J Ind Microbiol Biotechnol (2014) 41:1571–1580
DOI 10.1007/s10295-014-1486-4

SIMB
Society for Industrial Microbiology and Biotechnology

Genome organization and assessment of high copy number and increased expression of pectinolytic genes from *Penicillium griseoroseum*: a potential heterologous system for protein production

Janaina Aparecida Teixeira · Guilherme Bicalho Nogueira · Marisa Vieira de Queiroz · Elza Fernandes de Araújo

Received: 16 September 2013 / Accepted: 8 January 2014 / Published online: 7 August 2014
© Society for Industrial Microbiology and Biotechnology 2014

Abstract The fungus *Penicillium griseoroseum* has the potential for application on an industrial scale as a host for the production of homologous and heterologous proteins, mainly because it does not produce some mycotoxins or secrete proteases under the growth conditions for pectinase production. However, for the fungus to be used effectively as an expression heterologous system, an understanding of the organization of its genome, as well as the mechanisms of gene expression and protein production, is required. In the present study, the size of the *P. griseoroseum* genome was estimated to be 29.8–31.5 Mb, distributed among four chromosomes. An analysis of *plg*1 and *pgg*2 pectinolytic genes expression and copy number in recombinant multi-copy strains of *P. griseoroseum* demonstrated that an increase in the number of gene copies could increase enzyme production, but the transcription could be affected by the gene integration position. Placing a copy of the *plg*1 gene under the control of the *gpd* promoter of *Aspergillus nidulans* yielded a 200-fold increase in transcription levels compared to the endogenous gene, and two copies of the *pgg*2 gene produced an 1100-fold increase compared with the endogenous gene. These results demonstrated that transcription, translation, and protein secretion in the fungus *P. griseoroseum* respond to an increased number of gene copies in the genome. The processing capacity and efficiency of protein secretion in *P. griseoroseum* are consistent with our premise that this fungus can be used for the industrial-scale production of several enzymes.

J. A. Teixeira · G. B. Nogueira · M. V. de Queiroz · E. F. de Araújo (✉)
Departamento de Microbiologia/BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG CEP 36570-000, Brazil
e-mail: ezfa@ufv.br

Keywords *Penicillium griseoroseum* · Genome organization · PFGE · Gene expression · Protein production · Pectinase

Introduction

The filamentous fungus *Penicillium griseoroseum* has been used as heterologous system for large-scale protein production [10, 15]. Wild-type *P. griseoroseum* CCT6421 is considered promising for the production of pectinases because this strain does not produce mycotoxins, such as patulin, citrinin, and ochratoxin A, under the culture conditions used for pectinase production [5–7, 10, 20]. In addition, this fungus does not secrete proteases into the culture medium under the pectinase production conditions [10]. These characteristics highlight the advantage of the use of *P. griseoroseum* as a host strain to produce proteins on an industrial scale. However, although an understanding of genome organization and gene regulation mechanisms are important requirements for the use of a host organism in the industrial production of metabolites, little is known about the organization of the *P. griseoroseum* genome.

Studies have sought to increase the production of pectinolytic enzymes by *P. griseoroseum* for use in the fruit juice and wine industries [6, 20]. To evaluate the organization and regulation of genes encoding polygalacturonase (PG) EC. 3.2.1.15 and pectin lyase (PL) EC 4.2.2.10 in *P. griseoroseum*, Ribon et al. [25] and Bazzolli et al. [7] isolated and characterized two genes encoding PG (*pgg*1 and *pgg*2) and two genes encoding PL (*plg*1 and *plg*2), respectively. These genes are present as single copies in the genome, are regulated at the transcriptional level, and are induced by pectin and repressed by glucose [8, 26]. The authors reported that the transcriptional levels of *pgg*2 and

*plg*1 genes are higher than those of *pgg*1 and *plg*2, demonstrating that the *plg*1 and *pgg*2 genes are responsible for producing PL and PG in *P. griseoroseum*, respectively [8, 26, 32].

The construction of strains encoding multiple copies of target genes or gene-silenced strains has been successfully employed to increase protein productivity in host strains, including several species of *Aspergillus, Penicillium*, and *Trichoderma* as well as yeasts such as *Pichia pastoris* [11, 18, 21, 34]. Recombinant strains of *P. griseoroseum* with increased PL and PG production have been obtained using integrative expression vectors containing the target gene under the control of a strong constitutive promoter. These recombinant strains have additional copies of the *plg*1 and *pgg*2 genes that are under the control of a strong constitutive promoter for the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and the termination region of the gene encoding tryptophan synthase (*trp*C) from *Aspergillus nidulans* [3, 4, 9, 31, 32]. The recombinant strains of *P. griseoroseum* exhibited a 15-fold increase in the enzymatic activity of PG and a 132-fold increase in PL activity compared with the wild-type strain when cultured in sucrose or sugar cane juice [3, 9, 31, 32].

Genetic and physiological studies of isolated *P. griseoroseum* CCT6421 have highlighted its advantages for use as an expression heterologous system for protein production. The aim of the present study was to increase knowledge of the genomic organization and gene regulation of recombinant strains of *P. griseoroseum.* Specifically, this study estimated the genome size, the number of chromosomes, and the genomic location of the *plg*1 gene in *P. griseoroseum*. After determining the number of copies of the *plg*1 and *pgg*2 genes in recombinant strains of *P. griseoroseum* with increased production of pectin lyase and polygalacturonase, it was possible to infer a relationship between the increased gene copy number and increased enzyme production and to verify the effect of genome position on the expression of these genes. In addition, the expression pattern of the *plg*1 and *pgg*2 genes under the control of the *gpd* promoter of *A. nidulans* was evaluated in the double-recombinant strain T20.

Materials and methods

Microorganisms and growing conditions

The wild-type strain of *P. griseoroseum* CCT6421, which was isolated at the Universidade Federal de Viçosa, and the mutant strain *P. griseoroseum* PG63 *nia*D^{-}, which was obtained by Pereira et al. [23] were used in this study. The recombinant strains (T) that overproduce PL, PG, or PL and PG were obtained by Araújo et al. [3], Cardoso et al. [9] and Teixeira et al. [31]. These recombinant strains were previously characterized and showed ectopic integration of the expression vectors in the genome [9, 31, 32]. The mutant strain *P. griseoroseum* PG63 *nia*D^{-} has a 122-bp deletion in the *nia*D gene, which encodes nitrate reductase [23].

