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Cloning and sequence analysis of a polygalacturonase-encoding gene from the phytopathogenic fungus *Sclerotinia sclerotiorum*

(Pectin degradation; endopolygalacturonase; nucleotide sequence; sequence comparisons; secondary structure; promoter; gene family)

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SUMMARY

The phytopathogenic fungus *Sclerotinia sclerotiorum* produces a number of extra-cellular pectin-degrading enzymes. We have cloned and determined the complete sequence of a gene (*pg1*) encoding an endopolygalacturonase (PG1). The coding region consists of a non-interrupted 1143-bp open reading frame. *S. sclerotiorum pg1* was compared to other fungal PG-encoding genes. Basic transcription control sequences were identified in the 5′ non-coding region.The deduced amino acid (aa) sequence (380 aa) of the enzyme is compared to seven fungal PG sequences and shows a high level of identity (41.5 to 59.8%). Predicted secondary structures were compared, revealing a similar protein organization most probably in antiparallel β sheets. Hybridization analysis using a *pg1* 0.65-kb *Bam*HI fragment as a probe allowed the identification of seven different recombinant phages from a genomic library. Analysis of the hybridizing restriction fragments suggests that PG-encoding genes are organized as a family.

INTRODUCTION

The role of pectin-degrading enzymes in the infection process of phytopathogenic fungi is poorly understood, despite the importance of fungal diseases as the major cause of economic loss in plant agriculture. *S. sclerotiorum* is an ubiquitous fungus able to infect a wide range of plants and to secrete a complete set of enzymes to macerate plant tissues (Riou et al., 1991). Biochemical studies have demonstrated that *S. sclerotiorum* secretes several molecular forms of hydrolases that attack pectin but which differ in their isoelectric point and molecular weight (Riou et al., 1992a). An analytical and immunological study revealed a temporal sequence in the production of pectic enzymes and isoenzymes during growth in pectic polymer cultures, corresponding to the induction of several endo and exoenzymes (Riou et al., 1992b). Enzyme activities in non-induced glucose-grown cultures were associated with polygalacturonase and pectinmethylesterase isoforms which were produced constitutively (Riou et al., 1992b). However, nothing is known about the structure or the regulation of the genes that encode these enzymes in *S. sclerotiorum*.

Information concerning pectinase-encoding genes of saprophyte fungi is mainly derived from studies on *Aspergillus* (Bussink et al., 1990; 1992; Ruttkovski et al., 1991; Kitamoto et al., 1993). There have been fewer molecular genetics study of pectinases from phytopathogenic fungi. Polygalacturonase genes from *Cochliobolus carbonum* (Scott-Craig et al., 1990) and from *Fusarium moniliforme* (Caprari et al., 1993) have been cloned.

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Abbreviations: aa, amino acid(s); bp, base pair(s); kb, kilobase(s) or 1000 bp; nt, nucleotide(s); ORF, open reading frame; PG, polygalacturonase; *pg*, gene encoding PG; *S.*, *Sclerotinia*; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6; X or Xaa, any aa.

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The aim of this study was the isolation and the characterization of a gene called *pg1* encoding an endopolygalacturonase from the phytopathogenic fungus *S. sclerotiorum*.

EXPERIMENTAL AND DISCUSSION

(a) Characterization of the gene encoding PG1

The sequence of the PG1-encoding gene revealed a 1143-bp ORF (Fig. 1) which contained no intron. The nt sequence encodes a polypeptide of 380 aa with a calculated M_r of 37849 that has a high Ser, Gly and Thr content (35%).

The N-terminal sequence of the *S. sclerotiorum* PG1 polypeptide strongly suggests that the encoded protein is post-transcriptionally cleaved, releasing a putative signal peptide of 17 aa. There is a Lys^{41}-Arg^{42} sequence which is the substrate for KEX 2 endoprotease. Thus, PG1 may be synthesized as a preproprotein that matures by a two-step process. The deduced PG1 sequence has two potential *N*-glycosylation sites situated at aa 314 and 361 (Fig. 1).

