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ORIGINAL ARTICLE

Differential expression of *plg* genes from *Penicillium griseoroseum*: *plg*1 a pectinolytic gene is expressed in sucrose and yeast extract

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Abstract

Aims: To study the regulation of the *plg*1 and *plg*2 genes of *Penicillium griseoroseum*, in order to identify the industrial potential of their products in alternative carbon sources that are cheaper and widely available in Brazil.
Methods and Results: RT-PCR and Northern blot were used to investigate if *plg*1 and *plg*2 expression is under influence of catabolic repression, ambient pH and cAMP. Results demonstrated that the genes were differentially regulated depending on the carbon sources in the culture medium and pH. Sucrose, a noninducing carbon source of the pectinolytic system, was able to promote *plg*1 transcription but only when yeast extract was added into the culture medium.
Conclusions: The *plg* genes are differentially expressed. The *plg*1 gene is more attractive for industrial use due to its expression in alternative carbon sources like sucrose and yeast extract.
Significance and Impact of the Study: In recent years, industries have been trying to replace the toxic conventional treatments employed in these processes by more eco-friendly enzyme treatment. Alternative carbon sources will be tested with the aim to reduce the costs associated to pectin lyase production in Brazil.

Introduction

In nature, micro-organisms have been endowed with vast potentials such as the production of an array of enzymes, some of them commercially exploited over the years. Pectinases comprise a group of complex enzymes involved in the hydrolysis of pectic substances that are really relevant for the industry since they have a share of 25% in the global sales of food enzymes (Singh *et al.* 1999).

Pectinase-encoding genes have long been cloned due primarily to the huge industrial relevance of this class of food-grade enzymes. To date there are several commercial preparations of pectic enzymes routinely being used (Kashyap *et al.* 2001), but there is still a need for novel enzymes with specific biochemical and physical properties that meet industrial demands. In addition, the understanding of gene regulation can dramatically increase the successful application of pectinase technology. Although acidic pectinases are traditionally known for their application in fruit juice processing the use of alkaline pectinases have proven to be advantageous in industrial scale (Hoondal *et al.* 2000) and will probably expand the use of pectinases into other areas of application.

Commercial pectinase preparations are produced from fungal micro-organisms, mainly by *Aspergillus niger* strains (Couto and Sanromán 2006). Among the filamentous fungi, some of the genus *Penicillium* are good producers of pectinases (Alaña *et al.* 1990). However they have never been reported as a primary source for large-scale production of pectic enzymes.

The pectinase production by *Aspergillus* strains has been proposed using different solid agricultural and agro-industrial residues as substrates such as wheat bran, soy bran, cranberry and strawberry pomace, coffee pulp and coffee husk, husk, cocoa, lemon and orange peel, orange bagasse, sugar cane bagasse, wheat bran, sugar cane bagasse and apple pomace (Couto and Sanromán 2006).

Our group has concentrating efforts on the study of pectinases secreted by *Penicillium* for more than a decade. The work started in 1989 when Baracat *et al.* analysed several strains of filamentous fungi, with the purpose of obtaining enzymes for textile fibres degumming and food industry application (Baracat *et al* 1989). *Penicillium griseoroseum* was selected because of its appreciable pectinase production, especially pectin lyase, and absence of cellulase. Pectin lyase (EC 4·2·2·10) (PNL) is unique among all pectinases due to its intrinsic capability to use highly esterified pectin, which is particularly advantageous in the fruit juice industry since no methanol is formed in the course of enzyme action and hence the specific aroma is not affected (Alaña *et al.* 1991).

A programme for genetic improvement of *P. griseoroseum* started with the establishment of an efficient transformation system (Queiroz *et al.* 1998) followed by the characterization of pectin lyase coding gene (Bazzolli *et al.* 2006). Despite the existence of PNL multigene families in several filamentous fungi (Harmsen *et al.* 1990; Fraissinet-Tachet *et al.* 1995), only two genes were found in the genome of *P. griseoroseum*. The *plg*1 gene exhibits some properties that have never been reported before for other PNL genes such as the induction by citric pectin and the production of a protein with an alkaline pI value; those have interesting potential application for the hydrolysis of pectin in fruits industry. Alkaline pectinases have been proven to be effective as bioscouring agents of cotton fabrics (Hoondal *et al.* 2000; Ranveer *et al.* 2005).

