Journal of
Microbial & Biochemical Technology

Monteiro et al., J Microb Biochem Technol 2015, 7:1
DOI: 10.4172/1948-5948.1000173

Research Article Open Access

Trichoderma reesei Mycoparasitism against *Pythium ultimum* is coordinated by G-alpha Protein *GNA1* Signaling

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Abstract

Trichoderma reesei (*Hypocrea jecorina*) is widely explored in industry and its potential for using in agriculture as a biocontrol agent against phytophatogenic fungi has just began to be investigated. We have investigated the involvement of G proteins during mycoparasitism against plant pathogens. Here we described the role of *GNA1*, a G-alpha protein that belongs to α_i group in Cell Wall Degrading Enzymes (CWDEs) production by *T. reesei* during antagonism against *Pythium ultimum*. For that, two mutants were used: Δ*gna1* and *gna1*QL (=constitutively activated version of *GNA1*). The *gna1*QL mutant of *T. reesei*, like the parental TU-6, inhibited the growth of *P. ultimum* in plate confrontation assay and grew faster than the parental TU-6 while the Δ*gna1* did not grow over *P. ultimum*. Scanning electron microscopy showed that the *gna1*QL mutant promoted more morphological alterations of *P. ultimum* cell wall than the parental TU-6 while the Δ*gna1* caused no effects. The mutant Δ*gna1* showed less CWDEs activity than *gna1*QL and TU-6 during *in vitro* cultivations. The *gna1*QL mutant showed a better performance in production of CWDEs such as endochitinase, N-Acetyl-β-D-glucosaminidase (NAGase), lipase and acid phosphatase, after 72 hours of incubation. However, the parental TU-6 showed higher cellulase activity than *gna1*QL and Δ*gna1*. The intracellular content of cAMP in the strains after 72 hours of incubation in the presence of *P. ultimum* cell wall was: *gna1*QL (79.85 ± 12), Δ*gna1* (268.65 ± 8.5) and TU-6 (109.70 ± 9.2) pmol/mg protein. RT-qPCR results showed a low level of transcripts of mycoparasitism-specific genes in Δ*gna1* strain. We therefore suggest that the production of some CWDEs during mycoparasitism by *T. reesei* against *P. ultimum* can be mediated by *GNA1* activity or cAMP levels.

Keywords: Degrading enzymes; G-protein; Mycoparasitism; *Trichoderma reesei*

Introduction

The potential of the genus *Trichoderma* as biocontrol agents of plant disease was first recognized by Weindling in the early 1930s [1], which described the mycoparasitic action of *T. lignorum* (later renamed as *T. virens*) on *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and its beneficial effects in control of plant pathology. Since then, the genus has been extensively investigated as an antagonist of soil-borne plant pathogens as an alternative to the use of chemical fungicides [2]. *T. reesei* (*Hypocrea jecorina*), in particular, is widely used for industrial applications such as pulp and paper and biomass degradation for cellulosic ethanol [3]. However, *T. reesei* has been also employed as a biocontrol agent [4-6].

Biological control by *Trichoderma* is known as a combination of different mechanisms, among which the most important are: competition for nutrients, production of volatile and non-volatile antibiotics, coiling around the host, and production of hydrolytic enzymes [7]. The mechanism that involves the action of hydrolytic enzymes is called mycoparasitism [2] and results in penetration of the cell wall of the host fungus and utilization of its cellular contents [7]. Mycoparasitism studies have generally focused on the production of chitinases, β-1,3-glucanases, and proteases [8-10], all of which are closely related to the cell wall composition of the pathogen [11]. We previously reported that other enzymes, such as phosphatases and lipases, are involved in mycoparasitism [5]. Furthermore, using proteomic approaches, we recently also identified a role for α-mannosidase and arabinofuranosidase (ABFase) in mycoparasitism [12].

Pythium is a genus of parasitic oomycete and some species are among the most aggressive soilborne pathogens, causing seed rot and seedling damping-off in many crops [13]. *P. ultimum* is a ubiquitous plant pathogen and one of the most pathogenic of the genus and because that, their genome was sequenced [14]. Mycoparasitism of *P. ultimum* by *Trichoderma* involves fungus-fungus interaction and host-pathogen cross-talk with participation of G proteins [15-18], protein kinases [19] and signaling molecules such as cyclic AMP [20]. However, the elucidation of the signaling pathways underlying mycoparasitism is still opened [21].

