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SHORT COMMUNICATION

The minimal regulatory region necessary for the expression of the *Penicillium griseoroseum plg*1 gene

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Abstract The expression of the *Penicillium griseoroseum plg*1 gene is induced by citric pectin and repressed by glucose. In this work, the minimal region of the *plg*1 gene promoter essential for expression in pectin and sucrose plus yeast extract was identified by using constructs containing the *gfp* ORF under control of the *plg*1 gene promoter. The fragment A (283 bp) is essential for *plg*1 expression in sucrose plus yeast extract. Fragment B (309 bp plus 184; core promoter) was critical for expression in pectin and abolished the catabolic repression by glucose. Therefore, the fragment of 776 bp (fragment A and B) is essential for the expression of the *plg*1 gene in natural inducing conditions (pectin as carbon source) and in sucrose plus yeast extract. The fragment B is a promising minimal promoter usable for heterologous expression in filamentous fungi, since genes that contain it could be activated by the presence of peel from citric fruits (which contains citric pectin) and are not affected by glucose in these agricultural by-products.

Keywords *Penicillium griseoroseum* · Pectin lyase · Minimal promoter region · Green fluorescent protein

Penicillium griseoroseum CCT6421 is a saprophytic fungus that secretes a vast number of extracellular enzymes that are involved in the degradation of pectin, a plant cell-wall complex heteropolymer (Baracat et al. 1989). Due to the great diversity in the structure of pectin, there are different groups of pectinases that are involved in pectin degradation (Yadav et al. 2008; Glinka and Liao 2011). Pectin lyase (PNL) (EC 4.2.2.10) is a microbial pectinase with biotechnological applications in the textile, food, and paper industries and in plant pathogenesis (Jayani et al. 2005; Ziemiński et al. 2012). PNL enzymes are able to degrade highly esterified pectin without previous action of other enzymes, and methanol is not produced as a by-product (Alaña et al. 1991; Gummadi and Kumar 2005).

Generally, the expression of pectinase genes is transcription-regulated by various environmental factors, including pH and the carbon source of the culture medium. Previous studies conducted by our team showed that non-conventional substrates, such as sugar cane bagasse and oats, are also usable carbon sources for pectin lyase production by *P. griseoroseum* (Minussi et al. 1998; Cardoso et al. 2010; Gonçalves et al. 2012).

Gene activation is promoted by pectin and other pectic components such as polygalacturonic acid, arabinose, and rhamnose, while inhibition is induced by glucose and sucrose (Bussink et al. 1991; Fawole and Odunfa 2003). Reports show that the promoter region of most pectic genes contains recognition sequences for ubiquitous proteins involved in the control of expression of fungal genes such as CreA, PacC, and Hap2-3-4 (de Vries et al. 2002).

In this study, expression plasmids containing deletions in the regulatory region of the *plg*1 gene derived from *P. griseoroseum* have been constructed to determine the functionality of putative *cis*-elements. The effects of such deletions associated with those generated by inducer and repressor substances on the expression of the green fluorescent protein (*gfp*) reporter gene have been investigated. The study of the regulation pectinase genes should provide some clues in the field of industrial application and help to establish a gene expression model in fungi. In our previous work, we demonstrated that *plg*1 expression is complex and is regulated by the

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carbon source, the pH, and methylxanthines (stress conditions) (Bazzolli et al. 2006). Therefore, this work aimed to identify the minimal promoter region of the sequenced *plg*1 that is essential for the simultaneous expression of this gene in pectin and sucrose plus yeast extract, a non-conventional carbon source for pectinase gene.

The strains used in this study were: (1) *P. griseoroseum*, a wild strain previously isolated from the seeds of woody plants at the Universidade Federal de Viçosa (Viçosa, MG, Brazil) and deposited at the Tropical Culture Collection André Tosello (Campinas, SP, Brazil; accessing number CCT 6421), and for the construction of the *plg*1 promoter::*gfp* strains; and (2) the spontaneous mutant strain *P. griseoroseum* PG63 that harbors a deletion of 122 bp in the structural region of the gene coding for nitrate reductase (*nia*D) (Pereira et al. 2004).

