Identification of Cucumber Mosaic Virus Subgroup I Isolates from Banana Plants Affected by Infectious Chlorosis Disease Using RT-PCR

Zora Singh, Western Australian State Agricultural Biotechnology Centre, School of Biological and Environmental Sciences, Murdoch University, Perth, Western Australia 6150; **R. A. C. Jones,** Western Australian Department of Agriculture, Baron-Hay Court, South Perth, Western Australia 6151; and **M. G. K. Jones,** Western Australian State Agricultural Biotechnology Centre, School of Biological and Environmental Sciences, Murdoch University, Perth, Western Australia 6150

ABSTRACT

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Reverse transcription-polymerase chain reaction (RT-PCR) assays, restriction enzyme analysis, and comparison of nucleotide sequences were used to identify isolates of cucumber mosaic cucumovirus (CMV) that belong to subgroup I from infectious chlorosis–affected banana plants. Infected samples were from irrigated banana plantations at Carnarvon in the semiarid Gascoyne region of central Western Australia, and from Kununurra in the northern tropical Kimberley region. An RT-PCR assay with primers designed in a conserved region of the 3′ end of the CMV coat protein gene amplified a 486- to 488-bp DNA fragment from infected banana samples. CMV was detected in leaf blades, midrib, and pseudostem of infected plants, but not in asymptomatic plants. Restriction enzyme analysis of PCR products using *Eco*RI and *Msp*I showed that the samples tested from diseased banana were infected with CMV subgroup I isolates. This result was confirmed by sequencing PCR-amplified products, which showed 98% homology to sequences of CMV subgroup I isolates but only 76% homology to sequences of CMV subgroup II isolates. The RT-PCR is a reliable assay to detect CMV in banana plants and, when combined with restriction enzyme digestion, provides a simple means of identifying the CMV subgroup present.

Additional keywords: heart rot, *Musa*, sheath rot

Cucumber mosaic cucumovirus (CMV) is a multicomponent virus with a single-stranded positive-sense RNA genome consisting of three RNA species plus a fourth subgenomic RNA (17). Numerous isolates differing in host range and pathogenicity have been reported. They fit into two major serotypes, subgroups I and II. Subgroup I, typified by isolate DTL, occurs predominantly in the tropics and subtropics, while subgroup II, typified by isolate ToRS, is prevalent in temperate regions (5).

Infection with CMV is found in a wide range of crops, including banana, in irrigated plantations at Carnarvon in the semiarid Gascoyne region of central Western Australia. This region produces 9% of the Australian banana crop, and average banana fruit yields are among the highest in the world (2,14). Infectious chlorosis disease of banana was first reported in New South Wales, Australia, in 1930 (11), and later in South and Central America, the Caribbean, India, and the Philippines (1,3,13,17). In Western Australia, it was first reported in 1988 (2). Infectious chlorosis disease of bananas caused by CMV (11,12), which has been confused with zinc deficiency in bananas at Carnarvon in the past, occurs in most plantations, with up to 10% of plants affected. Symptoms include yellow streaking or mosaic patterns on leaves, often with a curling of leaf margins. In cool weather, rotting of heart leaves and the center of the pseudostem (heart rot or sheath rot) also occurs. Symptoms are often restricted to suckers. Fruits do not normally form on infected plants, but when they do they develop necrotic spots and rings. Infected banana planting stock and infected alternative host plants in banana plantations constitute the primary sources of infection. CMV is readily aphid-transmitted to and from banana (1).

Polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) assays (8,22,23), which involve the enzymatic amplification of a DNA fragment defined by two oligonucleotide primers, have been used to diagnose a number of plant viruses (e.g., 15,20,21), including CMV (19,30).

The aims of this work were (i) to develop an RT-PCR assay for detection of CMV in diseased banana plants and (ii) to identify and characterize CMV isolates from banana. RT-PCR, restriction enzyme analysis of amplified PCR fragments, and comparison of nucleotide sequences were used to characterize CMV isolates.

Address correspondence to M. G. K. Jones.

