Generation of cDNAs Specific to Lettuce Infectious Yellows Closterovirus and Other Whitefly-Transmitted Viruses by RT-PCR and Degenerate Oligonucleotide Primers Corresponding to the Closterovirus Gene Encoding the Heat Shock Protein 70 Homolog

T. Tian, V. A. Klaassen, J. Soong, G. Wisler, J. E. Duffus, and B. W. Falk

First, second, third, and sixth authors: Department of Plant Pathology, University of California, Davis 95616; fourth and fifth authors: U.S. Department of Agriculture ARS, 1636 E. Alisal St., Salinas, CA 93915.
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ABSTRACT

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Oligonucleotide primers were designed based on nucleotide sequences corresponding to the conserved phosphate 1 and 2 motifs contained within the closterovirus-encoded heat shock protein 70 (HSP70) homolog. These primers were used with RNAs extracted from virus-infected plants and reverse-transcriptase polymerase chain reaction to generate specific cDNAs for lettuce infectious yellows closterovirus (LIYV) and for four additional whitefly-transmitted viruses for which corresponding nucleotide sequence data were unavailable: tomato infectious chlorosis virus (TICV), cucurbit yellow stunting disorder virus (CYSDV), beet pseudo-yellows virus (BPYV), and lettuce chlorosis virus. The resulting cDNAs of approximately 600 bp, corresponding to LIYV, TICV, CYSDV, and BPYV, were cloned, and the nucleotide sequences were determined. Computer-assisted analysis of the deduced amino acid sequences showed that all exhibited significant similarity to the HSP70 proteins in general and to the HSP70 homologs encoded by closteroviruses in particular. Comparative alignments showed the amino acid sequences for LIYV, TICV, BPYV, and CYSDV were more similar to each other than to the corresponding regions for the HSP70 homologs of three aphid-transmitted closteroviruses. Digoxigenin-11-UTP–labeled transcripts were generated from each cloned cDNA and used in RNA and dot blot hybridization analyses. Probes for LIYV, TICV, BPYV, and CYSDV hybridized only with double-stranded RNAs or extracts of plants infected with the corresponding virus.

A number of viruses that cause yellowing symptoms in plants are transmitted by specific whitefly vectors. The best known of these is lettuce infectious yellows closterovirus (LIYV), which is transmitted by the sweet potato whitefly, *Bemisia tabaci* Gennadius (13). During the early 1980s, LIYV occurred in epidemic proportions and caused losses totaling $8 million to lettuce, sugar beet, melon, and squash crops in the southwestern United States in a single season (13). In addition to LIYV, other whitefly-transmitted yellowing viruses include tomato infectious chlorosis virus (TICV, transmitted by *Trialeurodes vaporariorum* Westwood [14,32]), beet pseudo-yellows virus (BPYV, transmitted by *T. vaporariorum* [25]), cucurbit chlorotic spot virus (CCSV, transmitted by *T. vaporariorum* [33]), sweet potato sunken vein virus (SPSVV, transmitted by *B. tabaci* [9]), cucurbit yellow stunting disorder virus (CYSDV, transmitted by *B. argentifolii* Bellows & Perring [20]), and lettuce chlorosis virus (LCV, transmitted by *B. tabaci* and *B. argentifolii* [15]). In recent years, it has been suggested that these viruses represent an emerging threat to worldwide agriculture (8,17). Their incidences, as well as the incidences of their specific vectors, are expanding, particularly in temperate regions. Because many of these viruses have overlapping geographic as well as plant host ranges and can cause similar symptoms in many of their host plants, the ability to rapidly identify and differentiate these viruses is highly desirable.

With the exception of LIYV, these viruses are not generally well characterized. Many have been suggested as possible closteroviruses (6–8,16,25,32), but the individual viruses have been somewhat difficult to study, due in part to their obligate vector transmissibility to host plants, their phloem limitation in the host, their overall low titer within infected plants, and the difficulty in purifying sufficient quantities of their filamentous, threadlike flexuous virions to allow molecular characterization and development of serological and nucleic acid-based probes. Of these viruses, the complete nucleotide sequence has been reported for only LIYV (23). However, recent work with TICV, CYSDV, and SPSVV suggest that these viruses are similar to LIYV and that they are bipartite closteroviruses (E. Rodriguez-Cerezo, *personal communication*; [26,32]). BPYV recently has been partially characterized, and limited nucleotide sequence data are available (8). Although the complete nucleotide sequence of CCSV has been determined, it is not accessible (33). Limited characterization combined with the emerging importance of these viruses demonstrates the need for nucleic acid and serologically based methods and reagents to accurately identify and directly compare the various whitefly-transmitted RNA yellowing viruses.

