PROGRESS TOWARDS A DEFINITIVE DIAGNOSTIC TEST FOR SUGARCANE MOSAIC VIRUS INFECTION

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Abstract

A new, rapid and sensitive protocol is reported for diagnosis of sugarcane mosaic virus (SCMV) using small (2 cm^2) samples of leaf tissue as starting material. The procedure involves total RNA isolation followed by reverse transcription and amplification by the polymerase chain reaction (RT-PCR) of a substantial portion (884bp) of the coat protein gene of SCMV. This can be followed by corroborative amplification of a smaller region (360bp) within the first amplified fragment (nested PCR) to reduce the potential for false positives. Results have shown that both the 884bp and 360bp fragments are reproducibly amplified from infected leaf but not from control (visually symptom free) material of the same variety. Comparison of the sequence of the larger fragment to the international database has confirmed the identity of the source material as SCMV. So far the method has successfully diagnosed SCMV in infected sugarcane from two widely separated regions of KwaZulu-Natal: from the midlands (Eston) and from the extreme north coast (Pongola). This augurs well for its eventual application as a reliable and definitive test for SCMV regardless of strain. In addition, there is the potential to use sequence information from the larger fragment for specific strain identification. This could, in turn, lead to the design of tests for specific strain diagnosis.

Introduction

Sugarcane mosaic potyvirus (SCMV) is one of the most widely distributed and important pathogens of sugarcane worldwide, causing the disease known simply as mosaic (Koike and Gillaspie, 1989). In South Africa, under conditions of severe mosaic infection in a susceptible variety, reduction in sucrose yield may be as high as 42% (Bailey and Fox, 1987). Despite selection and use of the more resistant varieties, combined with adoption of planting strategies devised to minimise infection (Bailey and Fox, 1980), mosaic and the potential for yield loss persist, particularly in certain geographic regions of the industry. Hence, in relation to both future field control of the pathogen and the national and international movement of germplasm, there is a need for a method of SCMV detection which is sensitive, rapid and unambiguous.

In turn, improved diagnosis of mosaic could have a direct impact on the development of new resistant varieties. The identification of potentially useful germplasm is dependent on accurate assessment of phenotype during selection. At least 40% of all germplasm has to be discarded due to mosaic susceptibility. At present the SCMV resistance or susceptibility phenotype is established through field trials at sites of high infection. Such trials are laborious and time consuming; furthermore, their success depends upon the development of visual symptoms which are not an obligate expression of pathology (Cronjé *et al.*, 1994).

Detection of viruses in infected plant tissue depends upon the precise identification of low levels of viral molecules in a complex molecular background. The most specific and sensitive diagnostic methods are those which exploit sequence information in the viral genome itself. Particular advantages are afforded by techniques based on the polymerase chain reaction (PCR) which are exceptionally sensitive and quick. However, diagnostic PCR requires the design of specific primers; therefore some knowledge of genomic sequence is a prerequisite. In addition, since all potyviruses have single (positive) stranded RNA genomes, PCR amplification must be preceded by reverse transcription (RT) to create a second, complementary strand of DNA against which the reverse primer of the primer pair can bind.

The best sequence characterised region of the genome of the SCMV group of strains comprises the 3' terminal genes for the nuclear inclusion protein encoding polymerase (NIb) and capsid (coat) protein (CP) (Gough *et al.*, 1987; Frenkel *et al.*, 1991). On the basis of homologies within these published sequences, PCR primer pairs and successful diagnostic tests have been devised for one strain of SCMV (SCMV-SC) prevalent in Australia (Smith and van de Velde, 1994) as well as more broadly for potyviruses in general (Pappu *et al.*, 1993). To date, however, no PCR detection techniques have been developed or tested for diagnosis of SCMV strains occurring in South Africa.

This paper reports a new small scale protocol developed for reverse transcription and PCR amplification (RT-PCR) of a substantial portion (884bp) of the coat protein gene of SCMV followed by corroborative amplification of a smaller region (360bp) within the first amplified fragment (nested PCR). Principles of the method are given here but not detailed methodology. The procedure involves several short steps: (1) preparation of total RNA from leaf tissue using hot phenol extraction, (2) partial purification of RNA via spun column separation, (3) reverse transcription and (4) PCR. So far the method has successfully diagnosed SCMV in infected sugarcane from two widely separated regions of KwaZulu-Natal: from the midlands (Eston) and from the extreme north coast (Pongola).