Penicillium griseoroseum shows an interesting PNL expression pattern that can be economically attractive to industry because enzyme activity can be detected in minimal medium with added sucrose supplemented with yeast extract even in the absence of pectic compounds (Baracat-Pereira *et al.* 1994; Minussi *et al.* 1998). Detailed investigation demonstrated that the same effect could be achieved when yeast extract was replaced by cAMP leading to the conclusion that yeast extract has at least one substance that contributes to a high intracellular level of cAMP and favours PNL production (Baracat-Pereira *et al.* 1999). Signal transduction via cAMP has also been implicated in the regulation of a variety of process in pathogenic and saprophytic fungi, including virulence, morphogenesis, sexual development, and utilization of endogenous and exogenous carbon sources (Pall 1981; Kronstad *et al.* 1998; Barhoom and Sharon 2004; Jeraj *et al.* 2005). A similar mechanism was described to be responsible for the activation of a cascade of signal transduction that culminates in the production of citric acid and xylanases in other fungi (Al Obaidi and Berry 1980; Morosoli *et al.* 1989; Legisa and Bencina 1994; Gradisnik-Grapulin and Legisa 1997; Flipphi *et al.* 2003).

In the search for decreased production costs of microbial enzymes of commercial interest it seemed motivating that a fungus strain could secrete PNL induced by sucrose, a more economic substrate widely available in Brazil. Previous studies conducted by our team showed that nonconventional substrates, like sugar cane bagasse and oats, are also viable substrates for pectin lyase production by *P. griseoroseum*.

In this work we investigated at a molecular level the effects of carbon sources and ambient pH on the expression of the *plg* genes to find out which is the most suitable gene for use in a genetic strain improvement. We also determined whether alkaloids known to inhibit cyclic phosphodiesterase affected the expression of the *plg*1 gene in the aim to show the correlation between cAMP signalling and gene expression to broaden, in the future, the substrates used for PNL production.

Materials and methods

Strains and growth conditions

Penicillium griseoroseum was isolated from forest seeds and has been deposited in the Coleção de Culturas Tropicais André Tosello, Brazil (CCT 6421). Culture maintenance and inoculum production have been reported earlier (Brumano *et al.* 1993). For the experiments carried out in this study, 9-day-old conidia were used to inoculate 50 ml of minimal medium (Pontecorvo *et al.* 1953). Erlenmeyer flasks were agitated at 150 RPM and 25°C. The mycelium was harvested by filtration through a nylon gauze after 24 h, washed thoroughly with sterile distillated water and transferred to buffered minimal medium [6·98 g l^{-1} KH_2PO_4, 5·44 g l^{-1} K_2HPO_4, 1·0 g l^{-1} $(NH_4)_2SO_4$, pH 6·8] containing 0·4% (w/v) of a carbon source and supplemented with $MgSO_4{\cdot}7H_2O$ (0·05 g l^{-1}). Cultures were incubated in an orbital shaker at 150 RPM and 25°C, for different time according to each experiment. Harvested mycelium was quickly frozen in liquid nitrogen and maintained at –80°C until RNA extraction.

pH-shift experiments

Penicillium griseoroseum was precultured in minimal medium containing 0·4% glucose for 24 h, washed with sterile water and then transferred to fresh medium with initial pH value of 4·5, 5·5, 6·3, and 6·8. Two media were tested: an unbuffered [2·0 g l^{-1} KH_2PO_4, 7·0 g l^{-1} K_2HPO_4, 1·0 g l^{-1} $(NH_4)_2SO_4$ supplemented with $MgSO_4{\cdot}7H_2O$ (0·05 g l^{-1})] and buffered minimal medium [6·98 g l^{-1} KH_2PO_4, 5·44 g l^{-1} K_2HPO_4, 1·0 g l^{-1}