The 260 nt in the 5′-non coding region of the *pg1* gene were investigated for various promoter consensus sequences. A number of possible transcription control sequences were identified. Three C+T-rich regions were detected, 56, 90 and 137 bp upstream from the ATG start codon. The longest region is situated at nt −90 and is composed of 17 consecutive pyrimidine bases. A putative

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                                                                                           CTAGAATTGCAGCTTCAACA  -241
CAGCATCAATTACTTTGGCTAATTGCTGGTCAGGTTGCACGGGTTTACCGTTGTCGACTTGTGGAGAGCTGACGGTGCGATTTAACGGTGTCCCACTTTAAATCTTCCCCAAATTTCAAT  -121
CTCGTCTTGACATATATAAGCTTCCAATACCTTCTTTTCTTTCCTCATCTCATCATCACTTACATTCTTTTCCATACTGTCTAGTATTACATTCCTTAAATAACAACTCTACTAGCCAAT  -1
ATGGTTCACATTCTTTCCTCGGCCTTGAGCCTCCTTCGGCTGGGCGCAGCTGTGTCTGCCGCTCCAGCACCAGCACCAACTGCTGCACCAAACGTTGCCGATGCTCTTGCAGCAGTCGAG  120
 M  V  H  I  L  S  S  A  L  S  L  L  R  L  G  A  A  V  S  A  A  P  A  P  A  P  T  A  A  P  N  V  A  D  A  L  A  A  V  E   40
AAGAGGGCCGGTTCCTGCACCTTCTCGGATCCAGCGGTGCCGCTGCCTGCCATCAAGTCCAAGGCTTCCTGCGCAACCATCGTCATCAGCGCCGTCGCTGTTCCATCCGGAACCACTCTC  240
 K  R  A  G  S  C  T  F  S  D  P  A  V  P  L  P  A  I  K  S  K  A  S  C  A  T  I  V  I  S  A  V  A  V  P  S  G  T  T  L   80
GACTTGACTGGCCTCAAATCTGGTACCCACGTTGTCTTCGAGGGTACTACTACCTTCGGTTACGAAGAGTGGTCCGGTCCTCTCGTCTCCGTCTCCGGAACTGACATCACCGTTACCGGT  360
 D  L  T  G  L  K  S  G  T  H  V  V  F  E  G  T  T  T  F  G  T  E  E  W  S  G  P  L  V  S  V  S  G  T  D  I  T  V  T  G  120
GCCTCCGGAAGCGTTCTCGATGGTAACGGAGCCAAGTACTGGGATGGCAAGGGTACCAACGGTGGAAAGACCAAGCCAAAGTTCTTCTACGCTCACTCCTTGAAGGGCAAGTCCTCCATC  480
 A  S  G  S  V  L  D  G  N  G  A  L  Y  W  D  G  K  G  T  N  G  G  K  T  K  P  K  F  F  Y  A  H  S  L  K  G  K  S  S  I  160
AACAACGTCAAGATCTTGAACTCCCCAGTCCAAGTTTTCTCCATCAACTCTGCCTCTGGTCTTACTCTCTCTGGTATCACTATCGACAACTCTGCCGGTAACTCCCTCGGACACAACACT  600
 N  N  V  K  I  L  N  S  P  V  Q  V  F  S  I  N  S  A  S  G  L  T  L  S  G  I  T  I  D  N  S  A  G  N  S  L  G  H  N  T  200
GATGCTTTCGATGTTGGATCTTCCACTGATATCACCATCTCCGGCGCCAACGTTCAAAACCAAGATGACTGTCTCGCTATCAACTCCGGTACCGGCATCACCTTCACCGGTGGAACCTGC  720
 D  A  F  D  V  G  S  S  T  D  I  T  I  S  G  A  N  V  Q  N  Q  D  D  C  L  A  I  N  S  G  T  G  I  T  F  T  G  G  T  C  240
TCCGGTGGTCACGGTCTCTCCATCGGATCCGTCGGTGGACGTTCCGACAACGTTGTCTCTGATGTTATCATTGAATCCTCCACCGTCAAGAACTCCGCCAACGGTGTTCGCATCAAGACT  640
 S  G  G  H  G  L  S  I  G  S  V  G  G  R  S  D  N  V  V  S  D  V  I  I  G  S  S  T  V  K  N  S  A  N  G  V  R  I  K  T  280
GTTTCCGGTGCCACTGGATCCGTCTCCGGCGTTACCTACAAGGACATCACCCTCTCCGGTATTACCAGCTACGGTGTTGTCATCGAGCAAGATTACGAGAACGGTTCCCCAACTGGAAGG  960
 V  S  G  A  T  G  S  V  S  G  V  T  Y  K  D  I  T  L  S  G  I  T  S  Y  G  V  V  I  E  Q  D  Y  E  N  G  S  P  T  G  K  320
CCAACCTCCGGTGTTCCCATCACTGGTGTCACTCTCTCCAACGTTCACGGTACCGTCTCTTCCTCCGCTACCAACGTTTACGTTCTCTGCGCCAAGTGCTCTGGCTGGACCTGGGATGTT  1080
 P  T  S  G  V  P  I  T  G  V  T  L  S  N  V  H  G  T  V  S  S  S  A  T  N  V  Y  V  L  C  A  K  C  S  G  W  T  W  D  V  360
AACGTTACTGGTGGTAAGACCTCCACCAAGTGCGCTGGTCTCCCATCTGGTGTCAAGTGTTAAATAGTTTAACACGGCTACTTTCAAGCACTGGATTCCACTAGGAAATCAATTGTAGAG  1200
 N  V  T  G  G  K  T  S  T  K  C  A  G  L  P  S  G  V  K  C  ●                                                           380

