

DEVELOPMENT OF A DNA-BASED DIAGNOSTIC METHOD TO DETECT SUGARCANE BACTERIAL PATHOGENS WITH EMPHASIS ON *CLAVIBACTER XYLI* SUBSP. *XYLI*

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Abstract

A polymerase chain reaction (PCR) protocol was developed that specifically detects *Clavibacter xyli* subsp. *xyli* (*Cxx*), the causal agent of ratoon stunting disease of sugarcane. Two primers were developed, namely T₂ and T₄, to amplify the intergenic spacer region of 16S-23S ribosomal DNA of probably all sugarcane bacterial pathogens, including *Cxx*, *Xanthomonas campestris* pv *vasculorum*, the causal agent of gumming disease and *X. albilineans*, the causal agent of leaf scald. The advantages of this approach are that one set of primers can be used for the detection of several pathogens, and that the main pathogens present can be identified based on the length of the amplified fragment. Alternatively, the amplified fragment can be recovered and the precise sequence can be used for identification. The method can also be used to demonstrate mixed infections.

Based on a multiple sequence alignment, two *Cxx*-specific primers (T₈ and T₉) were designed. These primed the specific amplification of a 237 bp DNA product from genomic DNA samples of *Cxx*. The 237 bp product was also amplified directly from cultured *Cxx* cells and from *Cxx*-infected sugarcane xylem sap. For the amplification of *Cxx* in infected sugarcane xylem sap, 0.8% (w/v) polyvinylpyrrolidone was added to the sap prior to amplification. This provides a quick diagnostic method for the detection of *Cxx* in xylem sap without prior extraction of DNA.

Introduction

Sugarcane is susceptible to a wide range of diseases caused by various fungi, bacteria, viruses and phytoplasmas, many of which can cause substantial reductions in yield. Diseases also have a great impact on sugarcane breeding programmes. Every year approximately 40% of new genotypes are discarded in the third selection stage at the South African Sugar Association Experiment Station (SASEX) because of susceptibility to diseases, and a further 40% are discarded in the fourth selection stage. Latent or symptomless infections may allow the build-up and spread of diseases. The ability to diagnose a wide range of pathogens with sensitive and reliable methods, even in apparently symptomless plants, is essential. Diagnostic methods should provide a combination of sensitivity, speed, reliability and high output to serve as

useful diagnostic tests for pathogens.

Ratoon stunting disease (RSD), leaf scald and gumming are three of the most economically important diseases of sugarcane world-wide. RSD is caused by the bacterium *Clavibacter xyli* subsp. *xyli* (*Cxx*) (Davis *et al.*, 1984), while gumming and leaf scald are caused by *Xanthomonas campestris* pv *vasculorum* (*Xcv*) (Cobb, 1983; Dye, 1978, as recorded by Ricaud and Autrey, 1989), and *Xanthomonas albilineans* (*Xa*) (Ashby, 1929; Dowson, 1943, as recorded by Ricaud and Ryan, 1989). Diagnosis of RSD is difficult because of the absence of specific symptoms. Phase contrast microscopy (PCM) and serologically based detection methods including immunofluorescence microscopy (IFM) and the evaporative binding enzyme immunoassay (EB-EIA) have so far been used for diagnosis at SASEX. These techniques allow a definite positive diagnosis, but negative results from relatively small samples of stalks do not guarantee the absence of the disease in the field. Until recently, diagnosis of leaf scald and gumming at SASEX was based on visual symptoms and culturing, after which the Gram stain and several tests including gelatine liquefaction, starch hydrolysis, nitrate reduction and carbohydrate tests were conducted. Although this technique was useful, it took two to three weeks to complete the diagnosis.

The polymerase chain reaction (PCR) has become popular for detecting and identifying pathogens. PCR protocols have been developed for other sugarcane pathogens, including Fiji disease virus and sugarcane mosaic virus (Smith *et al.*, 1994), and *Xa* (Honeycutt *et al.*, 1995). For diagnosis of bacterial pathogens the variable regions within the ribosomal RNA (rRNA) operons have been used for the selection of PCR oligonucleotide primers for a range of species. Intra-species 16S RNA gene variation is small for most described bacterial pathogens but the intergenic spacer region between the 16S and 23S genes (Figure 1) is sufficiently variable for species-specific primers to be designed (Barry *et al.*, 1991).

Presently little is known about strain variation in *Cxx*, *Xcv* and *Xa* in South Africa. Information on this can be obtained by analysis of the 16S-23S spacer region sequences.

