Detection of Sugarcane Mosaic Virus and Fiji Disease Virus in Diseased Sugarcane using the Polymerase Chain Reaction

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ABSTRACT

Smith, G. R., and Van de Velde, R. 1994. Detection of sugarcane mosaic virus and Fiji disease virus in diseased sugarcane using the polymerase chain reaction. Plant Dis. 78:557-561.

The reverse transcription–polymerase chain reaction (RT-PCR) was adapted for detection of sugarcane mosaic potyvirus (SCMV) and Fiji disease fijivirus (FDV) in total nucleic acid extracts from diseased sugarcane. The denaturation method developed for RT-PCR of FDV-specific double-stranded RNA (dsRNA) was suitable for the amplification of SCMV RNA and also amplified SCMV-specific replicative form (double-stranded) RNA. RT-PCR amplification of total nucleic acid extracts from SCMV-infected sugarcane yielded a 359-bp product and detected the presence of the virus in a $1:10^4$ dilution of a solution containing the total nucleic acids extracted from 250 mg of SCMV-infected tissue. Four primer pairs, selected to prime the synthesis of different regions of the segmented dsRNA genome of FDV, were evaluated; the oligonucleotide pair FDV7F and FDV7R, which prime the synthesis of a 450-bp fragment, gave the best result. FDV was detected in a $1:10^7$ dilution of a solution containing the total nucleic acids extracted from 250 mg of FDV-infected sugarcane tissue. Both viruses could be RT-PCR amplified from the same sample by including both primer pairs in the reaction solutions (duplex RT-PCR). The viruses were identified in the sample by the difference in size of the synthesized products, and the identity was confirmed by Southern blot hybridization with the appropriate probe.

Additional keywords: diagnosis, *Saccharum*

Sugarcane mosaic potyvirus (SCMV) and Fiji disease fijivirus (FDV) are important pathogens of sugarcane (*Saccharum* L. interspecific hybrids) in Australia, causing mosaic and Fiji disease respectively (2,15,16). SCMV belongs to the large and economically important potyvirus group and has long, flexuous virions, each containing one molecule of positive sense single-stranded RNA which is polyadenylated at the 3' end. The coat protein coding region of SCMV strain SC has been cloned and sequenced (3), and diagnostic cDNA probes have been developed to this region (13). FDV, type species of the plant reovirus subgroup 2 (Fijivirus) (5), has a segmented genome consisting of 10 linear molecules of double-stranded RNA (dsRNA) with the molecular mass of the segments ranging from 1.1×10^6 to 2.9×10^6 (1.7 to 4.4 kbp) with a total genome size of about 20×10^6 (30 kbp) (10). Diagnostic cDNA probes have been developed for the detection of FDV in sugarcane (12,13), and the sensitivity of detection has been significantly enhanced by reverse transcription–polymerase chain reaction (RT-PCR) amplification of target dsRNA prior to probing (14).

Both SCMV and FDV are of concern during the movement and quarantine of sugarcane germ plasm into and within Australia, as well as movement between and within many other countries. Symptoms of viral infection can take some time to develop, and ratooning of the plant is sometimes necessary to induce symptoms of FDV infection. Previous studies have shown that detection of these viruses in infected sugarcane plants is limited by the sensitivity of the probes and is influenced by the site from which the sample is removed (13). The potential of RT-PCR to enhance detection of FDV in sugarcane has already been demonstrated (14), while PCR-based tests have been applied to increase the sensitivity of detection of potyviruses such as plum pox virus (PPV) (18). Primer pair FDV7 primes the synthesis of three products from the FDV genome (14), which may result in a loss of sensitivity of detection. Because limited sequence data from other regions of the FDV genome were available, other primer pairs were selected for comparison with FDV7. Here, we report the conditions for routine RT-PCR amplification of SCMV- and FDV-specific RNA from samples of diseased sugarcane for the sensitive detection of these two viruses, as well as the RT-PCR amplification of diagnostic regions of these two viruses from the same sample (duplex RT-PCR).

Accepted for publication 17 December 1993.