If the whitefly-transmitted yellowing viruses are closteroviruses, they should share the closterovirus genome organization and composition, including the closterovirus-specific heat shock protein 70 (HSP70) homolog gene (1,12). Based on this hypothesis, we

Corresponding author: B. W. Falk; E-mail address: bwfalk@ucdavis.edu

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designed specific oligonucleotide primers that correspond to conserved motifs within the closterovirus HSP70 homolog. We used reverse-transcriptase polymerase chain reaction (RT-PCR) to generate cDNAs corresponding to LIYV, TICV, BPYV, CYSDV, and LCV. The resulting cDNAs for LIYV, TICV, BPYV, and CYSDV were cloned, and their nucleotide and deduced amino acid sequences were determined and compared. We also generated probes from each cloned cDNA and used them to identify and differentiate the respective whitefly-transmitted viruses in extracts of infected plants.

MATERIALS AND METHODS

Virus and vector maintenance. Colonies of *B. tabaci*, *B. argentifolii*, and *T. vaporariorum* were maintained as previously described (10,13,25). LIYV was maintained by transmission to lettuce (*Lactuca sativa* L. 'Summer Bibb') plants by *B. tabaci*; BPYV was transmitted to *Nicotiana clevelandii* Gray plants by *T. vaporariorum*; CYSDV was transmitted to *Cucumis sativus* L. 'National Pickling' plants by *B. argentifolii*; LCV was transmitted to *N. clevelandii* plants by *B. tabaci*; and TICV was transmitted by *T. vaporariorum* to *Lycopersicon esculentum* Mill. plants. Beet yellows closterovirus (BYV) was maintained by transmission to *Tetragonia expansa* Murr. plants by *Myzus persicae* Sulzer, and citrus tristeza closterovirus (CTV) double-stranded RNA (dsRNA) was provided by A. Rowhani (University of California, Davis).

RNA extraction and analysis. Total RNAs were extracted from healthy and virus-infected plants by methods described previously (18), and dsRNAs were extracted as described by Valverde et al. (31). Total or dsRNAs were analyzed by polyacrylamide or glyoxal-dimethyl sulfoxide denaturing agarose gel electrophoresis as described (19). RNA hybridization analysis was done with dsRNAs and denaturing agarose gel electrophoresis, followed by capillary transfer in 10× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0) to Hybond N (Amersham Corp., Arlington Heights, IL) membranes (29). Samples for dot blot analysis were prepared based on the RNA dot blot protocol for Hybond N, with minor modifications. Tissues (0.15 g) were ground in extraction buffer (0.056

Phosphate 1 Motif

	Amino Acid Sequence
BYV	G L D F G T T
CTV	G L D F G T T
LIYV	G L D F G T T

	Nucleotide Sequence
BYV	5' GGT TTA GAC TTC GGC ACC ACT 3'
CTV	5' GGT TTA GAC TTC GGT ACC ACG 3'
LIYV	5' GGC TTA GAT TTC GGT ACT ACT 3'
HSP-P-1 Primer	5' GGN TTA GAN TTC GGN ACN AC 3'

Phosphate 2 Motif

	Amino Acid Sequence
BYV	F G G G T F D
CTV	F G G G T F D
LIYV	F G G G T F D

	Nucleotide Sequence
BYV	5' TTC GGA GGT GGA ACG TTT GAC 3'
CTV	5' TTC GGC GGT GGG ACT TTT GAT 3'
LIYV	5' TTT GGA GGA GGT ACC TTT GAT 3'
HSP-P-2 Primer	3' AAN CCN CCN CCN TGN AAA CT 5'

Fig. 1. Comparison of amino acid sequences within the conserved phosphate 1 and 2 motifs of the closterovirus heat shock protein 70 homologs and design of corresponding primers for reverse-transcriptase polymerase chain reaction. Partial amino acid sequences containing identical amino acid sequences are shown for the respective motifs. The corresponding nucleotide sequences for each virus are shown below the amino acid sequences. BYV = beet yellows closterovirus (accession X73476); CTV = citrus tristeza closterovirus, Florida isolate T36; and LIYV = lettuce infectious yellows closterovirus (accession U15441). The primer (HSP-P-1 and HSP-P-2) nucleotide sequences are given; N indicates the degenerate position where the primer included A, G, C, or T. HSP-P-1 is of positive polarity, and HSP-P-2 is complementary to the corresponding virus nucleotide sequence.

