BUREAU OF SUGAR EXPERIMENT STATIONS

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FINAL REPORT
SRDC PROJECT BS48S
ENHANCEMENT OF THE SENSITIVITY OF GENE PROBES FOR VIRAL DISEASES OF SUGARCANE BY THE POLYMERASE CHAIN REACTION

by

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SD94006

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1.0 SUMMARY

The sensitivity of gene probes for two important viral pathogens of sugarcane, sugarcane mosaic virus (SCMV) and Fiji disease virus (FDV), has been significantly enhanced by a version of the polymerase chain reaction (PCR). This version, known as RT-PCR, involves a reverse transcription (RT) step to synthesise DNA from the RNA template prior to the PCR amplification. RT-PCR increased the level of sensitivity of detection by 10^3-fold for SCMV and by up to 10^6-fold for FDV. The RT-PCR conditions for routine detection of SCMV and FDV in sugarcane tissues were determined, but the glasshouse trial was not completed. Project benefits and outcomes are:

- very sensitive and specific tests for two important sugarcane pathogens
- methodology developed for routine detection of SCMV and FDV
- considerable experience and expertise in the development of PCR-based tests
- molecular diagnostic tests are available for international collaboration on detection of sugarcane pathogens
- experience and data to commence BS86S `Construction of synthetic Fiji disease virus resistance genes for use in sugarcane'
- publication of a paper describing the first application of RT-PCR to detection of a reovirus (FDV) in plant samples

2.0 BACKGROUND

Fiji disease virus occurs in New South Wales and southern and central Queensland, while sugarcane mosaic virus has occurred everywhere but has only been important in the last 30 years in parts of southern Queensland. They have caused serious economic loss to the Australian sugar industry by affecting yield, restricting the use of agronomically superior cultivars, and causing various control measures to be applied. It is essential that Fiji disease is not spread by the accidental introduction of asymptomatic diseased cane to the disease-free areas of the Burdekin and northern Queensland. In addition, it is important that sugarcane mosaic virus is not spread from southern Queensland to other areas in the state.

BSES scientists in collaboration with Associate Professor Dale of the Centre for Molecular Biotechnology, QUT, produced DNA probes for detection of FDV and SCMV in SRC project BS10S `The production and evaluation of gene probes for diagnosis of sugarcane mosaic virus and Fiji disease virus'. These probes were not sensitive enough to detect the presence of the virus in asymptomatic sugarcane. The polymerase chain reaction (PCR) is a patented technique developed to enhance the level of sensitivity of detection of nucleic acids. PCR can be controlled so that only specific segments of DNA, or RNA in modified PCR, are amplified. PCR can detect extremely small amounts of viral DNA or RNA and amplify it to a level where it can be detected by a DNA probe, thereby increasing the sensitivity of diagnostic tests involving DNA probes. PCR is a relatively new technique but has been extensively applied in medical research for detection of pathogens (eg mycobacteria, HIV), diagnosis of genetic disorders (eg sickle cell anaemia, β-thalassemia) and identification of forensic samples (eg hair, sperm). The application of PCR to enhance the sensitivity of DNA probes for the detection of FDV and SCMV in sugarcane involves the adaption of techniques and protocols used in medical and veterinary research.

3.0 OBJECTIVES

- Adapt the polymerase chain reaction to improve the sensitivity of gene probes for Fiji disease virus and sugarcane mosaic virus.
- Develop the routine methodology for PCR detection.
- Determine the effectiveness of enhanced sensitivity on detection of viral nucleic acids in cane.

4.0 INTRODUCTORY TECHNICAL INFORMATION

Sugarcane cultivars from New South Wales and southern and central Queensland must be quarantined before transfer to north Queensland for use as parents or testing as potential commercial varieties, mainly to prevent the spread of Fiji disease. A quarantine period of 2 years is considered necessary to allow development of morphological symptoms of viral infection as Fiji disease can have a long latent period, especially on moderately resistant cultivars. The time that cultivars spend in quarantine adds considerably to the cost of producing new commercial cultivars as they can not be utilised as parents in plant breeding programs.