MATERIALS AND METHODS

Primers. The Fiji disease virus clones pFDV7 and pFDV59 contain cDNA inserts of approximately 500 bp each in the *Sma*I site of plasmid pUC12 and hybridize within the 1.7-kbp doublet and the 3.7-kbp triplet bands of the FDV genome (Fig. 1), respectively (G. R. Smith, *unpublished*). The plasmid template was prepared by the method of Hattori and Sakaki (4). The ends of the cDNA inserts were sequenced by the dideoxy chain termination method with T7 DNA polymerase (Sequenase 2.0, United States Biochemical [USB]) or Taq DNA polymerase (TaqTrack, Promega), ^{35}S-ATP (Amersham), and M13 universal or reverse primers (USB or Promega). The FDV-specific PCR primers derived from the sequence data, including the FDV7 pair previously described (13), are listed in Table 1.

The SCMV primers were selected from the sequence of the SCMV strain SC coat protein coding region (3). SCMV- and FDV-specific primers were synthesized on either a Millipore 7500 DNA synthesizer or an Applied Biosystems Model 391 PCR-Mate DNA synthesizer. Oligo$(dT)_{16}$ and random hexamer primers were purchased from Perkin-Elmer Cetus (PEC).

Nucleic acid preparations, reverse transcription, PCR, blotting, and detection. Total nucleic acid extracts from sugarcane samples were prepared as previously described (14). Reverse transcription with Maloney-murine leukemia virus reverse transcriptase (MMLV) (PEC) or Superscript from Bethesda Research Laboratories (BRL) (Maloney-murine leukemia virus reverse transcriptase RNase H- [MMLV-SS]), was performed as described (13) in 50 mM KCl, 10 mM Tris-Cl, pH 8.3, buffer containing 200 U of enzyme, 5 mM $MgCl_2$, 2.5 mM each dNTP, 1 U RNase

Table 1. Primers and conditions for reverse transcription–polymerase chain reaction amplification of sugarcane mosaic virus and Fiji disease virus RNA

Primer pair	Sequence	Product size	Annealing/extension temperature for maximum sensitivity	
			Temperature (C)	Dilution[a]
S400-551 S400-910	5′-ACA CAA GAG CAA CCA GAG AGG 5′-AGT CAA AGG CAT ACC GCG CTA	359	60	$1:10^4$
FDV7F FDV7R	5′-CCG AGT TAC GGT CAG ACT GTT CTT 5′-CAA GTG GTG ACG AAA TGA TGG CAG	450	60	$1:10^7$
FDV727F FDV727R	5′-CTG GCA TTA TTA CGC TAA ACG TCT TGT 5′-GAT CTA CTA GAA ACG CAT TTG GAG CAT	360	60	$1:10^6$
FDV59F FDV59R	5′-TCA AGC TCA AGA GAA AGC AGT GGG 5′-CCT CCG CAA TTG GCA TAC CTC AAA	400	60	$1:10^6$

[a] Dilution of original solution containing total nucleic acids extracted from 250 mg of tissue.

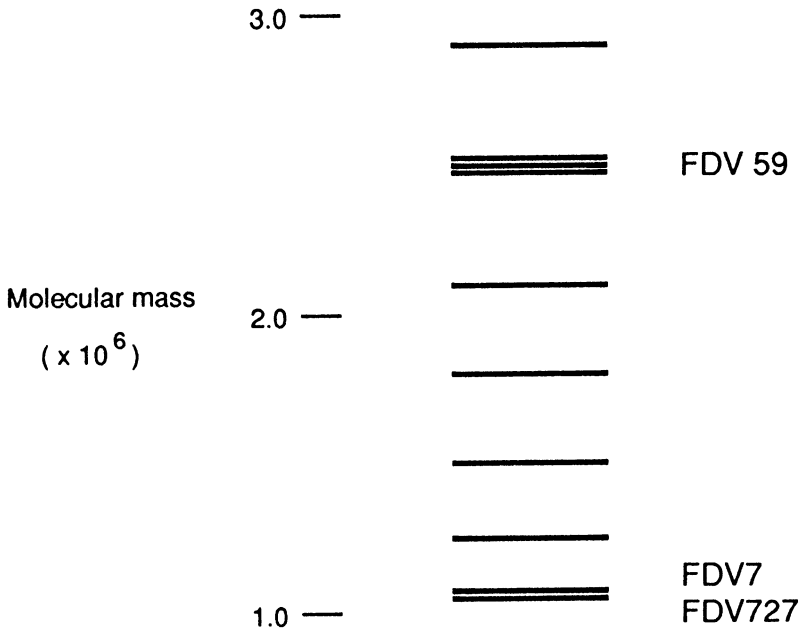

Fig. 1. Fiji disease virus genome organization. FDV-specific probes hybridize within segments 2,3,4 (FDV59) or 9,10 (FDV7, FDV727).

