid extract of individual insects with primers P1V and P5C. A DNA fragment of about 440 bp was amplified, and it had the same mobility as that of the fragment amplified from whiteflies fed on TYLCV-infected tomato (Fig. 3). The identity of the amplified TYLCV DNA fragments was confirmed by hybridization with a TYLCV DNA probe and by restriction enzyme analysis (not shown).

Threshold of TYLCV detection in plants and whiteflies by PCR. We attempted to determine the threshold of TYLCV detection in whiteflies by mixing DNA from a single viruliferous whitefly with DNA from whiteflies reared on uninfected plants, so as to achieve serial 10-fold dilutions of the viruliferous insect DNA. After a 1:1,000 dilution, the PCR signal obtained with primers P1V and P5C was still very strong (not shown); however, further dilutions were not practical because of the large number of insects needed. Alternatively, DNA from a single viruliferous whitefly and from an infected tomato plant were subjected to serial dilutions (10^{-1}–10^{-11}) in water. TYLCV DNA was amplified from plant and insect DNA diluted up to 10^{-9} (Fig. 4). No signal was obtained with dilutions of 10^{-10} and 10^{-11}.

DISCUSSION

We have shown that PCR enabled us to amplify the complete genome of TYLCV from DNA extracts of infected tomato plants. PCR was also used to amplify TYLCV DNA fragments from nucleic acid extracts of individual whiteflies.

Because the tomato yellow leaf curl disease is present in a wide belt of tropical and subtropical regions from western Africa to Taiwan (6), it is important that phytopathologists and breeders are able to identify, type, and compare the local TYLCV isolates. DNA sequence comparison of TYLCV from Italy, Israel, and Thailand has shown that these isolates have between 71 and 77% nucleotide identity (9), much less than is expected from isolates of the same virus. This heterogeneity may be an obstacle to the breeding for TYLCV resistance. The ability to amplify the complete genomic molecule of TYLCV from plant extracts will help identify new TYLCV isolates and perform phylogenetic analyses of TYLCV worldwide. This should also be possible, by using degenerate primers, where sequences have diverged. The capsid protein of the Israeli, Italian, and Thai TYLCV isolates present three conserved regions between amino acids 11–17, 67–76, and 221–246. Sequences from these three regions may provide sites for primers that will facilitate the analysis of the heterogeneous TYLCV isolates.

TYLCV DNA can be detected in a single insect by Southern blot hybridization, with a threshold of detection in the range of 0.5 pg of DNA or 300,000 viral genome copies (17). PCR amplified viral sequences to detectable levels in DNA from a single whitefly diluted up to 10^{-9}. Theoretically, a single viruliferous insect could be detected in a population of several million individuals. In our studies, we detected one infected insect in 1,000 individuals.

PCR amplification of TYLCV in a single whitefly has allowed us to identify a TYLCV infection of beans in open fields. Although beans have been grown near tomato fields for many years, TYLCV infection of these plants was not shown until 2 yr ago (*unpublished*).

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