Materials and methodological principle

Anchored oligo-dTs [5'-$(T)_{17}$A-3', 5'-$(T)_{17}$C-3' and 5'-$(T)_{17}$G-3'] for priming first strand synthesis of cDNA from the 3' poly-A terminus of SCMV genomic RNA and three specific primers, BHf, GSf and GSr, were synthesised locally (DNA Synthesis Laboratory, Biochemistry Department, University of Cape Town and Department of Virology, Medical School, University of Natal). The potential use of these primers to reverse transcribe and amplify fragments of the SCMV genome at its 3' end, including the 5' region of the coat protein gene, is shown in Figure 1. All three specific primers are based on the SCMV-SC coat protein sequence of Frenkel *et al.* (1991):

BHf is a forward 24-mer primer. The 5' end binds 135bp upstream of the start of the mature coat protein, within the CP open reading frame (ORF).

BHf: 5'-AGC-TCC-ATA-TAT-TGC-AGA-AAC-AGC-3'

GSf is a forward 21-mer primer, identical to S400-551 of Smith and van de Velde (1994) which binds within the core region of the CP gene.

GSf: 5'-ACA-CAA-GAG-CAA-CCA-GAG-AGG-3'

GSr is a reverse 21-mer primer, identical to S400-910 of Smith and van de Velde (1994). Its 5' end binds 200bp upstream from the point of CP termination.

GSr: 5'-AGT-CAA-AGG-CAT-ACC-GCG-CTA-3'

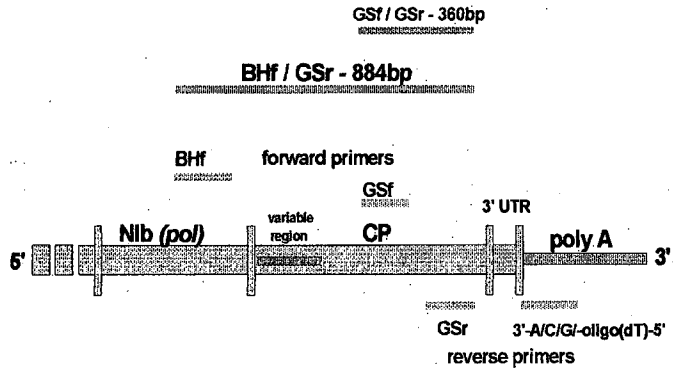

FIGURE 1. Combination of primers for RT-PCR amplification of part of the coat protein open reading frame in the 3' region of the sugarcane mosaic virus genome.

First-strand cDNA synthesis may be achieved by reverse transcription primed either by tethered oligo-(dT) primers or by the specific primer GSr. Subsequently the 3' region of the genome may be amplified by PCR using dual combinations of specific primer. Primer pair BHf and GSr amplifies an 884bp fragment of the genome of the sugarcane strain of SCMV (SCMV-SC) which includes the region encoding the amino terminus of the coat protein. Primer pair GSf and GSr amplifies a 360bp fragment within the 884bp product of BHf and GSr (nested PCR). Primer designations and designs are as described in the text. NIb (*pol*) = nuclear inclusion gene encoding RNA dependent RNA polymerase; CP = coat protein gene.

Results and discussion

Typical results of PCR following first strand synthesis of DNA from total RNA extracted from sugarcane leaf material are shown in Figure 2. Results show that primer pair BHf and GSr amplifies an approximately 880bp fragment from virally infected leaf material from Eston but not from uninfected (symptomless) leaf material of the same variety (NCo376) (Figure 2A). Results also show that the fragment generated from the virally infected material is better amplified following reverse transcription with GSr (Figure 2A, lanes 8 and 9) compared with reverse transcription with oligo$(dT)_{17}$A/G/C (Figure 2A, lanes 10 and 11). Nested PCR using primer pair GSf and GSr results in amplification of the expected 360bp fragment within the approximately 880bp product of BHf and GSr (Figure 2B), thereby providing further evidence that the fragment is derived from the SCMV coat protein sequence. Comparable results to those shown in Figure 2 have been obtained with samples of variety ZN82-3003 from Pongola showing visual symptoms of SCMV. A comparison of the sequence of the larger amplified fragment to the international database has confirmed the identity of the source material as SCMV.

To date, application of the new RT-PCR technique has resulted in clear and fully reproducible diagnostic signals from infected leaf material from two different host varieties in two widely separated geographic regions of the industry. This augurs well for its eventual application as a reliable and definitive test for SCMV regardless of strain. Work is in progress to establish how universally the test can be applied for the diagnosis of SCMV in South Africa and neighbouring territories.