The purpose of this project was to develop a set of primers to permit the sensitive and accurate identification of bacterial pathogens in sugarcane. It was hypothesised that if a set of

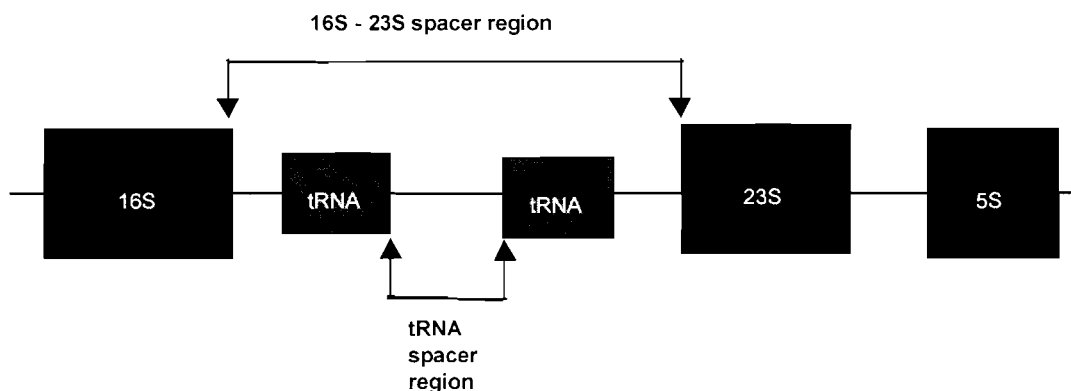


Figure 1. Generalised illustration of the ribosomal genes situated on the rRNA operon.

primers could be developed that would allow the amplification of any sugarcane bacterial pathogen, specific primers could be designed for the identification of different species. A set of primers targeting the highly variable spacer region between the 16S and 23S ribosomal RNA operon of bacteria was developed for this purpose. After aligning 16S-23S intergenic spacer region sequences, a set of highly specific primers was developed for the detection of *Cxx*.

Materials and Methods

Bacterial strains and culture conditions

Cxx isolates were maintained on a sugarcane medium (MSCm) (Davis *et al.*, 1980), modified by Brumbley (personal communication¹). The cultures were incubated for 3-4 weeks at 24°C. Plants expressing gumming symptoms were collected from two areas where the disease is common, Eston in the KwaZulu-Natal Midlands and Pongola in northern KwaZulu-Natal. Plants showing leaf scald symptoms were also collected from Pongola. The cultures were maintained on Wilbrink's agar (Ricaud and Autrey, 1989) at 30°C. Growth occurred within three days and cells were subcultured every week for a maximum of 3 weeks.

Genomic DNA extraction

Genomic DNA was extracted from bacterial cultures as described by van Antwerpen (1999). Bacterial cultures of *Cxx*, *Xa* and *Xcv* were also used directly in PCR. The colonies were scraped from the agar surface and suspended in autoclaved filter-purified water, and 0.5 µl was used as template in PCR reactions depending on the concentration of the bacterial cells (Hiraishi, 1992).

Primer design, DNA amplification and sequencing of the spacer region between the 16S and 23S rRNA genes

Primer pair T₂ forward (5'TAGGACTAAGTCGTAA-CAAGG3') and T₄ reverse (5'CIITGCCAAGGCATC-CACC3') was used to amplify the intergenic spacer region between the 16S and 23S rRNA genes of all the bacterial

species used in this study and was designed by aligning known bacterial sequences obtained from the Genbank database. DNA fragments obtained with primer pair T₂ and T₄ were amplified and the cycling parameters used were as described by van Antwerpen (1999). The PCR products were resolved in a 2% (w/v) agarose gel and visualised under UV light. PCR products were purified from the agarose gel using a Qiagen QIAquick Gel Extraction Kit after electrophoresis. The purified DNA served as template for sequencing reactions.

Sequencing of 16S/23S amplified fragments

PCR-based sequencing reactions were carried out in a thermal cycler (Touchdown temperature cycler; HYBAID, Teddington, England) with ABI PRISM™ dye terminator cycle sequencing using the Perkin Elmer Ready Reaction kit with AmpliTaq R DNA Polymerase. DNA was amplified and the cycling parameters were used as described by van Antwerpen (1999). The extension products were precipitated, denatured and transferred to an autosampler tube. The samples were loaded in the ABI Prism Genetic Analyser for analysis.

Alignment of sequences and development of *Cxx*-specific primers

Sequences obtained were compared with sequences in the Genbank sequence databank for identification. Sequences for each bacterial species were compared to determine whether different strains of the species exist and a consensus sequence for each species was determined. The consensus sequences of all species were aligned and compared with each other by using the DNASIS computer program version 2.1 (Hitachi Software Engineering Co Ltd, 1994). A pair of *Cxx*-specific primers, namely T₈ forward (5'TTGTCCAGGCGCCGGATCTGAGACAGTACT3') and T₉ reverse (5'TGCTCGGTCCACT3') were designed.

Specific amplification of *Cxx*

Amplification reactions were performed in a 15 µl reaction volume for primer pair T₈ and T₉ as described by van Antwerpen (1999). A reaction mixture with sterile filter-purified water served as the negative control. Cycling parameters were used as described by van Antwerpen

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(1999). The specificity of primer pair T_8 and T_9 was tested using genomic DNA from 41 bacterial isolates from sugarcane, including *Xcv* and *Xa*. A range of DNA concentrations containing 5, 50, 100 and 200 ng of bacterial genomic DNA were used as template in PCR. These primers were also tested on xylem sap extracted directly from stalks infected with *Cxx*, *Xa* and *Xcv*. Ten-fold serial dilutions were used as template in the PCR.