de $(NH_4)_2SO_4$, pH 6·8], both containing citric pectin (0·4%) or sucrose (0·4%) and yeast extract (0·06%) as carbon sources. Cultures were kept under agitation in an orbital shaker at 150 rev min^{-1}, 25°C, for 24 h prior to collection. Mycelia were quickly frozen in liquid nitrogen and kept at –80°C for posterior RNA isolation. Supernatant had pH measured after mycelia collection (24 h). Catabolite repression was studied by inoculating the fungus in minimal medium, pH 6·8, containing 0·4% citric or apple pectin, glucose, fructose, or galactose. Mycelia were harvested after 24 and 36 h of growth and freeze-dried for RNA extraction.

Effects of modulators of intracellular cyclic nucleotide monophosphate on the expression of the *plg*1 gene

The mycelium was harvested after incubation for 24 h, separated by filtration, washed several times with distilled water and placed in buffered medium supplemented with $MgSO_4 \cdot 7H_2O$ (0·05 g l^{-1}). The following substances purchased from Sigma were added: 2 mmol l^{-1} sodium fluoride (NaF), 5 mmol l^{-1} caffeine, and 5 mmol l^{-1} cAMP dibutyryl. An equal quantity of ethanol was added into the respective control to eliminate a possible interference. Minimal medium and minimal medium supplemented with sucrose, yeast extract, or sucrose and yeast extract were the controls. Cultures were incubated at 25°C and shaken at 150 rev min^{-1} for 18 and 24 h when mycelia were collected and stored at –80°C for further analysis.

Total RNA isolation and Northern blot analysis

Total RNA from *P. griseoroseum* was isolated according to Sambrook *et al.* (1989). Samples containing 15 μg of total RNA were denatured and submitted to electrophoresis in a 1·2% (w/v) agarose gel containing formaldehyde. The gels were transferred to nylon blotting membrane (Duralon; Stratagene®) and fixed by UV cross-linking. Membrane was hybridized at 63°C, for 16 h and washed with 2× standard saline citrate (SSC), 0·1% sodium dodecyl sulfate (SDS) (20 and 10 min) and 1× SSC, 0·1% SDS (10 min). The probe consisted of the cDNA clones of *plg*1 (AF502279) and *plg*2 (AF502280), radiolabelled with [α-^{32}P] dATP as suggested by the Random Prime It II Labeling System kit (Stratagene). Membrane was exposed to autoradiography film for 5 days, at –80°C with intensifying screen. The partial sequence of the γ-actin gene from *P. griseoroseum* was used as internal loading control. For re-hybridization, the membrane was washed in 0·5% SDS, at 65°C, for 4 h, in order to remove hybridized probe. To certify reproducibility RNA was extracted from mycelium obtained from three independent experiments.

Reverse transcriptase polymerase chain reaction

The Reverse Transcription System (Promega) with oligo (dT) and 2·0 μg of RNA DNAse-free was used in transcription reactions incubated at 42°C, for 1 h and 15 min. The primers used for amplification of *plg*1 and *plg*2 genes were, respectively, 5′-TAGAAACTACCAACTCCCAACATG-3′ (PI*plg*1) and 5′-TCCGCACGAGTAAATCACACTC-3′ (PF*plg*1) and 5′-CTATATCAACTTCGAAAATG-3′ (PI*plg*2) and 5′-AGGTACACGTTCCAGTAGTGG-3′ (PF*plg*2). The reaction contained 5 μl of the RT reaction, 10 pmol of each pair of primer, 25 pmol of each dNTP. The PCR was performed on a PTC-100 (MJ Research, Inc.) with machine adjusted for 40 cycles consisting of denaturing for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min 30 s, at 72°C, with a final 7 min, 72°C extension period at the end of the PCR. The γ-actin gene from *P. griseoroseum* was amplified with the primers 5′-ACACCTTCTACAACGAGCTG-3′ and 5′-GGAAGCTCGTAGGACTTCTC-3′ as control. Total DNA from the fungus was also amplified to guarantee that RNA was DNA-free. After amplification, the products were analysed on a 1·5% agarose gel stained with ethidium bromide. The PCR fragments were eluted from the gel, cloned into pGEM-T Easy vector (Promega) and sequenced to confirm product's identity.