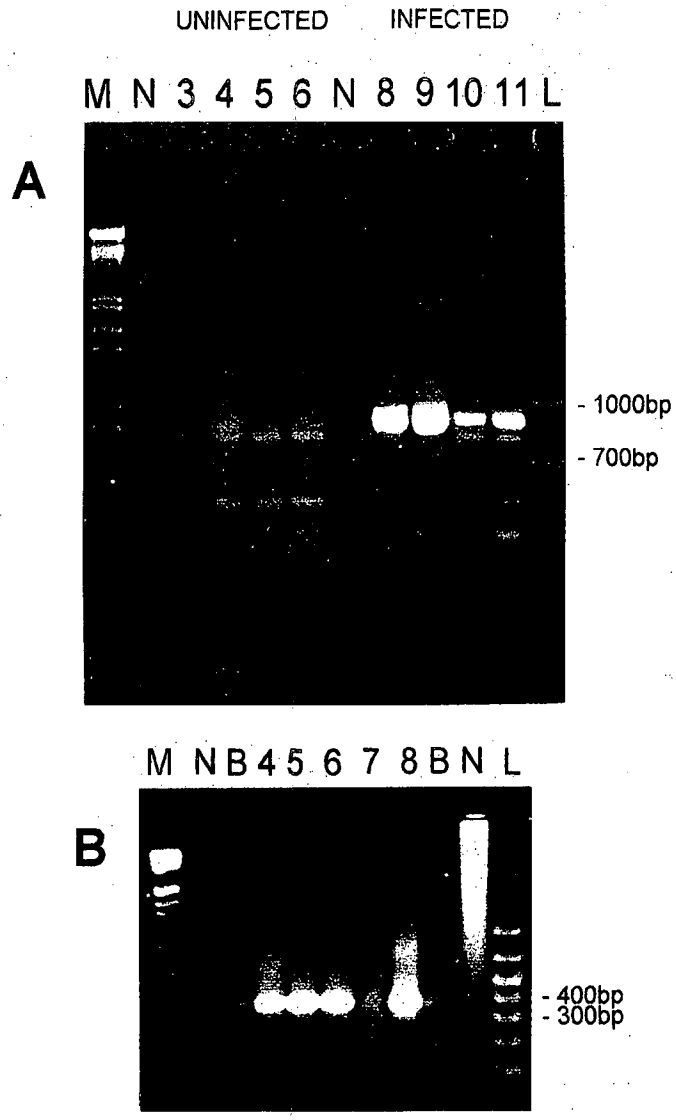

FIGURE 2. Agarose gel electrophoresis of RT-PCR generated fragments of the coat protein gene of sugarcane mosaic virus.

A. Specific PCR amplification of an approximately 880bp fragment with primers BHf and GSr using reverse transcribed crude RNA extracts from uninfected leaves (lanes 3-6) and infected leaves (lanes 8-11) of NCo376 from Eston. *Lanes 3,4 and 8,9*: template was 1µl (left) or 5µl (right) total RNA, reverse transcribed with GSr as primer. *Lanes 5,6 and 10,11*: template was 1µl (left) or 5µl (right) total RNA, reverse transcribed with oligo$(dT)_{17}$A/G/C as primers.

B. Reamplification of a portion of the 880bp fragment, using primers GSf and GSr, generating a nested PCR subfragment of approximately 360bp. Template was prepared by resolubilisation of a small excised sample of the 880bp gel fragment in 200µl of buffer and concentrations were varied from 1µl of a 1/1000 dilution (lane 4) to 1µl of a 1/10 dilution (lane 8).

[B = blank (unoccupied) lane; N = no template control; M = Lambda-Eco RI/Hind III molecular weight markers; L = BiomarkerLow 50-1000bp ladder (Bio Ventures Inc.)].

The RT-PCR method requires only a small amount of material to be sampled (2 cm^2 of leaf tissue) and the full analysis, including nested PCR, can be completed in less than 24 hours. The protocol would lend itself quite well to multiple sample analysis. In addition, it offers two major advantages over the procedure of Smith and van de Velde (1994). Firstly, it incorporates an optional but simple nested PCR step which considerably reduces the potential for false positives. Secondly, it

generates a large fragment of the SCMV coat protein open reading frame (ORF) including the region encoding the amino terminal of the protein in which most of the strain specific variability occurs. This fragment could be isolated routinely and subjected to further analysis by being cloned in *Escherichia coli* and sequenced. Comparison with sequence information in international databases would allow accurate strain identification. Knowledge of the prevalent SCMV strains could, in turn, lead to the design of tests for specific strain diagnosis.

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