All plasmid constructs were derived from pAN52-1-GFP carrying the SGFP-TYG version of the green fluorescent protein (GFP) under control of the glyceraldehyde 3-phosphate dehydrogenase gene (*gpd*A) promoter and the terminator region of the tryptophan synthase gene (*trp*C) from *Aspergillus nidulans*. From pAN52-1-GFP (6.4 kb), the plasmid p*plg*1-*gfp*786 was constructed, containing the *gfp* gene under regulation of the whole *plg*1 regulatory region (Bazzolli et al. 2006). The promoter region used was 786 bp, but the full promoter sequenced was approximately 2,000 bp. Located within this regulatory region (786 bp), there are unique restriction sites for the enzymes *Nco*I, *Ssp*I and *Sma*I. This main fragment was subdivided in the fragments: A: the region between −786 and −503, *Nco*I/*Ssp*I (283 bp); and B: the region between −503 and −184, *Ssp*I/*Sma*I fragment (319 bp) (Fig. 1).

The plasmid p*plg*1-*gfp*786 was digested with the enzymes restriction listed above according to the manufacturer's instructions (Promega, Madison, WI, USA). The fragments of interest were recovered using the GFX PCR DNA and Gel Band Purification Kit (Amersham Life Sciences, Amersham, UK), and the DNA fragments and plasmid were ligated using T4 DNA ligase (Promega). The resulting plasmids, pNSpΔ283 and pSpSΔ319, corresponding to the deletion of fragments A and B, respectively, were confirmed by sequencing. Then, the plasmids were used to transform competent *Escherichia coli* DH5α. The plasmid pAN52-1-GFP was used as a fluorescence control. All constructs were used in association with plasmid pNPG1 containing the *nia*D gene (Pereira et al. 2004) to co-transform *P. griseoroseum* protoplasts, including p*plg*1-*gfp*786, which contains the complete *plg*1 regulatory region (Fig. 1).

The transformation of protoplasts of *P. griseoroseum* PG63 was performed as described by Teixeira et al. (2011). Transformed colonies cultivated on minimal medium (MM) (Pontecorvo et al. 1953) were stable, and the formation of sectors was not observed. The transformants were confirmed by PCR using specific primers for the *gfp* gene (5'-AGGGCG TGGAGCAGTTCACC-'3, GFP1 and 5'-CCTCGATGTTGT GGCGGATC-'3, GFP2). A DNA fragment of 512 bp was generated by the amplification of *gfp*, the gene from transformants harboring the plasmids pNSpΔ283 and pSpSΔ319, thus demonstrating the presence of the *gfp* gene (data not shown).

All transformants were single-spore purified and microcultivated directly on glass slides for subsequent analysis by fluorescent microscopy. The culture media consisted of buffered minimal medium (pH 6.8) supplemented with different carbon sources such as 0.4 % pectin, 0.4 % glucose, 0.4 % pectin plus 0.4 % glucose, or 0.4 % sucrose plus 0.06 % yeast extract. After 48 h at 25ºC, the slides were examined under an Olympus (Hamburg, Germany) model BX-60 fluorescent microscope equipped with a 460–480 nm excitation filter set. Images were captured with an Olympus model U-CMAD-2 camera and subsequently edited using the image analyzer program Image Pro® Plus version 4.0 (Medial Cybernetics, Silver Spring, MD, USA).

Different deletions of the 5'upstream region of the *plg*1 gene were performed to identify the minimal region that is required for the proper regulation and expression of the *plg*1