inhibitor (PEC) (1), and 10 pmol of each primer. Primers were boiled (100 C) with the template for 5 min, then quenched on ice. Conditions for reverse transcription in 20 μl of reaction solution were room temperature (ca. 20 C) for 10 min, 42 C for 60 min, 99 C for 5 min, and 5 C for 5 min. An 80-μl aliquot of reaction buffer containing 50 mM KCl, 10 mM Tris-Cl, and 2 mM $MgCl_2$ (unless varied), pH 8.3, (1) containing the FDV-specific primers (10 pmol each) and AmpliTaq (2.5 U), was added to the 20-μl solution from the reverse transcription step. PCR conditions were 95 C for 2 min followed by 30 cycles of 95 C for 1-min denature, variable (50, 55, 60, or 67 C) for 1-min anneal-extension, and 60 C for 7-min final extension. Magnesium ion concentration was varied by the addition of aliquots of a stock 25 mM solution of $MgCl_2$ to PCR buffer II (50 mM KCl, 10 mM Tris-Cl, pH 8.3) (1). Electrophoresis of products, alkaline blotting, and detection with the appropriate Bionick (BRL) labeled probe using a Photogene kit (BRL) were as previously described (14). Total nucleic acid extracts were serially diluted in Milli-Q (Millipore) water as described (14) for use in sensitivity end point assays.

Duplex RT-PCR of both viruses. Solutions for reverse transcription contained 5 pmol of each primer (FDV7F, FDV7R, S400-551, and S400-910) in a total volume of 20 μl. Conditions were as described above. For PCR, the RT solution was added to 80 μl of solution containing 5 pmol of each primer and thermal cycled as described above.

RESULTS

Primer design. Approximately 300 bases of each FDV-specific insert were sequenced, and primer pairs were selected from the sequence data (Table 1). The G + C ratio of the sequenced regions varied between 36 and 44%, so that selection of primer pairs with a G + C ratio of 50% was difficult. The G + C ratios of the selected FDV-specific primer pairs were either 40 or 50%, with the ratio within the pair the same. The SCMV-specific primer pair S400-551 and S400-910 was selected to amplify the central region of the coat protein coding region of SCMV (Fig. 2), a region that appears to be highly conserved at the amino acid level between SCMV strains (9).

RT-PCR amplification of the SCMV-specific RNA. SCMV-specific RNA was RT-PCR amplified from total nucleic acid extracts of virus-infected sugarcane using the boil/quench primer-template annealing technique developed for RT-PCR of FDV-specific dsRNA. Reverse transcription with MMLV appeared to yield more product than RT with MMLV-SS (Fig. 3). Primer S400-910 anneals to the single positive sense viral RNA of SCMV. Primer S400-551 anneals to the negative strand which occurs in replicative form (double-stranded) RNA. Reverse transcription reactions which contained one of the S400 primers (either S400-551 or S400-910) yielded cDNA from which the expected sized product was synthesized in the subsequent PCR step, although less product was produced in RT-PCR solutions that only contained S400-551 in the RT phase. Priming the reverse transcription phase with oligo$(dT)_{16}$ or random hexamers prior to PCR with the S400 pair resulted in similar amounts of DNA product to that produced by reverse transcription reactions primed with the S400 pair.

Effect of annealing/extension temperature and magnesium ion concentration. The three FDV primer pairs (FDV7, FDV727, and FDV59) and the SCMV-specific primer pair (S400) RT-PCR amplified FDV- or SCMV-specific RNA, respectively, from total nucleic acid extracts at PCR annealing and extension temperatures between 50 and 67 C, and magnesium ion concentrations between 1.0 and 5.0 mM. The optimal annealing and extension temperature was 60 C (Table 1). Magnesium ion concentration did not appear to be critical to efficient RT-PCR amplification of FDV-specific sequences (*data not shown*), although there did appear to be less product in the FDV7 pair primed