The introduction of Fiji disease into north Queensland or the Burdekin district would cause the same upheaval in the variety situations as occurred in southern Queensland in the 1970s and 80s and in the Mackay district during the 1980s. The transfer of varieties into the northern areas of the State should have a virtually nil risk factor. DNA probes for the detection of FDV and SCMV in quarantined cane were previously developed, but sensitivity was inadequate for this purpose. PCR would increase the sensitivity of detection of these DNA probes, reduce the risk of releasing asymptomatic diseased cane and reduce the time that varieties are held in quarantine. This would allow faster transfer of productive commercial or parental varieties across quarantine barriers.

5.0 RESEARCH METHODOLOGY

A brief outline of the methodology follows. Full details are given in Smith *et al* (1992) and Smith and Van de Velde (in press).

Primers

The four FDV-specific cDNA inserts in the DNA probes produced in SRC Project BS10S were sequenced, and five primer pairs selected from the data (Table 1). Each pair consisted of a forward (F) and a reverse (R) primer. The SCMV primers were selected directly from the sequence data of Frenkel *et al* (1991). The primers constituting the pair were matched for G+C ratio and length so that the theoretical T_m (5400-551 and 5400-910) (melting temperature) of each primer within the pair was the same.

Table 1

Characteristics of SCMV- and FDV-specific primers

Primer pair	Size (-mer)	G+C ratio	T_m (°C)	Expected size of product (bp)
S400	21	0.52	64	359
FDV7	24	0.50	72	450
FDV727	27	0.41	76	360
FDV29	30	0.30	78	1 000
FDV55	24	0.46	70	300
FDV59	24	0.50	72	400

Nucleic acid preparations

Total nucleic acids were extracted from leaf samples by grinding the tissue, frozen in liquid nitrogen, in a buffer containing STE, SDS, β-mercaptoethanol, phenol and chloroform. After centrifugation, the aqueous phase was re-extracted with phenol:chloroform and sodium acetate and recentrifuged. Total nucleic acid extract (TNAE) was precipitated from the final aqueous phase with sodium acetate and ethanol and collected as a pellet by centrifugation.

When necessary, FDV dsRNA was purified from diseased leaves showing abundant galls by the method of Skotnicki *et al* (1986), using CF11 cellulose column liquid chromatography but omitting the DNAse treatment.

Reverse transcription

Two reverse transcriptases, MMLV and rTth, were investigated. MMLV has only reverse transcriptase activity whilst rTth has both reverse transcriptase and DNA polymerase activity, and can be directed by changing the metal ion enzyme co-factor. Reverse transcription also requires `priming'; and the specific PCR primers, random hexamer primers and oligo dT primers, were tested.

Polymerase chain reaction, gel electrophoresis and Southern blotting
An aliquot of PCR reaction mix containing the DNA polymerase `AmpliTaq' was added to the reverse transcription solution, and placed in a Perkin-Elmer Cetus Thermal cycler. A two step cycle was selected in which the anneal/synthesis steps were combined. After 35 cycles the DNA products were electrophoresed through a 1 or 2% agarose gel, stained, photographed and transferred to a nylon membrane with sodium hydroxide. The membrane was then hybridised with the appropriate probe that had been previously labelled with biotin. The blots were developed with the PhotoGene non-radioactive chemiluminescent system, and results were recorded on Kodak X-Omat film.

Sensitivity
Preparations were serially diluted in Milli-Q (Millipore) quality water and aliquots were added to the RT mix as described.

Effect of annealing/extension temperature and magnesium ion concentration on amplification
Four different annealing/extension temperatures were investigated for each primer (50, 55, 60 and 67^{o}C). The effect of magnesium ion concentration between 1.0 and 5.0 mM on the PCR product was investigated.

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

Glasshouse trial
Twenty pots, each containing three single eye setts of either NCo310 (susceptible to FDV, moderately susceptible to SCMV) or Q95 (susceptible to SCMV, moderately susceptible to FDV) were established in the containment glasshouse at the Eight Mile Plains Pathology Farm. Maize plants cv. Iochief were inoculated with SCMV to provide inoculum for infection. Adults of the planthopper vector *Perkinsiella saccharicida* were collected from the field and established on FDV infected stools within a cage. After 2 months, the subsequent generation of insects was used to infect the test cane with FDV. The `scotch-brite' method was used to infect plants with SCMV.