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MATERIALS AND METHODS

Plant samples and CMV isolates. Leaf samples of banana *Musa* sp. AAA 'Cavendish' group cv. Williams showing symptoms of infectious chlorosis were collected from different plantations in Carnarvon and from one plantation at Kununurra in the northern, tropical Kimberley region of Western Australia (Fig. 1). Isolates SN, LY, LI, LD, and LW of CMV subgroup II, originally obtained from infected narrow-leafed lupins (*Lupinus angustifolius*) or subterranean clover (*Trifolium subterraneum*), and CMV subgroup I isolate SL from subterranean clover came from previous work (9,10,30). Virus cultures were maintained in subterranean clover cv. Woogenellup or *Nicotiana glutinosa*; CMV isolates from banana were maintained in *N. glutinosa.*

CMV indexing on *N. glutinosa* and by enzyme-linked immunosorbent assay (ELISA). Sap was extracted from banana leaves in (1:2 wt/vol) 0.1 M phosphate buffer, pH 7.2, containing 0.1% (vol/vol) thioglycollic acid and mechanically inoculated in the presence of celite to four *N. glutinosa* plants. Indicator plants were grown at 15 to 20°C in a glasshouse. Inoculation tests were repeated three times.

Fig. 1. Map of Western Australia showing the location of banana growing areas at Carnarvon and Kununurra.

Symptoms caused by CMV isolates from banana were compared with symptoms caused by CMV isolate SL (subgroup I) and CMV LW, LY, LI, LD, and SN isolates (subgroup II) maintained in *N. glutinosa.*

To detect CMV by ELISA, banana leaf samples were ground (1 g per 5 ml) in 0.5 M sodium citrate, pH 6.8, containing 5 mM EDTA, 20 g of polyvinyl pyrrolidone per liter, 1.5 ml of Tween 20 per liter, and 2 ml of thioglycollic acid per liter, and the extracts were tested by DAS-ELISA as described previously (9), using 0.5 mg of *p*-nitrophenyl phosphate per ml in 10 ml of diethanolamine per liter, pH 9.8, as substrate. Absorbance (A_{405}) values were measured in a Titertek Multiskan photometer (Flow Laboratories, Australian Biosearch, Perth, Western Australia). The polyclonal CMV antiserum used was supplied by the late R. I. B. Francki, Waite Agricultural Research Institute, Adelaide, South Australia. It readily detects isolates belonging to both subgroups of CMV (10).

RNA extraction, reverse transcription, and PCR amplification. Total nucleic acids, including RNA, of CMV were extracted from infected and healthy banana tissue using a modification of previously described procedures (26,30). Five-millimeter-diameter disks were obtained with a cork borer from leaf blade, midrib, and pseudostem material. The disks were placed in 1.5-ml microcentrifuge tubes and macerated with a glass rod in 500 µl of extraction buffer (50 mM Tris HCl, pH 8.5, 10 mM EDTA, and 200 mM NaCl), vortexed for 5 s, and centrifuged at 14,000 × *g* for 1 min. After centrifugation, the aqueous phase was transferred to a new microcentrifuge tube containing 500 µl of phenol:chloroform:isoamyl alcohol (50:50:1) and then treated as described previously (30).

The reverse transcriptase reaction and PCR amplifications were done using the primers and the protocol described by Wylie et al. (30). Using CMV primer 1 (downstream): 5′-GCCGTAAGCTGGATGGACAA-3′ and CMV primer 2 (upstream): 5′-TATGATAAGAAGCTTGTTTCGCG-3′, the predicted length of the amplified DNA fragment is 499 to 502 bp for CMV subgroup II isolates and 486 to 488 bp for CMV subgroup I isolates. Ten microliters of the amplified PCR products was separated on a 2% agarose gel in TBE buffer (24) with ethidium bromide (1 µg of buffer per ml). A ladder of 123 bp and digests of øX174 RF by *Hae*III and of Lambda DNA by *Hin*dIII were used as size markers.

Restriction digests of PCR products. PCR-amplified fragments were digested by restriction endonucleases *Bam*HI, *Hin*dIII, *Eco*RI, and *Msp*I (Bethesda Research Laboratories, BRL) using 6-µl amplified DNA fragments diluted twofold in sterile distilled water, 2 µl of appropriate buffer (10X, BRL), and 0.7 µl of enzyme. All digestions were at 37°C for 2 h. Digests were analyzed by gel electrophoresis as described earlier. Fragment sizes were estimated from size markers and from sequenced PCR products.