GGGGAATGGAGAAAGGGGTTCGAGCCCGGCTGCACCCAGTTGTCAATTCTTTGTACATATAGCTTCTCT                                                    1269
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Fig. 1. Nucleotide sequence of the *S. sclerotiorum pg1* gene and deduced aa sequence. Putative TATA box is indicated by a star, putative CAAT boxes by open triangles. C+T-rich regions are underlined. A+T-rich regions are indicated by broken lines. The stop codon is indicated by a filled circle, a putative polyadenylation signal is underlined. Putative glycosylation sites are double underlined. The downward arrow indicates a possible target for the KEX endoprotease. The nt sequence data for *pg1* have been assigned the accession No. L12023 in the GenBank database. The PG1-encoding gene was cloned using a 1.5-kb cDNA fragment of the *pg* gene from *A. niger* strain RH5344 as a probe. This probe was radiolabelled and used to screen 5×10^4 clones of a genomic library of *S. sclerotiorum* prepared by ligation of fragments generated by partial *Mbo*I digestion with the arms of λEMBL3, produced by *Eco*RI and *Bam*HI treatment (hybridization was overnight in $5 \times$ SSC/50% formamide/0.1% SDS at 37°C with a final wash in $2 \times$ SSC/0.1% SDS at room temperature). After a second round of screening, DNA was isolated from twelve recombinant phages, digested with *Sal*I, which separates phage DNA from the fungal insert, and again hybridized with the cDNA *pg* probe. Among the twelve phage DNAs analyzed, eight hybridized to the probe. DNA from clone 9.1a containing one 2.3-kb *Sal*I hybridizing fragment, was chosen for further studies. This fragment was subcloned in pUC18 and sequenced by the dideoxy chain-termination method (Sanger et al., 1977), using T7 DNA polymerase (Pharmacia) and phage M13 universal primers according to the manufacturer's recommendations.

TATA box (TATATAA) was identified at nt −108. Three additionnal A+T-rich regions were located at nt −25, −130 and −144. Four possible CAAT boxes were identified at nt −234, −124, −97 and −5. The sequence surrounding the ATG start codon is in agreement with the Kozak consensus sequence (CCACCATGGC; Kozak, 1981). No typical polyadenylation signal (AATAAA) could be identified within the 126 bp sequenced of the 3′-untranslated region, but a similar motif AAATCAAA was detected 42 bp downstream from the stop codon, at nt 1186 (Fig. 1).

Analysis of the codon usage of the *pg1* sequence showed that only 45 out of the 61 sense codons are used, with a third position preference for pyrimidines over purines. Codons ending in C are preferred and represent 49% of the codons used, except for Val, Gly and Asp, where T is preferred.