Results

Effect of substrate and pH on gene expression

In order to determine whether the expression of the pectin lyase encoding genes *plg*1 and *plg*2 from *P. griseoroseum* are submitted to global regulations, we first searched consensus binding sequences of fungal transcription factors in their promoter region (Bazzolli *et al.* 2006). Putative sites for CreA, the main catabolite repressor in filamentous fungi, were seen in the regulatory regions of *plg*1 (AF502279) and *plg*2 (AF502280) genes. The results indicate that *plg*1 and *plg*2 suffer catabolite repression by glucose although *plg*1 expression was detected when cultivation was done in sucrose and yeast extract. The results obtained with the carbon-medium shift experiments showed that expression of the *plg*1 and *plg*2 genes was differentially regulated depending on the carbon sources in the culture medium. Carbon catabolite repression is known as the inhibition of induced protein synthesis in response to the presence of a favourable carbon source like glucose and is frequently associated to transcription repression (Dowzer and Kelly 1991). Initially, gene expression was evaluated by Northern blot (data not shown) but since no hybridization signal could be detected upon the use of *plg*2 as probe even after several attempts, a more sensitive approach was employed. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis detected transcripts of *plg*1 in cells grown on citric pectin and apple pectin but transcription was substantially reduced when glucose was added into the media (Fig. 1a). Easily metabolizable sugars such as glucose, fructose, and sucrose totally repressed *plg*1 expression. Interestingly, polygalacturonic acid had no effect on the *plg*1 gene, while sucrose and yeast extract, two substances that are not considered inducers of any pectic system, had a significant impact on gene expression. The *plg*2 gene showed the same expression pattern as *plg*1 in citric and apple pectins but at lower levels and transcription was also affected by glucose (Fig. 1b). In opposition to *plg*1, *plg*2 was poorly expressed in galacturonic acid and no transcripts were detected in sucrose and yeast extract. As expected, actin was expressed under all conditions and its signal intensity was used as a measure of the actual amount of RNA present in each lane on the blot (Fig. 1c).

It has been reported that *P. griseoroseum* produces PNL using sucrose as carbon source as long as yeast extract is added into the medium (Baracat-Pereira *et al.* 1994). These conditions were used to investigate gene expression. The *plg*1 gene was first detected from 1 to 2 h and an intense expression from 18 to 36 h was also demonstrated after which time expression progressively decreased (Fig. 2). *plg*2 expression was never detected under the conditions used, indicating that *plg*1 gene is the only gene expressed under the cultivation in sucrose and yeast extract (data not shown).

Binding sites for PacC (Tilburn *et al.* 1995), regulator of gene expression by pH, were detected in the upstream region of *plg*1. For this reason, we decided to investigate the regulation of *plg*1 expression by ambient pH in buffered and unbuffered media containing 0·4% citric pectin or 0·4% sucrose plus yeast extract as carbon source (Fig. 3). Culture supernatant was collected and pH was measured after 24 h of growth. The *plg*1 transcript level was maximal at a pH initial value of 6·8 in pectin-containing media. *plg*1 expression responded negatively to unbuffered media confirming that the pH of the medium strongly controls gene expression. The pH values did not change during the course of the experiment in buffered medium. The acidic pH seen in unbuffered medium containing citric pectin in 24 h can be explained by the release of acid monomers and polymers during pectin degradation. Interestingly, expression was seen in the presence of sucrose and yeast extract regardless of the pH value although a higher level was detected in pH 6·3. All studies were further conducted with the fungus grown on buffered media (pH 6·8). The *plg*2 gene was not