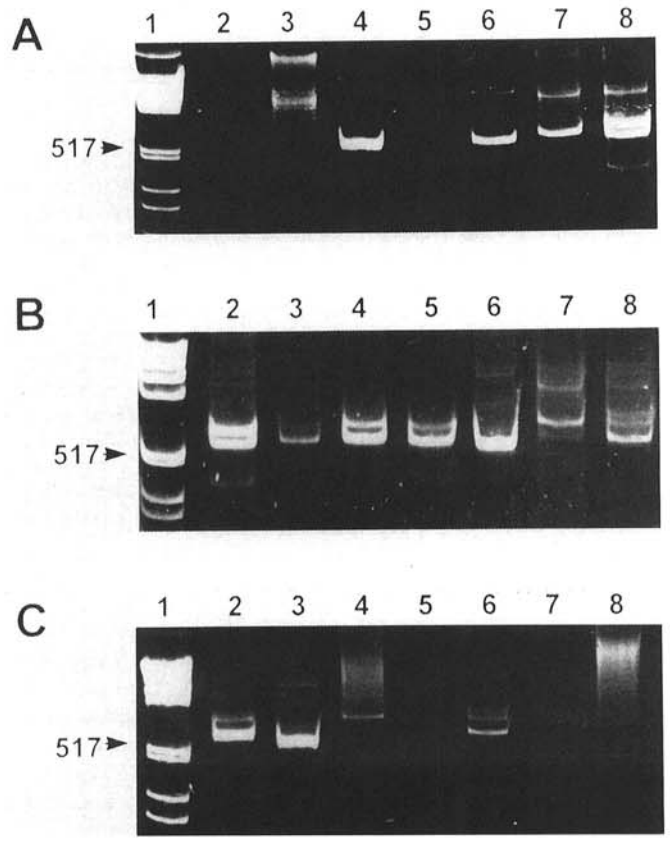

Fig. 2. Polyacrylamide gel electrophoresis analysis of reverse-transcriptase polymerase chain reaction (RT-PCR)-generated cDNAs corresponding to various whitefly-transmitted viruses. **A through C,** lane 1, DNA size markers, arrows indicate the 517-bp marker. **A,** Total RNAs from healthy and virus-infected plants were used for reverse transcription with primer HSP-P-2. Complementary DNAs were amplified with primers HSP-P-2 and HSP-P-1. Lanes 2 and 3, RT-PCR–generated DNAs from healthy *Nicotiana clevelandii* and *Cucurbita pepo* plants, respectively. Lane 4, PCR-generated DNA for lettuce infectious yellows closterovirus (LIYV) cloned DNA, pSP6. Lanes 5 through 8, RT-PCR–generated cDNAs for beet pseudo-yellows virus (BPYV)-infected *N. clevelandii* plants, LIYV-infected *Lactuca sativa* plants, tomato infectious chlorosis virus (TICV)-infected *Lycopersicon esculentum* plants, and cucurbit yellow stunting disorder virus (CYSDV)-infected *C. pepo* plants, respectively. **B,** RT-PCR–generated cDNAs from double-stranded RNAs (dsRNAs) extracted from plants infected with CYSDV, citrus tristeza closterovirus, TICV, beet yellows closterovirus, LIYV, BPYV, and lettuce chlorosis virus (LCV), lanes 2 through 8, respectively. **C,** RT-PCR–generated cDNAs from dsRNAs extracted from healthy and virus-infected plants. Lanes 2 through 8, RT-PCR products for dsRNAs of TICV, LIYV, zucchini yellow mosaic potyvirus-infected *C. pepo* plants, tobacco mosaic tobamovirus-infected *N. tabacum* plants, LCV, healthy *Lycopersicon esculentum* plants, and healthy *C. pepo* plants, respectively.

M MOPS [morpholinepropanesulfonic acid], 0.014 M sodium acetate, 0.003 M EDTA, pH 7.0) followed by phenol/chloroform clarification and centrifugation (12,000 × *g* for 5 min). One hundred microliters of the aqueous phase was removed and diluted with 150 µl of deionized formamide/formaldehyde (75/9%), and samples were heated at 65°C for 5 min. Samples were diluted with an equal volume of 20× SSC, and two replicate 100-µl samples were spotted on Hybond N membranes, baked at 80°C for 1 h, and used for hybridization analysis.

RT-PCR. Oligonucleotide primers were designed based on conserved regions of the phosphate 1 and 2 motifs of the HSP70 homologs encoded by the genomes of CTV, BYV, and LIYV (Fig. 1; [5,21,23]). Oligonucleotide primers were designed so each primer contained 20 nucleotides with degeneracies (any of the four possible nucleotides) at the nonidentical positions. The phosphate 1 primer (HSP-P-1) had four degeneracies and was of positive polarity; the phosphate 2 primer (HSP-P-2) was complementary to the coding region and had five degeneracies (Fig. 1).

RT-PCRs were done with total RNAs and dsRNAs as templates. RNAs were denatured with methyl mercuric hydroxide, and reverse-transcription reactions were done in 20 µl at 37°C for 1 h, with 200 ng of primer HSP-P-2 and MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD [23]). Five microliters of the reverse-transcription reaction was removed and used for PCR. A cloned LIYV cDNA that corresponds to the LIYV RNA 2 HSP70 homolog coding region (pSP6 [23]) was used as a positive control template. PCR reactions were done in 100 µl, with 3 mM $MgCl_2$, 200 µM dNTPs, 200 ng each of primers HSP-P-1 and HSP-P-2, and 1 unit of *Taq* polymerase. PCR reactions were done by first incubating samples at 94°C for 4 min, followed by 26 cycles at 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min. A final incubation at 72°C was done for 10 min. Resulting DNAs were analyzed by polyacrylamide gel electrophoresis (22).