Sequencing and computer analysis. Sequencing of amplified PCR products corresponding to a fragment of the CMV coat protein gene was done by the dideoxy-chain termination reaction (25). Templates for sequencing were prepared using the Magic PCR Preps DNA purification system (Promega Corporation, Note 34, 1991, Madison, WI). Sequencing was done twice in both directions using PCR cycle sequencing reagents (Applied Biosystems, Melbourne, Victoria, Australia) and an ABI 273 DNA sequencer. The sequences obtained were analyzed and compared with published data using programs of the University of Wisconsin Genetics Computer Group (GCG) and Intelli Genetics (IG) via satellite linkage to the University of Georgia.

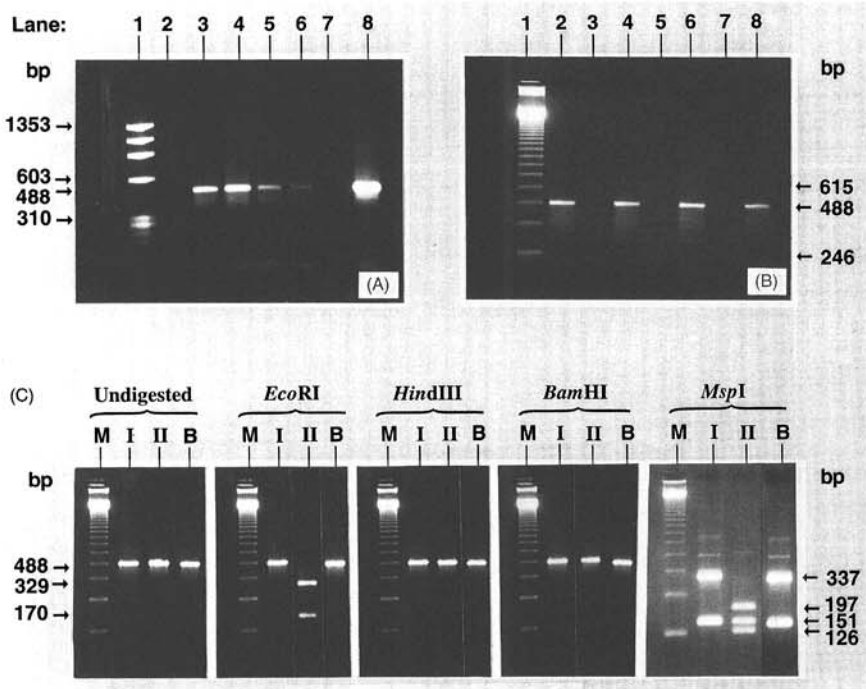

Fig. 2. Reverse transcription–polymerase chain reaction (RT-PCR) assays of cucumber mosaic cucumovirus (CMV). **(A)** Assays of CMV from banana leaf blades (isolate MR): lane 1, marker øX174 *Hae*III digest; lanes 2 to 6, plant extract containing viral RNA from infected leaf blade diluted 1, 32, 64, 128, and 256 times, respectively; lane 7, control from leaf blade of healthy leaf; lane 8, positive CMV control (isolate LY). **(B)** Assays of CMV from infected banana samples from different plantations: lane 1, 123 bp ladder marker; lanes 2 and 6, isolates from different plantations from North River Road (isolate NRR) and McGlade Road (MR), Carnarvon; lane 4, isolate from Kununurra (BK2); lanes 3, 5, and 7, healthy plants from the same respective locations; lane 8, CMV positive control (isolate LY). **(C)** RT-PCR of CMV followed by restriction enzyme digestion. Each panel consists of a 123-bp ladder marker lane (M), amplified cDNA of CMV subgroup I (isolate SL), CMV subgroup II (isolate LY), and banana isolate MR (B). The treatments (undigested or treated with restriction enzymes *Eco*RI, *Hin*dIII, *Bam*HI, and *Msp*I) are indicated above each panel. (The *Msp*I digestions were not quite complete, as weak bands of full-length amplified DNAs are also present.)

RESULTS

Symptoms in *N. glutinosa*. Eight isolates of CMV from banana and CMV subgroup I isolate SL caused obvious mottling and leaf distortion. Subgroup II isolates LY, LW, LI, LD, and SN caused mild mosaic and limited leaf distortion.