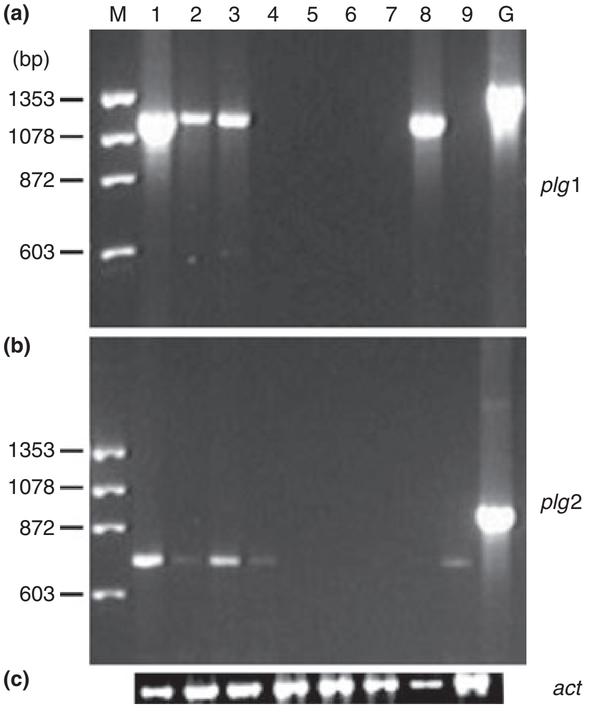

Figure 1 Effect of catabolite repression on the expression of *plg*1 and *plg*2 genes of *Penicillium griseoroseum*. The fungus was grown for 24 h on minimal medium containing citric pectin (1), citric pectin and glucose (2), apple pectin (3), apple pectin and glucose (4), glucose (5), fructose (6), sucrose (7), sucrose and yeast extract (8), galacturonic acid (9). Total DNA (G). RT-PCR was done with specific primers for *plg*1 (a) and *plg*2 (b). The γ-actin gene was the internal control (c).

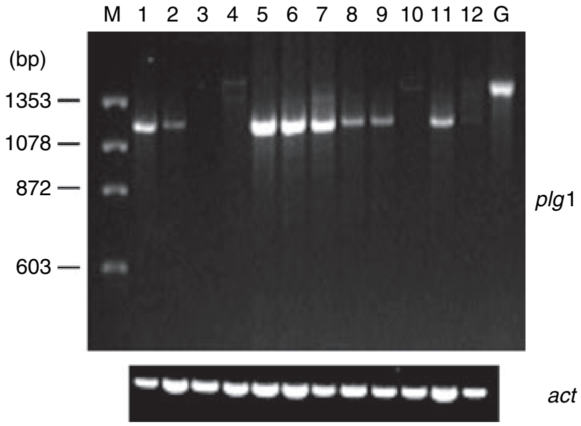

Figure 2 RT-PCR analysis showing the expression pattern of *plg*1 gene from *Penicillium griseoroseum* in sucrose-containing media supplemented with yeast extract. Mycelia were collected at 1 h (1), 2 h (2), 4 h (3), 6 h (4), 18 h (5), 24 h (6), 36 h (7), 48 h (8), 60 h (9), 72 h (10), 76 h (11), and 96 h (12). Total DNA amplified with gene specific primers (G). The γ-*actin* gene was the internal positive control (γ-*act*). Phage PhiX 174/*Hae* III was used as size marker (M).