The G proteins are a family of guanine nucleotide-binding proteins

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Received October 07, 2014; **Accepted** December 01, 2014; **Published** December 08, 2014

Citation: Monteiro VN, Steindorff AS, Almeida FBR, Lopes FAC, Ulhoa CJ, et al. (2014) *Trichoderma reesei* Mycoparasitism against *Pythium ultimum* is coordinated by G-alpha Protein *GNA1* Signaling. J Microb Biochem Technol 7: 001-007. doi:10.4172/1948-5948.1000173

that relay signals from cell surface receptors to intracellular effectors. The involvement of signal transduction pathway components such as G proteins in control of CWDE expression and coiling processes has been suggested [5,17,18,22]. The *GNA1*, G-alpha protein that belongs to α_i group of the fungal G-proteins was already cloned from *T. reesei* and a mutant carrying a constitutively activated version of and *GNA1* (*gna1*QL) and *GNA1* deletion (Δ*gna1*) is available [23,24].

The aim of this study was to test the role of the G-alpha protein *GNA1* in antagonism of *P. ultimum* by *T. reesei* and in the CWDE production induced by *P. ultimum* cell wall as well. Our findings provide possible functions for *GNA1* in mycoparasitism-related processes and suggest an overlapping function in the regulation of mycoparasitism-related genes with another G protein (GNA3) previously described.

Materials and Methods

Microorganisms and culture conditions

The uridine auxotrophic *T. reesei* TU-6 mutant strain (ATCC MYA-256), strain PFG1 (=TU-6 retransformed with pyr4 gene), strain expressing constitutively activated version of *GNA1* (*T. reesei gna1*QL) and a *gna1* deletion strain (Δ*gna1*) were obtained from the Institute for Chemical Engineering (Vienna University of Technology, Research Area Gene Technology and Applied Biochemistry, Vienna, Austria) [24]. *P. ultimum* was obtained from Laboratório de Fitopatologia (Universidade de Brasília, Brasília, Brazil). The microorganisms were maintained on MEX medium (3% malt extract and agar 2% w/v) supplemented with 10 mmol.L^{-1} uridine (Sigma-Aldrich Co., Wisconsin, USA) in case of TU-6.

For production of CWDEs, we have used a mycelium replacement system in 200 mL of minimal medium as described by [22] supplemented with 0.1% (w/v) peptone and 5 g/L of previously purified cell wall from *P. ultimum* as carbon source. The experiments were conducted with three biological replicates. After 24, 48 and 72 hours of incubation the mycelia were harvested by filtration through filter paper and the culture filtrate were used as a source of enzymes. Fungal mycelia were kept at -80°C and used for cAMP analysis and total RNA isolation. The culture filtrate was kept in an ice bath and the filtration was conducted in a cold chamber to avoid cellulase activity.

Biolog Phenotype Microarray analysis

The global carbon assimilation profiles were evaluated using the Biolog Phenotype MicroArray technique [25], with the Biolog FF Microplate. The *T. reesei* strains were grown in 2% malt extract agar under ambient laboratory conditions with diffuse day light at 25°C. The inocula were prepared after conidial maturation (2-3 days), by rolling a sterile, wetted cotton swab in the area containing the conidia. The conidia were suspended in 16 ml of sterile phytagel (0.25% Phytagel, 0.03% Tween 40) in disposable borosilicate tubes (20 mm×150 mm). The spore solution was mixed manually for 5 seconds and adjusted to a T590 of 75% ± 3%. Next, 100 µl of spore solution was transferred to each well of a Biolog FF Microplate. The microplates were kept in the dark at 25°C. The mycelial growth was assessed by measuring the A750 at 12 h, 24 h, and 48 h. Each *Trichoderma* strain was analysed in 3 independent experiments, using different inocula. Two-Way ANOVA was used to compare the carbon assimilation between strains. Bonferroni post-tests were used to compare replicate mean by each carbon source and compare to parental TU-6. The statistics tests were performed using GraphPad Prism software version 5.00. Only p-values<0.05 were considered as significant [26].

Dual culture tests and scanning electron microscopy (SEM) analysis

Discs of 5 mm diameter from minimal medium (MM) [(w/v), $MgSO_4.7H_2O$ 0.1%, KH_2PO_4 1%, $(NH_4)2SO_4$0.6%, tri-natriumcitrate. $2H_2O$ 0.3%, glucose 1%, 50X trace elements solution 1 volume, agar-agar 1%] were taken from the edge of actively growing colonies of fresh fungal cultures and placed on the surface of the MM plate at a spacing of 4 cm. The plates were incubated at 28°C, and after 4 and 7 days mycelial samples from the interaction region and after contact region were collected and examined by scanning electron microscopy (SEM) [9].