CMV detection by ELISA and RT-PCR. CMV was detected in 47 leaf blade samples from different infectious chlorosis–affected banana plants from 46 plantations at Carnarvon and one at Kununurra by ELISA (one sample per plantation). Twenty-nine of these samples were tested further by RT-PCR in three experiments.

In experiment 1, RT-PCR of 12 of these samples produced an expected single band of 486 to 488 bp (Fig. 2) from leaf midrib and pseudostem extracts diluted up to 256-fold and from leaf blade extracts diluted up to 64-fold (Fig. 2A). No products were detected from extracts of 12 symptomless banana plants. Purified CMV-RNA and extracts from CMV-infected *N. glutinosa* each yielded a PCR product similar in size to that of the 12 samples.

In experiment 2, leaf samples of five other CMV-infected and five symptomless banana plants from different plantations were separated into leaf blade, leaf midrib, and pseudostem and tested by RT-PCR. The CMV-specific product was detected in each of the five diseased samples, in leaf blade extracts diluted up to 64-fold, and in undiluted midrib and pseudostem extracts.

In experiment 3, extracts of the leaf blade, midrib, and pseudostem of single

CMV-infected and symptomless banana plants from each of 11 plantations near Carnarvon and from one near Kununurra were tested. The RT-PCR assay yielded a band of 486 to 488 bp from ELISA-positive plants, whereas no band was recorded from healthy plants (Fig. 2B, data shown from three locations only).

Restriction digests of PCR products. Representative undigested and *Eco*RI, *Bam*HI, *Hin*dIII, and *Msp*I digested PCR-amplified DNA fragments from banana MR and defined CMV subgroup I (isolate SL) and CMV subgroup II (isolate LY) are shown in Figure 2C. PCR products from samples of five CMV-infected banana plants (MR, NRR, B1, B6, B12) from different plantations were not digested by *Eco*RI or *Bam*HI, were slightly reduced in size (by 9 bp) when digested with *Hin*dIII (due to a *Hin*dIII site in primer 2), and yielded two bands of 335 to 337 and 151 to 152 bp when digested by *Msp*I (Fig. 2C). In contrast, the 499 to 502 bp PCR products of CMV isolates LY, LW, and SN, when digested with *Eco*RI, yielded two bands of 329 to 332 and 170 bp, were not digested with *Bam*HI, were reduced by 9 bp when digested with *Hin*dIII, and on digestion with *Msp*I, yielded three visible bands of 197, 151, and 126 bp and a sequence-predicted band of 25 to 28 bp. When subgroup I (isolate SL) was digested as above, the fragment patterns obtained were identical to those from the banana isolates of CMV (Fig. 2C).

Comparison of nucleotide sequences. The sequences of the amplified CMV coat protein gene segments of CMV isolates from banana (Fig. 3) included the 3′ end of the open reading frame ending at position 363 (marked by an arrow) and continued into the 3′ untranslated region. Comparison of the consensus sequence with published sequences showed 98, 97, and 97% homology with CMV-Y, O, and C strains of CMV subgroup I, respectively (6,7,18). In contrast, comparison with CMV subgroup II strains Q and WL indicated 77 and 75% homology, respectively. Sequence comparison of amplified CMV coat protein gene segments (Fig. 3) from four CMV isolates from banana revealed base changes at position 41 in two isolates (B6 and BK2), leading to a change in the coded amino acid from asparagine to serine and isoleucine, respectively. Isolate BM had two additional bases (A and G) in the noncoding region at positions 413 and 426, respectively.

DISCUSSION

On electrophoresis of RT-PCR products from CMV-infected banana plants, CMV RNA extracted from banana leaf blade, midrib, and pseudostem gave single bands of 486 to 488 bp. Dilution of extracts from leaf blades improved reliability, possibly by diluting inhibitory sap components, but dilution was not necessary with extracts from midrib and pseudostem. Dilution of inhibitory substances from host extracts was reported necessary for detection by RT-PCR of CMV from lupin seeds (30), plum pox virus in leaves of stone fruit trees (29), and bean yellow mosaic virus in gladiolus corms and leaves (27,28).