(b) Primary structure analysis, comparison with other PG sequences and secondary structure predictions

The PG1 sequence was compared to seven fungal PG sequences: PGI, PGII and PGC from *Aspergillus niger* N400, PG from *A. niger* RH5344, PGNI from *C. carbonum,* PG from *A. oryzae* and PG from *F. moniliforme* (Fig. 2). The deduced aa sequences are of similar sizes (from 362 to 383 aa). The eight PG sequences share an overall 20% identity (77 perfect matches among 398 aligned residues). The degree of similarity increases up to respectively 59.8, 58.8, 55, 56.3, 57.8, 41.5 and 59.2%, when PG1 from *S. sclerotiorum* is compared to PG1, PGII and PGC from *A. niger* N400, PG from *A. niger* RH5344, *A. oryzae* and *F. moniliforme*, and PGNI from *C. carbonum.* Eight Cys and eighteen Gly residues are found among the conserved aa. This might suggest the presence of important disulfide bridges and of highly flexible regions (loops) in these regions.

The positions of the potential *N*-glycosylation sites (Asn Xaa Ser/Thr) are not conserved among the fungal *pg* genes. The four *A. niger* sequences contain an *N*-glycosylation site at aa 263 (with respect to the *S. sclerotiorum* sequence), which is not found in *C. carbonum, A. oryzae, F. moniliforme* or *S. sclerotiorum* sequences. The *N*-glycosylation site situated at aa 314 in the *S. sclerotiorum* sequence is found only in *A. niger* PGI and *A. oryzae* PG, and the site situated at aa 361 is not found in any of the other sequences (Fig. 2). An extensive search using the PROSITE database (Bairoch, 1992) reveales the presence of a motif, CXGGHGXSIGSVG (aa 239–251) that is specific for this family of fungal enzymes. When this comparison was extended to higher plants and bacteria, the extent of this motif was reduced to GHGXSXGS, where X is always a hydrophobic residue. The positively charged residues in the region Arg^{277}-Ile^{278}-Lys^{279}, which

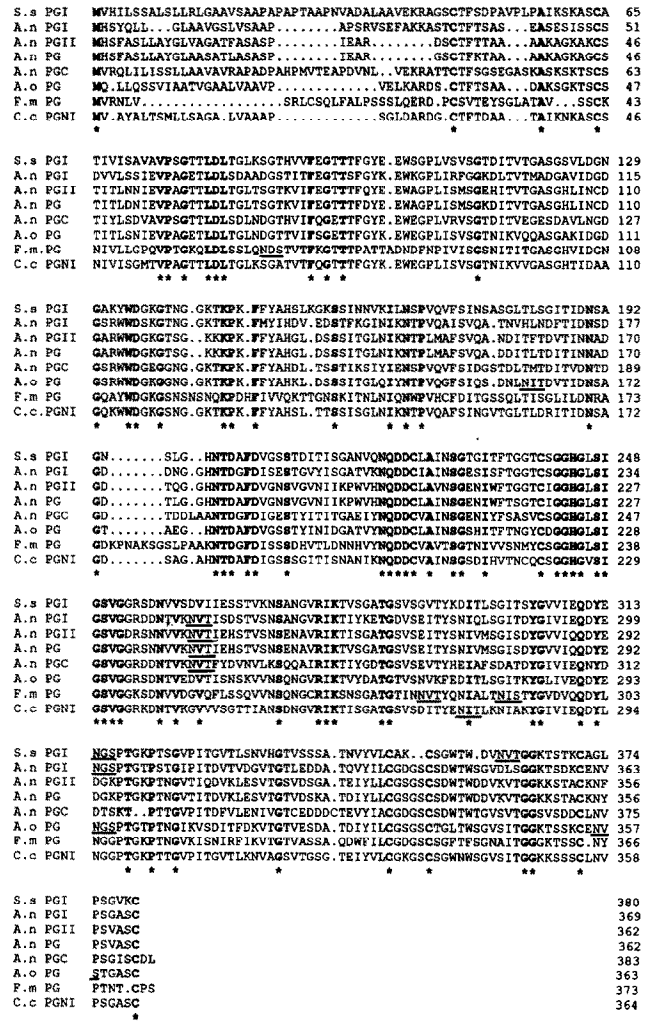

Fig. 2. Comparison of the deduced aa sequence of the PG1 of *S. sclerotiorum* (S.s) with the PG sequences of *A. niger* (A.n) N400 (PGI, PGII, PGC), *A. niger* (A.n) RH344 (PG), *C. carbonum* (C.c) (PGNI), *A. oryzae* (A.o) (PG) and *F. moniliforme* (F.m) (PG). All the protein sequences analysis have been performed with the help of the ANTHEPROT suite of programs (Geourjon and Deléage, 1994). The multiple alignment has been carried out with the Clustal program (Higgins and Sharp, 1988). Gaps were introduced into the sequences to achieve optimal alignment and are represented by dots. Residues that are conserved amongst all six sequences are in bold letters and are indicated by asterisks. The putative *N*-glycosylation sites are underlined.