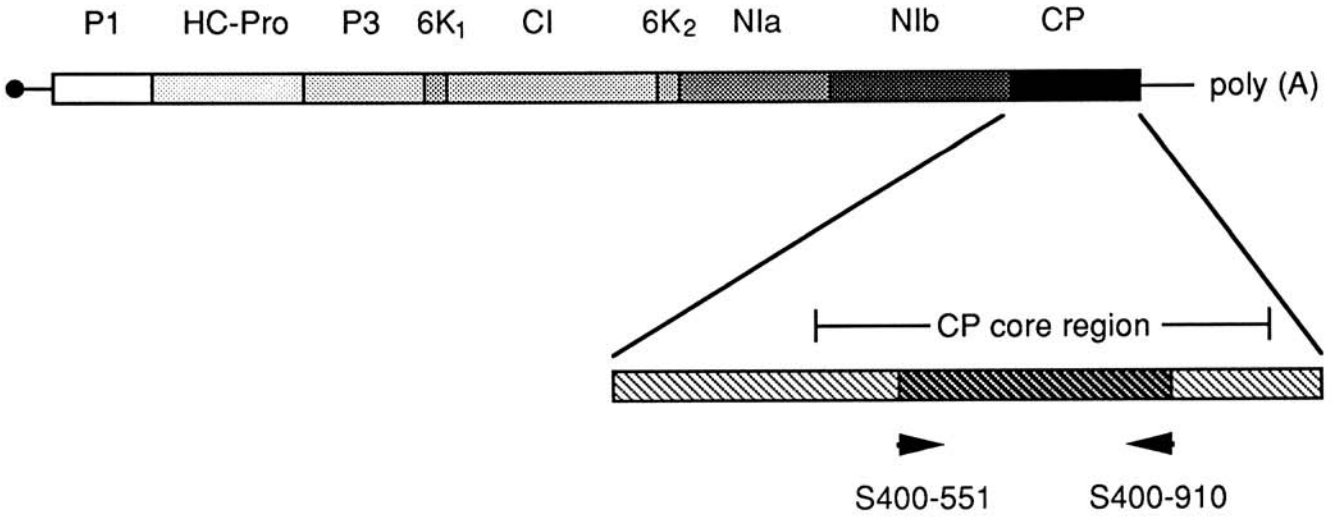

Fig. 2. Position of annealing sites of S400 primers within the coat protein coding region of sugarcane mosaic virus (SCMV). The position and relative sizes of the other proposed SCMV coding regions are based on the genetic map of plum pox virus (8). The core region of the coat protein is as defined (17). Abbreviations: P1, first protein; HC-Pro, helper component-protease; P3, third protein; $6K_1$, putative 6k peptide; CI, protein with RNA helicase activity; $6K_2$, second putative 6k peptide; NIa, nuclear inclusion 'a' protein (VPg and protease); NIb, nuclear inclusion 'b' protein (presumed RNA polymerase); and CP, coat protein. The genome has a 5'-terminal genome linked protein (VPg) and a 3' untranslated region between the coat coding region and the poly(A) tail.

PCR reactions with 1.0 mM magnesium ion. The SCMV-specific sequence was amplified in reactions containing magnesium ion concentrations between 1.5 and 4.0 mM, primed with the S400 oligonucleotide pair; no RT-PCR amplification occurred at 1.0 mM.

Sensitivity. The limit of detection of SCMV in dilutions of the original total nucleic acid extract as an equivalent sample of tissue was 400 ng ($1:10^4$ dilution of original extract), while the limit of detection for FDV was 0.4 ng of tissue ($1:10^7$ dilution of original extract).

Duplex RT-PCR of both viruses. SCMV- and FDV-specific cDNA were amplified from the same sample by RT-PCR (Fig. 4). Under the conditions used, considerably more FDV-specific product was produced than SCMV-specific product. Southern blots of the products hybridized with both probes (Fig. 4A), pS400 (Fig. 4B), or pFDV7 (Fig. 4C) confirmed the origin of the DNA fragments.