RNA isolation and RT-qPCR

Total RNA was isolated from the mycelia by grinding with a mortar and pestle under liquid nitrogen, followed by extraction using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions and digested with DNase I (Invitrogen). Total RNA (5 µg) from each pooled sample was reverse transcribed into cDNA in the presence of oligo(dT) and ramdom hexamer primer in a volume of 20 µl using the Maxima™ First Strand cDNA synthesis kit (Fermentas). The synthesized cDNA was diluted with 80 µl of water and used as a template for real-time PCR. Reactions were performed in the iQ5 real-time PCR system (Bio-Rad). Each reaction (20 µl) contained 10 µl of MAXIMA® SYBR-green PCR Master Mix (Fermentas), forward and reverse primers (500 nM each, Table 1), cDNA template, and nuclease free water. PCR cycling conditions were 10 min at 95°C (1 cycle), 15 s at 95°C followed by 1 min at 60°C (40 cycles), and a melting curve of one min at 95°C followed by 30 s at 55°C and a final ramp to 95°C with continuous data collection (1 cycle) to test for primer dimers and nonspecific amplification. The tef1α transcript was used as internal references to normalize the amount of total RNA present in each reaction (Table 1). The expression level of the genes was calculated from the threshold cycle according to the 2-ΔΔCT method [27]. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA (1, 0.1, 10^{-2} and 10^{-}

Primers for qPCR (5' to 3')			
genes	**Acession Trire2**	**Forward**	**Reverse**
cbh1	123989	CCGAGCTTGGTAGTTACTCTG	GGTAGCCTTCTTGACTGAGT
gluc83	121746	CAGCAAGCTCAACAACGTCAAGGT	TCCATGAGGCAATGTTGGCGTTTC
lip1	106405	GATTCCTTCGAAGGCTCCTTG	ACGAAGTTGCGGATGTTCTTG
ap1	71566	TCTTTGCCATCTTGCTGACC	GAGAAGACGGGTGAGTATTGG
nag1	21725	AATGGAGTGCCGATCATCAC	TTGTGCTGAAGATGGACTGC
chit42	80833	GGACATCACTCATGTCATCTACTC	GACATCGTTCCAGGAATCATCC
tef1	46958	CCACATTGCCTGCAAGTTCGC	GTCGGTGAAAGCCTCAACGCAC

Table 1: List of genes selected for differential expression analysis under mycoparasitic conditions by qPCR. The primers pairs used were designed based on the sequences of *T. reesei* available in the JGI database (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

^{3}). Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. The experiment was conducted with three repetitions for each sample and results were compared by one-way ANOVA with Dunnett's posttest (α=5%) to analyze the differences between conditions related to control sample (TU-6) using GraphPad Prism version 5.00 for Windows.

Enzyme assays

Cellulase activity was measured as filter paper activity (FPase) as described bydo Nascimento Silva and co-workers. One unit of enzyme activity was defined as the formation of 1 µmol of reducing sugars per minute under the conditions of the assay [5]. Endochitinase activity was measured with a colorimetric method using chitin as substrate [8]. One unit of enzyme activity was defined as the amount of enzyme which release 1 µmol N-acetylglucosamine in 1 h at 37°C. The β-1,3-Glucanase activity assay was performed as described previously [28] using laminarin (Sigma) as substrate. The amount of reducing sugar releases from laminarin was determined as described previously [29]. NAGase, Lipase and acid phosphatase activities were determined using the colorimetric method, using the respective p-nitrophenyl-derivated (Sigma-Aldrich Co., Wisconsin, USA) as a substrate. Enzyme activity was assayed by measuring the rate of formation of ρ-nitrophenol from substrate. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol ρ-nitrophenol in 1 min under reaction conditions [26].

The experiments were conducted with three repetitions for each sample and results were compared by one-way ANOVA with Dunnett's post-test (α=5%) to analyze the differences between conditions related to control sample (TU-6) using GraphPad Prism version 5.00 for Windows.

Measurement of intracellular cAMP levels

Intracellular cAMP levels were determined using adirect cAMP enzyme immunoassay kit (Sigma-Aldrich Co., Wisconsin, USA) according to the manufacturer's instructions. cAMP concentration was related to the protein content of the sample. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Sigma-Aldrich Co., Wisconsin, USA). The measurements were conducted using the mycelia of *T. reesei* after 72 hs of incubation in presence of *P. ultimum* purified cell wall.

Cell wall purification of *P. ultimum*

Quantities from 10 to 20 agar plates (PDA) containing mycelium of the *P. ultimum* was inoculated into 1 L flasks containing 500 ml of liquid medium MYG. These flasks were incubated at a temperature of 28°C under constant stirring of 160 rpm in a rotary shaker for 7 days. The mycelium was harvested by filtration through Whatman 01 filter paper and used in the purification wall. The mycelium was ground to powder in liquid nitrogen in a mortar and pestle. After soaking, the mycelia were treated with urea (8 M w/v). Then the cell wall extracts were centrifuged for 15 minutes under rotation 10,000 rpm, the supernatant was preparations discarded, and the precipitates rinsed with distilled water. The precipitates obtained after the washings above were homogenized with a solution of ammonium hydroxide (1 M v/v), centrifuged for 30 min at 10,000 rpm, and the precipitates rinsed with distilled water as described previously. The last wash the precipitates were resuspended in formic acid (0.5 mol L^{-1}) and again centrifuged and washed with distilled water as mentioned above. In the last washing, the pH was adjusted to pH 6.0 and the precipitates obtained from *P. ultimum* lyophilized and used as a source inducing.