may be candidates for ionic interactions with the carboxylate groups of the substrate (Bussink et al., 1992) are also found in *S. sclerotiorum* PG1. This sequence is also conserved in prokaryotes and in higher plants. An extensive search for this pattern in a number of databases (SwissProt 27, PIR 38 and GenBank 79) revealed that it is very specific (no other sequence bearing this motif was found), and could be considered as the second signature of this enzyme family.

Secondary structure predictions (Fig. 3) of the PG1 sequence clearly suggest that this protein belongs to the all β structural class of proteins, if the signal peptide segment (aa 1 to 42) is not taken into account. The deduced

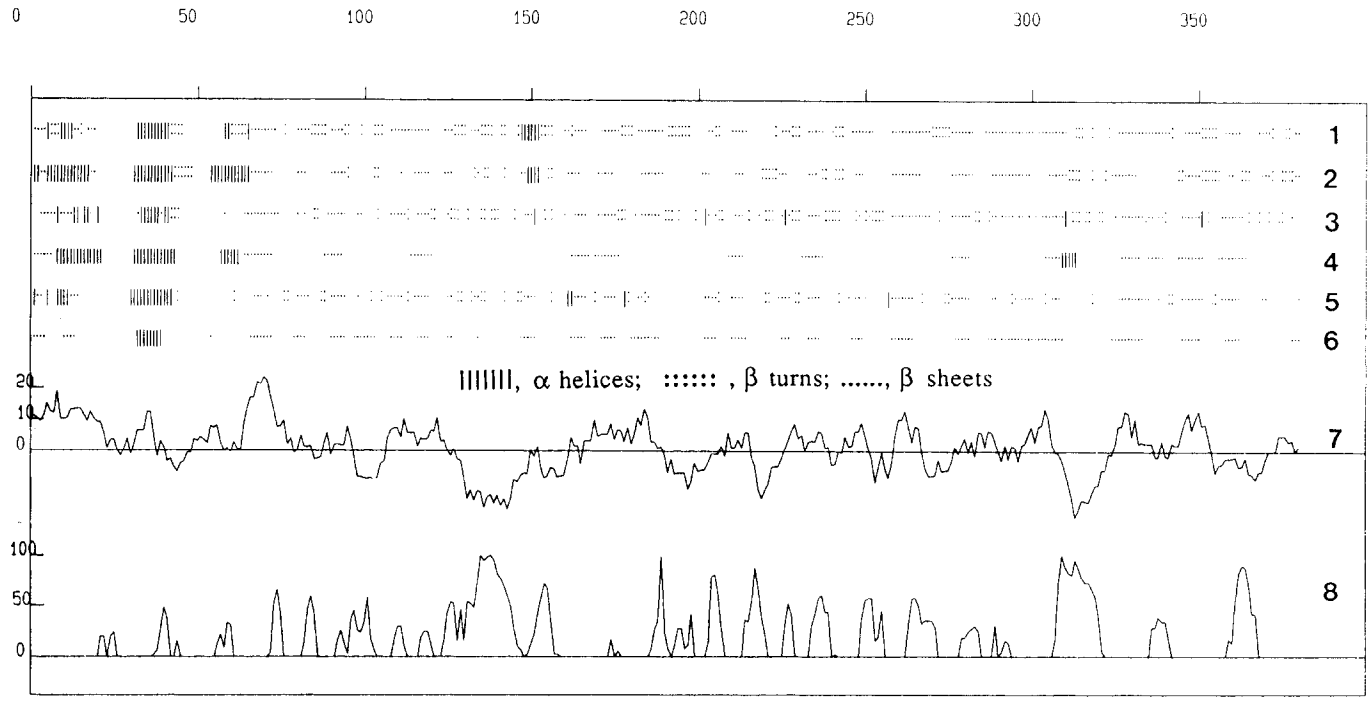

Fig. 3. Predicted secondary structure of *S. sclerotiorum* PG1. The blanks in the sequence represent coil segments. The numbered scale indicates the position of residues in the sequences. The secondary structure (lines 1 to 6, respectively) has been predicted, respectively, according to GOR II (Gibrat et al., 1987), GOR I (Garnier et al., 1978), HOMOLOGUE (Levin et al., 1986), Chou and Fasman prediction method (1978), 'Double prediction method' (Deléage and Roux, 1987) and SOPM (Geourjon and Deléage, 1994). The hydropathy profile (line 7) has been generated by the Kyte and Doolittle method (1982). The antigenicity profile (line 8) is that of Parker et al. (1986).

β sheet structures predicted by all the methods are most likely to be antiparallel since sheet-turn-sheet motifs are predominant along the sequence. The two largest peaks of hydrophylicity, and antigenicity (Fig. 3) are located in regions that are predicted to be either in β turns or in coil regions suggesting that these stretches could be the loop segments. The agreement between the different predictive schemes is excellent indicating that the quality of the predictions should be good.

The secondary structures of all other known fungal endopolygalacturonases sequences were also analysed, revealing that they are made up of β sheets, indicating a common folding pattern for this type of enzyme. Although no folding pattern can be derived from secondary structure prediction alone, it seems that this family could have a β barrel folding pattern, made up of 15 to 18 β strands.

(c) Evidence for a polygalacturonase family of genes in *Sclerotinia*