6.0 DETAILED RESULTS

Major results of the project are outlined below. Fully detailed results are given in Smith *et al* (1992) and Smith and Van de Velde (in press).

Primer design
Approximately 300 bases of the FDV-specific cDNA inserts were sequenced. The G+C ratio of the sequenced regions varied between 36 and 44%, so that selection of primers with a G+C ratio of 50% was difficult (Table 1). The five FDV primer pairs had G+C ratios between 30 and 50%, although the ratio within the pair was the same. For those primer pairs with low G+C ratios, the length of the primer was increased to 30 bases to increase the theoretical strand melting temperature. The expected size of the PCR product was predicted based on the size of the cDNA insert (Table 1).

The SCMV-specific primer pair S400-551 and S400-910 were selected to amplify the central region of the coat protein coding region of SCMV, a region of amino acid sequence that appears to be highly conserved between SCMV strains (McKern *et al*, 1991).

RT-PCR amplification of FDV-specific RNA
No amplified product was evident if the standard protocol (Anon, 1991) was followed. Only when the primers were boiled/quenched with the template was the expected sized product synthesised. No DNA product was produced in RT-PCR reactions containing TNAE from healthy sugarcane nor in the various RT-PCR controls. No DNA products were synthesised in reactions primed with the FDV55 primer pair. The FDV29 primer pair directed synthesis of a 450 bp product rather than an expected 1 000 bp product. The FDV7 primer pair directed synthesis of the expected 450 bp product, as well as minor 270 and 150 bp products.

The products of RT-PCR primed with the pairs FDV7, FDV727, FDV29 and FDV59 were proven to be FDV-specific by Southern blotting and hybridisation with the appropriate FDV-specific probe. In general, magnesium ion concentration did not appear to be critical to efficient amplification of FDV-specific sequence, and concentrations between 1.5 and 5.0 mM were equally effective. Some primer pairs were more efficient in the RT-PCR amplification of FDV dsRNA sequence from samples. The best primer pair FDV7, at an annealing/extension temperature of 60^{o}C, could detect the presence of FDV in 0.4 ng (0.4 x 10^{-9} g) of tissue (Table 2). The same primers detected 100 ag (1 x 10^{-16} g) of purified FDV dsRNA. These represent respectively a 10^{6}-fold and 10^{4}-fold increase in sensitivity over the best DNA probes.

RT-PCR amplification of SCMV-specific RNA
Using the boil/quench method of priming the reverse transcription of FDV dsRNA, the SCMV-specific sequence was RT-PCR amplified from TNAE of infected plants. RT could be successfully primed with the specific primers, either individually or as the pair, random hexamers or oligo dT primers. The SCMV-specific sequence was amplified in reactions containing magnesium ion concentrations between 1.5 and 4.0 mM. No product was evident in RT-PCR reactions containing 1.0 mM magnesium ion. The best annealing/extension temperature for detection of SCMV in sugarcane was 60^{o}C, with the RT-PCR protocol detecting the presence of the virus in 400 ng of tissue (Table 2). This is 10^{3}-fold more sensitive than that achieved with the DNA probe pS400.

Table 2

Limit of detection of FDV and SCMV in diluted total nucleic acid extracts

Primer pair	Product size (bp)	Minimum tissue weight in which virus detected			
		Annealing/extension temperature (°C)			
		50	55	60	67
S400	359	400 µg	400 µg	400 ng	40 µg
FDV7	450	4 ng	nt	0.4 ng	40 ng
FDV727	360	40 ng	nt	4 ng	40 ng
FDV29	450	40 ng	nt	40 ng	40 µg
FDV55	-	-	-	-	-
FDV59	400	400 ng	nt	4 ng	40 ng

Duplex RT-PCR
SCMV- and FDV-specific sequences were RT-PCR amplified in solutions containing the four primers and SCMV and FDV RNA. Under the conditions used, considerably more FDV-specific product was produced in preference to the SCMV-specific product. Southern blots of the DNA products probed with either pS400 or pFDV7 confirmed the origin of the synthesised DNA.