Sensitivity of the Cxx-specific PCR test

The sensitivity of PCR using the *Cxx*-specific primer pair T_8 and T_9 was determined by using a dilution series of the bacterial DNA (50 ng, 5 ng, 0,5 ng, 50 pg, 5 pg and 0,5 pg) and bacterial cells diluted in water, using a ten-fold dilution series from 10^1 to 10^6 . Purified genomic DNA from *Cxx* was mixed with an excess of 60 ng sugarcane genomic DNA: 50 ng, 5 ng, 0,5 ng, 50 pg, 5 pg and 0,5 pg of *Cxx*.

Detection of Cxx in sugarcane xylem sap

Cxx cells were diluted in healthy xylem sap using a ten-fold dilution series from 10^1 to 10^6 . The xylem sap was used as template for the PCR test using the *Cxx*-specific primers under the conditions described above. However, to increase the sensitivity of the test, samples of xylem sap containing 0,8% (w/v) polyvinylpyrrolidone (PVP) were tested against xylem sap samples without PVP.

The efficacy of the PCR protocol to detect *Cxx* in sugarcane xylem sap was compared with IF, PCM and EB-EIA. Nine hundred and sixty-eight sugarcane stalks were collected from fields in different areas of the South African sugar industry. Xylem sap was extracted from stalk pieces using positive pressure and the sap from five stalks was pooled in one tube. A 0,5 μ l aliquot from each tube was then used as template in the DNA amplification reactions.

Results

Fragments obtained with primer pair T_2 / T_4

The total number of base pairs for the sequenced product from *Cxx* was 611 bp, of which 143 were adenosine, 149 cytosine, 169 guanosine and 150 thymine, with a GC content of 51,96%. When amplifying DNA from *Xcv*, different sized fragments ranging from 586 bp to 623 bp were amplified from different isolates. Three types of sequences were obtained for *Xcv*. These sequences were compared with known sequences on the Genbank database and it was confirmed that they belonged to the *X. campestris* group of pathogens. Two types of sequences were obtained for *Xa*.

Selection of Cxx-specific primers

Sequences obtained from fragments amplified from *Cxx* genomic DNA using primer pair T_2 and T_4 were aligned and compared with each other in order to design a primer pair (T_8 / T_9) specifically for *Cxx*.

Specificity of primer pair T_8 and T_9

Primers T_8 and T_9 primed the amplification of a 237 bp DNA

product from genomic DNA samples of all the different *Cxx* isolates used. No amplification products were observed in 41 other bacterial isolates, including *Xanthomonas* and *Pseudomonas* species from sugarcane. The specificity of primer pair T_8 and T_9 was also tested using genomic DNA from *C. xyli* subsp. *cynodontis* (*Cxc*), *C. michiganense* subsp. *michiganense* (*Cmm*), *C. michiganense* subsp. *insidiosum* (*Cmi*), and *C. michiganense* subsp. *nebraskense* (*Cmn*). DNA from *Cxc* was amplified and resulted in a 237 bp fragment. No amplification products were obtained from the other *Clavibacter* species used. The T_8 and T_9 primers could therefore distinguish between all the *Clavibacter* species in this study, except *Cxc*.

Detection limits of the primer pair T_8 / T_9 in the PCR diagnostic test

Cxx cells from a pure broth culture were centrifuged and diluted in a ten-fold dilution series in water and used in PCR to determine the sensitivity of the primers. In this test, the primers could detect the bacteria in dilutions of up to one million-fold when 10 μ l was used as template in the PCR. Dilutions of the genomic DNA of *Cxx* were also used to determine the sensitivity of the primers in the PCR. The *Cxx*-specific primers could amplify the DNA from a concentration as low as 50 pg, and when *Cxx* DNA was mixed with 60 ng of healthy plant DNA, the primers detected the *Cxx* DNA at a concentration as low as 0,5 ng.

Detection of Cxx in sugarcane xylem sap

As mentioned above, primer pair T_8 and T_9 primed the amplification of the 237 bp DNA product directly from cultured cells and also from xylem sap from *Cxx*-infected stalks. To increase the sensitivity of the test, 0,8% (w/v) polyvinylpyrrolidone (PVP) was added to the xylem sap (Pan *et al.*, 1998).

Comparison between PCR and other diagnostic tests

The efficacy of the PCR protocol to detect *Cxx* in sugarcane xylem sap was compared with other diagnostic tests used at SASEX. In a first test, EB-EIA, IFM and PCM were compared with PCR. From a total of 91 samples, PCR detected 12 positive samples, IFM detected 15 positive samples and PCM 10 positive samples. Only four samples tested positive by all four methods. PCR did not detect *Cxx* in nine samples that were found to be positive using the other detection methods. EB-EIA did not detect seven positive samples; IFM did not detect one positive sample and PCM did not detect 10 of the samples that had been identified as positive by the other methods. From these results it was apparent that PCR was not highly sensitive.

In a second comparison, attempts were made to increase the sensitivity of PCR by freezing and thawing, varying the volume of template and varying the addition of PVP to the template. In this second test PCR was compared with EB-EIA.

A total of 151 samples were tested in the second comparison, 88 of which came from Swaziland and 63 from Umfolozi. EB-EIA detected four positives in the Swaziland samples,

while PCR detected none. Immunofluorescence microscopy confirmed that the four diagnosed positive by EB-EIA were positive. The xylem sap of these samples was a brownish colour, indicating the presence of substances that might have suppressed the PCR amplification of *Cxx*. In the 63 samples from Umfolozi, PCR detected 24 positive samples, whereas EB-EIA detected 21 positives. The minigel (Southern Cross Biotechnologies), in which 96 samples can be run simultaneously, is shown in Figure 2.

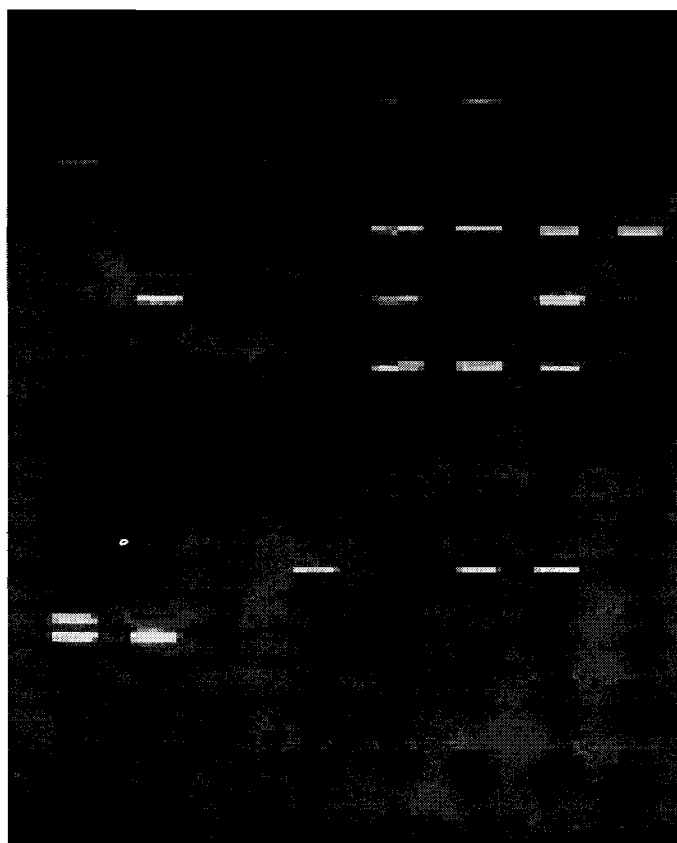


Figure 2. PCR amplification products of *Cxx* cells in xylem sap using primer pair T8 and T9 in the 96-well minigel system. Empty lanes represent negative results.

Discussion

The direct loss in income due to the incidence of diseases in the South African sugar industry is estimated to be approximately R120 million per annum (personal communication²). However, this value does not include indirect losses resulting from the rejection of high yielding varieties due to disease susceptibility. More than 60% of promising varieties are discarded from the variety selection programme at SASEX, some of which might otherwise have high yielding potential.

RSD and leaf scald are often difficult to detect due to a lack of diagnostic symptoms or latency. There are no methods

available for the chemical control of bacterial diseases in sugarcane, and the production of healthy planting material under sanitary conditions is one of the most important control measures. Currently, PCM and the EB-EIA test are used to monitor the RSD situation in the sugar industry and ensure the health of seedcane stocks. PCR-based detection methods specific for *Cxx* and other bacterial pathogens would assist in the monitoring and prevention of spread of diseases. PCR can also be used to determine whether different strains of bacterial pathogens exist.

Target site used for PCR amplification in this study

The intergenic spacer region between the 16S and 23S rRNA genes has been considered an ideal region for developing specific PCR primers that can differentiate bacteria, because a high percentage of sequence variation occurs in this region (Li and De Boer, 1995). This area of the prokaryotic genome is also easily accessible because of the conserved nature of the surrounding 16S and 23S rRNA genes (Barry *et al.*, 1991).

Variation between bacterial species and strains

The T₂ and T₄ primers that were developed for the amplification of sugarcane bacterial pathogens successfully amplified the 16S-23S spacer regions of *Cxx*, *Xcv* and *Xa*. The advantages of this approach are that one set of primers can be used for the identification of bacterial pathogens by recovering and sequencing of the amplified fragment.

Sequences obtained with primers pair T₂/T₄

The size of the amplified fragment, when using *Cxx* genomic DNA and primer pair T₂/T₄, was 611 bp. This differs from the size of the fragments obtained by Pan *et al.* (1998); 566 bp for *Cxx* and 546 bp for *Cxc*, using primer pair G1/L1. This difference might be because the South African isolates differed from those used by Pan *et al.* (1998), and also because of the difference in the length of the amplified fragments obtained with primer pairs G1/L1 and T₂/T₄. Heterologous sequences of the 16S/23S spacer regions are found in the multiple copies of the rRNA operon within other bacterial species (Gurtler and Stanisich, 1996). However, *Cxx* contains only one rRNA operon (Sathyamoorthy *et al.*, 1991) and the spacer region between the 16S and 23S genes of *Cxx* does not contain any tRNA genes (Fegan *et al.*, 1998). This is consistent with the spacer regions of other Gram-positive organisms with high G+C content (Fegan *et al.*, 1998), excluding the possibility that an extra tRNA exists in the current *Cxx* sequence. It can be concluded that there might be strain differences between different *Cxx* isolates, and this requires further investigation.

The species *X. campestris* represents a complex group of plant-pathogenic bacteria that are similar in morphology but differ in host range. Accurate identification of plant pathogenic *X. campestris* species is difficult and time consuming (Gilbertson *et al.*, 1990). When using primer pair T₂/T₄ for the amplification of *Xvc* genomic DNA, it was found that different sized fragments were amplified for different iso-

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lates. The sizes of the T₂/T₄ amplified fragments obtained in this study ranged between 540 and 626 bp. A possible explanation for the different types of sequences obtained for *Xcv* in this study might be that there are different *Xcv* strains present in sugarcane plants. The determination of whether different strains of *Xcv* exist in sugarcane is a subject for future study.

A 619 bp product was amplified with primer pair T₂/T₄ from *Xa*. In both types of sequences obtained for *Xa* in this study, several small deletions and inclusions were found as well as point mutations, suggesting the existence of different strains of this species. Honeycutt *et al.* (1995) and Davis *et al.* (1997) also reported the existence of different strains of *Xa*.

Implications of different strains of pathogens for plant breeding

The occurrence of different strains of bacterial pathogens has implications for plant breeding. Currently, some promising new varieties are discarded in the later stages of selection or even after release because of their susceptibility to a specific pathogen. Should different strains of a pathogen be known to exist, new varieties could be screened against all known strains in the early stages of selection, thus saving time and resources. Characterising resistance to individual strains would allow for the maintenance of resistance genes in breeding populations. These could be exploited in the breeding programme through 'gene stacking', by crossing individuals carrying the different resistance genes. An advantage of such an approach is that useful germplasm is not discarded on the basis of susceptibility to a single strain of a pathogen.

Development of specific primers

By aligning and comparing the sequences obtained from fragments amplified from genomic DNA of *Cxx*, *Xcv* and *Xa* using primer pair T₂/T₄, it was possible to design a pair of primers specific for *Cxx*. Primer pair T₈ and T₉ was designed and a 237 bp fragment was amplified from genomic DNA as well as from *Cxx* cells. The primers also amplified a 237 bp fragment directly from sugarcane xylem sap. Sequencing these fragments confirmed that it was *Cxx*. The primers were tested against different isolates of other *Clavibacter* species (*Cmm*, *Cmi*, *Cmn*, *Cxc*), as well as *Xcv*, *Xa*, *Pseudomonas* species and several unknown bacterial species isolated from sugarcane plants. The primers were highly specific to *Cxx* and did not amplify DNA from other bacterial species; they were nevertheless not able to distinguish between *Cxx* and *Cxc*. However, *Cxc* is a xylem-inhabiting endophyte of Bermuda grass (*Cynodon dactylon*) and has not been found naturally in sugarcane (Davis *et al.*, 1984).

The detection limit for *Cxx* by the PCR protocol in this study was 5 pg of genomic bacterial DNA. However, when the bacterial DNA was mixed with an excess of sugarcane DNA, the detection limit was 0.5 ng of bacterial DNA (one hundred-fold less sensitive). PCR could also be performed

directly on *Cxx* cells from culture and from *Cxx*-infected sugarcane xylem sap. *Cxx* cells could be detected in a million-fold dilution of *Cxx* in xylem sap when PVP was added to the sap. It appeared that the PCR test was more sensitive when xylem sap was used as template rather than plant DNA.

Comparison of the PCR test with other diagnostic tests

A limiting factor in applying the PCR protocol for the detection of *Cxx* in sugarcane xylem sap is the presence of PCR inhibitors, such as polysaccharides or phenolic compounds (Henson and French, 1993). Various reagents have been included in the PCR buffer by researchers, such as PVP and polyethylene glycol (PEG) (MacKenzie *et al.*, 1997), PVP and Ficoll (Pan *et al.*, 1998) to minimise the possible effect of inhibitors in DNA extracts from plants. In this study, 0.8% (w/v) PVP added to the xylem sap increased the sensitivity of the PCR protocol considerably. *Cxx* cells could be detected in a million-fold dilution, thus improving the detection limit one hundred times. When different volumes of the dilutions were used as template in the PCR it was found that 0.5 µl of xylem sap was adequate as template.

A comparison was made between the four methods used for the detection of *Cxx*, namely PCR, IFM, EB-EIA and PCM. PCR was more sensitive in detecting *Cxx* than EB-EIA and PCM. Although the sensitivity of IFM and PCR appeared to be similar, it is accepted that PCR is more accurate in detecting *Cxx* because the test is based on a specific DNA sequence of *Cxx*.

Conclusions

The aim of this study was to develop a DNA-based diagnostic test using PCR for the detection of pathogens in sugarcane and specifically for *Cxx*. This was accomplished with the development of primer pairs T₂/T₄ and T₈/T₉.

It was demonstrated that significant sequence heterogeneity exists within the eubacterial 16S/23S spacer region at the genus and species levels. The size and number of fragments produced by amplification of the 16S/23S spacer region can be a useful tool in identifying species of bacteria. However, the sizes of fragments from the three bacterial species used in this study did not differ sufficiently to make recognition possible in 2% agarose gels, and the amplified fragment was therefore recovered and the precise sequence was used for identification.

The strategy used has the advantage of providing specific DNA primers for any micro-organism. Furthermore, the rapid technique requires no previous information on the molecular biology of the micro-organism of interest. The use of DNA sequences in the spacer region between the 16S and 23S rRNA has shown that efficient and specific primers can be generated that are able to readily distinguish between closely related eubacteria.

A specific primer pair (T₈ and T₉) was designed for the detection of *Cxx* in sugarcane DNA or sugarcane xylem sap

using the 16S/23S spacer region. The methodology provides a rapid, sensitive assay for the detection of *Cxx* in sugarcane. Ninety-six samples can be analysed in a thermal cycler in a 35-cycle PCR run, using a three-temperature programme in only 2½ hours. A special minigel system (Southern Cross Biotechnologies) in which 96 samples are analysed simultaneously, is used for the diagnostic test. The interpretation of the test results is simple and relies on the presence or absence of a single 237 bp fragment. It takes five to 10 minutes for the fragments to be resolved in the agarose gel. The overall time taken to conduct this diagnostic test is less than three hours.

It is not envisaged that the *Cxx*-specific PCR test will replace the serological-based detection methods currently used at SASEX for the mass diagnosis of RSD. However, PCR is the method of choice for the detection of *Cxx* in RSD transmission trials and diagnosis in quarantine.

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ALLELIC VARIATION IN THE GENE ENCODING PYROPHOSPHATE DEPENDENT PHOSPHOFRUCTOKINASE (PFP) IN SUGARCANE

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Abstract

The enzyme pyrophosphate dependent phosphofructokinase (PFP), considered to play an important role in sucrose metabolism in plants, comprises two protein subunits encoded by separate genes. Part of the β -subunit gene was used as a model in investigating allelic variation in the polyploid genetic environment of sugarcane. The gene fragment was amplified by the polymerase chain reaction (PCR) from various total genomic DNA samples and a number of individual DNA molecules cloned randomly from each source. Sequence analysis of clones has demonstrated that two strikingly different alleles are present in ancestral *Saccharum* species as well as modern hybrid varieties.

Introduction

Sugarcane is a highly heterozygous and genetically cumbersome crop (Aljanabi, 1998). This is at least in part because it is highly polyploid, having a suspected base chromosome number of 10 and between 110 and 130 chromosomes in the 2n state, contributed by two major ancestral species, *Saccharum officinarum* and *Saccharum spontaneum* (D'Hont *et al.*, 1996). The number of alleles of important genes and the degree to which those alleles vary in the complex genetic environment of sugarcane is of considerable interest because of the potential for exploitation of genetic variation through breeding, and manipulation of gene function via genetic engineering. In addition, such information might throw light on the general speciation and evolution of polyploids, about which little is known (Soltis *et al.*, 1992). The working hypothesis for this study was that allelic variation can best be established by targeting a single copy gene (i.e. a gene with one copy per haploid genome), the argument being that such a gene should be present in multiple allelic copies in sugarcane. The gene encoding the enzyme pyrophosphate dependent phosphofructokinase (PFP) is such a gene (Blakeley *et al.*, 1992). PFP is considered to play an important role in sucrose metabolism in plants (Dancer and ap Rees, 1989). Part of the gene encoding the β -subunit of the enzyme was adopted as the study model. Using genomic DNA from different sugarcane sources as template, the gene fragment was amplified by the polymerase chain reaction (PCR) with primers designed to complement conserved regions of the gene. Amplified fragments were ligated into plasmid vectors and individually cloned in *E. coli* for comparative sequencing analysis.

Materials and Methods

Primers

Forward primer B1 was designed from a region of high homology identified by comparison of known PFP gene sequences from castor bean and potato while reverse primer B8 was designed from a sugarcane PFP sequence (Sarita Groenewald; PhD thesis, University of Natal, 1998, and personal communication):

B1 (20bp) (5' to 3') ATI-GAT-TTC-ATI-CCI-GAG-GT

B8 (21bp) (5' to 3') CCA-TCA-GTG-ATG-TCA-ATG-CTG

The predicted product of B1/B8 amplification is a region of the PFP gene representing exons 10, 11 and 12 and their flanking introns, approximately 1200bp in size in all.

Gene amplification, cloning and sequencing

Genomic DNA was extracted from *Saccharum officinarum* (Black Cheribon), *Saccharum spontaneum* (Coimbatore) and a commercial variety (N21) as described by Harvey and Hockett (1998). PCR reactions contained, in a final volume of 50 μ l: 10mM Tris-HCl, 50mM KCl, 1,5mM MgCl₂, 50 μ g gelatine, 0,33mM deoxynucleoside triphosphates (dNTPs), 0,4 μ M primers B1 and B8, 1U Taq DNA polymerase (Boehringer Mannheim) and 75ng genomic DNA, at a final pH of 8,3. Thermal treatment was 94°C for 1,5 min (1 cycle), 94°C for 45 sec, 45°C for 30 sec and 72°C for 2 min (10 cycles) and 94°C for 30 sec, 40°C for 30 sec and 72°C for 2 min (30 cycles) followed by 72°C for 2 min then 35°C for 5 min. PCR products were purified by electrophoresis through 1% (w/v) agarose gels in TBE buffer (45mM tris-borate, 1mM EDTA, pH 8,0) followed by excision of bands visualised by conventional ethidium bromide staining and gel removal by spun column (GenElute, Supelco). PCR fragments were ligated into the pGEM[®]-T Easy plasmid vector (Promega) and cloned in *E. coli* strain JM109 according to manufacturer's instructions. Cloned plasmids were extracted from bacterial cells and purified using column separation kits (Nucleobond AX20, Macherey-Nagel) while estimations of quality and quantity were made both spectrophotometrically at 260nm and by visual observation following gel electrophoresis. Plasmid inserts were used with either B1 or B8 primer in cycle sequencing reactions according to standard dye terminator chemistry methodologies (Big Dye Kit, Perkin Elmer). Reaction product analysis was accomplished by capillary electrophoresis using a Perkin Elmer ABI Prism 310 Genetic Analyzer.

Sequence analysis

Sequences were edited manually to remove plasmid vector components. PFP homology was measured by nucleotide comparison of individual sequences to genes registered in international databases using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.*, 1990). Sequence overlaps, alignments and other data manipulations were accomplished using Perkin Elmer software Sequence Navigator 1.0.1 and/or the software programme DNASIS for Windows v2.1.

Results and Discussion

Two PCR amplification products of approximately 1250bp and 1100bp in size were obtained consistently from the genomic DNAs of all three sources: Black Cheribon, Coimbatore and N21. The fragments were designated PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ respectively. In the case of Coimbatore, a third product of approximately 950bp was sometimes obtained. Only PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ were cloned and sequenced in this study. B1 generated sequences were used for BLAST homology searches. All scores obtained were above the significance threshold of 80 and ranged from 119 to 522 (mean = 411), indicating that all gene fragments obtained by PCR were identifiable as PFP. Where possible, forward and reverse sequences were integrated using Contig Manager, an overlap function of the software programme DNASIS, to provide full length frag-

ment sequences. Results of cloning, sequencing and BLAST homology searches of fragments PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ from all three germplasm types are summarised in Table 1.

To investigate sequence differences between the two size species PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀, representative full length sequences obtained from commercial variety N21 (one sequence of each type, Table 1) were compared with each other in an initial analysis. Results showed that the size differences between the two fragments was due to sequence variations throughout their lengths rather than a single major insertion or deletion in one of the fragments. Homologous cDNA sequences of the castor bean PFP β -gene (exon regions 10, 11 and 12), assumed to be relatively conserved across species, were used to determine the positions of exon and intron regions in the sugarcane gene fragments. Exon regions of the two sugarcane variants showed 77-78% nucleotide homology while, as expected, intron regions showed greater variability with 48-59% nucleotide homology.

Having used N21 data to establish the existence of two variants of the gene, apparently associated with a size difference between the B1 and B8 primer sites, the data obtained for Black Cheribon and Coimbatore were analysed. For each of the germplasm sources, sequences clustered into one of two distinct sequence types, shown by consensus sequence comparisons to correspond closely to the two allelic variants

Table 1. Sequencing results and PFP homology scores for cloned gene fragments obtained by PCR from genomic DNA of *Saccharum officinarum* (Black Cheribon), *Saccharum spontaneum* (Coimbatore) and the commercial variety N21.

GERMPLASM SOURCE	FRAGMENT	CLONE NAME	B1 (FORWARD) GENERATED SEQUENCE LENGTH (bp)	B1-SEQUENCE BLAST SIGNIFICANCE SCORE FOR PFP	B8 (REVERSE) GENERATED SEQUENCE LENGTH (bp)	OVERLAP REGION (bp)	FULL, LENGTH SIZE (bp)	
BLACK CHERIBON	PFP.B1/B8 ₁₂₅₀	BC.T#1	643	499	668	285	1026	
		BC.T#3	662	513	643	272	1033	
		BC.T#4	556	352	366	-	-	
		BC.T#5	650	322	670	66	1254	
		BC.T#6	370	277	606	-	-	
		BC.T#10	651	495	651	93	1209	
		BC.T#11	660	119	653	283	1030	
		BC.T#12	650	495	687	129	1208	
		BC.T#13	640	495	637	21	1256	
		PFP.B1/B8 ₁₁₀₀	BC.B#17	530	522	392	-	-
			BC.B#18	532	522	432	-	-
			BC.B#21	475	504	390	-	-
			BC.B#24	485	331	653	91	1047
COIMBATORE	PFP.B1/B8 ₁₂₅₀	CO.T#4	743	489	-	-	-	
		CO.T#7	522	250	532	24	1030	
		CO.T#8	461	197	465	-	-	
N21	PFP.B1/B8 ₁₂₅₀	N21.T#10	777	495	715	273	1219	
		N21.B#9	735	522	686	395	1026	

BC, Black Cheribon; CO, Coimbatore; T, top gel fragment, 1250bp; B, bottom gel fragment, 1100bp. BLAST scores above 80 indicate significant homology to known PFP genes.

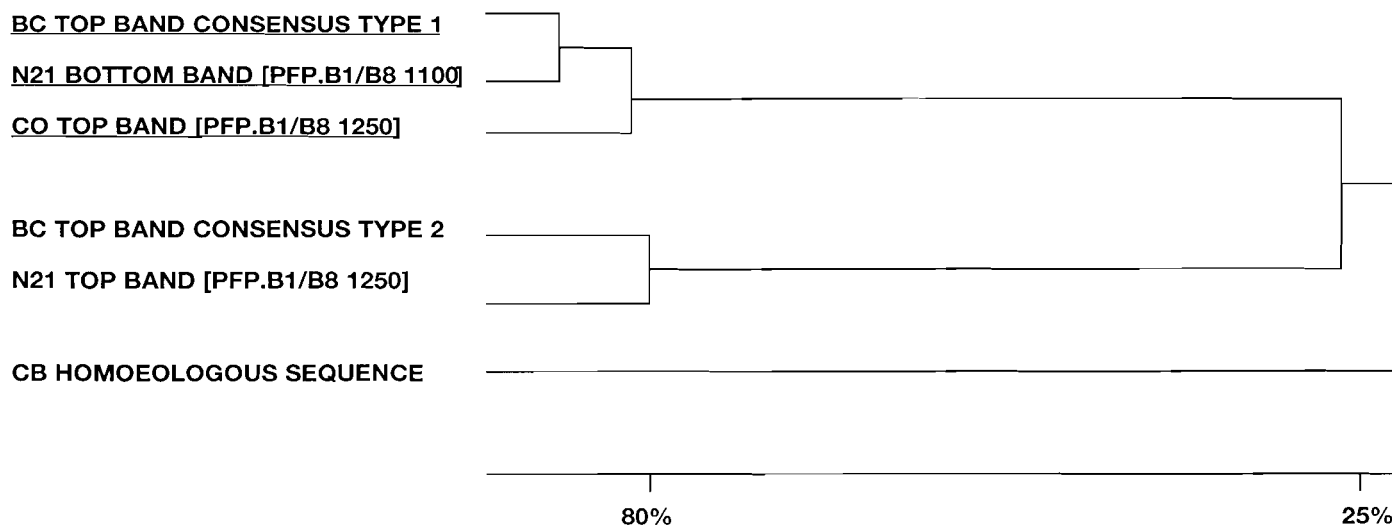


Figure 1. Homology tree derived from sugarcane PFP gene fragments and the equivalent sequence from castor bean. All contributing sequences represent full length B1/B8 PFP fragments. Consensus sequences types 1 and 2 were each based on three full length fragments and were obtained in Sequence Navigator. The tree was derived in DNASIS. BC, Black Cheribon; CO, Coimbatore; CB, castor bean (*Ricinus communis*). Scale shows relative homology values in %.

identified in N21. However, correlation between sequence type and size species of origin was not tight, suggesting that contamination of the PFP.B1/B8₁₂₅₀ and PFP.B1/B8₁₁₀₀ fragment populations had occurred at the point of gel band excision. This is not unlikely, as it is recognised that DNA fragments such as these, differing in size by only 150bp, may appear separate visually but not be resolved perfectly by agarose gel electrophoresis. Future preparative separation would be accomplished more precisely in polyacrylamide gels.

Relationships between the various sugarcane PFP gene fragment sequences and the homoeologous sequence from castor bean are illustrated in Figure 1. Two distinct allelic clusters are identifiable among the sugarcane sequences. It is notable that the degree to which they differ from each other is almost as great as the degree to which each differs from castor bean, a dicotyledonous plant not closely related to sugarcane. Although the analysis is based on limited numbers of cloned fragments and only single sequence runs, the result is sufficiently striking to suggest that the variations do reflect significant allelic differences in the genome. The high level of distinction and the fact that both gene forms occur in the ancestral germplasm of sugarcane suggest that the alleles diverged early in the evolution of the genus *Saccharum*.

Confirmation of this work and further investigations into PFP gene variants and their expression is being undertaken.

Acknowledgements

The authors thank Deborah Carson of the Biotechnology

department at the SASA Experiment Station and Dr Sarita Groenewald of the Institute for Plant Biotechnology, University of Stellenbosch, for practical advice, helpful discussions and appraisal of the manuscript.

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