Eight recombinant phages were isolated after screening of the genomic library with the *A. niger* RH5344 *pgII* cDNA probe and one of these clones was shown to contain the *pg1* gene. The relationships between the eight putative *pg* clones were investigated. DNA from all eight clones was extracted and digested with *Sal*I which releases the fungal insert from the λEMBL3 arms, and with *Eco*RV and *Bam*HI which, respectively, cut once and three times in *pg1*. After Southern transfer, these DNAs were hybridized with a *pg1* 0.65-kb *Bam*HI fragment as a probe. Analysis of *Sal*I digests revealed that all the hybridizing fragments are of different sizes, and are larger than the 2.3-kb fragment containing the *pg1 gene*. The restriction analyse with *Bam*HI and *Eco*RV showed that three of the phage DNAs are similar to the phage containing the *pg1* gene, while the other phages exhibited different restriction profiles. These results suggest that *S. sclerotiorum* might contain a family of PG-encoding genes.

Partial sequencing of the phages DNAs exhibiting similar *Bam*HI and *Eco*RV restriction profiles than the *pg1* gene (L. Fraissinet-Tachet, unpublished data), revealed differences in the nt sequences indicating that they correspond to different genes. These data are in agreement with previous biochemical studies. Different exoPG (Riou et al., 1992a) and endoPG (Keon and Waksman, 1990) have been purified from *S. sclerotiorum*. The N-terminal sequence of PG1 (Ala Gly Ser Cys Thr Phe Ser Asp Pro Ala Val Pro) differs from the equivalent sequences determined for an exoPG (Ser Val Asp Ser Phe Ile Ala Xaa Glu Pro Ile Ala) and an endoPG (Ala Thr Thr Gly Thr Phe Ser Gly Ser Ser Gly Ala), indicating the presence of multiple PG-encoding genes. Moreover, contrary to the data described by Marciano (1982), we

have shown that under our growth conditions, *S. sclerotiorum* can produce numerous proteins which are able to degrade pectic substrates: PG and pectinmethylesterase activities have been resolved in nine and four different forms, respectively (Riou et al., 1992b). The existence of gene families would explain the multiplicity of these pectic enzymes in *S. sclerotiorum*.

(d) Conclusions

(*1*) The *S. sclerotiorum pg1* gene encoding endoPG has been isolated and sequenced. The deduced aa sequence shows strong similarities to endoPG from saprophytic and phytopathogenic fungi.

(*2*) An extensive database search revealed that the most conserved regions, sequences CXGGHGXSIGSVG (aa 239–251) and RIK (aa 277–279) are also found in the fungal endoPGs and in the plant exoPGs, they can thus be considered as specific signatures of the PG.

(*3*) The predicted secondary structure suggests that this protein belongs to the β structural class of proteins.

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