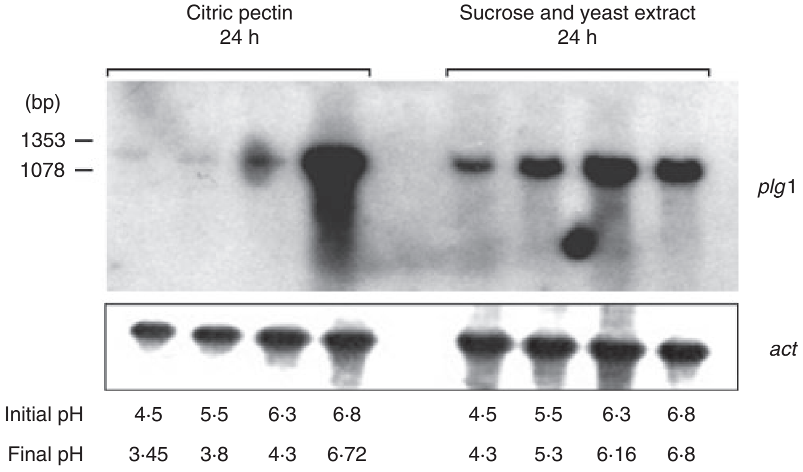

Figure 3 Effect of pH on the expression of the *plg*1 gene. *Penicillium griseoroseum* was cultivated on unbuffered and buffered media containing citric pectin or sucrose as carbon source. Yeast extract was added into the sucrose-containing medium. Radiolabelled *plg*1 was employed as probe. Equivalent loading of total RNA was confirmed by hybridization with the γ-*actin* gene.

considered in this experiment because of its poor expression in pectin and sucrose.

The role of yeast extract on *plg*1 gene expression

Since the *plg*1 gene was expressed on sucrose and yeast extract but not on sucrose only, a study was done to verify the role of yeast extract on its expression. RT-PCR was chosen as an approach due to its sensitivity. When sucrose or glucose was added into the minimal medium as the sole carbon source no transcript was seen in contrast to the condition when the media were supplemented with yeast extract (Fig. 4). However, *plg*1 expression was higher in the presence of both yeast extract and sucrose. In this experiment, the presence of the transcript was also evaluated at 36 h to verify the influence of catabolic repression on the results obtained for glucose. There has been a suggestion that yeast extract positively influences pectin lyase production by rising intracellular levels of cAMP (Baracat-Pereira *et al.* 1999) an effect also seen for methylxanthines on the production of pectin lyase of *P. griseoroseum* (Minussi *et al.* 1997). We checked this assumption by growing *P. griseoroseum* in the presence of substances known to activate the transduction cascade via cAMP, with or without yeast extract (Fig. 5). Dibutyril cAMP did not affect the expression of the *plg*1 gene but transcripts accumulated under yeast extract. Also, NaF induced gene expression but only when caffeine was present. The expression of *plg*1 on sucrose and caffeine (i), sucrose, dcAMP and yeast extract (ii), sucrose, NaF and yeast extract (iii), sucrose, caffeine and yeast extract (iv) and sucrose, NaF and caffeine (v) was similar to the control (sucrose and yeast extract). These data indicate that high levels of cAMP are not the solely responsible for gene expression and that more complex mechanisms must be involved.

Discussion

It is well established that pectic enzymes are used in the textile and food industries for clarification and depectinization of fruit juices, wines, oil olive, and paper treatment. In recent years, industries have been trying to replace the toxic conventional treatments employed in these processes by more eco-friendly enzyme treatment (Ranveer *et al.* 2005).

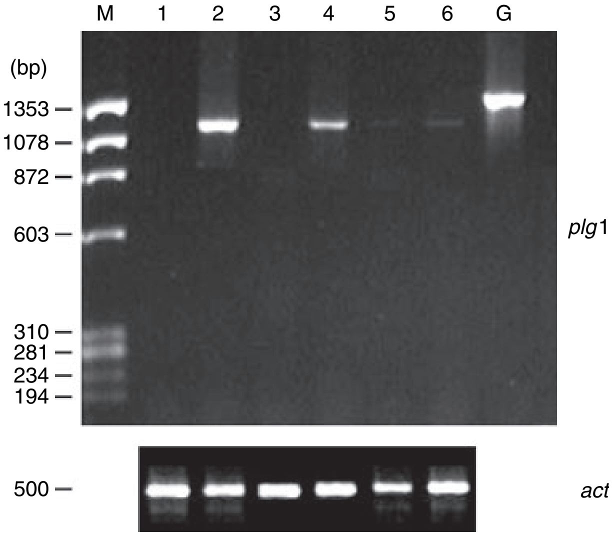

Figure 4 Effect of yeast extract on *plg*1 expression. Total RNA was extracted from mycelia grown on buffered media (pH 6·8) with added sucrose (1), sucrose and yeast extract (2), glucose (3), glucose and yeast extract (4), glucose and yeast extract –36 h (5) and yeast extract (6). Total RNA was used in RT-PCR reactions with specific gene primers. Total DNA amplification (G). The γ-*actin* gene was the internal positive control (γ-*act*).