Results

Deletion of *gna1* leads to a loss in antagonism ability of *T. reesei* against *P. ultimum*

In order to understand the role of *GNA1* in the antagonism of *T. reesei* against *P. ultimum*, we performed a direct dual culture confrontation tests monitoring the growth of *T. reesei* (TU-6, *gna1*QL and Δ*gna1*) over *P. ultimum* during 7 days. The possible modification on cell wall ultrastructure of *P. ultimum* was evaluated by scanning electron microscopy. Figure 1 shows that both *T. reesei* TU-6 and the *gna1*QL mutant inhibited the growth of *P. ultimum* in plate confrontation within 3 days. However, the mutant *gna1*QL grew faster than the parental TU-6. SEM showed changes in cell wall morphology and growth of *P. ultimum* in the interaction zone with *T. reesei* 72 hours after contact (4 days after inoculation) (Figure 1). TU-6, identified by smaller diameter mycelia, produces holes characteristic of CWDEs production in *P. ultimum* cell wall though it also showed a wrinkled appearance after 3 days of growth. On the other hand, the mutant *gna1*QL produced more holes than TU-6, indicating that it displays a higher efficiency of antagonism/CWDEs production. As can be observed in SEM analysis, the mutant Δ*gna1* did not cause any effect in *P. ultimum* cell wall (Figure 1), indicating that *GNA1* plays an important role on antagonism ability, principally in coiling and CWDEs production.

Deletion of *gna1* affects the metabolism and protein secretion in *T. reesei*

Since Δ*gna1*strain did not overgrow in dual culture confrontation tests, we performed the global carbon assimilation by Biolog Phenotype MicroArray technique to evaluate the hole of *GNA1* in *T. reesei* metabolism (supplementary material). In general, Δ*gna1* strain showed a decreasing in carbon assimilation, excepted for glycogen, that showed a statistically significant increase ($P<0.001$) when compared with either the parental TU-6 and for the *gna1*QL. No significant difference ($P>0.05$) was found between TU-6 and Δ*gna1* in assimilation of L-Phenylalanine, β-Cyclodextrin, L-Asparagine, Stachyose, Uridine, Maltitol, L-Threonine, L-Serine, L-Sorbose, L-Proline, N-acetyl-D-Mannosamine, α-Methyl-D-Galactoside, among others (supplementary material). The constitutively activation of *GNA1*, on the other hand, did not affect drastically the metabolism of *T. reesei*. Interestingly, most of carbon affected assimilation ($P<0.001$) were carbohydrates when compared *gna1*QL and TU-6, as follow: α-Cyclodextrin, Dextrin, α-D-Glucose, D-Mannose, Sucrose, D-Xylose, D-Melezitose, Maltotriose, Turanose, D-Ribose, L-Arabinose, D-Raffinose, and D-Sorbitol. Comparisons of metabolic profile between *T. reesei* TU-6 and strain PFG1 (=TU-6 retransformed with pyr4 gene) did not show significant difference ($P>0.05$) in any carbon source tested (supplementary material). Furthermore, no differences were observed in grow rate on plates between strains (data not shown). Due that, all experiments were conduct with TU-6 as reference and any difference between strains were considered based on carbon assimilation and not on direct growth capacity.

The intracellular level of cAMP in the strains after 72 hours of incubation in presence of *P. ultimum* cell wall was: *gna1*QL (79.85 ± 12), Δ*gna1* (268.65 ± 8.5) and TU-6 (109.70 ± 9.2) pmol/mg protein. No significant difference was observed between TU-6 and *gna1*QL, although Δ*gna1* showed a high content in cAMP levels. This result is typical for Gαi deletion and was already reported by Rocha-Ramírez and Reithner and their co-workers showed that *GNA1* is capable to inhibit the adenylate cyclase [15,17].

The content of extracellular protein in *gna1*QL was not significantly different with TU-6 (63.5 µg. mL^{-1} ± 8.23 and 83.6 µg. mL^{-1} ± 6.28 respectively), suggesting that the mutation in *GNA1* did not affect the rate of protein production. However, when the *gna1* gene was deleted, the mutant produced less protein than TU-6 (36.6 µg. mL^{-1} ± 6.17).

GNA1 regulates the expression of CWDEs genes in *T. reesei*

In an effort to understand how *GNA1* regulates the CWDEs production we performed quantitative PCR (RT-qPCR) to access gene expression profile of *T. reesei* (strains TU-6, *gna1*QL, and Δ*gna1*) during *in vitro* mycoparasitism (Figure 2). The results showed that in general, all genes encoding CWDEs analyzed in this study had low transcripts levels when compared with either the TU-6 and for the mutant *gna1*QL, suggesting a close relationship between *GNA1* activity and expression of CWDEs genes. The cbh1 gene was 100-fold more expressed in the mutant *gna1*QL in comparison to the TU-6 in 48 hours of culture and decreased drastically after 72 hours (Figure 2). Another gene of great importance in mycoparasitism is gluc83 that encodes to a glucanase [30]. The transcript levelof gluc83 was the same in TU-6 and mutant *gna1*QL after 48 hours of cultivation, however, the transcript level of gluc83 in the mutant *gna1*QL decreased by 1.5-fold after 72 hours of cultivation (Figure 2). Since *P. ultimum* has a large amount of β-1,3-glucans in their cell wall, this result is relevant and indicates that the expression of gluc83 was being regulated directly or indirectly by *GNA1* and not by cAMP, whereas in 72 hs intracellular cAMP levels in the mutants are opposite. The expression of other genes such as nag1, Lip1, chti42 and ap1, which encode respectively for Nagase, lipase, chitinase and acid phosphatase, were also evaluated. The transcript level of four genes showed similar after 72 hours of cultivation in the mutant *gna1*QL compared to TU-6 (Figure 2). Interestingly, the transcript levelof Lip1, in the mutant *gna1*QL, showed approximately 10-fold higher within the first 24 hours, compared to the TU-6 (Figure 2). This finding is important because it shows a possible mechanism for transient regulation by *GNA1* in the initial degradation of *P. ultimum* cell wall.

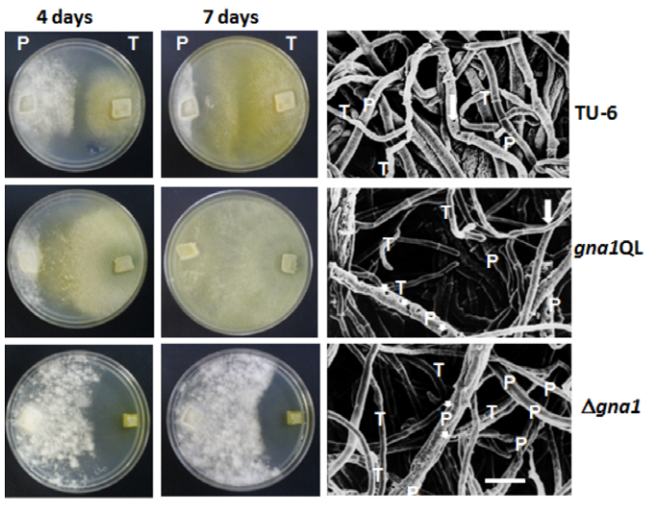

Figure 1: Photographs of dual culture tests and scanning electron microscopy between *T. reesei* (TU-6), *T. reesei* (*gna1*QL) and *T. reesei* (Δ*gna1*) and *P. ultimum*. T=*Trichoderma*; P=*P. ultimum*; Bar=10 µm. Arrows show the contact between T and P. Arrowheads indicate the holes caused by interaction between T and P. SEM analysis was carried out at 1,800X.

The mutant *gna1*QL exhibited a high activity of CWDEs during "*in vitro*" mycoparasitism

Regarding to mycoparasitism, only the fact that *T. reesei* shows a high or low CWDEs gene expression is not guarantee to biocontrol being successful or unsuccessful. For this reason, we assayed the follow CWDEs activity: cellulase (FPase), glucanase (β-1,3), NAGase, lipase, chitinase, and acid phosphatase. Figure 3 shows that TU-6 showed a high cellulase activity (10.3 U. mL^{-1}) followed by *gna1*QL (6.46 U. mL^{-1}) and Δ*gna1* did not show cellulase activity ($p \leq 0.001$). The mutant *gna1*QL exhibited a high endochitinase ($p \leq 0.01$) and NAGase ($p \leq 0.001$) activities in comparison with TU-6, showing approximately 2-fold more activity for both enzymes (Figure 3) while Δ*gna1* mutant showed a low endochitinase activity. Reithner and co-workers reported a less chitinase activities and reduced nag1 and ech42 gene transcription in Δtga1 mutant of *T. atroviride*, thus supporting our results [17]. Furthermore, figure 3 shows that the *gna1*QL mutant produces β-1,3-glucanase at a higher level than the parental TU-6 ($p \leq 0.001$) after 48 hours. However, no difference was observed after 72 hours (2.3 U.mL^{-1} and 1.8 U.mL^{-1} for *gna1*QL and TU-6 respectively). Δ*gna1* mutant showed low activity of β-1,3-glucanase (0.74 U.mL^{-1}). Since the presence of lipids and phosphate in cell wall have been described for a number of fungi [31], the activities of lipase and acid phosphatase were also investigated. Figure 3 shows that lipase activity in *gna1*QL (2.23 U.mL^{-1}) was higher than in TU-6 (1.37 U.mL^{-1}) ($p \leq 0.001$) whereas Δ*gna1* mutant showed much less activity (0.52 U.mL^{-1}). The role of lipids in fungal cell walls has not been elucidated. However, we can infer from our study that although the mutant *gna1*QL has a high gene expression of Lip1 in the first 24 hours, the highest enzyme activity was reached only after 72 hours. The data suggest a long process of post-translational modifications and secretion of lipase and it can be influenced by *GNA1*.The activity of acid phosphatase is shown in Figure 3. The *gna1*QL mutant showed a high acid phosphatase activity (11.25 U.mL^{-1}) when compared with TU-6 (4.88 U.mL^{-1}) ($p \leq 0.001$) and with Δ*gna1* mutant (1.24 U.mL^{-1}). Phosphate has been identified in almost all fungal cell walls analyzed. It ranges from 0.1 to 2% of the cell wall's dry weight [31]. Here we described that the formation of this enzyme can be regulated by *GNA1*.

Taken together our results demonstrated that *GNA1* protein could regulate the formation of CWDEs directly or indirectly. Furthermore, no direct correlation between gene expression and enzyme activity was observed, taking into account the time points analyzed.

Discussion

The study of *T. reesei*, a typically industrial fungus, as a biocontrol against *P. ultimum* has just started [4-6] when compared with *T. harzianum* or *T. atroviride*. Although there is a consensus in the mode of action of *Trichoderma* during the mycoparasitic process, the molecular and biochemical basis of this process is still unclear and some aspects like CWDEs gene expression and secondary metabolites production must be studied in more detail [6,13]. Many reports suggest the participation of signal cascade components such as G proteins, cAMP and MAP kinase in control of mycoparasitism [19]. We have therefore tested the involvement of the G-alpha protein *GNA1* of *T. reesei* in antagonism against *P. ultimum* and in CWDE production during mycoparasitism as well. The *gna1*QL mutant has a single amino acid modification (Q204L) in the *GNA1* protein, which impairs the intrinsic GTPase activity and leads to constitutive activation of this protein [32]. A *gna1* deletion strain was obtained by replacement of the coding region with the *H. jecorina* pyr4-gene conferring uridine

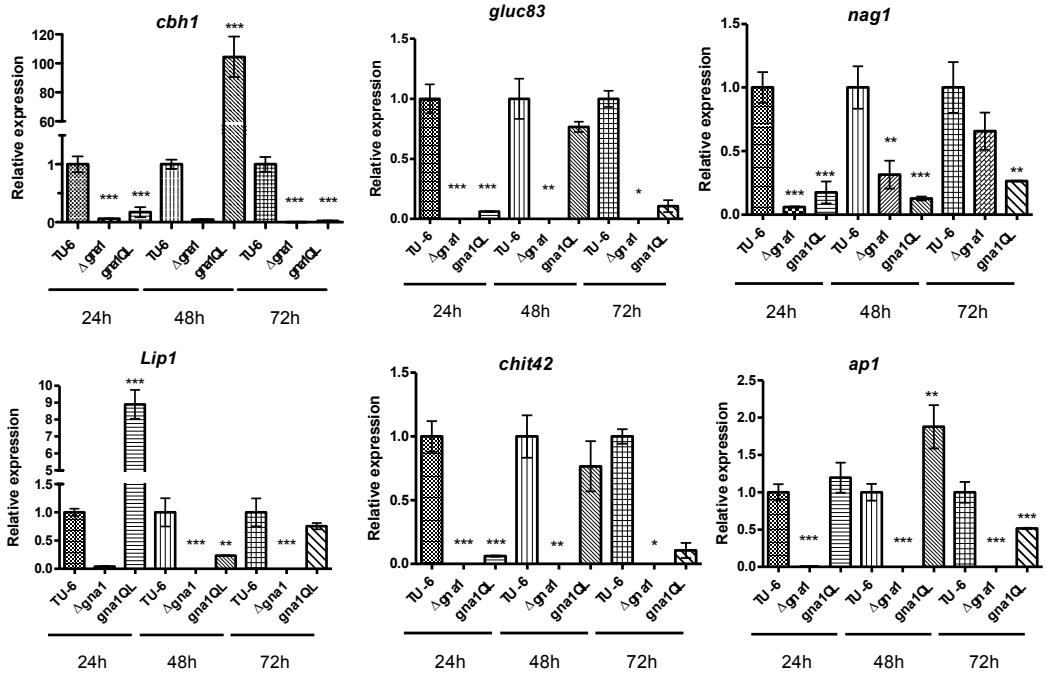

Figure 2: Differential expression analysis and quantification of transcript levels of biocontrol-related genes expressed by *T. ressei*(TU-6; *gna1*QL and Δ*gna1*) under mycoparasitic conditions against *P. ultimum* after 24, 48 and 72 hours of cultivations. The data were presented as fold change using the $2^{-\Delta\Delta Ct}$method. The results were compared by one-way ANOVA with Dunnett's posttest (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

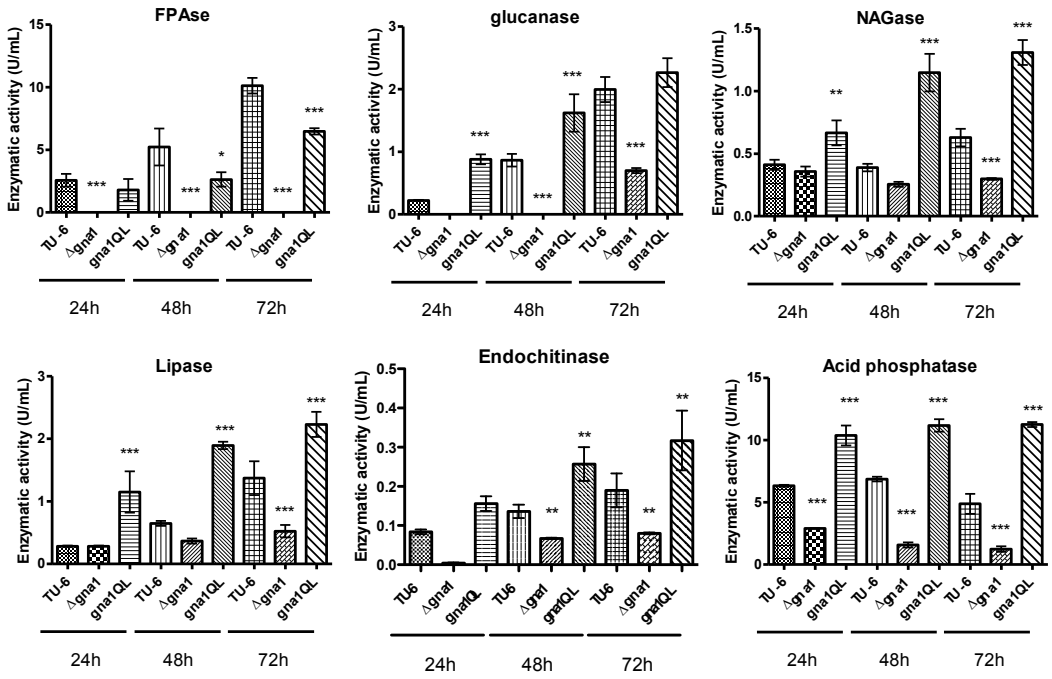

Figure 3: Enzymatic activity of cellulase (FPAse), endochitinase (chit42), NAGase (nag1), β-1,3-glucanase (gluc83), Lipase (lip1), Acid phosphatase (ap1) after 24, 48 and 72 hours of incubation of *T. reesei* (TU-6), *T. reesei* (*gna1*QL) and *T. reesei* (Δ*gna1*) with *P. ultimum* cell wall as the carbon source. Enzyme activity was assayed by the colorimetric method as indicated in the materials and methods section. In all cases, the standard deviation values were smaller than 5% of the mean values of triplicate.

prototrophy. These strains were already studied and these G proteins are involved in cellulase formation and mediate a tolerance of osmotic and oxidative stress linked with as light as carbon source [23,24].

Rocha-Ramírez and co-workers reported that a similar *GNA1*, Tga1 of *T. atroviride* is involved in both coiling and conidiation (primordial factors in antagonism process). Furthermore, strains that expressed an antisense version of the gene were hypersporulating

and coiled at a much lower frequency in the biomimetic assay [15]. Reithner and co-workers also reported that tga1 gene deletion in *T. atroviride* resulted in a complete loss of overgrowth of *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* during direct confrontation as well a decreasing in chitinase formation [17]. Our results are in accordance with that, showing that Δ*gna1* loss the capacity of overgrowth of *P. ultimum* (Figure 1). Additionally, we showed that *GNA1* influences the formation of cellulase, glucanase, chitinase, lipase and acid phosphatase as well, suggesting that *GNA1* are involved in mycoparasitism. Moreover, we observed that an activated mutant protein with no GTPaseactivity (*gna1*QL) did not affect the sporulation and coiled at a higher frequency. Opposite results were reported to *T. virens* since ΔtgaA mutants (homologue to Tga1) were not effect on growing and sporulation, compared with wild type. However, ΔtgaA mutants showed a reduced ability to colonize *S. rolfsii sclerotia*, whereas they were fully pathogenic against *R. solani* [16]. These results support the claim that different species of *Trichoderma* display completely different strategies to antagonize their host/prey [6] and also suggest a phytopathogenic specific response by *Trichoderma*, which can act in the production of lytic enzymes, secondary metabolites/antibiotics or simply competing for nutrients.

Seibel and co-workers reported that cellulase gene transcription was abolished in Δ*gna1*mutant on cellulose in light and enhanced in darkness. Our experiments were performed in day-light conditions. However, Seibel and co-workers showed that mutants expressing a constitutively activated *GNA1* did not transmit the essential inducing signal for cellulase formation induced by cellulose, suggesting that the signal transduction of cellulase formation is complex and involves also GNA3 and light-carbon source dependence [23,24]. Although TU-6 produced higher cellulase activity, there is no guarantee that TU-6 is the best mycoparasitic antagonist against *P. ultimum* (Figure1). The antagonism of *P. ultimum* by *T. reesei* seems not to require cellulase gene expression since the negative cellulase mutant QM9978 overgrew *P. ultimum* on plate confrontation assays as well as protecting the plant against pathogens [4]. However, production of enzymes as cellulases and acid phosphatase by *T. reesei* are important mechanism taken together in biocontrol [5,6].

Most phytopathogenic fungi have chitin and β-1,3-glucan as the main structural components. However, *Pythium* spp. shows approximately 82% of β (1 → 3), (1 → 6)-D-glucans and 18% of β (1 → 4), together with a low chitin content (less than 1%) [31]. These findings support the idea that β-1,3-glucanase plays an important role in mycoparasitism against *P. ultimum* and now we have evidence that the regulation of the formation of this lytic enzyme by *T. reesei* can be linked with G proteins and/or cAMP. However, the elucidation of the mechanism that link cAMP to chitinases and glucanases production is still unclear since do Nascimento Silva and coworkers showed that gna3QL, that rises the cAMP level, showed a similar behavior of *gna1*QL, instead Δ*gna1*mutant that shows a high intracellular cAMP content [5]. These facts could be explained since the G protein pathway is involved in many cellular processes that share signaling molecules as cAMP. Thus, the response to G protein actionis not a single linear sequence of cAMP pathway that was already reported to act as a positive as negative effector of endoglucanase and NAGase induction, in *T. reesei* and *T. harzianum* respectively [22,33]. In *T. virens*, on the other hand, low levels of cAMP by deletion of an adenylate cyclase-encoding gene (tac1) leads a reduction on growth and secondary metabolite production as well, impaired sporulation, and principally, and a loss in capacity to overgrow host fungi like *S. rolfsii*, *R. solani*, and *Pythium* sp. [34].

The role of acid phosphatase in mycoparasitism has also been suggested and seems to be involved in nutrient competition [5,35] also reported a high level of activity using gna3QL for acid phosphatase, suggesting that the increase in these enzymes activity during mycoparasitism is not dependent of cAMP levels but by the activity of *GNA1* or GNA3. However, more studies are needed to check this hypothesis, since the metabolism of phosphate is a complex process and involves also regulation of pH [36]. This study demonstrated that the production of CWDEs such as endochitinase, β-1,3-glucanase, lipase and acid phosphatase is regulated by *GNA1* protein. As a consequence, mutation as *gna1*QL showed to improve the antagonism against *P. ultimum* in confrontation assays while the Δ*gna1* mutant was not capable to antagonize *P. ultimum*. The study contributes to understand the role of G-proteins in mycoparasitism and in biological control field by *Trichoderma*. Other analyses such as antifungal compound formation, competition for nutrients during *in-vivo* biocontrol and carbon catabolite repression in the mutants needs to be elucidated.

Taking the results together, cAMP can stimulate coiling/recognition in *Trichoderma*, so the cAMP pathway seems to have antagonistic roles in mycoparasitism-relevant coiling response. However, the direct action of *GNA1* or GNA3 can also regulate the expression of mycoparasitism related genes independently of cAMP. In this sense, more detailed studies including signals recognizing by *Trichoderma* receptors and downstream targets signaling cascades will be necessary to understand the network of antagonism and mycoparasitic interaction.

Acknowledgement

This work was supported by a biotechnology research grant to C.J.U. (CNPq and FUNAPE/UFG) and FINEP / CT-AGRO / Ação Transversal - 01.07.0551.00. R.N.S is supported by The State of São Paulo Research Foundation (FAPESP) (proc. 2010/15683-8). The authors thank Dr. Sônia Nair Baó (UnB/Cel) for her support with scanning electron microscopy.

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