To obtain the inoculum, the strains were grown at 25 °C for 7 days in a Petri dish containing enriched potato dextrose agar (PDA) culture medium, and the conidia were resuspended in a solution of Tween 80 (0.2 % v/v). To analyze the enzymatic activity and expression of the *plg*1 and *pgg*2 genes, 10^6 conidia/ml were obtained from the recombinant and PG63 strains and inoculated in a mineral-buffered (pH 6.8) minimal medium containing 6.98 g/l K_2HPO_4, 5.44 g/l KH_2PO_4, and 1.0 g/l $(NH_4)_2SO_4$ and supplemented with 1.1 g/l $MgSO_4{\cdot}7H_2O$, 10 g/l sucrose, and 0.6 g/l yeast extract. The strains were cultured in 250-ml Erlenmeyer flasks in a volume of 100 ml and maintained under constant agitation at 150 rpm and a temperature of 25 °C for 120 h. Samples were collected at intervals of 12 h. The mycelium used for total RNA extraction and the determination of the mycelial dry weight was obtained by filtering the cultures through a 400 mesh/in^2 sieve (37 μm mesh size). Three replicates were performed for each experiment.

Protoplast preparation and analysis by pulsed-field gel electrophoresis (PFGE)

Protoplasts were obtained according to the method proposed by Dias et al. [12] with modifications. A 5 ml volume of osmotic stabilizer (10 mM phosphate buffer, 0.6 M KCl) and 30 mg of Lysing Enzymes L1412 (SIGMA®) was added to 800 mg of mycelium and incubated at 80 rpm and 30 °C for a period of 3–4 h. The protoplasts were then filtered, washed with osmotic stabilizer, and centrifuged at 2300*g* for 5 min. A suspension of 10^8 protoplasts/ml was mixed with an equal volume of 1.4 % agarose solution at 50 °C. The blocks of agarose containing the protoplasts were transferred to microtubes containing 1 ml of NDS solution (1 % *N*-lauroyl sarcosine, 50 mM EDTA, pH 8.0, 10 mM Tris–HCl) and 50 μl proteinase K (20 mg/ml) and incubated at 50 °C for 48 h. The blocks were washed twice in 50 mM EDTA (pH 8.0) at 50 °C for 30 min, and the tubes containing the blocks were stored at 4 °C until use.

The gel was prepared with 0.8 % chromosomal-grade agarose (Bio-Rad) in 0.5× TBE (45.0 mM Tris–borate, 1.0 mM EDTA, pH 8.0). The buffer was changed every 48 h and maintained at constant temperature (10 °C). The separation was performed in a CHEF-DRII system from Bio-Rad with a current of 1.2 V/cm and pulses divided into three blocks: B1 8400–13000 s, 75 h; B2 3300–5400 s, 90 h; B3 2000–2700, 45 h. After chromosome separation, the gel was treated with 100 ml of distilled water containing 5 μl of ethidium bromide solution (10 mg/ml) for

30–60 min and then placed in distilled water for at least 30 min. The gel was then analyzed, and the image was scanned with the Eagle Eye II (Stratagene) photo-documentation device.

DNA extraction and Southern blot analysis

For karyotype analysis using the telomeric regions of *P. griseoroseum*, total DNA was extracted following Specht et al. [29] with modifications [31]. The total DNA was cleaved with the restriction enzymes *Bam*HI, *Eco*RI, and *Sma*I, and the restriction fragments were separated by electrophoresis in a 0.8 % agarose gel. Then, the total DNA and chromosomal DNA obtained by PFGE were transferred to a nylon membrane (Duralon-UV™, Stratagene) by capillary action as described by Sambrook et al. [27]. Prior to the transfer, both gels were treated with denaturing (1.5 M NaCl, 0.5 M NaOH) solution for 1 h, followed by neutralization (0.5 M Tris–HCl pH 7.0; 1.5 M NaCl) solution for 1 h.

The membrane containing the total DNA restriction fragments was hybridized for 18 h at 38 °C with a fragment of the telomeric region from *Botrytis cinerea* in vector pTel13 as a probe [16]. The labeling of the probe and the detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche®, Roche Applied Science), following the manufacturer's recommendations.

The membrane containing the chromosomal DNA was hybridized for 18 h at 65 °C with the coding region of the *plg*1 gene [7] radiolabeled with a [α-^{32}P]d-ATP probe; the Random Prime IT Labeling Kit was used for radiolabeling (Stratagene). The membrane was washed twice in 2× SSC, 0.1 % SDS for 20 min and twice in 1× SSC, 0.1 % SDS for 10 min at 65 °C. The membrane was exposed to XOMAT K film (Kodak), at −80 °C for 12 h, and the film was developed.

Real-time PCR analysis

Total RNA extraction and analysis of plg1 and pgg2 gene expression

Total RNA was extracted with TRIzol T9424 reagent (TRI Reagent, Sigma®). The mycelium was ground in liquid nitrogen, and 200 mg was transferred to a centrifuge microtube containing 1.0 ml of TRIzol and 150 µl of 20 % PVP-40 (polyvinylpyrrolidone 40, Sigma®). After homogenization, the mixture was centrifuged at 15000*g* for 20 min, and the supernatant was transferred to another tube containing 200 µl of chloroform. After homogenization, the mixture was centrifuged at 15000*g* for 20 min, and the aqueous phase was transferred to a fresh tube containing 250 µl of precipitating solution (0.8 M sodium citrate, 1.2 M sodium chloride) and 250 µl of isopropanol. The mixture was incubated on ice for 10 min and then centrifuged at 15000*g* for 10 min. The supernatant was discarded, and the pellet was washed with 80 % ethanol. The pellet was resuspended in 40 µl of diethylpyrocarbonate (DEPC) water and stored at −80 °C.

The cDNA synthesis was performed from 1.0 µg total RNA using the Im Prom-II™ Reverse Transcription System (Promega) kit according to the manufacturer's instructions. To quantify *plg*1 and *pgg*2 gene expression, the cDNA samples obtained from the PG63 and recombinant T20 strains of *P. griseoroseum* [31] were used. The γ-actin gene from *P. griseoroseum* was used as an endogenous control. The primers used for γ-actin gene amplification were act1_F (5′-TGGGAGCTTCGGTCAAGAGA-3′) and act1_R (5′-ACCAACTGGGACGACATGGA-3′). The relative quantification was performed according to the $2^{-\Delta\Delta CT}$ method proposed by Livak and Schmittgen [17]. The tests for amplification efficiency and experiment validation were performed as described by Pfaffl [24]. The experiments were repeated three times for each point tested using three preparations of total RNA obtained from independent biological samples.