Cloning, generation of probes, and hybridization analyses. RT-PCR–generated cDNAs for LIYV, TICV, CYSDV, and BPYV were cloned into pGEM-T (Promega, Madison, WI). RT-PCR–generated DNAs were mixed directly with T-tailed pGEM-T at a 1:1 molar ratio, and ligations were done per the manufacturer's instructions. The resulting plasmids were transformed into *Escherichia coli* DH5α, and cells were placed on Luria-Bertani plates containing 100 µg of ampicillin per ml (29). Recombinant colonies were selected, and plasmids were purified, digested with *Sac*II and *Nde*I, and analyzed by polyacrylamide gel electrophoresis (29).

Plasmids containing cDNAs of the predicted size (~600 bp) were used to generate probes for use in RNA and dot blot hybridization assays. Plasmids were digested with *Nde*I, and digoxigenin-11-UTP–labeled (DIG-labeled) transcripts were generated by T7 RNA polymerase and the Genius 4 RNA labeling kit (Boehringer Mannheim, Indianapolis, IN), except each reaction also contained 20 units of RNasin (Promega). Opposite polarity probes were generated by digesting plasmids with *Nco*I, and DIG-labeled transcripts were generated by SP6 RNA polymerase. Hybridization reactions were done overnight at 55°C, with 30 to 50 ng of DIG-labeled transcript per ml of hybridization solution (23). Blots were washed, and hybridization reactions were detected with Lumigen PDD as described in the Genius System Users' Guide (Boehringer Mannheim).

Nucleotide sequence analyses. Nucleotide sequences of the cloned RT-PCR–generated DNAs for LIYV, CYSDV, TICV, and BPYV were determined by dideoxy sequence analysis (30) with the Sequenase kit (United States Biochemical Corporation, Cleveland). Deduced amino acid sequences were compared for similarity by the nonredundant sequence database at the National Center for Biotechnology Information, Bethesda, MD and BLASTP (2,3). Further comparisons were done by PileUp and Gap software from the University of Wisconsin Genetics Computer Group (GCG) package (11).

RESULTS

RT-PCR. When the deduced amino acid sequence of the LIYV HSP70 homolog was compared with the corresponding proteins of aphid-transmitted BYV and CTV, the phosphate 1 and 2 motifs were highly conserved (23). These motifs contain sequences of 9 and 12 amino acids, respectively, that are shared among LIYV, BYV, and CTV (23). Comparison of the nucleotide sequences for the corresponding coding regions showed there was also a high degree of nucleotide sequence identity. There were sequences of seven amino acids in each motif in which the corresponding nucleotide sequences showed only four changes in the region corresponding to the phosphate 1 motif and five changes in the region corresponding to the phosphate 2 motif for the three viral sequences (Fig. 1). Therefore, oligonucleotide primers were designed to be identical to the nucleotide sequence corresponding to the phosphate 1 motif (HSP-P-1), to be complementary to the sequence corresponding to the phosphate 2 motif (HSP-P-2), and to the degenerate at nucleotide positions that were not identical (Fig. 1).

Initial PCRs to test the HSP-P-1 and HSP-P-2 primers were done with LIYV RNA 2 cloned cDNA (pSP6) as the template. We consistently amplified DNA of the expected size (~600 bp) from the LIYV cDNA template (Fig. 2A, lane 4). DNAs of ~600 bp also were generated by RT-PCR when total RNAs from LIYV-, TICV-, and CYSDV-infected plants were used as templates; how-

