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Differential expression of polygalacturonase-encoding genes from *Penicillium griseoroseum* in different carbon sources

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A second polygalacturonase-encoding gene (*pgg2*) of *Penicillium griseoroseum* was cloned and consists of an opening reading frame of 1107 bp after removal of two introns. The gene encodes a protein of 369 amino acids with a predicted molecular mass of 38.3 kDa. The deduced protein sequence exhibited high homology with other fungal endopolygalacturonases. A polymerase chain reaction (PCR)-based strategy was used to study the expression patterns of *pgg1* and *pgg2* genes under different culture conditions and our results show that both genes are regulated by the carbon source at the transcriptional level. The *pgg1* transcript was detected at 76 h of fungal growth in pectin while the *pgg2* transcript was also induced by sucrose. The addition of yeast extract to glucose medium abolished the repressive effect of glucose, suggesting that the transcription of these genes is controlled by different mechanisms.
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Introduction

Pectinases degrade polysaccharides of the plant cell wall, are produced by diverse organisms, and participate in different biological processes. Phytopathogenic fungi secrete polygalacturonases (PGs) to help them colonize host tissues during infection [9]. The oligogalacturonides released by PG act as signal molecules to induce defense responses in plants [7]. Recently, PGs have been associated with fruit ripening, cell wall modification, and flower and leaf abscission [14,16]. However, a large amount of relevant research on pectinases is due to their biotechnological value in the beverage and food industries. For example, pectinases are used in fruit processing and are responsible for the specific degradation necessary for smooth textures [13]. Enzymatic depectinization is also important in the production of clear juices and concentrates and is currently used to increase wine quality [18].

Several PG-encoding genes have been cloned from fungi and PG multigenic families have been identified [8,9,21]. However, the four isoforms reported for *Fusarium moniliforme* are produced by a single gene [6]. Until now, there are few reports explaining the reason for this PG isoform diversity and their significance for the organisms [17,25].

Our group has been studying the physiological aspects that influence pectinase production by *Penicillium griseoroseum.* We have investigated the effect of different inducers in the enzymatic activity [3,19]. Alternative carbon sources, like sugar cane juice, have been examined to reduce the production costs of PG and pectin lyase [20]. In previous studies, we reported the isolation of the first PG-encoding gene (*pgg1*) from *P. griseoroseum* [22]. Here we describe the characterization of a second PG gene (*pgg2*) and the temporal expression of both genes, which are regulated at the transcriptional level. Also, the effect of other carbon sources, like sucrose and glucose, on PG production has been investigated. We expect that our studies will provide us the experimental basis for a strain improvement program, where the fungus would secrete large amounts of PG when cultivated in medium containing inexpensive carbon sources. These overproducing strains could be used in the textile industry for the degumming of crude textile fibers as an alternative for the chemical process normally used.

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Materials and methods

Microorganism and culture conditions

P. griseoroseum (strain CCT 6421; Coleção de Culturas Tropicais André Tosello, Campinas, Brazil) was isolated from forest tree seeds. Culture maintenance and inoculum production were the same as described previously [4]. Five-day-old conidia were inoculated into Erlenmeyer flasks containing 100 ml of minimal medium (g l^{-1} KH_2PO_4 3.4; K_2HPO_4 6.8; $(NH_4)_2SO_4$ 1.0, pH 6.3), supplemented with 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 0.6 g l^{-1} yeast extract when mentioned. Pectin (Sigma P9135, St. Louis, MO), glucose, or sucrose was added as carbon source to a final concentration of 3 g l^{-1}. Flasks were incubated at 25°C, on a rotatory shaker (150 rpm), and the cells were harvested as indicated below.

Cloning of the pgg2 gene

A 2.2-kb *Xba*I fragment containing the second PG gene was isolated from a genomic library as previously described [22]. Both strands were sequenced with universal (Promega, Madison, WI) or specific primers, using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The

sequencing reactions were analyzed on ABI Prism 310 automatic DNA sequencer (Applied Biosystems).

