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Cloning and Sequence Analysis of the Endopolygalacturonase Gene from the Pitch Canker Fungus, *Fusarium circinatum*

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Abstract. The fungus *Fusarium circinatum* causes pitch canker disease on mature pine trees and root rot and damping-off of pine seedlings. Endopolygalacturonases (endoPGs) play a major role during penetration of plants by fungi. Digestion of the pectic polysaccharides in the plant primary cell walls is one of the earliest functions of endoPGs during infection. The research objective was to clone and characterize the gene encoding endopolygalacturonase in *F. circinatum*. A 970-bp DNA fragment was cloned by using degenerate PCR amplification from *F. circinatum* DNA. Sequence data for this fragment were used to design specific primers for use in genome walking to amplify and sequence the remaining portion of the *F. circinatum* endoPG gene (*Fcpg*). The amino acid sequence predicted from this gene showed 90% and 87% similarity to *Fusarium oxysporum* and *Fusarium moniliforme* endoPGs, respectively.

Endopolygalacturonases (poly[1,4-α-D-galacturonide] glycanohydrolase, EC 3.2.1.15) are the first cell wall-degrading enzymes that are produced by fungal pathogens during infection [4, 5]. Therefore, endoPGs play a role in the establishment of fungal pathogens. Several features of the endoPGs indicate a multiple role for them during pathological and physiological events characterized by targeted degradation of the cell wall [7]. EndoPGs digest plant cell walls to produce small oligosaccharides that are assimilated by the fungus. They also release oligogalacturonides that function as elicitors for plant defense responses [5]. The levels of elicitor-active oligogalacturonides increase in vitro upon interaction with plant cell proteins, called polygalacturonase-inhibiting proteins (PGIPs). This may trigger the hypersensitive response in incompatible plant–pathogen interactions [5, 7].

The fungus *Fusarium circinatum* is the causal agent of an important disease known as pitch canker on mature pine trees. In South Africa, it causes root rot and damping off in pine seedlings [12]. The role of endoPGs in the pathogenicity of *F. circinatum* has never been considered. The aim of this study was to clone and sequence the gene that encodes for endoPGs in *F. circinatum*. This forms part of a concerted effort to expand the base of knowledge pertaining to the physiology and biology of the pitch canker fungus.

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Materials and Methods

Fungal culture, growth conditions, and DNA isolation. *Fusarium-circinatum*, isolate MRC6213, was obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) and was cultured in malt extract broth at 25°C in a 250-ml conical flask for 10 days without shaking. Fungal mycelium was harvested by filtration and freeze-dried. Total genomic DNA was isolated from the fungal mycelia according to the protocol of Raeder and Broda [8].

PCR amplification and cloning. DNA manipulations were done according to standard protocols in Sambrook et al. [10]. All PCR reactions were done in a Hybaid Omnigene (UK) thermocycler. Degenerate primers FPG1 (GA(CT)AA(CT)GA(CT)TT(CT)(GA)A(CT)CC(GCT)AT(CT)) and FPG2 (CA(AGCT)GT(AG)TT(AGCT)GT(AGC)GG(AG)TA(AG)TT(AG) [1] were used to amplify endoPG fragments. The PCR products were cloned into the polylinker region of pGEM-T-Easy vector (Promega, UK).

Plasmid DNA was isolated from recombinant bacterial clones and sequenced by using SP6 (TAATACGACTCACTATAGGG) and T7 (TATTTAGGTGACACTATAG) primers with an ABI Prism model 377 sequencer (Perkin-Elmer Inc, USA). The sequence was used to design *F. circinatum* endoPG-specific primers FcPG1 (GGGG-