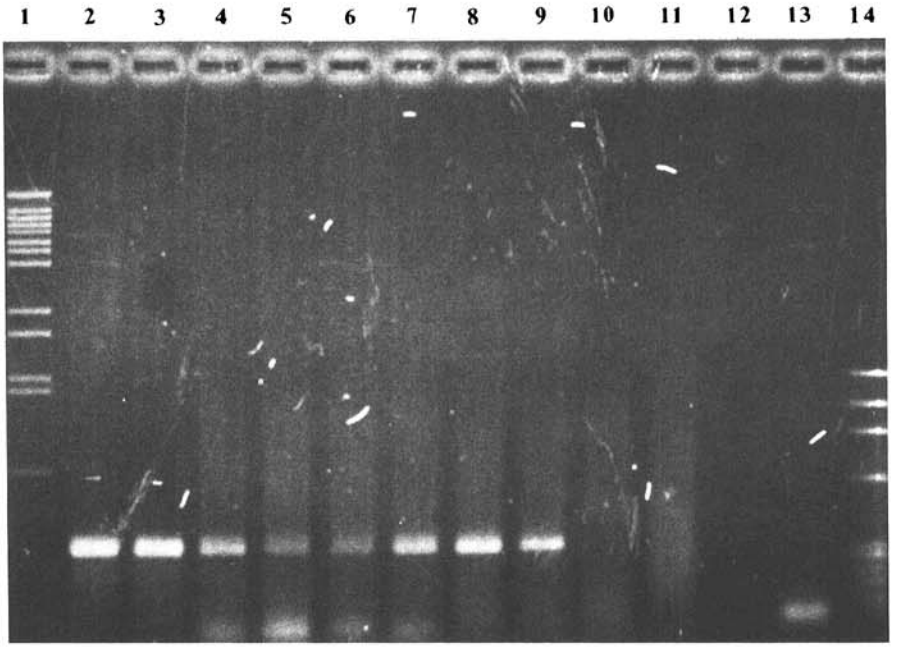

Fig. 3. The effect of different reverse transcription primers and enzymes on reverse transcription–polymerase chain reaction (RT-PCR) amplification of sugarcane mosaic virus (SCMV) specific RNA. Lane 1, λ/*Bst*EII DNA markers (150 ng total DNA); lanes 2,3, PCR positive control (pS400 plasmid) S400 primers, Perkin-Elmer Cetus's Maloney-murine leukemia virus (PEC MMLV); lane 4, SCMV in total nucleic acid extracts (TNAE), S400 primers, PEC MMLV; lane 5, SCMV in TNAE, S400 primers, Bio-Rad Laboratories (BRL) MMLV-SS; lane 6, SCMV in TNAE, S400-551; lane 7, S400-910; lane 8, oligo$(dT)_{16}$; lane 9, random hexamers; lane 10, TNAE from healthy plant, S400 primers; lane 11, PCR control no AmpliTaq; lane 12, PCR control pS400, no primers; lane 13, PCR control no template, S400 primers; lane 14, $\phi\chi174$/*Hae*III DNA markers (150 ng total DNA). Unless otherwise stated, RT reactions contained PEC MMLV (lanes 6–13).

DISCUSSION

Sugarcane mosaic potyvirus and Fiji disease fijivirus specific RNA sequence can be reliably amplified by RT-PCR from crude total nucleic acid preparations of infected sugarcane. The primer/template–boil/quench method developed for RT-PCR amplification of FDV dsRNA (14) was used to amplify SCMV-specific single-standed RNA and replicative form (double-stranded) RNA. This priming technique may be applicable to a wide range of samples from virus-infected plants, possibly increasing the sensitivity of detection by priming more of the viral-specific RNA present in the sample for reverse transcription and subsequent PCR amplification. This priming technique also appears to give the most consistent amplification of sugarcane bacilliform badnavirus DNA from sugarcane samples (K. S. Braithwaite, *personal communication*). Oligonucleotide primers were selected which directed synthesis of DNA fragments of the expected size. All the primer pairs gave the best results in reactions with the temperature of the annealing/extension phase at 60 C. The RT-PCR amplification of SCMV- or FDV-specific RNA from total nucleic acid extracts worked over a range of magnesium ion concentrations. Therefore, the S400 and FDV7 primer pairs should be able to be applied with confidence to sugarcane samples for detection of these two important pathogens.