RNA isolation

Total fungal RNA was isolated after 24, 48, 72, 76, 85, and 96 h of incubation from mycelia cultivated in medium containing pectin as the carbon source [24]. To analyze the effect of different carbon sources on the expression of the genes under study, total RNA was prepared from mycelia grown for 24 and 76 h in pectin, glucose or sucrose medium supplemented or not with yeast extract. Genomic DNA was eliminated by treatment with RNase-free DNase I (Promega). Total RNA was prepared from three independent sets of experiments and used in reverse transcription polymerase chain reaction (RT-PCR) reactions (below) to ensure the reproducibility of the results.

RT-PCR and cDNA synthesis

The Reverse Transcription System kit (Promega) was employed for the synthesis of the first-strand cDNAs. The reaction was carried out at 42°C for 75 min in a 20-μl reaction mixture using 1 μg of total RNA and an oligo(dT) primer according to the manufacturer's instructions. Specific primers for *pgg1* and *pgg2* were synthesized for use in the following PCR amplifications: 5′-TTTCATATTTTCTTTATC-3′, 5′-AAAATTCG-CAGAATTTAGCAGCTA-3′ for *pgg1* (PIPG1 and PFPG1, respectively), and 5′-GATCGGACACCTACTTAAAA-3′ and 5′-CTGTTTATTGATCACCATTTGATC-3′ for *pgg2* (PIPG2 and PFPG2, respectively). Five microliters of the RT reaction was used in a PCR reaction containing 10 pmol each of the oligonucleotides PIPG1 and PFPG1 or PFPG1 and PFPG2, in a volume of 25 μl. Forty thermal cycles were employed, each consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 90 s, and a final 7-min extension step at 72°C. Primers for the γ-actin gene of *P. griseoroseum* were employed in each reaction to test the integrity of the RNA. The PCR products were electrophoresed in a 2% high-resolution agarose gel. To check if the bands were due to genomic contamination, *P. griseoroseum* genomic DNA was amplified with the same PCR mix and analyzed in the same gel. The reverse transcription reactions were also used to generate *pgg1* and *pgg2* cDNA. All RT-PCR products were cloned into pGEM-T easy vector (Promega) and sequenced as described above.

The GenBank accession numbers for the PG cDNA from *P. griseoroseum* are AF195791 (*pgg1*) and AF195790 (*pgg2*). The genomic sequence of *pgg2* has received the accession number AF195113.

Results

Cloning of the pgg2 gene

The *P. griseoroseum pgg2* gene has an opening reading frame of 1107 bp encoding a protein of 369 amino acids. Comparison of genomic and cDNA sequences of the *pgg2* gene confirmed the existence of two 58-bp introns. Intron 2 has the 5′ and 3′ splice sites consistent with the conserved sequences 5′ GTAaGT and (C/t)AG 3′ for fungal introns [1]. The 5′ splice site of the first intron (GTAGAT) resembles this consensus sequence. The deduced amino acid sequence has two potential glycosylation sites (Asn–X-Thr/Ser) at Asn 300 and Asn 338.

The amino acid sequence identity between the cDNA of *pgg1* and *pgg2* was 60%. The amino acid sequence identity between *pgg2* and PG genes from *P. digitatum* [11], *P. janthinellum* [15], *P. expansum* [12], and *Aspergillus niger* (*pgaII*) [5] was 84%, 64%, 66%, and 50%, respectively.

Differential expression of the pgg1 and pgg2 genes

The temporal patterns of expression of *pgg1* and *pgg2* were examined by RT-PCR analysis with gene-specific primers. The *pgg1* gene was expressed under inducible conditions at 76 h of incubation (Figure 1A). When PCR products were analyzed by electrophoresis, the *pgg1* transcript was barely detectable at other time points. The *pgg2* gene had a different expression profile in inducing medium (Figure 1B) as the transcript level remained approximately constant over time. These data are consistent with Northern analysis previously done (data not shown). For this reason, the time points 76 and 24 h were chosen to detect the presence of *pgg1* and *pgg2* transcripts, respectively, in different carbon sources supplemented or not with yeast extract. Results show that *pgg1* is expressed only in the presence of pectin with or without yeast extract (Figure 2A). However, glucose was the only condition tested that repressed *pgg2* (Figure 2B). When yeast extract was added to the glucose medium, lower expression was seen compared to the other conditions tested. Also, *P. griseoroseum* genomic DNA was amplified and analyzed on the same gel as the RT-PCR reactions. Due to the intron's presence, the different size of the