Adaptor: 5′ CTA ATA CGA CTC ACT GGG CTC GAG CGG CGG CCC GGG CAG GT 3′
3′H_2N-CCG GTC CA-P 5′

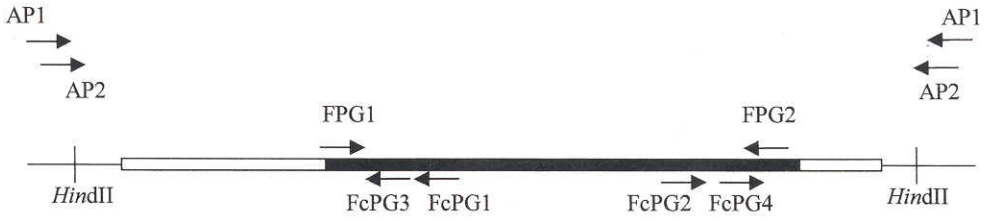

Fig. 1. Genome-walking strategy. The shaded box represents a 970-bp fragment of *F. circinatum* endoPG, and the open box depicts an unknown endoPG region to be sequenced. The restriction enzyme *Hin*dII cleaves in the region outside the endoPG coding region. Arrows point to the direction of amplification. Names of primers are indicated. Primers AP1, AP2 bind in the adaptor region.

GAAAATACTTTGGG), FcPG2 (GGTATATTCTGTGTGGTG), FcPG3 (CCACTAATGACAATGGGG) and FcPG4 (GGTGGTGG-TAAGACTAGC).

Genome walking and sequence analysis. Genome walking protocol was modified from Siebert et al. [11]. To amplify the regions on both the 5′ and 3′ ends of the partial *Fcpg* sequence, genomic DNA (3 μg) of *F. circinatum* was digested for 24 h with enzyme *Hin*dII (20 units). The digested fragments were resuspended in 20 μl of water. Ten microliter of the digested fragments was ligated to 5 μl adaptors (50 pmol/μl) for 16 h at 10°C. The structure of the adaptors is illustrated in Fig. 1. Ligated DNA was purified over Qiagen PCR clean-up columns and diluted 100-fold.

A primary PCR reaction was performed with primer pairs AP1 (GATCATAATACGACTCACTATAGGG), FcPG1 or AP1, FcPG2. A secondary PCR reaction was performed in the same way as the primary PCR reaction by using primer pairs AP2 (AATAGGGCTCGGGCGG), FcPG3 or AP2, FcPG4 for 20 cycles (genome-walking strategy on Fig. 1). Distinct PCR fragments were purified from agarose gels and were cloned into pGEM-T-Easy vector. Plasmids with inserts were sequenced with T7 and SP6 primers. Sequences were analyzed with Sequence Navigator (Perkin-Elmer) and were used to draw UPGMA trees with computer package PAUP. Fcpg DNA and peptide sequence alignments were produced by using the CLUSTAL program (http://www.2.ebi.ac.uk). Secondary structure prediction plots were obtained using a web-based SOPMA tool (*http://www.expasy.ch/tools/#secondary/*).

Genbank accession number. The sequence data for *Fusarium circinatum* endopolygalacturonase was deposited in the Genbank database. The accession number is AF207825.

Results

Cloning and sequence analysis of *Fcpg*. The sequence of a PCR fragment of *F. circinatum* genomic DNA obtained with primers FcPG1 and FcPG2 was found to be 970 bp in size and 85% identical to that of the endoPG of *F. oxysporum*. By genome walking towards the 5′ and 3′ ends, distinct fragments of about 500 bp and 300 bp respectively were amplified. Sequence analysis of the cloned fragments showed that they had overlapping stretches with the previously sequenced 970-bp endoPG fragment. In total, 1561 bp of DNA sequence was obtained (data not shown). This part of the genome contained the full coding region for an endoPG. This was supported by the high similarities with other *Fusarium* endoPGs, i.e., 90% and 87% similar to *F. oxysporum* and *F. moniliforme*, respectively [3, 6].

Fusarium circinatum endoPG is encoded by a gene 1332 bp in length, and from sequence comparison with published endoPG sequences of *Fusarium* spp., *Fcpg* has four introns. Intron sizes were consistent with those of most filamentous fungi, between 49 and 85 bp [9]. Some putative promoter motifs were present in the 5′ non-coding region.