The primer pair FDV7F and FDV7R, which amplifies the expected 450-bp

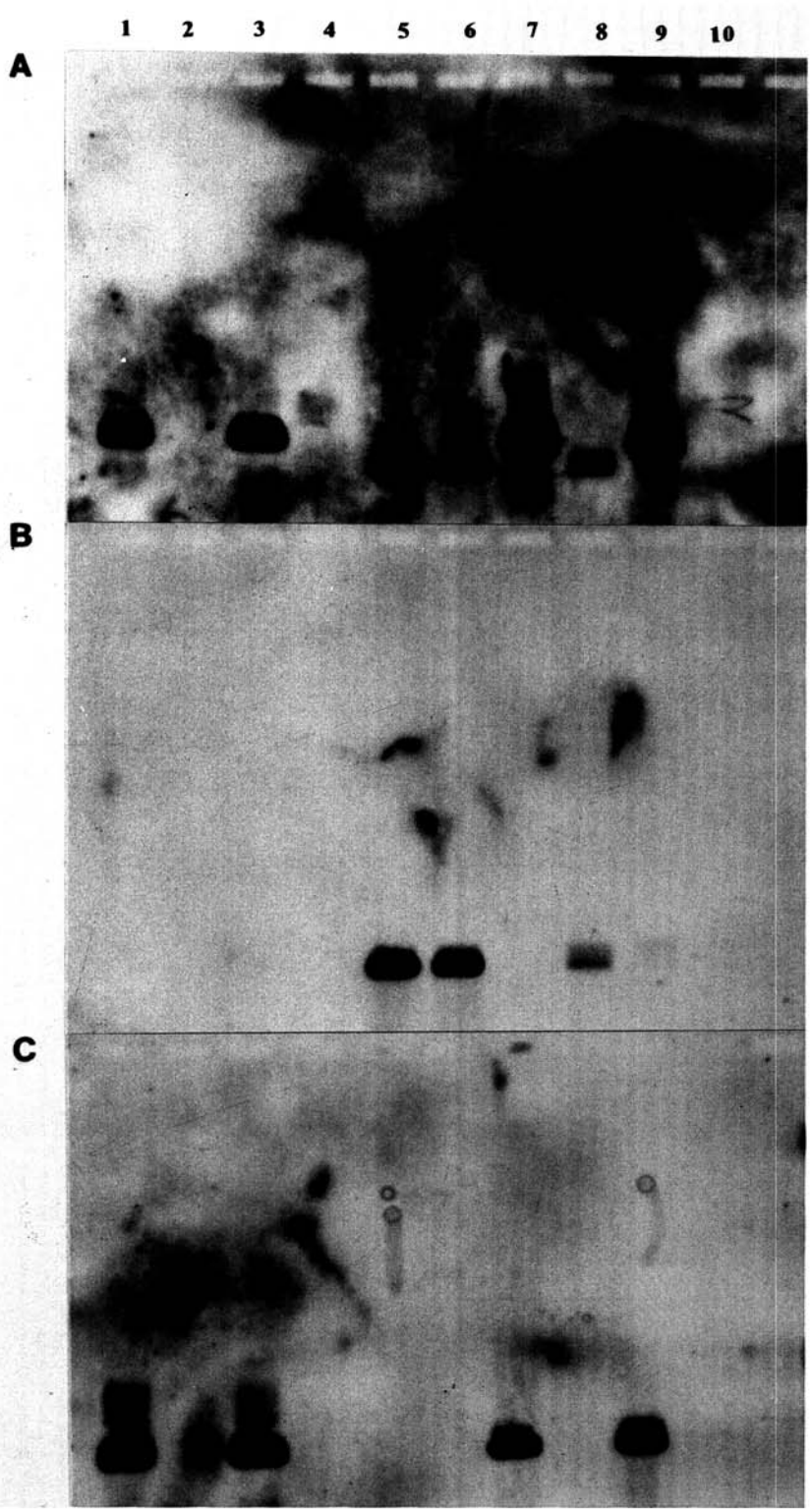

Fig. 4. Duplex reverse transcription–polymerase chain reaction (RT-PCR) of Fiji disease virus (FDV) and sugarcane mosaic virus (SCMV) from sugarcane samples. **(A)** Southern blot of RT-PCR products from FDV7 and S400 primed reactions probed with biotinylated pFDV7 and pS400. **(B)** Southern blot of RT-PCR products from S400 and FDV7 primed reactions probed with biotinylated pS400. **(C)** Southern blot of RT-PCR products from S400 and FDV7 primed reactions probed with biotinylated pFDV7. Lanes 1–9 contain total nucleic acid extracts from virus-infected sugarcane. Lane 1, FDV7 primers and FDV Total nucleic acid extracts (TNAE); lane 2, FDV7 primers and SCMV TNAE; lane 3, FDV7 primers and FDV/SCMV TNAE; lane 4, S400 primers and FDV TNAE; lane 5, S400 primers and SCMV TNAE; lane 6, S400 primers and FDV/SCMV TNAE; lane 7, FDV7/S400 primers and FDV TNAE; lane 8, FDV7/S400 primers and SCMV TNAE; lane 9, FDV7/S400 primers and FDV/SCMV TNAE; lane 10, PCR control FDV7/S400 primers and no template.