The oligonucleotides were designed with Primer Express software (Applied Biosystems, California, USA) and were based on the *plg*1 (AF502279.1) and *pgg*2 (AF195113.2) gene sequences deposited in the database of the National Center for Biotechnology Information (NCBI). The following sequences of the oligonucleotides used in this study are listed: plg1B_F (5′-GGACAT GATCTGGATCGAATCA-3′); plg1B_R (5′-TGGAGATAG TGACACGCTTGGA-3′); pgg2A_F (5′-GAACATCTCC GGCCTTAACG-3′); pgg2A_R (5′-CCTCGCTGTTGTCG ATGGT-3′);

Determination of the copy number of the plg1 and pgg2 genes

The plasmids pAN52plg1 (6.75 kb) and pAN52pgg2 (6.55 kb), which contain the *P. griseoroseum* genes *plg*1 (1.39 kb) and *pgg*2 (1.19 kb), respectively, were used to prepare the DNA standard curve. Plasmid DNA was extracted with the Wizard® Plus SV Minipreps, DNA Purification System kit (Promega). The quality and concentration of total DNA and of the plasmid samples were determined spectrophotometrically at 260/280 nm. The absolute number of plasmid copies was obtained from the equation proposed by Whelan et al. [36]. The plasmid DNA samples were diluted at a 1:10 ratio. Eight serial dilutions in the range of 4×10^1 to 4×10^7 copies/µl of plasmid pAN52plg1 and 6×10^1 to 6×10^7 copies/µl of plasmid pAN52pgg2 were used to construct the standard curves. The equation $y = ax + b$ (R^2), which was obtained from the calibration curve, was $y = -3.32x + 39.924$ (0.99) for the *plg*1 gene and $y = -3.31x + 37.076$ (0.99) for the *pgg*2 gene.

The experiments for relative and absolute quantification were conducted in a 7300/7500 real-time PCR system (Applied Biosystems, California, USA). All PCR reactions were performed with 0.2 μM of each oligonucleotide and SYBR Green PCR Master Mix (Applied Biosystems, California, USA) at a final concentration of 1× in a final volume of 25 μl per reaction.

Genome size estimate for P. griseoroseum

The genome size was estimated based on the *C* value, which was calculated as described by Wilhelm et al. [37]. The amount of DNA corresponding to the size of a haploid genome (C value) was derived from the ratio of DNA mass (*m*, as determined by UV absorbance) and the copy number of the target sequence (*N*, determined by real-time PCR), i.e., $C = m \times N^{-1}$. The genome size was calculated by $\Gamma = C \times N_A \times M_{bp}^{-1}$, where N_A is Avogadro's number (6.022×10^{23} mol^{-1}) and M_{bp}^{-1} is the average molecular weight of a base pair (660 g mol^{-1}).

Enzymatic trials of pectin lyase and polygalacturonase

Pectin lyase activity was determined via absorbance at 235 nm as described by Albersheim and Killias [2]. One unit of PL (U) was defined as the amount of enzyme required to produce a nanomole of $\Delta^{4,5}$ galacturonate per minute of reaction. A coefficient of molar extinction of 5550 M^{-1} cm^{-1} was used for the calculation [1].

PG activity was determined by measuring reducing sugars with the DNS (dinitrosalicylic acid) method described by Miller [19]. One unit of PG (U) was defined as the amount of enzyme required to produce a micromole of galacturonic acid per minute of reaction.

Results

Estimation of genome size, the number of chromosomes, and chromosomal location of the *plg*1 gene of *P. griseoroseum*

The estimation of the size of the haploid genome by real-time PCR requires the absolute quantification of a single-copy gene in the genome. The *plg*1 [7] and *pgg*2 [25] genes studied here are present as single copies in the genome of the wild-type strain of *P. griseoroseum* and were used to estimate the genome size of this species. The estimated genome size of the wild-type strain of *P. griseoroseum* in three independent experiments ranged from 29.8 to 31.5 Mb (Table 1).

The karyotype of *P. griseoroseum* was determined by PFGE and exhibited a pattern consisting of four chromosomal bands with an estimated size of 11.44 Mb (I), 9.22 Mb (II), 6.24 Mb (III), and 4.12 Mb (VI), which were calculated based on the banding pattern of *Schizosaccharomyces pombe* (Fig. 1a). The size of the *P. griseoroseum* genome estimated by PFGE was approximately 31.0 Mb.

The chromosomal location of the *plg*1 gene and the integration pattern of the copies of the expression vector pAN52plg1 were determined from the hybridization of the chromosomes from the PG63 and recombinant T105 strains of *P. griseoroseum*, which were separated by PFGE and transferred to a nylon membrane. The membrane was hybridized with the fragment containing the *plg*1 coding region labeled with [α-^{32}P]d-ATP. The results indicated that the *plg*1 gene is located on chromosome II (9.22 Mb) of *P. griseoroseum* and that the bands observed for the recombinant strain T105 may be due to ectopic integration of pAN52plg1 with chromosomes II, III, and IV (Fig. 1b). The position of the bands was measured by using a scale ruler in the original PFGE and in the autoradiography of the hybridization.

The number of chromosomes in *P. griseoroseum* was confirmed by hybridization of the telomeric chromosomal region. The total DNA of the wild-type strain was cleaved with restriction enzymes and hybridized with a probe containing a 225-bp fragment corresponding to the telomeric region of *B. cinerea* present in the vector pTel13 (Fig. 2). For each enzyme tested, a pattern of eight marked bands was observed, with two bands corresponding to a chromosome. Therefore, the total number of four chromosomes was confirmed from the band profile obtained from hybridization.