The banana plantations of the Carnarvon and Kununurra regions of Western Australia are among the most isolated in the world, and it is of interest to study the strain compositions of virus diseases present in such areas. Restriction analysis of PCR products provides a simple routine method to determine the CMV subgroup present. The restriction enzyme digest patterns obtained from banana CMV isolates and from a known CMV subgroup I isolate were those expected for CMV subgroup I isolates, whereas three isolates belonging to CMV subgroup II gave the patterns expected for subgroup II. The sequence information obtained from the 3′ end of the open reading frame and part of the 3′ untranslated region allowed differentiation between CMV subgroups. Computer analysis of the DNA sequence derived from banana isolates showed 97 to 98% homology with CMV subgroup I and 75 to 77% homology with CMV subgroup II. Moreover, the sequences of banana CMV isolates from Western Australia were more homologous to each other than to sequences of other published subgroup I isolates (4,16,18) with only a few base changes. On the basis of restriction digest analysis of the PCR products from CMV isolates from banana (using *Eco*RI and *Msp*I) and their nucleotide sequences, CMV-induced infectious chlorosis of banana in Western Australia is caused by CMV subgroup I. No subgroup II was found, although it has frequently been detected in the cooler southwestern corner of the state in other species (10,30). This is consistent with published information that CMV subgroup I isolates are found predominantly in the subtropics and tropics where Carnarvon and Kununurra are located, respectively (5).

We did not directly compare ELISA with RT-PCR in routine testing for CMV in bananas, but we suggest that RT-PCR may be an appropriate test where detection of very low levels of infection is required in large-scale propagation of CMV-free planting material.

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B1   TAT GAT AAG AAG CTT GTT TCG CGC ATT CAA ATT CGA GTT AAT CCT TTG CCG AAA TTT GAT  60
      Y   D   K   K   L   V   S   R   I   Q   I   R   V   N   P   L   P   K   F   D  20
B6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ... ... ... ... ... ...
BK2  ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ...
MR   ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ... ... ...

B1   TCT ACC GTG TGG GTG ACA GTT CGT AAA GTT CCT GCC TCC TCG GAC TTA TCC GTT GCC GCC  120
      S   T   V   W   V   T   V   R   K   V   P   A   S   S   D   L   S   V   A   A  40

B1   ATC TCT GCT ATG TTC GCG GAC GGA GCC TCA CCG GTA CTG GTT TAT CAG TAT GCT GCA TCT  180
      I   S   A   M   F   A   D   G   A   S   P   V   L   V   Y   Q   Y   A   A   S  60

B1   GGA GTC CAA GCT AAC AAC AAA TTG TTG TAT GAT CTT TCG GCG ATG CGC GCT GAT ATA GGT  240
      G   V   Q   A   N   N   K   L   L   Y   D   L   S   A   M   R   A   D   I   G  80

B1   GAC ATG AGA AAG TAC GCC GTC CTC GTG TAT TCA AAA GAC GAT GCA CTC GAG ACG GAC GAG  300
      D   M   R   K   Y   A   V   L   V   Y   S   K   D   D   A   L   E   T   D   E  100
                                                        *

B1   CTA GTA CTT CAT GTT GAC ATC GAG CAC CAA CGC ATT CCC ACA TCT GGG GTG CTC CCA GTC  360
      L   V   L   H   V   D   I   E   H   Q   R   I   P   T   S   G   V   L   P   V  120
          ↓
B1   TGA TTCCGTGTTCCCAGAACCCTCCCTCCGATTTCTGTGGCGGGAGCTGAGT-TGGCAGTTCTGC-TATAAACTGTCTG 437
B6   ... ..................................................-...........-...........
BK2  ... ..................................................-...........-...........
MR   ... ..................................................A...........G...........

B1   AAGTCACTAAACGTTTTACGGTGAACGGGTTGTCCATCCAGCTTACGGC  488
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Fig. 3. Sequence of reverse transcription–polymerase chain reaction (RT-PCR) amplified cDNA fragments of cucumber mosaic cucumovirus (CMV) banana isolates B1, B6, BK2, and MR. PCR fragments include the 3′ end of the coat protein gene, and part of the 3′ untranslated region. The sequence starts in the coat protein gene and terminates at position 363 as marked by an arrow: the corresponding amino-acids are given beneath the nucleotide sequence of isolate B1. Sequences vary at position 41 as indicated; for isolate MR, there are two additional bases (at positions 413 and 426) in the 3′ untranslated region, as indicated.

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