region as well as 150- and 270-bp fragments (14), gave the best results for detecting FDV. The FDV727 primer pair was selected in the expectation that the sensitivity of detection could be increased if only the expected product (360 bp) was synthesized. However, this oligonucleotide pair was 10-fold less sensitive than the FDV7 pair for detection of FDV in total nucleic acid extracts. The cause of this difference in sensitivity is unclear but may be due to the primers, as the selection of efficient and specific primers from nucleotide sequence is somewhat empirical (11). The SCMV-specific primers were designed to anneal to nucleotide sequences within the central region of the coat protein coding region. A number of strains of SCMV are characterized, with only strains A (strain SC[3]) and J (maize dwarf mosaic virus-A [MDMV-A]) recorded in Australia (7). The S400 primers were designed in anticipation of amplifying all strains of SCMV that may be present in sugarcane germ plasm. The central portion of the coat protein of SCMV strains shows strong homology at the amino acid level (9), although the degree of homology at the nucleotide level is unknown, as the coat protein region of strain A (=SC) is the only reported sequence (3). SCMV-specific cDNA was PCR amplified following reverse transcription primed with a variety of oligonucleotides. Oligo$(dT)_{16}$, which hybridizes to the polyadenylated 3′ tail; random hexamers; S400-910, which primes the SCMV positive sense ssRNA strand; and the S400 pair all resulted in approximately the same amount of DNA after PCR. The S400-551 primer hybridizes to the negative strand of SCMV, which is only present in the replicative form (double-stranded) RNA of this virus. S400-551 primed reverse transcription of cDNA for subsequent PCR amplification. Priming the negative strand as well as the positive strand did not appear in this instance to significantly increase the amount of SCMV-specific DNA produced by RT-PCR, as dsRNA is a small portion of the SCMV-specific total RNA (J. L. Dale, *personal communication*).

RT-PCR amplification of viral-specific RNA prior to Southern hydridization with biotinylated probes was more sensitive than Northern or manual blotting for detection of virus in sugarcane samples. RT-PCR was at least 10^3-fold more sensitive for detection of SCMV and up to 10^6-fold more sensitive for detection of FDV in total nucleic acid extracts from infected plants (13). In template dilution experiments in which the products of RT-PCR solutions, which originally contained diminishing amounts of original virus-infected sugarcane extracts, were blotted and probed, there did not appear to be an increase in sensitivity in the Southern blot over the agarose gel. This was possibly the

result of efficient PCR amplification of cDNA present in low concentrations to a level visible on an ethidium bromide stained gel, with the reverse transcription step being template concentration dependent; or alternatively, the specific activity of the probes is low. The difference in the level of sensitivity of detection between the two viruses is probably the result of three factors: 1) the amount of virus in the plant; 2) ssRNA may have been degraded by RNase prior to reverse transcription (dsRNA is highly resistant to RNase degradation in 150 mM NaCl [1× SSC, which is 0.15 M NaCl plus 0.015 M sodium citrate][6], and the NaCl concentration used in the extraction buffer during this study was 100 mM [1× STE]); and 3) for dsRNA, two priming sites exist for primer-specific reverse transcription, doubling the potential cDNA available for subsequent PCR amplification. Sugarcane mosaic virus and Fiji disease virus are major concerns to the Australian sugar industry because of the demonstrated crop losses caused by these viruses, and because of the restrictions imposed on the movement of germ plasm. RT-PCR amplification of viral-specific RNA sequences from infected sugarcane primed by the S400 and FDV7 primers is a reliable, sensitive, and specific technique for detecting SCMV and FDV infection in sugarcane. The primer pairs can be used in duplex so that two viruses can be detected in the same RT-PCR solution, which may be important for the economics and logistics of screening germ plasm. The method described here can now be used for the practical screening of germ plasm for the presence of these two important pathogens of sugarcane.

ACKNOWLEDGMENTS

We thank James Dale for his useful comments and critique of this work, and we gratefully acknowledge the financial and logistical support of the Bureau of Sugar Experiment Stations and the Sugar Research and Development Corporation.

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