Glasshouse trial
The test plants were successfully established but problems with inoculum preparation and vectors delayed their infection until too late in the year. During this time it was also decided that a better experimental design would be needed to sample the cane with the very sensitive RT-PCR technique. This trial will need to be redesigned and re-established to test the efficacy of the diagnostic test under controlled conditions.

7.0 DISCUSSION OF RESULTS

A modification of the polymerase chain reaction (RT-PCR) was successfully adapted to detection of FDV and SCMV RNA in sugarcane. The RT-PCR enhancement of sensitivity over the relevant DNA probe for detection of the viruses in sugarcane tissue is 1 000 (10^3) fold in the case of SCMV, and 10^6-fold in the case of FDV, depending on the primers and PCR conditions used. The adaption of the PCR technique and the improvement in sensitivity successfully completed the first objective of the project.

Conditions for routine detection were determined, and initial research was completed on simultaneous detection of SCMV and FDV. For optimal detection of FDV, the best primer pair was FDV7 using PCR anneal/extension at 60°C, and the PCR was relatively unaffected by magnesium ion concentration between 1.5 and 5 mM. For detection of SCMV, the optimal anneal/extension temperature was also 60°C, and again magnesium ion

concentration was not critical over the range 1.5 to 5 mM. Reverse transcription of SCMV RNA could be primed with the specific primers, random hexamer primers or oligo dT primers. This successfully completed the second objective by developing the routine methodology for PCR detection of these two viruses.

SCMV and FDV can be reliably and sensitively detected in relatively crude total nucleic acid preparations of infected sugarcane by RT-PCR. However, a glasshouse trial to determine the effectiveness of the RT-PCR test for detecting the presence of the virus in asymptomatic infected cane could not be completed. This was due to problems with inoculation and sampling strategy. Consequently, the third objective has been only partially achieved.

8.0 RECOMMENDATIONS

- More research is essential before these tests can be routinely applied to test quarantined cane. (A preliminary application to SRDC on this topic was submitted but was not successful.)
- Encourage the testing and evaluation of these probes overseas to determine their value for detecting these viruses in different situations.
- Utilise the expertise developed in this project to develop other PCR-based diagnostic tests for sugarcane pathogens.
- Further test the possibility of duplex RT-PCR for economical screening of sugarcane germplasm.

These sensitive, specific diagnostic tests are not yet in routine application to test quarantined sugarcane as more research is necessary to evaluate a sampling strategy. This research should be given high priority as successful implementation of routine testing will increase the security of quarantine and provide useful data for the application of other diagnostic tests.

International collaboration on development and testing may be facilitated with these two tests. The RT-PCR protocol and primer sequence for detection of FDV has been published and we are making the FDV probes available to the first overseas scientists. The RT-PCR protocol and SCMV-specific primer sequences have been supplied to overseas scientists on a confidential basis. Information from the United States indicates that the SCMV primers are detecting strains of SCMV not present in Australia, an important result for application of this test to foreign varieties held in quarantine. The possibility of international collaboration for developing diagnostic tests for sugarcane pathogens should be pursued.

The expertise and techniques developed in this project should be utilised to develop other PCR-based tests for sugarcane pathogens. Research to develop PCR-based tests for rsd and sugarcane bacilliform virus (SCBV) has already commenced, and should be considered for sugarcane mild mosaic virus (SCMMV), which has just been identified in Australia.

This project demonstrated that duplex RT-PCR can be used to detect two distinctly different viruses in the same sample. Duplex RT-PCR would be an economical, effective method to screen germplasm, but more research is necessary to ascertain the level of sensitivities and

other PCR parameters.

9.0 INTELLECTUAL PROPERTY

No patentable developments were made in this project. The primer sequences for RT-PCR amplification of FDV dsRNA have been published, while the SCMV-specific primer sequence will be published shortly.

10.0 PUBLICATIONS

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