Analysis of the copy number of genes *plg*1 and *pgg*2 and the activity of pectin lyase and polygalacturonase in recombinant strains of *P. griseoroseum*

The number of copies of the *plg*1 and *pgg*2 genes in recombinant strains of *P. griseoroseum* with high PL and PG

Table 1 Estimate of the haploid genome size of the wild-type strain of *P. griseoroseum*

Target	Sample concentration (ng/μl)	Calibration curve[a] $y = ax + b$ (R^2)	Target copy number	*C* (pg)	Γ (bp)
*plg*1	15	$-3.32x + 39.924$ (0.99)	4.6×10^5	0.033	29.8×10^6
	45	$-3.32x + 39.924$ (0.99)	1.33×10^6	0.034	31.0×10^6
*pgg*2	12	$-3.31x + 37.076$ (0.99)	3.57×10^5	0.034	30.7×10^6
	24	$-3.31x + 37.076$ (0.99)	6.95×10^5	0.035	31.5×10^6

[a] C_T curve versus the log of copy number. *a*, slope; *b*, intercept; R^2, correlation coefficient

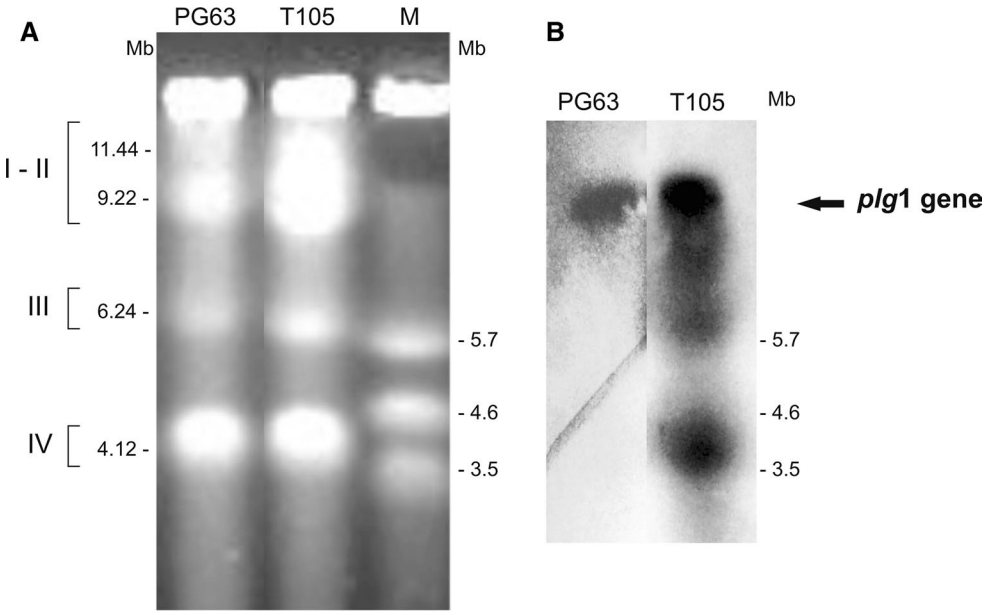

Fig. 1 Molecular karyotypes of *P. griseoroseum* PG63 and the recombinant T105 strain and the location of the *plg*1 gene. **a** Pulsed-field gel electrophoresis (PFGE) analysis revealing the chromosome distribution in *P. griseoroseum* PG63 and the recombinant T105 strain. **b** Characterization of the *plg*1 gene by DNA hybridization. The *arrow* indicates the location of an endogenous *plg*1 gene on chromosome II of *P. griseoroseum*. M, *Schizosaccharomyces pombe* size marker (5.7, 4.6, and 3.5 Mb)

production was determined by absolute quantification with real-time PCR. Table 2 shows the copy number for the *plg*1 and *pgg*2 genes, which were calculated for the wild-type, mutant PG63 *nia*D^{-}, and recombinant (T) *P. griseoroseum* strains and normalized by the number of copies of these genes in the wild-type strain. The *P. griseoroseum* PG63 *nia*D^{-} strain was used to validate the qPCR technique used to determine the copy number because this strain has the same number of copies of the *plg*1 and *pgg*2 genes as the wild-type strain.

A correlation between the number of copies of the *plg*1 or *pgg*2 genes under the control of the *gpd* promoter and the activity of PL or PG, respectively, was observed in the recombinant strains. The enzymatic activity of PL was not linearly correlated with the increase in the *plg*1 gene copy number (Fig. 3a, b). Although the R^2 (0.71) was high in general, the T09 strain showed decrease in PL activity. The same was observed with PG, the activity was not linearly correlated with the *pgg*2 gene copy number (Fig. 3c, d). Differences in the activity of PG could be observed in strains that had the same number of copies of the *pgg*2 gene, such as strains T3.4 and T20, in which the copy of the *pgg*2 gene was controlled by the *gpd* promoter. Strain T20 (34966 U/ml) exhibited significantly greater PG activity than strains T3.4 (16727 U/ml). Strain T146 (36486 U/ml), which contained two copies of the *pgg*2 gene, exhibited the same or higher activity than the strains containing three copies of the integrated gene. This indicates that the copy position in the genome may affect gene expression.

In order to confirm the integrity of the constructs vectors expression, the DNA of the transformants was extracted, and PCR was performed with the gpdAF (5′-TATTTTC CTGCTCTCCCCACC-3′) and trpCR (5′-TGCTTCATCT CGTCTCCCGAA-3′) primers to determine the integration of pAN52plg1 and pAN52pgg2 into the recombinant strains genomes. We observed two fragments that correspond with the intact ectopic integrations of the *plg*1 and *pgg*2 genes under the control of the *gpd* promoter of *A. nidulans*. A 1.79 kb fragment corresponding to the final 200 bp of *gpd*A + 1390 bp *plg*1 + the initial 200 bp of *trp*C was observed for the strains contained the pAN52plg1. Another fragment of 1.59 kb was observed for those strains that contained pAN52pgg2 vector (data not shown).

Evaluation of *plg*1 and *pgg*2 gene expression in double-recombinant strain *P. griseoroseum* T20

The expression of the *plg*1 and *pgg*2 genes was analyzed to determine whether there were differences in expression due to the position in the genome or the number of copies. For this purpose, the double-recombinant strain T20 was selected because it has a single copy of the *plg*1 gene and two copies of the *pgg*2 gene, both of which are controlled by the *gpd* gene promoter from *A. nidulans* with different ectopic integration [31]. The strain *P. griseoroseum* PG63 was used as a control strain for the endogenous expression of the *plg*1 and *pgg*2 genes. Figure 4 shows the variation in *plg*1 and *pgg*2 gene expression, PL and PG activity, and the dry mycelial mass during 120 h of culture. By comparing the expression in strains PG63 and T20 and using the *plg*1 and *pgg*2 gene expression values from the PG63 strain as normalizers, it was observed that the additional copy of the *plg*1 gene in the T20 strain provided a 200-fold increase in expression relative to the endogenous gene, whereas two copies of the *pgg*2 gene provided a 1100-fold increase in expression with respect to the endogenous gene (Fig. 4a, b).