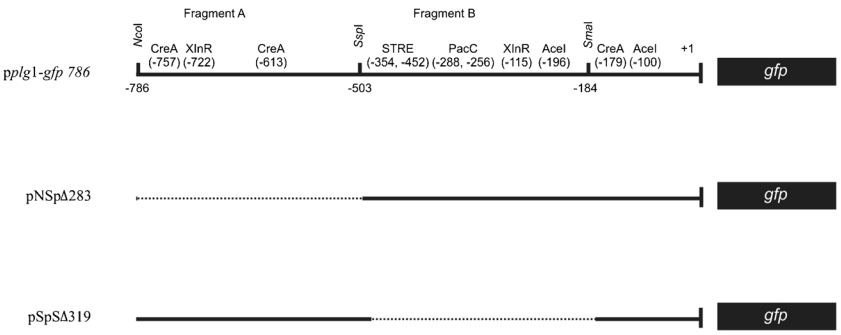

Fig. 1 Schematic representation of the entire regulatory region of the *plg*1 gene from *Penicillium griseoroseum* present in the reporter plasmid p*plg*1-*gfp*786 and its derivatives. *Fragment A* corresponds to the region −786 to −503 plus the core promoter (186 bp); *Fragment B* corresponds to the region −503 to −184 plus the core promoter (186 bp). In the sequence of each plasmid, the *numbers preceded by the delta sign* indicate the size of the deletion zones (bp). Each putative *cis-elements* and position are indicated in schematic representation. *Negative numbers* indicate the enzyme restriction sites in relation to the +1 translation start codon of the *plg*1 gene

gene. Two different fragments (A and B) were fused with a *gfp* reporter gene, and the constructed plasmids were used to transform the *P. griseoroseum* PG63 (*nia*-).

To further explore gene regulation, we fused different promoter fragments to a *gfp* reporter gene and studied GFP expression under various growth conditions. Figure 1 shows the cassettes used to transform *P. griseoroseum* PG63 mycelia. Fluorescence analysis was performed with fungal transformants grown under inductive and repressive conditions for *plg*1 expression, and the results are shown in Fig. 2. The transformant containing pNSpΔ283 (fragment B) expressed the *gfp* gene when cultivated in medium containing pectin (P) or pectin plus glucose (P + G) (Fig. 2e, g). This fragment abolishes the catabolic repression by glucose in the *plg1* gene. The transformant with pSpSΔ319 showed no fluorescence in all conditions tested. This plasmid lacks fragment A, which contains the CreA (−757, −613) and XlnR (−722) sites. Catabolic repression was verified in p*plg*1-*gfp*786 transformants containing both fragments A and B (Fig. 2c, d). The deletion of fragment A indicated the functionality of the two CreA binding sites (−613 and −757) from the *plg*1 regulatory region. All the CreA binding sites that have been characterized present two non-contiguous consensus sequences preceded by an AT-rich region, suggesting that CreA mediates repression by binding to two consensus sites (Takashima et al. 1996; Felenbok et al. 2001).

The deletion analysis of fragment B of 319 bp showed that this *plg1* regulatory region has an important function in the expression of this gene in pectin and sucrose plus yeast extract; therefore, fragment B is the minimal promoter sequence for this gene. Fragment B contains *cis*-elements [STRE (−354 e −452); PacC protein (−256 and −288), XlnR (−215) and ACEI (−196)] which are important for the expression of *plg*1, as indicated by the complete absence of GFP protein expression from the pSpSΔ319 construct, even in the presence of inducers such as pectin and sucrose plus yeast extract (Fig. 2i to l).

The results suggest that the induction of *plg*1 expression in the presence of sucrose plus yeast extract does not depend on the same regulatory region as that responsible for induction by pectin (Fig. 2). The separate fragments could not activate the *gfp* expression in cassettes contained only fragment A or B in