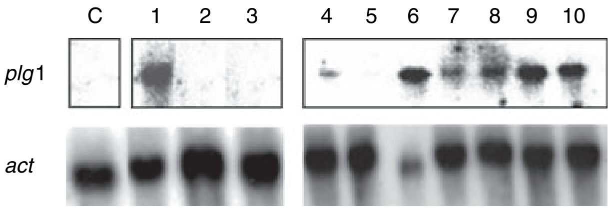

Figure 5 Effect of different modulators on *plg*1 expression. *Penicillium griseoroseum* was initially grown in mineral medium and then transferred to media containing different substances for 24 h. Total RNA extracted from mineral medium was used as control (C). The conditions used were as follows: sucrose and yeast extract (1); sucrose (2); yeast extract (3); sucrose and dcAMP (4); sucrose and NaF (5); sucrose and caffeine (6); sucrose, dcAMP and yeast extract (7); sucrose, NaF and yeast extract (8); sucrose, caffeine and yeast extract (9); sucrose, NaF and caffeine (10). Membrane was probed with the *plg*1 cDNA, stripped and then probed with the γ-actin gene.

Pectic enzymes have expressive industrial application and represents approximately 10% of world production of enzyme preparations. Commercial preparations are produced, in almost its entirely, from fungal sources. The genus *Aspergillus* has been the most commonly used for such production, especially *A. niger* (Couto and Sanromán 2006). In this work mineral medium was chosen for PNL production with the objective to standardize and quantify exactly the availability of nutrients for the fungus and the expression of the *plg* genes. However, other experiments conducted in our laboratory revealed that sugar cane bagasse is a promising substrate that can be applied for industrial purposes because of high levels of PNL1 produced (Piccoli-Valle *et al.* 2001a,b).

Despite the long lasting interest of plant pathology and biotechnology in fungal pectinases, the molecular mechanisms governing gene expression are still unknown. Two PNL genes (AF502279 and AF50280) were cloned in *P. griseoroseum* with special attention to *plg*1 that encodes an alkaline PNL (Bazzolli *et al.* 2006). Characterization of the pectic system at the genetic level may help optimize large-scale production and for this reason in this study we took a closer look into *plg*1 and *plg*2 expression. The gene *plg*1 was the one that best responded to the experiments here conducted. It suffers catabolite repression upon the addition of simple sugars such as glucose and fructose to media containing the natural inducers of PNL what is consistent with the expression profiles described for *A. niger* PNL genes (Vries *et al.* 2002). No hybridization signal was detected when total RNA was probed with *plg*2, even when the membrane was exposed to X-ray films for longer periods (data not shown). Due to the sensitiveness of the method, RT-PCR was employed for further assays and revealed that *plg*2 was only induced by citric pectin and apple pectin but in lower levels when compared to *plg*1. Because of its insignificant expression and the biochemical properties of PLG2 (an acidic pectinase) the following experiments focused on *plg*1.

A clear modulation of *plg*1 gene expression by external pH was observed, although a homologue of PacC, the pH regulatory transcription factor in *Aspergillus*, has not yet been identified in *P. griseoroseum*. The presence of several binding sites for PacC suggests a similar regulation to PNL genes from *A. niger*, *Glomerella cingulata* (teleomorph/; *C. gloeosporioides*) and *Aspergillus oryzae* (Templeton *et al.* 1994; Benen *et al.* 1996; Kitamoto *et al.* 2001a,b), that are better expressed in culture media near neutrality, especially when citric pectin is used as carbon source. This was also confirmed for some endopolygalacturonase genes from *Botrytis cinerea* (Wubben *et al.* 2000). Ambient pH is decisive for some pathogens like *Glomerella cingulata* (teleomorph: *C. gloeosporioides*) that need alkaline pH to express a high pectin lyase A activity and successfully colonize the host (Prusky and Yakoby 2003).