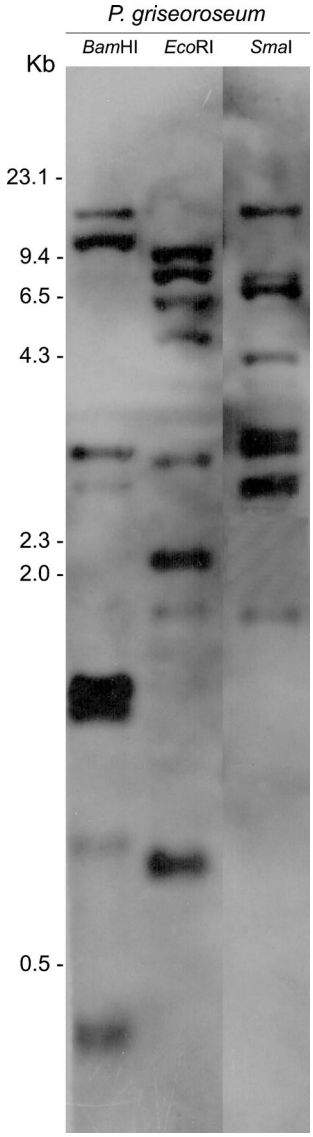

Fig. 2 Estimate of the chromosome number in the wild-type strain of *P. griseoroseum*. Autoradiography of the total DNA from *P. griseoroseum* cleaved with the enzymes *Bam*HI, *Eco*RI, and *Sma*I and hybridized with a DNA fragment containing the telomeric region of *B. cinerea,* which is present in vector pTel13

The activities of PL and PG that were detected in the supernatant of the culture increased gradually with time, with the highest detected PL activity (2367 ± 316 U ml^{-1}) occurring at 120 h (Fig. 4c) and the highest detected PG activity (35619 ± 6090 U ml^{-1}) occurring after 72 h of culture (Fig. 4d). The growth curves for strains PG63 and recombinant T20 are shown in Fig. 4e. The highest mycelial dry weight values were observed at 72 h of culture, with a decrease in mycelial dry weight after this time.

As expected, the temporal expression profiles of both *plg*1 and *pgg*2 genes were similar in the recombinant strain T20; transcript accumulation occurred at 24 h of culture, with a reduction in the amount of transcripts at 120 h (Fig. 5).

Table 2 Total copy number of the *plg*1 and *pgg*2 genes from the wild-type (W), mutant PG63, and recombinant (T) *P. griseoroseum* strains, including endogenous and ectopic copies. Values are normalized for the copy number of the *plg*1 and *pgg*2 genes in the wild-type strain of *P. griseoroseum*

Genes	Strain	Copy number
*plg*1	*P. griseoroseum* (W)	1
	PG63	1
	T47	1
	T16	2
	T20	2
	T70	2
	T09	3
	T24	5
	T105	5
*pgg*2	*P. griseoroseum* (W)	1
	PG63	1
	T143	2
	T146	2
	T3.3	3
	T3.4	3
	T20	3

Discussion

The present study describes, for the first time, the karyotype and genome size of the fungus *P. griseoroseum*, as well as the chromosomal location of the *plg*1 gene encoding pectin lyase. Furthermore, the expression of the *plg*1 and *pgg*2 genes in recombinant strains of *P. griseoroseum* with multiple copies of these genes was assessed. Several studies have shown that a combination of molecular techniques can facilitate a broad understanding of the genome organization and function of various model species and their industrial applicability. By combining PFGE (Fig. 1a), Southern blot hybridization (Figs. 1b, 2) and real-time PCR (Table 1), the present study demonstrates that *P. griseoroseum* has a genome of approximately 31.0 Mb that is divided into four chromosomes. These techniques also established the location of the *plg*1 gene on chromosome II of *P. griseoroseum* and of additional copies of the vector pAN52plg1 in the recombinant *P. griseoroseum* strain T105, which were integrated on chromosomes II, III, and IV. The estimated genome size of this species is in agreement with previous studies of other species in this genus, such as *Penicillium notatum* (32.1 Mb), *Penicillium chrysogenum* (32.19–34.1 Mb), and *Penicillium nalgiovense*