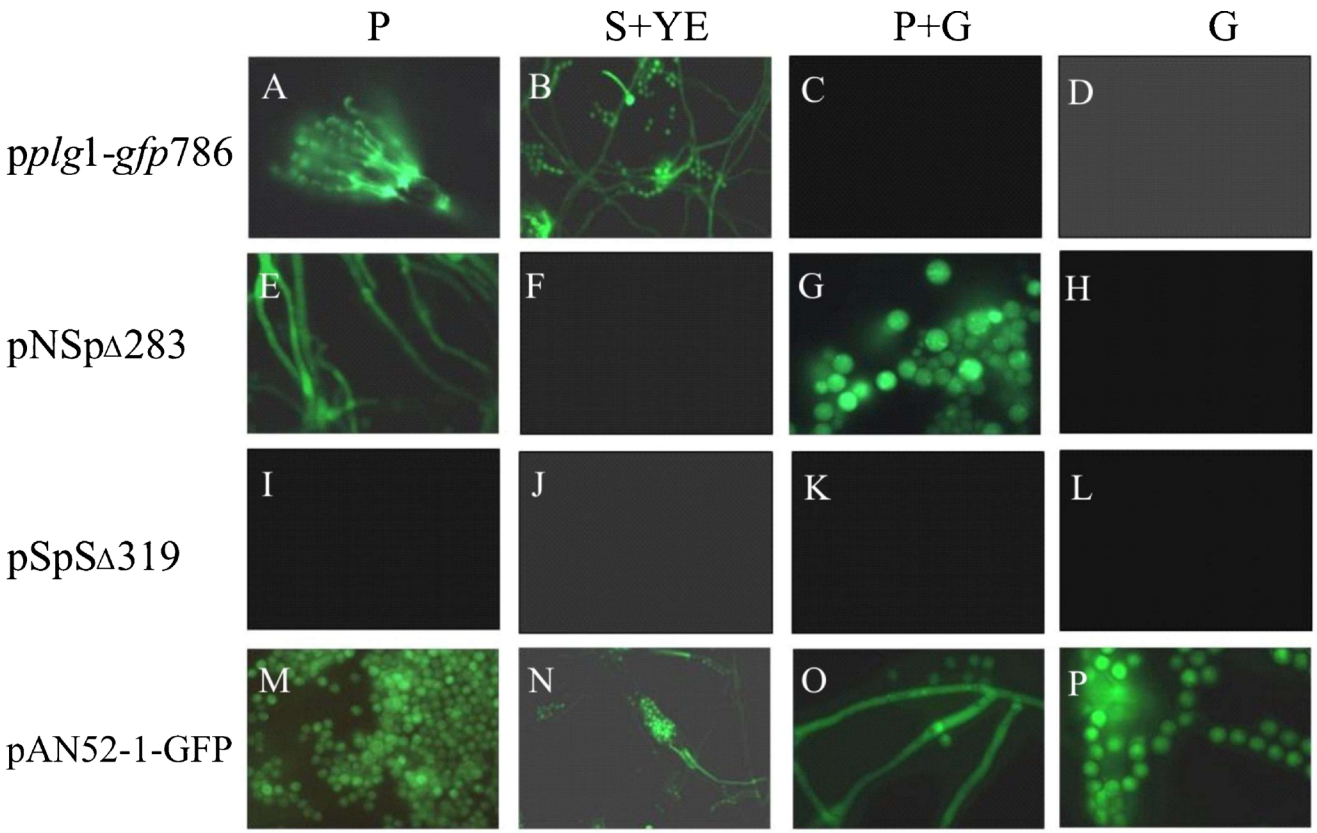

Fig. 2 Expression of the fluorescence of green fluorescent protein (GFP) in the mycelia of *Penicillium griseoroseum* transformants in which the reporter gene *gfp* is under the control of the regulatory region of the *plg*1 gene containing various deletions. A transformant containing the entire 786-bp fragment of the *plg*1 gene promoter (p*plg1-gfp*786) was used as control. Mycelia were incubated for 48 h in minimal medium supplemented with the carbon sources pectin (*P*), glucose (*G*), pectin plus glucose (*P+G*) and sucrose plus yeast extract (*S+YE*). pAN52-1-GFP, which contains the reporter gene *gfp* under the control of the constitutive promoter of the *gpd*A gene, was used as a fluorescence control

the presence of sucrose plus yeast extract, confirming the functionality of the 786-bp fragment as a minimal regulatory region for *plg*1 expression. The control strain transformed with the pAN52-1-GFP construct exhibited strong GFP fluorescence in all tested conditions. This analysis confirms that the *plg*1 gene is not regulated by elements such as the Upstream Activating Sequence (UAS).

The *plg*1 gene was chosen because of the unique properties of this enzyme compared to other fungal PNL genes, such as its expression in non-inducing media and preference for citric pectin (Bazzolli et al. 2006). The expression of *plg*1 in sucrose plus yeast extract depends on the presence of specific groups of putative *cis*-elements that are important for controlling PNL production. The 776-pb fragment (fragments A and B) is essential for the expression of the *plg*1 gene in natural inducing conditions, and in sucrose plus yeast extract, constituting the minimal region necessary for this expression. Knowledge concerning the regulatory mechanisms of *plg*1 could be used to optimize the industrial production of PNL, and to synthesize heterologous proteins in *P. griseoroseum*.

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