Penicillium griseoroseum has long been studied aiming to find alternative inducers for the industrial production of pectic enzymes (Baracat-Pereira *et al.* 1994; Minussi *et al.* 1996, 1998; Piccoli-Valle *et al.* 2001a,b). Minussi *et al.* (1998) described the viability of using small concentrations of sugar cane to induce PNL and PG production. This turned out to be an interesting feature since low cost substrates would be expected to greatly reduce production costs. The authors also showed that irrespective of the carbon source used enzyme activity was significantly increased by the addition of yeast extract into the medium. The higher level of *plg*1 transcript detected in sucrose and yeast extract from 18 to 36 h and the impossibility to detect *plg*2 expression even by RT-PCR confirm that the activity detected in the assays previously reported (Minussi *et al.* 1998) was probably due to PLG1 since there is no indication of other *plg* genes in *P. griseoroseum* (Bazzolli *et al.* 2006). A high level of transcript accumulation was seen every time yeast extract was added into the medium containing sucrose and glucose as carbon source. Although the regulatory region of *plg*1 has several putative CreA motifs the positive effect observed upon the assays conducted suggests the existence of a mechanism that counteracts the CreA-mediated repression.

Baracat-Pereira *et al.* (1999) suggested that small metabolites in yeast extract would induce PNL synthesis through cAMP-dependent pathway. This pathway seems to be important for gene regulation in other filamentous fungi (Kronstad *et al.* 1998; Rollins and Dickman 1998; Kiminori and Keller 2001; Shimizu and Keller 2001) but a more in-depth investigation is still necessary. We tested the influence of several substances known to affect different components of signal transduction pathway on expression of *plg*1. Caffeine and IBMX are cAMP phosphodiesterase inhibitors while NaF activates cyclate adenylate promoting an increase on the endogenous level of cAMP. Here, caffeine was the best substitute for yeast extract proving that this metabolite is really important for *plg*1 expression. It is probable that the presence of methylxantines in yeast extract and sucrose raises the level of cAMP and activate protein kinases responsible for the phosphorylation of a protein homologous to CreA, inactivating the protein. Also, caffeine is considered a stress factor that promotes the accumulation and maintenance of endogenous cAMP level (Hans and Thorner 1981). Upon the analysis of the cis-elements present on the regulatory region of the *plg*1 (AF502279) we found four CCCCT sequences that resemble the consensus site TTAG/CTAA described in *Saccharomyces cerevisiae* that is bound by members of the Yap protein family that are responsible for a variety of stress responses including oxidative, osmotic, and heat (Rodrigues-Pousada *et al.* 2005). However in *P. griseoroseum* they may help explain gene activation when the fungus was grown on stress conditions like the presence of caffeine, a methylxanthine that has been reported to inhibit growth in unicellular fungus like *Candida albicans* (Sabie and Gadd 1992). Besides the CCCCT sequences, two putative adjacent regulatory elements, GTGAACA (–592) and CAAT (–563), were also found. Together, these binding sites are called Filamentation and Invasion Response Element (FREs), and are needed for gene expression that depends on components of the MAP kinase-signalling pathway in dimorphic fungi (Madhani and Fink 1998). In *Colletotrichum lindemuthianum* the same motifs were found in the promoter of a polygalacturonase gene and deletions assays proved their importance for fungus pathogenicity (Herbert *et al.* 2002). So we can assume that the FRE-element is likely to activate transcription via cAMP, what explains its involvement on the production of PNL as previously demonstrated (Baracat-Pereira *et al.* 1999).

The pectinolytic system of *P. griseoroseum* can be used as a model for a comprehensive assessment of the basic mechanisms involved on gene expression in filamentous fungi, especially those related to signalling pathways. The characterization of the *plg* regulation is a first step towards the elaboration of strategies aiming the use of this system in industrial scale.

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