The peptide sequence of Fcpg has 374 amino acids and a molecular mass of 38.8 kDa with a theoretical pI of 6.63. There are two potential N-glycosylation sites of the Fcpg peptide sequence, while the endoPGs of *F. oxysporum* and *F. moniliforme* have three and four sites, respectively. These two N-glycosylation sites are conserved in all three *Fusarium* spp. The characteristic motifs, CXGGHGXSIGSVG and RIK [4], for fungal endopolygalacturonases were also observed in the predicted Fcpg peptide sequence. Secondary structure prediction plots showed that the Fcpg belongs into β-structural proteins class (results not shown).

Relatedness of *Fusarium* endopolygalacturonases. An unweighted pair group method with arithmetic averages (UPGMA) tree was produced by using the endoPG peptide sequence of the three *Fusarium* spp. and *Cryphonectria parasitica* (causal agent of chestnut blight) as outgroup (Fig. 2). The tree shows that the *F. circinatum* endoPG sequence is more closely related to that of *F. oxysporum* than it is to *F. moniliforme*.

Discussion

In this study, we report on the nucleotide sequence of *Fcpg*, the gene that encodes for the endopolygalacturonase in the pitch canker fungus *F. circinatum*. *Fcpg* occurs as a single copy in the genome (data not shown). This is consistent with previous studies on *F. oxysporum*

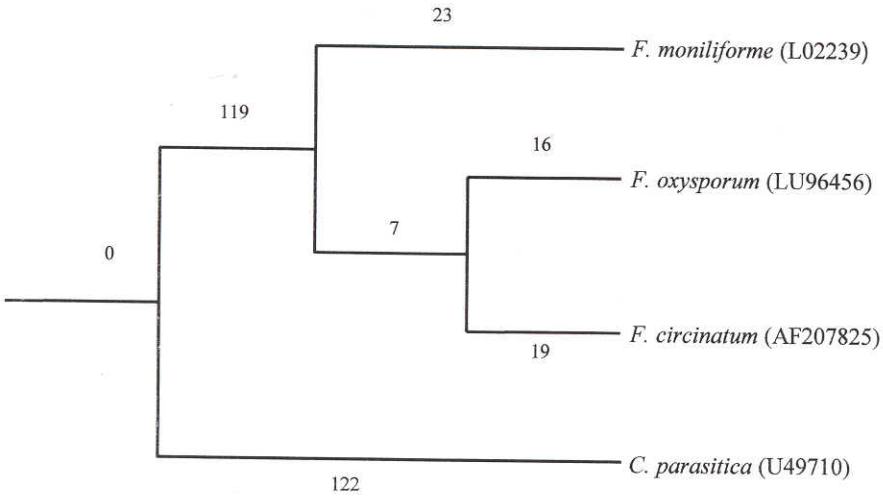

Fig. 2. A UPGMA tree produced from an alignment of peptide sequences of fungal endopolygalacturonases. The number of differences is indicated.

and *F. moniliforme* [2, 6]. The *F. moniliforme* endoPG enzyme has four different isoforms [3]. These are derived from differential glycosylations of one polypeptide. The fact that Fcpg has two potential N-glycosylations compared with four in *F. moniliforme* endoPG suggests that the number of isoforms in *F. circinatum* may be fewer than those in *F. moniliforme*.

The peptide sequence of Fcpg has an interesting characteristic in the signal peptide region. The endoPG signal peptide sequences of *F. moniliforme* and *F. oxysporum* are very different [6]. Alignment of endoPG signal peptide sequences shows that the Fcpg peptide has two motifs, PSSSLQERD and AIAALPAA. Remarkably, the PSSSLQERD motif is present in *F. moniliforme* endoPG and is absent in *F. oxysporum* endoPG. The AIAALPAA motif is present in *F. oxysporum* and is absent in *F. moniliforme* endoPG. Fcpg possesses both of these motifs. It is, therefore, possible to distinguish among the three *Fusarium* species by using only the signal peptide sequence. This may have implications in the evolution of endoPGs in *Fusarium* spp.

We have cloned and sequenced the endopolygalacturonase gene of *Fusarium circinatum* by using degenerate PCR and an elegant technique of genome walking. This gene has been shown to occur only once in the genome and is closely related to the endoPGs of other *Fusarium* spp.

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