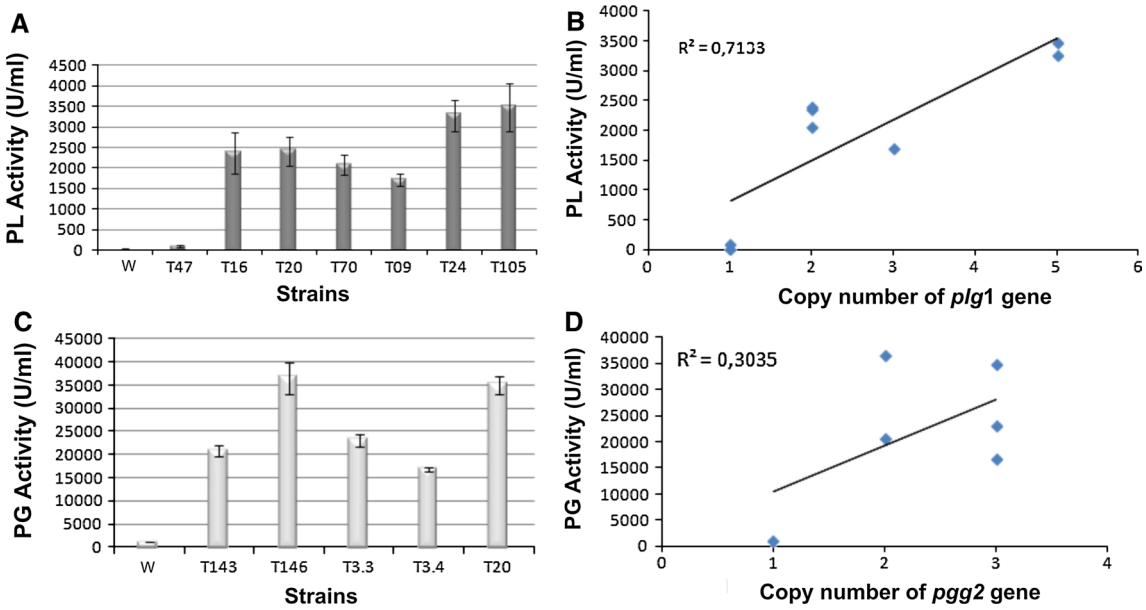

Fig. 3 Correlation of enzyme production and the number of copies of the *plg*1 and *pgg*2 genes under the control of the *gpd* promoter integrate in the genome of *P. griseoroseum* strains. PL (**a**) and PG (**c**) activity in the culture supernatant of recombinant strains (T) at 72 h. **b** Correlation between *plg*1 copy number and PL activity. **d** Correlation between *pgg*2 copy number and PG activity. The results represent the mean ± standard deviation from three independent experiments

(26.5 Mb) [13, 14, 33]. For the three species named above, PFGE estimates indicated that their genomes have similar sizes and are distributed on four chromosomes, which range in size from 4.0 to 5.0 Mb for the smallest chromosomes and 9.0 to 11.0 Mb for the largest.

In previous studies, Ribon et al. [25] and Bazzolli et al. [7] isolated and characterized the *pgg*2 and *plg*1 genes, respectively, and identified both as single-copy genes in the genome of this fungus. Based on this information, the genome size of *P. griseoroseum* was estimated by real-time PCR using the method proposed by Wilhelm et al. [37], which has been successfully used for several organisms. The techniques used in the present study produced similar results, indicating that the size of the *P. griseoroseum* genome varies between 29.8 and 31.5 Mb (Table 1). Although these techniques provide information on genome structure and organization, their limitations may result in under- or overestimates when used alone. Therefore, a combination of molecular techniques provides better accuracy.

An increase in enzyme production resulting from an increased number of copies of the target genes has been successfully demonstrated by several authors, particularly in species of *Aspergillus*, *Trichoderma,* and some yeast such as *P. pastoris*. The results of the present study demonstrated that increasing the copy number of the *plg*1 gene led to increased PL activity, but not in the linear correlation way. For the PG, activity was observed the same, but there was no linear correlation between the increased copy number of the *pgg*2 gene and PG activity, which suggests that the gene expression may be affected by the position of the gene in the genome. Since both genes are under control of the same regulatory region and responds to the same transcriptional factors. It has been reported that the variation in gene expression level for multi-copy strains may be due to the effect of genome position and not to the control of the regulatory promoter region [9, 11]. This finding may explain the reduction in PL and PG activities that was primarily observed for strains T09, T3.4, and T143 (Fig. 3a, c). However, for the strain T47 the gene exchange could be the explanation for the reduction in PL activity, since the expression cassette was intact in genome of this strain (Table 2). Analyze of SDS-PAGE has been done to verify the presence of the bands that corresponding to Plg1 (36 kDa) and Pgg2 (38 kDa) proteins, and in all strains were observed an intense band that match with PL and PG, except for recombinant strain T47 (data not shown). This indicates that the original location from the endogenous *plg*1 gene is not a transcriptionally active region.

Some researchers have used a strategy in which copies of target genes are inserted and increased in highly active regions of the genome, such as the ribosomal DNA locus, thereby obtaining increased protein production [18]. In addition, a growing number of studies have reported that an increase in protein production may not occur due to an insufficient supply of regulatory proteins or transcription factors that are essential to the process, or because of inadequate culture conditions [11, 21, 30]. The difference between the increase in gene expression of *pgg*2 and that