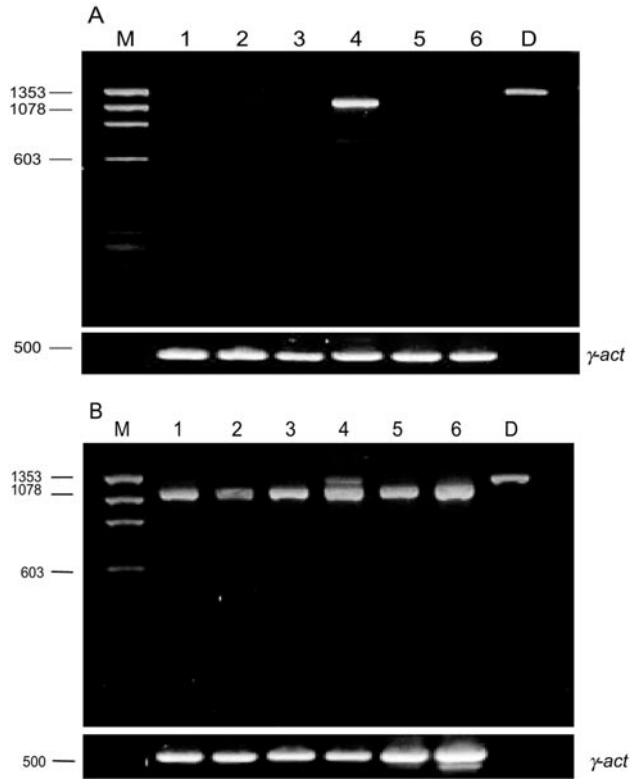

Figure 1 Temporal expression of the *pgg1* (A) and *pgg2* (B) genes detected by RT-PCR. Fungal RNA was isolated from mycelium grown in pectin-containing medium supplemented with yeast extract for 24 (1), 48 (2), 72 (3), 76 (4), 85 (5), 96 h (6), and employed in RT-PCR reactions. γ-Actin (γ-act) transcripts were used as an internal control. M: molecular size marker ϕX/*Hae*III. Product of genomic DNA amplification (D). The RT-PCR products were sequenced to confirm their identity.