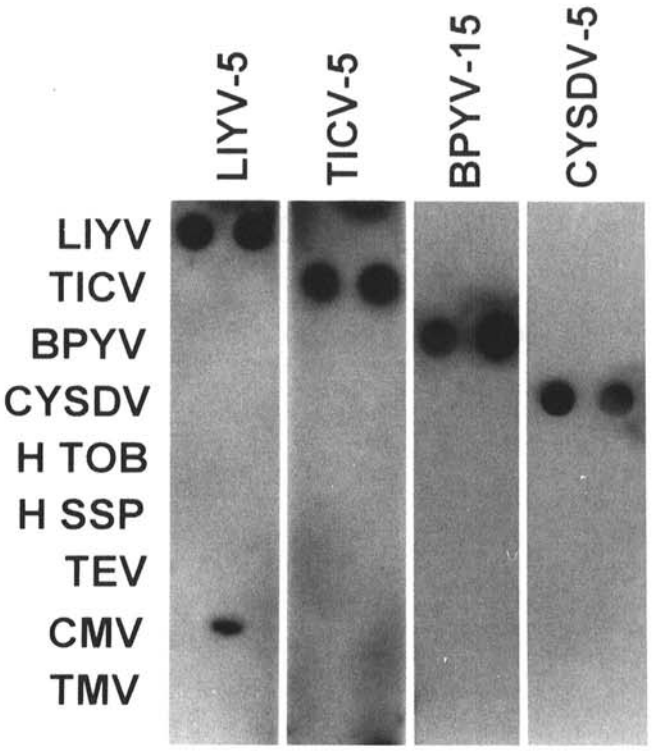

Fig. 3. Dot blot hybridization analysis of extracts from healthy and virus-infected plants with digoxigenin-11-UTP–labeled (DIG-labeled) RNA transcript probes. Transcripts were generated with T7 RNA polymerase and *Nde*I-digested reverse-transcriptase polymerase chain reaction clones for lettuce infectious yellows closterovirus (LIYV-5); tomato infectious yellows virus (TICV-5); beet pseudo-yellows virus (BPYV-15); and cucurbit yellow stunting disorder virus (CYSDV-5). Samples (horizontal rows) were applied to Hybond N membranes (Amersham) as four sets of paired replicate samples. The membrane was cut vertically into four identical replicates, and each was probed with the corresponding DIG-labeled probe (indicated at top). LIYV = LIYV-infected *Lactuca sativa* plants; TICV = TICV-infected *Lycopersicon esculentum* plants; BPYV = BPYV-infected *Nicotiana clevelandii* plants; CYSDV = CYSDV-infected *Cucurbita pepo* plants; H TOB = healthy tobacco (*N. benthamiana*) plants; H SSP = healthy pumpkin (*C. pepo* cv. Small Sugar) plants; TEV = tobacco etch potyvirus-infected *N. benthamiana* plants; CMV = cucumber mosaic cucumovirus-infected pumpkin plants; and TMV = tobacco mosaic tobamovirus-infected *N. benthamiana* plants.

ever, only minor amounts of product were obtained for total RNAs from BPYV-infected plants (Fig. 2A). Two DNAs of similar electrophoretic mobility (~600 bp) always were obtained for CYSDV (Fig. 2A, lane 8). Although we attempted to optimize RT-PCR conditions for using the degenerate primers, nonspecific products were sometimes obtained, particularly when using total RNAs from healthy or virus-infected plants. However, these were different in size and overall quality relative to the virus-specific cDNAs (Fig. 2A, lane 3).

When dsRNAs were used as the RT-PCR templates, the resulting cDNAs were similar to those obtained for total RNAs. However, we also consistently obtained an abundant DNA product for BPYV (Fig. 2B, lane 7), although it appeared to be of slightly slower electrophoretic mobility than the other ~600-bp RT-PCR–generated DNAs. Similar to the results for total RNAs, two closely migrating DNAs were seen for CYSDV and sometimes for other samples (e.g., TICV; Fig 2B, lane 4). When we included dsRNAs extracted from plants infected by the aphid-transmitted closteroviruses CTV and BYV, we were able to generate DNAs of similar size (Fig. 2B, lanes 3 and 5, respectively). When RT-PCR was performed with the HSP-P-1 and HSP-P-2 primers and dsRNA extracts from healthy plants, no distinct DNA products resulted (Fig. 2C, lanes 7 and 8). However, when dsRNAs for other viruses (tobacco mosaic tobamovirus and zucchini yellow mosaic potyvirus) were used as templates, some RT-PCR–generated DNAs were detected (Fig. 2C, lanes 4 and 5), but they were of various sizes and often variable in quantity.

Cloning RT-PCR products and RNA hybridization analysis. To determine whether the RT-PCR–generated DNAs were specific for each of the whitefly-transmitted viruses, we cloned the RT-PCR–generated cDNAs for LIYV, TICV, BPYV, and CYSDV. Analysis of the resulting transformants showed that several con-

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                phosphate 1
CYSDV     GLEFGTTFSTISSYVNGVMKVLKLNETEFIPTCLAITSNNDVV-VGGPAQVLS-NSDMPN
BPYV      GLEFGTTFSTISSFTNGEMKTLYVNNSPYIPTCLSISSEGDVI-IGSAAQVID-ESEVKS
LIYV      GLDFGTTFSTVSTLVNNSMYVLRLGDSAYIPTCIAITPGGEAI-IGGAAEVLS-GDDTPH
TICV      GLEFGTTFSTISAYVNGKMFELPLNNTTYISTCIAITSNSDAV-IGGAAEILS-SDVSPH
BYV       GLDFGTTFSSVCAYVGEELYLFKQRDSAYIPTYVFLHSDTQEVAFGYDAEVLSNDLSVRG
BYSV      GVDFGTTFSSVCVFNSGRLHVFKQQNSAYIPTCLFLYSDTMAMSFGYDAETASLDPNVKG
CTV       GLDFGTTFSTVAMATPSELVILKQFNSSYIPTCLLLHAEPNSVSYGYDAEYLA-ASGESG
Cons.     GUBFGTTFS$U.......U..&....$.@I.T.U.U......U..G..A..........
          HSP-P-1→