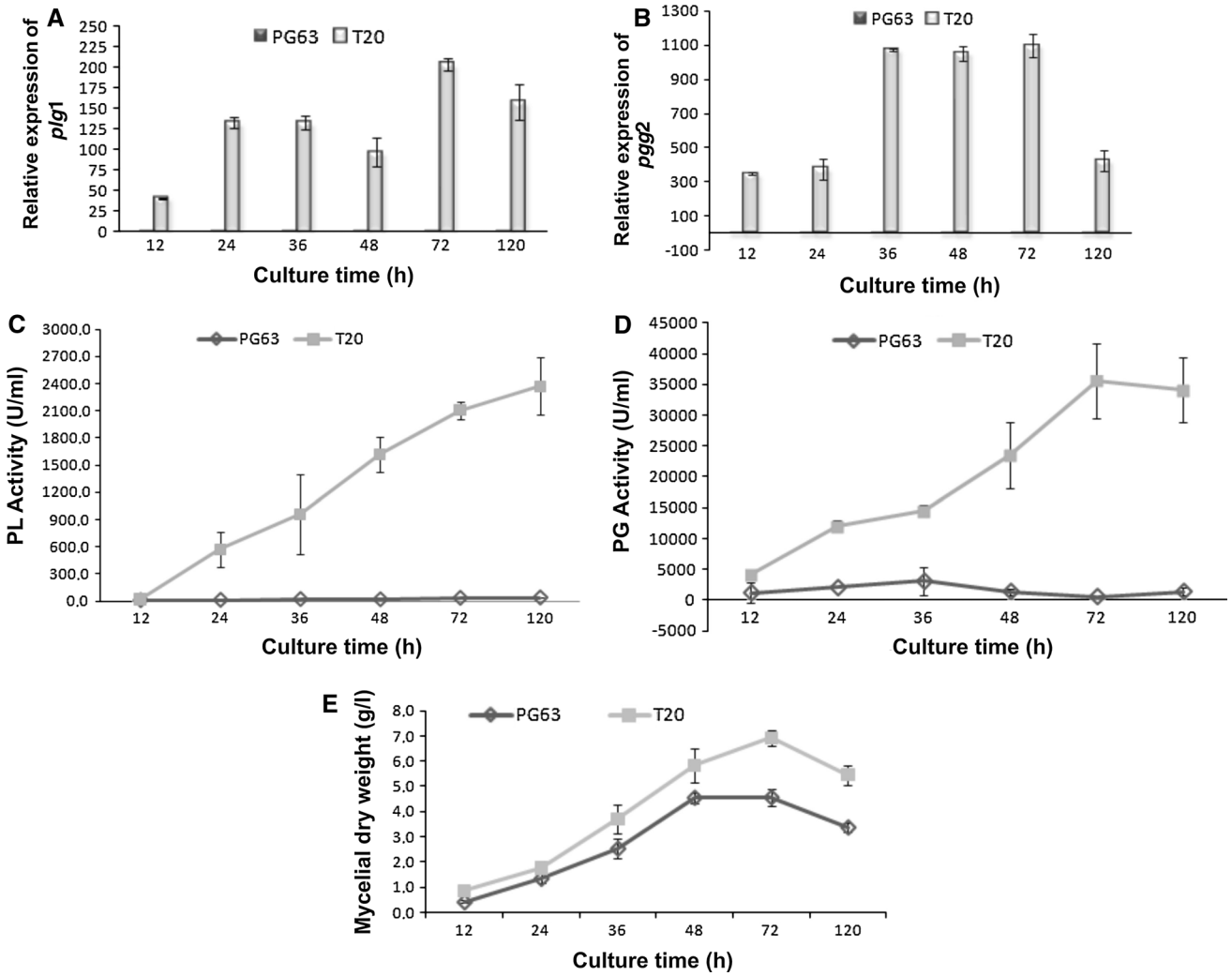

Fig. 4 Analysis of *plg*1 and *pgg*2 gene expression, PG and PL activity, and growth curves of the PG63 and recombinant T20 strains. Comparative analysis of recombinant strain T20 with PG63, showing the relative expression of genes *plg*1 (**a**) and *pgg*2 (**b**); the activity of PL (**c**) and PG (**d**) in the culture supernatant. Mycelial dry weight (**e**). The results represent the mean ± standard deviation of three independent experiments

of *plg*1, both of which were inserted into the genome of the recombinant strain T20, may be due to an effect of the position of these copies in the genome and not due to the 2:1 difference in the number of copies of the *pgg*2 and *plg*1 genes, respectively (Fig. 4a, b).

The analysis of *plg*1 and *pgg*2 gene expression in the recombinant strain T20 revealed a similar profile. This was an expected result because both the *plg*1 and the *pgg*2 gene are under the control of the *gpd* promoter gene of *A. nidulans* when inserted into the genome of strain T20 [31]. The level of *plg*1 and *pgg*2 gene expression decreased after 72 h of culture, concomitant with a reduction in the mycelial dry weight of the recombinant strain T20. These results can be explained by the depletion of the carbon source in the culture medium. Previous studies performed in our laboratory have demonstrated that, after 30 h of culture, sucrose and glucose residues cannot be detected. According to Silva-Udawatta and Cannon [28], during the consumption of the available carbon source, the synthesis of reserve carbohydrates, glycogen, and trehalose is initiated. However, for cells grown in batch fermentation systems, a reduction in the transcription of many genes has been observed when glycogen is metabolized [35]. Fungi and yeast studies in which the regulation of genes coding for glyceraldehyde-3-phosphate dehydrogenase was evaluated demonstrated that this gene is regulated in response to the carbon source and that the amount of transcripts decreased when the amount of glucose decreased [22, 38]. Although the *gpd* promoter of *A. nidulans* is constitutively expressed, nutrient exhaustion affects its regulation, causing decreased

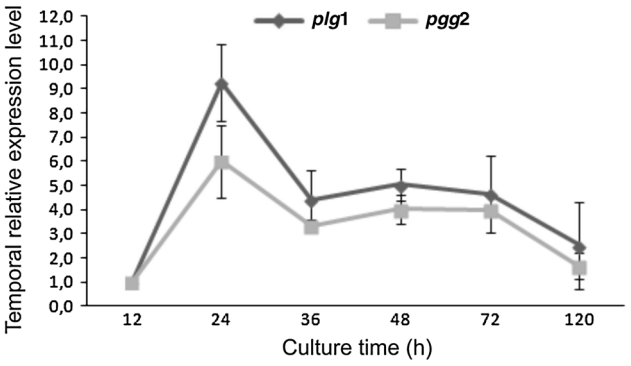

Fig. 5 Temporal relative expression levels of genes *plg*1 and *pgg*2 under control of the *A. nidulans gdp* promoter. Evaluation of the expression levels of *plg*1 and *pgg*2 in the mycelium of the recombinant strain T20 cultured for 120 h. The results represent the mean ± standard deviation of three independent experiments

expression following the stationary phase of mycelial growth in the recombinant strain *P. griseoroseum* T20.

Therefore, *P. griseoroseum* has great potential and suitable characteristics for use as a host strain in industrial applications because its genetic characteristics enable heterologous transformation and it possesses an efficient protein production and secretion system.

Acknowledgments This research was supported by the following Brazilian agencies: Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. Albersheim P (1966) Pectin lyase from fungi. Methods in Enzymol 8:628–635
2. Albersheim P, Killias U (1962) Studies relating to the purification and properties of pectin transeliminase. Arch Biochem Biophys 97(1):107–115
3. Araújo EF, Queiroz MV, Teixeira JA, Gonçalves DB, Bazzolli DMS, Ribon AO, Ribeiro JB (Inventores) (2007) Universidade Federal de Viçosa (Depositante). Linhagem recombinante geneticamente modificada *Penicillium griseoroseum* T20 com maior produção de pectina liase e poligalacturonase, cassetes de expressão para a construção da linhagem recombinante, preparação enzimática e processo para produção da preparação enzimática. Depósito de pedido de patente. PI 0705573-0
4. Araújo EF, Silva DO, Queiroz MV, Cardoso PG (Inventores) (2005) Universidade Federal de Viçosa (Depositante). Linhagem recombinante de *Penicillium griseoroseum* geneticamente modificada com aumento na produção de pectina liase. Depósito de pedido de patente. PI0502515-0
5. Baracat MC, Valentim C, Muchovej JJ, Silva DO (1989) Selection of pectinolytic fungi for degumming of natural fibers. Biotechnol Lett 11(12):899–902
6. Baracat-Pereira MC, Coelho JLC, Silva DO (1994) Production of pectin lyase by *Penicillium griseoroseum* cultured on sucrose and yeast extract for degumming of natural fibers. Lett Appl Microbiol 18:127–129
7. Bazzolli DMS, Ribon AOB, Queiroz MV, Araújo EF (2006) Molecular characterization and expression profile of pectin-lyase-encoding genes from *Penicillium griseoroseum*. Can J Microbiol 52(11):1070–1077
8. Bazzolli DMS, Ribon AOB, Reis KCP, Queiroz MV, Araújo EF (2008) Differential expression of *PLG* genes from *Penicillium griseoroseum*: *plg*1 a pectinolytic gene is expressed en sucrose and yeast extract. J Appl Microbiol 105:1595–1603
9. Cardoso PG, Ribeiro JB, Teixeira JA, Queiroz MV, Araújo EF (2008) Overexpression of the *plg*1 gene encoding pectin lyase in *Penicillium griseoroseum*. J Ind Microbiol Biotechnol 35(3):159–166
10. Cardoso PG, Teixeira JA, Queiroz MV, Araújo EF (2010) Pectin lyase production by recombinant *Penicillium griseoroseum* strain 105. Can J Microbiol 56(10):831–837
11. Chen X, Liang Y, Hua J, Tao L, Qin W, Chen S (2010) Overexpression of bacterial ethylene-forming enzyme gene in *Trichoderma reesei* enhanced the production of ethylene. Int J Biol Sci 6(1):96–106
12. Dias ES, Queiroz MV, Cardoso PG, Barros EG, Araújo EF (1999) Transformation of *Penicillium expansum* with a heterologous gene which confers resistance to benomyl. World J Microbiol Biotechnol 15:513–514
13. Färber P, Geisen R (2000) Karyotype of *Penicillium nalgiovense* and assignment of the penicillin biosynthetic genes to chromosome IV. Int J Food Microbiol 58(1–2):59–63
14. Fierro F, Gutiérrez S, Díez B, Martín JF (1993) Resolution of four large chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 Mb) in *Penicillium notatum* and chromosome I (10.4 Mb) in *Penicillium chrysogenum*. Mol Gen Genet 241(5–6):573–578
15. Gonçalves DB, Teixeira JA, Bazzolli DMS, Queiroz MV, Araújo EF (2012) Use of response surface methodology to optimize production of pectinases by recombinant *Penicillium griseoroseum* T20. Biocatal Agric Biotechnol 1:140–146
16. Levis C, Giraud T, Dutertre M, Fortini D, Brygoo Y (1997) Telomeric DNA of *Botrytis cinerea*: a useful tool for strain identification. FEMS Microbiol Lett 157:267–272
17. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2_{\mathrm{T}}^{-\Delta\Delta C}$ method. Methods 25:402–408
18. Marx H, Mecklenbrauker A, Gasser B, Sauer M, Mattanovich D (2009) Directed gene copy number amplification in *Pichia pastoris* by vector integration into the ribosomal DNA locus. FEMS Yeast Res 9:1260–1270
19. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
20. Minussi RC, Soares-Ramos JRL, Coelho JLC, Silva DO (1998) Sugar-cane juice induces pectin lyase and polygalacturonase in *Penicillium griseoroseum*. Revista de Microbiologia 29:246–250
21. Nevalainen HKM, Te'o VSJ, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. Trends Biotechnol 23:468–474
22. Pavlovic B, Horz W (1988) The chromatin structure at the promoter of a glyceraldehyde phosphate dehydrogenase gene from *Saccharomyces cerevisiae* reflects its functional state. Mol Cell Biol 8(12):5513–5520
23. Pereira JF, Queiroz MV, Lopes FJ, Rocha RB, Daboussi MJ, Araújo EF (2004) Characterization, regulation, and phylogenetic analyses of the *Penicillium griseoroseum* nitrate reductase gene and its use as selection marker for homologous transformation. Can J Microbiol 50(11):891–900
24. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45

25. Ribon AOB, Queiroz MV, Araújo EF (2002) Structural organization of polygalacturonase-encoding genes from *Penicillium griseoroseum*. Genet Mol Biol 25(4):489–493
26. Ribon AOB, Ribeiro JB, Gonçalves DB, Queiroz MV, Araújo EF (2009) Gel mobility shift scanning of pectin-inducible promoter from *Penicillium griseoroseum* reveals the involvement of a CCAAT element in the expression of a polygalacturonase gene. Genet Mol Biol 32(1):129–132
27. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
28. Silva-Udawatta MND, Cannon JF (2001) Roles of trehalose phosphate synthase in yeast glycogen metabolism and sporulation. Mol Microbiol 40(6):1345–1356
29. Specht CA, Dirusso CC, Novotny CP, Ullrich RC (1982) A method for extracting high molecular weight deoxyribonucleic acid from fungi. Anal Biochem 119(1):158–163
30. Teixeira JA, Corrêa TLR, Queiroz MV, Araújo EF (2014) pH-dependent effect of pectinase secretion in *Penicillium griseoroseum* recombinant strains. J Basic Microbiol 54:133–141
31. Teixeira JA, Gonçalves DB, Queiroz MV, Araújo EF (2011) Improved pectinase production in *Penicillium griseoroseum* recombinant strains. J Appl Microbiol 111:818–825
32. Teixeira JA, Ribeiro JB, Gonçalves DB, Queiroz MV, Araújo EF (2013) Overproduction of polygalacturonase by *Penicillium griseoroseum* recombinant strains and functional analysis by targeted disruption of the *pgg*2 gene. Appl Biochem Biotechnol 169:1965–1977
33. van den Berg MA, Albang R, Albermann K et al (2008) Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. Nat Biotechnol 26(10):1161–1168
34. Wang SW, Xing M, Liu G, Yu SW, Wang J, Tian SL (2012) Improving cellulase production in *Trichoderma koningii* through RNA interference on *ace*1 gene expression. J Microbiol Biotechnol 22(8):1133–1140
35. Werner-Washburne M, Braun E, Johnston GC, Singer RA (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. Microbiol Rev 57(2):383–401
36. Whelan JA, Russell NB, Whelan MA (2003) A method for the absolute quantification of cDNA using real-time PCR. J Immunol Methods 278(1–2):261–269
37. Wilhelm J, Pingoud A, Hahn M (2003) Real-time PCR-based method for the estimation of genome sizes. Nucleic Acids Res 31(10):e56
38. Wolff AM, Arnau J (2002) Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in *Mucor circinelloides* (syn. *racemosus*) and use of the *gpd*1 promoter for recombinant protein production. Fungal Genet Biol 35:21–29