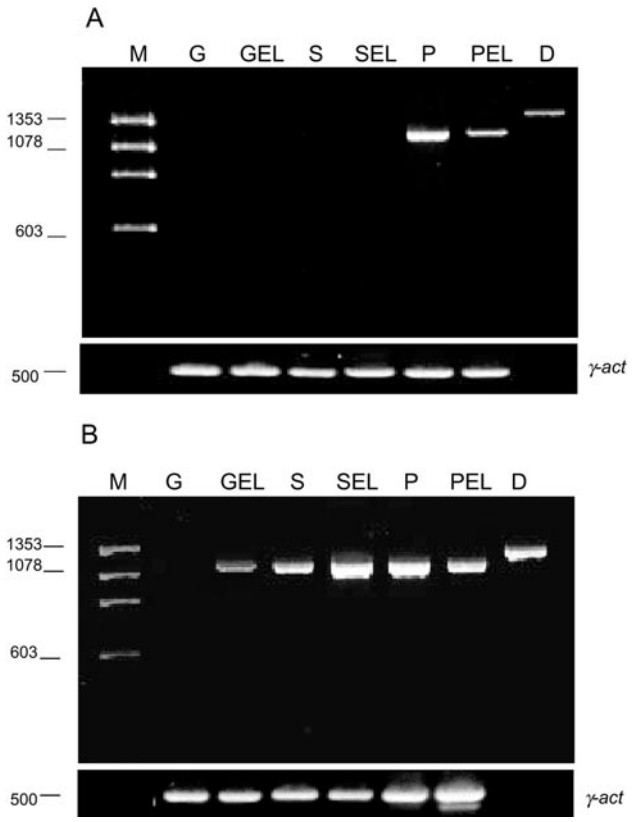

Figure 2 Expression of the *pgg1* and *pgg2* genes in media containing different carbon sources. Total RNA was extracted at 76 (A) and 24 h (B) from mycelium grown in glucose (G), sucrose (S), and pectin (P), supplemented or not with yeast extract (YE). Glucose and yeast extract (GEL), sucrose and yeast extract (SEL), pectin and yeast extract (PEL). γ-Actin (γ-act) transcripts were used as an internal control. M: molecular size marker ϕX/*Hae*III. Product of genomic DNA amplification (D). The RT-PCR products were sequenced to confirm their identity.

amplified product assured that no DNA was contaminating the RNA samples.

Discussion

Microorganisms that secrete pectinases have long been used in industry for different purposes. For the textile industry applications, the high levels of pectinase should be accompanied by little or no cellulase activity, since cellulase can reduce the fiber quality. As a starting point for our industrial strain improvement program, several isolates were assayed for their pectinase and cellulase activities [2]. *P. griseoroseum* was selected based on its induced pectinase production free of constitutive cellulases. We recently cloned the first PG-encoding gene from this fungus [22] and now report the characterization of a second gene, *pgg2*. The two genes so far characterized are probably endopolygalacturonase due to their high overall similarity with other fungal endopolygalacturonase sequences. The intron positions are conserved in *pgg1* and *pgg2*, despite their relatively low sequence similarity. This conservation of intron position was also observed in three PG genes from *Botrytis cinerea* [25], and the *pgaI* and *pgaII* genes from *A. niger* [5].

In this study, we used RT-PCR to verify the expression of *pgg1* and *pgg2*. Both PG-encoding genes are regulated at the transcription level; however, clear differences in expression were observed. The *pgg2* transcript level remained approximately constant over time while *pgg1* mRNA was detected only at 76 h. Pectin was the only inducer that triggered *pgg1* expression whereas expression of *pgg2* was only repressed upon incubation of mycelia on glucose. However, the addition of yeast extract could override the repressive effect of glucose. Baracat-Pereira *et al* [3] investigated the effect of yeast extract in pectin lyase production in *P. griseoroseum*. An organic substance of low molecular weight, soluble in the nucleotide fraction during extraction of yeast extract, was shown to induce pectin lyase synthesis when the fungus was cultured on sucrose. Their data supported that this substance could help to maintain a high intracellular concentration of cAMP, which would lead to enzyme synthesis. There is evidence that cAMP stimulates transcription by phosphorylating transcription factors, which would bind to *cis* elements activating gene expression [23]. A putative cAMP response element, as described by Fox *et al* [10], has been found in the promoter of the *pgg2* gene and is now being analyzed by eletrophoretic mobility shift assays. A putative binding site for the global regulator CREA overlaps a TATATAA element in the 5′ terminal region of *pgg1* and could explain the repression in the expression of this gene (unpublished data).

As seen from the results presented here, the two PG genes characterized so far in *P. griseoroseum* have their expression controlled by different mechanisms. The regulatory pathways that control the expression of other PG genes are still being investigated. However, their expression is altered by the carbon source present in the medium. For example, *B. cinerea* has an endopolygalacturonase family where genes are expressed with glucose, and polygalacturonic acid as carbon sources, although with some differences [25]. Bussink *et al* [5] reported the expression of the *pgaII* genes of *A. niger* and *A. tubigensis* to be strongly regulated by the carbon source.

Further studies will focus on characterization and regulation of other PG genes as well on a more detailed study of their promoters in a search for regulatory sequences that could explain a differential expression. Attempts to define an electroporation-based system for transformation of germinated conidia are now being done, aiming to obtain strains with multiple copies of the *pgg2* gene in the fungal genome. This gene was chosen based on its expression on a cheaper carbon source like sucrose. In the future, we expect that the results provided by our studies may provide more insight in the control of gene expression of filamentous fungi and contribute to obtain genetically engineered fungal strains for larger-scale PG production.

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