CYSDV     CYFYDLKRWVGVDSINYNVIKTKINPAYVTELRG------NDVYITGIDRGYTCTYTVKQ
BPYV      CYFCDLKRWVGVDATNFLVIKEKIKPLYVVKLVG------NDVYLTGVNKGFSCTYTVKQ
LIYV      CFFYDLKRWVGVDDNTFKFAMNKIRPKYVAELVE------GEVYLTGINKGFSIKLSVKQ
TICV      CFFYDLKRWVGVDEKSFDVAKKKIKPKYSVELKG------SEVYITGINKGYSTTLSVRQ
BYV       GFYRDLKRWIGCDEENYRDYLEKLKPHYKTELLKVAQSSKSTVKLDCYSGTVPQNATLPG
BYSV      GFFRDLKRWVGCDETNIEEYKSKLKPHYSVTLSNFGKGSRKIPTLGSYSGSVQMSGSLSG
CTV       SFYKDLKRWVGCTAKNYQTYLHKLSPSYKVIVKEFGTKSVPVPYLSPLNNDLGLSVALPS
Cons.     .@@.DLKRWUG..........KU.P.Y...U..........U..&...&.....U..

                                                        Connect 1
CYSDV     LILLYIETLVRLFSKVESITITSLNVSVPADYKCKQRMFMKSVCDSLGFSLRRIINEPSA
BPYV      LILLFIDTMVRLFSKTNNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSA
LIYV      LIKAYIETIVRLLASSYSLRVIDLNQSVPADYKNAQRLAARSVLKALSFPCRRIINEPSA
TICV      LIKAYIETIVKLFAKTFNLQIRDLNQSAPADYKCAQRLFARSVLNSLSFPCRRIINEPSA
BYV       LIATFVKALISTASEAFKCQCTGVICSVPANYNCLQRSFTESCVNLSGYPCVYMVNEPSA
BYSV      LIALFIQALVKSAAIEFKCECTELIVSVPANCDCMQRLFTENCVNLSGFTCVHMMNEPSA
CTV       LIASYAKSILSDAERVFNVSCTGVICSVPAGYNTLQRAFTQQSISMSGYSCVYIINEPSA
Cons.     LI..@...UU.............U..S.PA.....QR..........@....UUNEPSA

          ________          phosphate 2
CYSDV     AAIYFVSKY-PQYNNFLMYDFGGGTFDSSLIVRDGKYVTVVDTEGDSFLGGGTF
BPYV      AAIYSVSKY-PQHNYFIMYDFGGGTFDTSLITRDGQYVTVADTEGDSFLGGGTF
LIYV      AAVYCVSRY-PNYNYFLVYDFGGGTFDVSLIGKYKSYVTVIDTEGDSFLGGRDI
TICV      AAVYCVSRY-PEYDYFLVYDFGGGTF
BYV       AALSACSRIKGATSPVLVYDFGGGTFDVSVISALNNTFVVRASGGDMNLGGRDI
BYSV      AALSTCGRTDMSARNLLVYDFGGGTFDVSVLSSLNQTFTVRASGGDMNLGGRDV
CTV       AAYSTLPKLNSADKYLAVYDFGGGTFDVSIVSVRLPTFAVRSSSGDMNLGGRDI
Cons.     AA&....J.......&.UYDFGGGTFD.SUU......&.V..$.GD..LGG....
                    ←HSP-P-2
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Fig. 4. PileUP (University of Wisconsin Genetics Computer Group) alignments of deduced amino acid sequences (between the phosphate 1 and 2 motifs) of cloned cDNAs for reverse-transcriptase polymerase chain reaction clones for cucurbit yellow stunting disorder virus (CYSDV-5); beet pseudo-yellows virus (BPYV-34); and tomato infectious yellows virus (TICV-5). Lettuce infectious yellows virus (LIYV), beet yellows virus (BYV), beet yellow stunt virus (BYSV), and citrus tristeza virus (CTV) show amino acid sequences for corresponding regions of the HSP70 homologs (23). Regions corresponding to the phosphate 1, connect 1, and phosphate 2 motifs are indicated below labeled horizontal lines (5). Consensus sequences are indicated below, with U indicating bulky aliphatic residues (I, L, V or M); $ indicating threonine or serine; & indicating bulky hydrophobic residues (I, L, V, M, F, Y, or A); J indicating positively charged residues (K or R); @ indicating aromatic residues (F, Y, or W); and B indicating negatively charged residues (D or E). The deduced sequences for regions corresponding to those used in primer design are indicated in italics with HSP-P-1 and HSP-P-2 below.

tained plasmids with cDNAs similar in size to those generated by RT-PCR. Interestingly, we obtained cloned cDNAs that corresponded in size to each of the two products seen for the CYSDV RT-PCR–generated DNAs (data not shown).

To confirm that the plasmids contained virus-specific cDNAs, the recombinant plasmids were used to generate DIG-labeled transcripts for use as hybridization probes. Separate polarity transcripts were generated for each plasmid with SP6 and T7 RNA polymerases and were used in hybridization analyses with the virus dsRNAs and in dot blot assays with extracts from virus-infected plants. Hybridization analysis with dsRNAs showed that each of the probes was specific and hybridized only with dsRNAs for the corresponding virus (data not shown). When the probes were used in dot blot analyses of healthy and virus-infected plants, probes for a given virus hybridized only with extracts from plants infected with the corresponding virus (Fig. 3). Positive hybridization signals were obtained with probes of both polarities. No hybridization signals were seen for healthy control plant extracts or plants infected with other viruses (Fig. 3).

Nucleotide and amino acid sequence analyses. Because the above results confirmed that we had cloned specific cDNAs for each virus from the RT-PCR–generated cDNAs, we determined and compared the nucleotide and deduced amino acid sequences for each cDNA to determine whether the cloned sequences corresponded to regions encoding for HSP70 homologs. Nucleotide sequence analysis showed that the cloned cDNAs were 581 bp for LIYV (LIYV-5), 593 bp for TICV (TICV-5), 675 and 677 bp for two BPYV clones (BPYV-15 and BPYV-34, respectively), and 593 and 677 bp for two CYSDV clones (CYSDV-4 and CYSDV-5, respectively). Analysis of the LIYV-5 nucleotide sequence indicated that it lacked 12 nucleotides corresponding to the 5′ terminus of primer HSP-P-1. Comparison of BPYV-15 and BPYV-34 indicated three sequence heterogeneities. Two resulted in amino acid changes, whereas the third was a 2-bp deletion in BPYV-15; therefore, BPYV-34 was used for further sequence analysis. CYSDV-5 extended 84 nucleotides beyond the 3′ terminus of CYSDV-4; however, the sequences of both clones were identical in the overlapping regions, except for changes in the degenerate primer positions. CYSDV-4 had G, T, and C at nucleotide positions 3, 9, and 18, whereas CYSDV-5 had A, G, and G at the same positions. CYSDV-4 had A, T, and A at nucleotide positions 576, 585, and 588, whereas CYSDV-5 had T, A, and C at the same positions. The nucleotide sequences for BPYV-34, CYSDV-5, and TICV-5 were deposited with GenBank under accessions U67447, U67448, and U67449, respectively.

Computer-assisted translation of the nucleotide sequences showed that the cloned cDNA for each virus was representative of an open reading frame. Database comparisons of the deduced amino acid sequences by BLASTP showed that all exhibited significant similarity ($P < e^{-10}$) to the HSP70 homologs of BYV, CTV, and LIYV, as well as with other proteins of the HSP70 family, suggesting that the cDNAs for each virus were representative of the expected HSP70 homolog coding region. When these sequences and corresponding regions of LIYV, BYV, CTV, and beet yellow stunt closterovirus (BYSV) were aligned by PileUp, the presence of the conserved motifs was evident (phosphate 1, connect 1, and phosphate 2; Fig. 4). Because the 5′- and 3′-terminal regions for each clone resulted from the degenerate primers during RT-PCR, the terminal amino acid sequences for each cDNA were expected to correspond to the phosphate 1 and 2 motifs. LIYV-5, lacking 12 nucleotides of the HSP-P-1 primer, lacked 4 corresponding amino acids for the phosphate 1 motif. In addition, CYSDV-5 and BPYV-34 included 84 nucleotides beyond the HSP-P-2 primer site, corresponding to 28 amino acids beyond the phosphate 2 motif (Fig. 4). Examination of the two sequences beyond the phosphate 2 motif showed the C-terminal amino acid sequence to be GGGTF. This sequence also occurs within the phosphate 2 motif and corresponds to the region covered by primer HSP-P-2. Thus, the HSP-P-2 primer could have annealed at this location downstream of the nucleotide sequence corresponding to the phosphate 2 motif and initiated reverse transcription at this site, ultimately giving rise to the larger RT-PCR–generated DNAs for BPYV and CYSDV. Comparison of the deduced amino acid sequences for CYSDV-5 and BPYV-34 between the phosphate 2 motif and their C termini with similarly positioned sequences for the CTV, BYV, and LIYV HSP70 homologs showed that consensus amino acid residues (23) were present in the CYSDV-5 and BPYV-34 sequences, providing additional evidence that these clones are representative of a HSP70 homolog coding region and arose by primer HSP-P-2 binding at the second site during reverse transcription.

We also used Gap to compare the amino acid sequences corresponding to the regions between the phosphate 1 and 2 motifs. The sequences for the whitefly-transmitted viruses were more similar to LIYV and to each other than they were to the corresponding regions of the HSP70 homologs of CTV, BYSV, and BYV (Table 1).

DISCUSSION

The approach reported here, RT-PCR generation of virus-specific cDNAs with primers designed to correspond to conserved nucleotide sequences, previously has proven successful for studies on many other plant viruses, including potyviruses, luteoviruses, geminiviruses, and even aphid-transmitted closteroviruses (4,21, 24,27,28). Because closteroviruses are the only viruses of plants or animals known to contain a gene encoding for HSP70 homologs, this seemed to be a likely region of the closterovirus genome to target for RT-PCR. Indeed, Karasev et al. (21) demonstrated that for the aphid-transmitted closteroviruses BYV, BYSV, and CTV homologous nucleotide sequences within the HSP70 homolog gene (corresponding to HSP70 phosphate 1 and connect 2 motifs [the terminology used by Karasev et al. (21) was motifs A and E; we use the more recent terminology for HSP70 motifs suggested by Bork et al. (5)]) could be targeted in RT-PCR assays for generation of specific cDNAs. However, Karasev et al. (21) were unable to detect by RT-PCR cDNAs for the whitefly-transmitted LIYV and BPYV. Comparison of the nucleotide sequences for LIYV and

TABLE 1. Gap comparisons[a] between deduced amino acid sequences for regions between the heat shock protein 70 phosphate 1 and 2 motifs for whitefly- and aphid-transmitted closteroviruses[b]

	LIYV	TICV	CYSDV	BPYV	BYV	CTV	BYSV
LIYV	1						
TICV	0.71 (0.82)	1					
CYSDV	0.57 (0.72)	0.63 (0.77)	1				
BPYV	0.56 (0.73)	0.59 (0.75)	0.72 (0.85)	1			
BYV	0.37 (0.57)	0.41 (0.60)	0.37 (0.54)	0.35 (0.57)	1		
CTV	0.36 (0.55)	0.36 (0.53)	0.36 (0.53)	0.38 (0.54)	0.48 (0.62)	1	
BYSV	0.37 (0.57)	0.42 (0.59)	0.36 (0.55)	0.38 (0.57)	0.58 (0.73)	0.44 (0.62)	1

[a] Comparisons were made by the Gap program (University of Wisconsin Genetics Computer Group). Numbers show amino acid identity, and numbers in parentheses show similarity for each virus pair.

[b] LIYV = lettuce infectious yellows closterovirus; TICV = tomato infectious yellows virus; CYSDV = cucurbit yellow stunting disorder virus; BPYV = beet pseudo-yellows virus; BYV = beet yellows closterovirus; CTV = citrus tristeza closterovirus; and BYSV = beet yellow stunt closterovirus.

the aphid-transmitted closteroviruses showed a greater degree of nucleotide conservation for the regions corresponding to the phosphate 1 and 2 motifs than for other regions (including the connect 2 motif). Therefore, we designed oligonucleotide primers deduced from amino acid sequences for the conserved phosphate 1 and 2 motifs of the closterovirus-encoded HSP70 homologs. When the resulting primers were used in RT-PCR assays, we were able to generate cDNAs for LIYV, CTV, BYV, and four other whitefly-transmitted viruses: TICV, CYSDV, BPYV, and LCV.

Previous studies led to suggestions that TICV, BPYV, CYSDV, and LCV are like LIYV, whitefly-transmitted closteroviruses (E. Rodriguez-Cerezo, *personal communication*; [7,13,25,32]). Our data provide additional supporting evidence for this suggestion. Not only did we generate cDNAs for these viruses by RT-PCR and closterovirus degenerate primers, we cloned and characterized the cDNAs for CYSDV, TICV, and BPYV. Our data demonstrate that the genomes of these viruses contain regions coding for HSP70 homologs (at least between the phosphate 1 and 2 motifs). Although we did not attempt to clone the RT-PCR–generated DNA for LCV, it is highly likely that this DNA also corresponded to this region. Furthermore, our comparisons of the deduced amino acid sequences between the phosphate 1 and 2 motifs showed that this region of the HSP70 homolog is more similar among whitefly-transmitted LIYV, TICV, BPYV, and CYSDV than to the corresponding region of aphid-transmitted BYV, CTV, and BYSV HSP70 homologs. It remains to be seen whether the similarities and differences between aphid- and whitefly-transmitted closteroviruses prove to be consistent when more sequence information becomes available and is compared.

These results have immediate practical applications. Several whitefly-transmitted viruses that induce yellowing symptoms in plants are now emerging as economically important viruses (8,17). Based on their biological properties, many of these may prove to be whitefly-transmitted closteroviruses. The various whitefly vectors for these viruses are generally not easily differentiated morphologically, and all of these viruses have wide host ranges and cause similar symptoms on many of their common host plants (e.g., LIYV, CYSDV, TICV, and LCV cause similar symptoms in lettuce). Thus, visual identification/differentiation is not straightforward. We were able to use RT-PCR and degenerate primers to generate sequences and clone specific cDNAs for TICV, BPYV, and CYSDV—viruses for which no corresponding nucleotide sequence data were known but which were predicted to be closteroviruses. This approach also allowed us to develop reagents that have proven useful for differentiating plants infected by these viruses. It is likely that more whitefly-transmitted yellowing viruses will be discovered, and the RT-PCR approach used here may provide a means to rapidly gain important taxonomic information as well as provide a way to diagnose specific infections.

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