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Effect of a heterotrimeric G protein α subunit on conidia germination, stress response, and roquefortine C production in *Penicillium roqueforti*

Ramón O. García-Rico,^{1,3} Renato Chávez,^{1,4} Francisco Fierro,^{1,5} Juan F. Martín^{1,2*}

¹Institute of Biotechnology of León (INBIOTEC), León, Spain. ²Area of Microbiology, Faculty of Biological and Environmental Sciences, University of León, León, Spain. ³Department of Microbiology, Faculty of Basic Sciences, University of Pamplona, Pamplona, Colombia. ⁴Faculty of Chemistry and Biology, Santiago de Chile University (USACH), Santiago, Chile. ⁵Department of Biotechnology, Division of Biological Sciences and Health, Metropolitan Autonomous University Iztapalapa, Mexico City, Mexico

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Summary. Heterotrimeric G protein signaling regulates many processes in fungi, such as development, pathogenicity, and secondary metabolite biosynthesis. For example, the $G\alpha$ subunit Pga1 from *Penicillium chrysogenum* regulates conidiation and secondary metabolite production in this fungus. The dominant activating allele, *pga1*^{G42R}, encoding a constitutively active Pga1 $G\alpha$ subunit, was introduced in *Penicillium roqueforti* by transformation, resulting in a phenotype characterized by low sporulation and slow growth. In this work, the effect of the constitutively active Pga1^{G42R} $G\alpha$ subunit on conidial germination, stress tolerance, and roquefortine C production of *P. roqueforti* was studied. Pga1^{G42R} triggered germination in the absence of a carbon source, in addition to negatively regulating thermal and osmotic stress tolerance. The presence of the Pga1^{G42R} $G\alpha$ subunit also had an important effect on roquefortine C biosynthesis, increasing production and maintaining high levels of the mycotoxin throughout a culture period of 30 days. Together, the results suggest that G protein-mediated signaling participates in the regulation of these three processes in *P. roqueforti*. [Int Microbiol 2009; 12(2):123-129]

Keywords: *Penicillium roqueforti* · G-protein · roquefortine production · conidial germination · stress

Introduction

Heterotrimeric G binding proteins (G proteins) are key elements of signal transduction pathways in eukaryotes. G proteins are composed of three subunits: α , β , and γ , which remain inactive in the heterotrimeric state bound to a G protein-coupled receptor (GPCR), with a guanosine diphosphate (GDP) molecule bound to the $G\alpha$ subunit. The $G\alpha$ subunit

exchanges GDP for guanosine triphosphate (GTP) and dissociates from the $\beta\gamma$ dimer upon signal-mediated activation of the GPCR. Both the $G\alpha$ subunit and the $\beta\gamma$ dimer are then activated and independently interact with downstream effectors [16]. