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Molecular Analysis of an Endopolygalacturonase Gene from a *Eucalyptus* Canker Pathogen, *Cryphonectria cubensis*

PERCY M. CHIMWAMUROMBE[a,b], BRENDA D. WINGFIELD[a,b,*], ANNA-MARIA BOTHA[a,b] and MICHAEL J. WINGFIELD[b]

[a]*Department of Genetics, University of Pretoria, 74, Lunnon Road, Hillcrest, Pretoria 0002, South Africa;*
[b]*Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, 74 Lunnon Road, Hillcrest, Pretoria 0002, South Africa*

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***Cryphonectria cubensis* causes a serious *Eucalyptus* canker disease. Fungal cell wall degrading enzymes (CWDEs) are important during the early stages of interaction of the fungus with *Eucalyptus*. To improve our understanding of the molecular regulation of the interaction of *Eucalyptus* and *C. cubensis*, the relevant genes involved in this interaction should be identified, cloned and studied. The aim of this study was, therefore, to clone the endopolygalacturonase (endoPG) gene of *C. cubensis*. *C. cubensis* was grown on a medium supplemented with *Eucalyptus* cell wall extracts. Degenerate primers were designed to amplify part of the endoPG gene from *C. cubensis* genomic DNA. The resulting sequence was used to design specific primers for use in inverse PCR to amplify the entire endoPG gene of *C. cubensis* (*ccen-1*). The endoPG sequence of *C. cubensis* has 93% amino acid sequence similarity to that of the chestnut blight pathogen, *Cryphonectria parasitica*.**

Keywords: *Cryphonectria cubensis*; *Eucalyptus*; Endopolygalacturonases; Cell wall degrading enzymes

INTRODUCTION

The plant cell wall is a major barrier that many fungal pathogens must circumvent. To do this, fungi secrete a complex of cell wall degrading enzymes (CWDEs) that degrade the complex polymers making up cell walls (Mendgen *et al.*, 1996; Sexton *et al.*, 2000). CWDEs have a specific role in penetration and digestion of plant cell walls to release nutritive sugars that support pathogen growth. Individually, or as a group, CWDEs are potential virulence and pathogenicity determinants in fungal–plant interactions (Walton, 1994).

Pectin is one of the main constituents of plant cell walls. Pectin is comprised of alternating homogalacturonans and rhamnogalacturonans (Carpita and Gibeaut, 1993). To penetrate the pectin defence barriers, fungal pathogens secrete a variety of pectinases. These include exo/endopolygalacturonases and pectic lyases. Endopolygalacturonases (endoPGs) are secreted earlier than other CWDEs and are thus considered to be important in initiating pathogenesis (Albersheim and Anderson, 1971).

Cryphonectria cubensis is an ascomycetous fungus that causes a serious disease, known as *Cryphonectria* canker on *Eucalyptus* in plantations in the tropics and subtropics (Wingfield, 1999). This disease has seriously damaged seedling stands as well as clonal plantations, and is considered to be one of the most important threats to commercial *Eucalyptus* propagation (Wingfield, 1999). Understanding the role of endoPGs in pathogenesis could lead to the design of novel approaches to protect *Eucalyptus* from endoPG-mediated damage. In this study we report on the molecular characterisation of an endopolygalacturonase gene from *C. cubensis*.

MATERIALS AND METHODS

Fungal Culture and Growth Conditions

An isolate of *C. cubensis* known to be highly pathogenic to *Eucalyptus* and which is routinely

*Corresponding author. Address: Faculty of Natural and Agricultural Sciences, University of Pretoria, 74 Lunnon Road, Hillcrest, Pretoria 0002, South Africa. Tel.: +27-12-4203846. Fax: +27-12-4203947. E-mail: brenda.wingfield@fabi.up.ac.za

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used in disease screening trials (Van Heerden and Wingfield, 2000) was selected for use in this study. This isolate (CMW2113) is maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. The fungus was grown for 5 days on 2% malt extract agar plates at 25°C, in the dark. Five plugs (4 mm each) from the actively growing margins of cultures were inoculated into liquid medium. The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH, 3.0 g DL-Malic acid; 2.0 g NH_4NO_3; 1.0 g KH_2PO_4; 0.1 g $MgSO_4$ and was supplemented with 1% w/v *Eucalyptus* cell wall extracts as a carbon source in a litre (Errampalli and Kohn, 1995). Cell wall extracts were prepared as outlined by Mwenje and Ride (1997). The culture was incubated, with shaking, at 100 rpm at 25°C for 10 days. Mycelium was harvested by filtering through Whatman No. 1 filter paper and freeze-dried for storage. All the enzymes used in this study were obtained from Roche Biochemicals (Switzerland) unless stated otherwise.

DNA Extraction and Degenerate PCR Amplification

Freeze-dried fungal mycelium was ground to a fine powder under liquid nitrogen, using a mortar and pestle and sterile glass beads. Genomic DNA was isolated as previously outlined by Chimwamurombe *et al.* (2001). All standard DNA techniques were those described by Sambrook *et al.* (1989).

Degenerate primers cc2 (5′-TAY AAR GAR TGG GAR GGN CYN CTN ATH-3′) and cc4 (5′-NSW NCC RAT NSW NAG NCC TRG NCC NCC-3′) were designed from conserved regions of fungal endoPG genes to amplify part of the endoPG gene. In the PCR amplifications, the following reaction mixture was used: oligonucleotide primer cc2 (0.6 pmol/μl), oligonucleotide primer cc4 (0.6 pmol/μl), *C. cubensis* template DNA (25 ng), 1 mM dNTPs, 1.5 mM $MgCl_2$, 10X PCR buffer, and 5 units of *Taq* polymerase enzyme in 50 μl. PCR was performed for 30 cycles (one cycle = 1 min at 94°C, 1.5 min at 62°C and 1.5 min at 72°C). The reactions had an initial denaturation step of 4 min at 94°C and a final elongation step of 5 min at 72°C. The PCR products were separated on a 1% agarose gel stained with ethidium bromide and visualised under a UV transluminator. PCR fragments were purified from agarose gel and cloned into pGEM-T-Easy vector (Promega). Ligation mixtures were used to transform competent *E. coli* (JM109) cells.

Sequence Analysis, Southern Hybridisation and Inverse PCR

Sense and antisense strands of six candidate clones from degenerate PCR cloning were sequenced using the dideoxy-DNA chain-termination method on the ABI-377 Prism Automated sequencer (Perkin–Elmer, USA) using the BigDye Terminator Cycle Sequencing Reaction Kit (Perkin–Elmer, USA). The sequences were used in BLASTX searches and they were aligned to other published sequences in GenBank.

Genomic DNA of *C. cubensis* (10 μg) was separately digested for 16 h with restriction enzymes, *Eco* RI, *Hin*dIII, *Eco* RV, *Bam* HI and *Pst* I and blotted to nylon membranes (Roche Diagnostics, Switzerland). A 593 bp *C. cubensis* endoPG PCR fragment obtained from the degenerate PCR was DIG-labelled and used to probe the southern blots.

To amplify the remainder of the endoPG gene, a modified inverse PCR technique described by Ochman *et al.* (1990), was used. From the southern blots, it was known that a 3.5 kb fragment was generated with *Hin*dIII digestion (Fig. 2). The circularised template DNA was generated from genomic *Hin*dIII fragments, which were excised from the gel and purified. Specific primers icpg1 (5′-ACC GTC GAT AAC ATG TCC-3′) and icpg2 (5′-GGC GGT TCT TGC ACC GGA-3′) were designed from the 593-bp *C. cubensis* endoPG DNA fragment. In the inverse PCR reactions, the following reaction mixture was used: oligonucleotide primer icpg1 (0.6 pmol/μl), oligonucleotide primer icpg2 (0.6 pmol/μl), circularised *C. cubensis* template DNA (5 ng), 1 mM dNTPs, 1.5 mM $MgCl_2$, 10X PCR buffer, and 5 units of *Taq* polymerase enzyme in 50 μl. PCR was performed for 30 cycles (one cycle = 0.5 min at 94°C, 0.5 min at 50°C and 2 min at 72°C). The reactions had an initial denaturation step of 4 min at 94°C and a final elongation step of 5 min at 72°C. Inverse PCR fragments were purified from the agarose gel and cloned into pGEM-T-Easy vector. Ligation mixtures were used to transform competent *E. coli* (JM109) cells. Plasmid DNA was isolated from five candidate clones and was used in cycle sequencing with the BigDye kit (Perkin–Elmer, USA). The sequences were compared to the 593 bp fragment in order to identify the clone containing regions of overlap and hence gene of interest. The sequence data for *C. cubensis* endopolygalacturonase (*ccen-1*) has been deposited in Genbank database (accession number is AF360316).

RESULTS

A 593 bp fragment was obtained from degenerate PCR amplification and this had high homology to published endoPG sequences as revealed by BLASTX searches. The remaining 5′- and 3′-portions of the *C. cubensis* endoPG (*ccen-1*) were determined by sequencing the inverse PCR products. An additional 1335 bp fragment was sequenced from

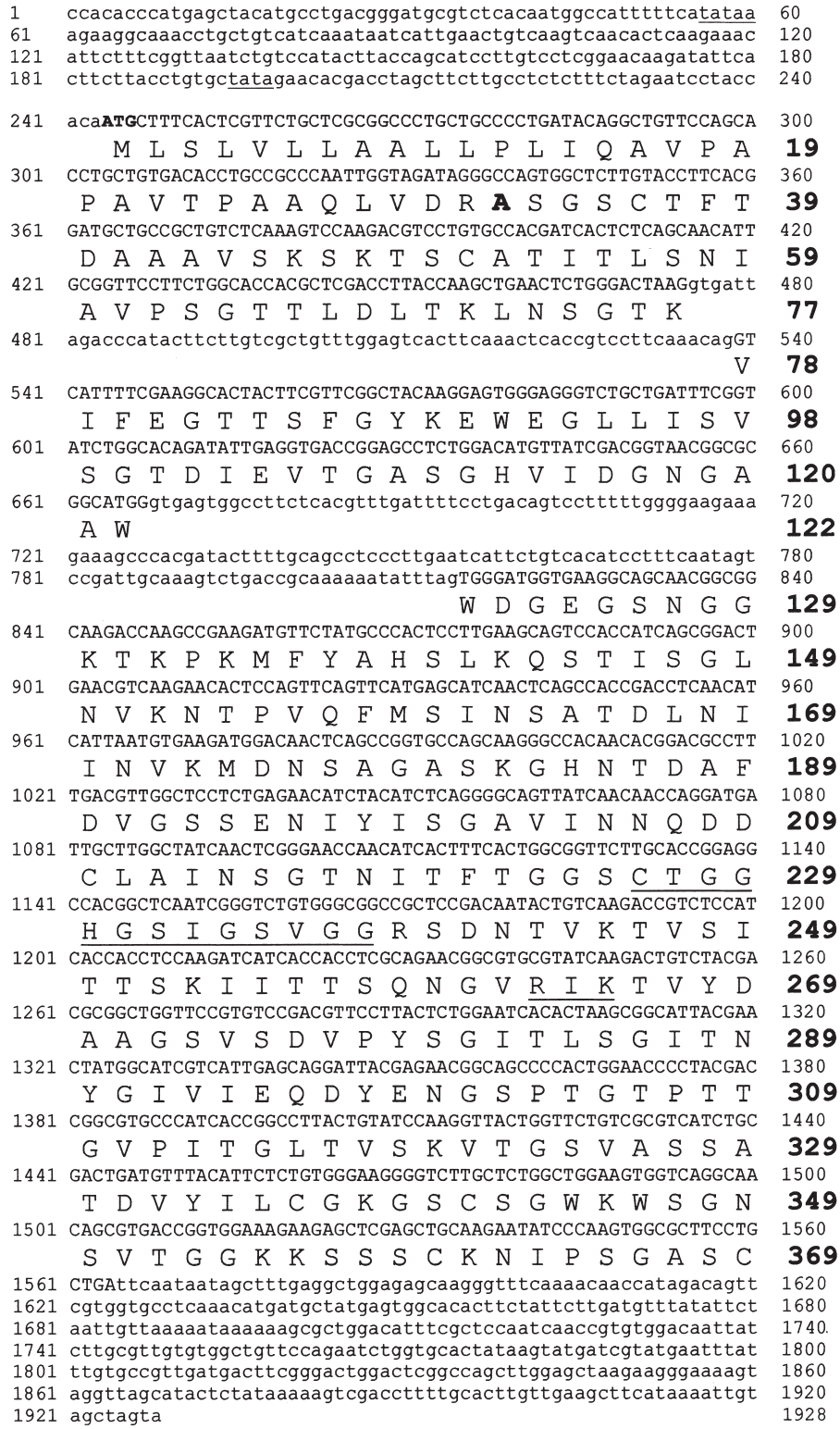

FIGURE 1 The nucleotide sequence and predicted amino acid sequence of the *C. cubensis* endoPG. The start codon is indicated in bold, the TATA signals are in small case and underlined. Introns are in small case and found within the coding region. The underlined amino acids indicate the characteristic motifs of endopolygalacturonases. The mature endoPG peptide begin at ala[32] indicated in bold.

the inverse PCR products and this sequence contained overlapping regions with the previously sequenced 593 bp fragment. The complete ccen-1 is encoded by a single open reading frame of 1320 bp (Fig. 1) that is interrupted by two introns that are 64 and 145 bp in size. A putative TATA signal was observed at position 194 on the DNA sequence (Fig. 1), although no CAAT signals were observed. Southern blot analysis of the *C. cubensis* genome using the 593 bp *ccen-1* fragment as a probe showed

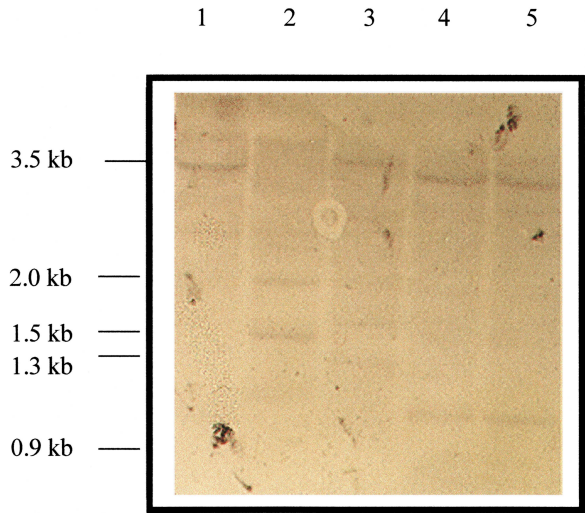

FIGURE 2 Southern blot analysis. The genomic DNA was digested with enzymes *Eco* RI (1), *Eco* RV (2), *Pst* I (3) *Bam* HI (4) and *Hin*dIII (5). Band sizes were estimated from a DNA marker (λDNA digested with *Eco* R1 and *Hin*dIII)

that there are at least two homologues of the *ccen-1* gene (Fig. 2). At least two hybridisation bands, at high stringency, were observed on the DNA blot of the genomic DNA, which was completely digested with five different restriction enzymes.

Ccen-l polypeptide is comprised of 369 amino acids that have a predicted molecular mass of 37.4 kDa and a calculated pI value of 6.42. There are two potential N-linked glycosylation sites. These are ^{218}NIT^{220} and ^{300}NGS^{302} (Fig. 1) and they are conserved in their relative position in the *Cryphonectria parasitica* endoPG (enpg-l) (Gao *et al.*, 1996). The mature *ccen-l* peptide commences at A^{32} (Von Heijne, 1986). Characteristic peptide endoPG signatures ^{226}CXGGHGXSIGSVG and ^{263}RIK were observed on the predicted ccen-1 sequence (Rambosek and Leach, 1987). Ccen-1 has 93% peptide sequence similarity to the *C. parasitica* endopg (Fig. 3) and the DNA sequences of these endoPGs have 87% identity. Web-based SOPMA for secondary structure prediction model showed that ccen-1 belongs to the β-structural proteins (data not shown).

DISCUSSION

In this study, we have successfully cloned and analysed an endopolygalacturonase gene from *C. cubensis*. To achieve this we used degenerate PCR and a modified inverse PCR technique. The modification involved cutting out a specified region of *Hin*dIII genomic digests using information obtained from Southern blot analysis. This allows for enrichment of circularised template DNA that contains the region of interest. This also results in a cleaner PCR product instead of smears, thus few PCR optimisation reactions are required.

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C.cubensis      MLSLVLLAALLPLIQAVPAPAVTPAAQLVDRASGSCTFTDAAAVSKSKTSCATITLSNIA 60
C.parasitica    MFSTLLLAALLPLIQAAPAPAVTPAAHLEDRASKSCTFTDAAAVSKSKASCATITLNNIA 60
                * *  ********** ********* * **** ************** ******* ***

C.cubensis      VPSGTTLDLTKLNSGTKVIFEGTTSFGYKEWEGLLISVSGTDIEVTGASGHVIDGNGAAW 120
C.parasitica    -PSGTTLDLTKLNSGTKVIFAGTTSFGYKEWEGPLISVSGTDIEVTGASGHVIDGNGAAW 119
                 ****************** ************ *************************

C.cubensis      WDGEGSNGGKTKPKMFYAHSLKQSTISGLNVKNTPVQFMSINSATDLNIINVKMDNSAGA 180
C.parasitica    WDGEGSNGGKTKPKMFYAHSLKQSTIHNLKVKNTPVQFMSINSATDLNVIDVTMDNSAGA 179
                ************************  * ***************** * * *******

C.cubensis      SKGHNTDAFDVGSSENIYISGAVINNQDDCLAINSGTNITFTGGSCTGGHG-SIGSVGGR 239
C.parasitica    SKGHNTDAFDVGSSENIYISGAVINNQDDCLAINSGTNITFTSGSCTGGHGLSIGSVGGR 239
                ****************************************** ******** ********

C.cubensis      SDNTVKTVSITTSKIITTSQNGVRIKTVYDAAGSVSDVPYSGITLSGITNYGIVIEQDYE 299
C.parasitica    SDNTVKTVSITNSKIIN-SQNGVRIKTVYDATGSVSDVTYSGITLSGITNYGIVIEQDYE 298
                *********** ****  ************ ****** ********************

C.cubensis      NGSPTGTPTTGVPITGLTVSKVTGSVASSATDVYILCGKGSCSGWKWSGNSVTGGKKSSS 359
C.parasitica    NGSPTGTPTTGVPITGLTVSKVTGSVASSATDVYILCGKGSCSGWKWSGNSVTGGKKSSS 358
                ************************************************************

C.cubensis      CKNIPSGASC 369
C.parasitica    CKNIPSGASC 368
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FIGURE 3 An amino acid sequence alignment of the endoPGs from *C. cubensis* and *C. parasitica*. The N-linked glycosylation sites are indicated in bold. Asterisks indicate similar amino acids between the two endoPGs.

The *ccen-1* gene has at least two homologues in the genome of *C. cubensis* and has a high sequence similarity to that of *C. parasitica* (enpg-1) (Gao *et al.*, 1996). High sequence similarity of endoPGs in related fungi is not surprising and has been reported previously in fungi (Posada *et al.*, 2000; Chimwamurombe *et al.*, 2001).

A comparison of *C. cubensis* and *C. parasitica* endoPGs revealed some interesting features. Our results have shown that the introns in the endoPG of *C. cubensis* are 64 and 145 bp in size, compared to those of *C. parasitica* that are 61 and 62 bp, respectively. The typical intron splices sites, GTPuNGT…NPuCTPuACN…PyAG, are conserved in *C. cubensis* as in other filamentous fungi (Rambosek and Leach, 1987). It would thus be possible to distinguish between the two fungi based on the size of the second intron. In addition, the endoPG peptide signature sequence CXGGHXSIGSVG show that the endoPG of *C. cubensis* lacks the sixth residue of the conserved motif because of a 3 bp deletion in its coding sequence. It was also observed that the two N-linked glycosylation sites are conserved in their relative sites on the endoPGs of *C. cubensis* and *C. parasitica* (Fig. 3). This implies that these enzymes may have similar glycosylation patterns.

The C-terminal portions of the endoPGs of *C. cubensis* and *C. parasitica* have no amino acid variations. However, variations exist in the N-terminal and middle domains. This may have implications in the substrate specificity of these enzymes as well as their differential ability to interact with polygalacturonase-inhibiting proteins (PGIPs).

We showed that *C. cubensis* endoPG exists as a multicopy gene; therefore the sequence data for the remaining members of this endoPG family are still to be determined. Furthermore, the role of the *C. cubensis* endoPG (*ccen-1*) in causing diseases, could subsequently be determined by a targeted-gene disruption approach, although the endoPG in *C. parasitica* has been shown not to be a significant factor in pathogenicity (Gao *et al.*, 1996). However, careful interpretation of this finding is required since only one of the endoPG homologues was disrupted and it is possible that the other homologues may be more relevant.

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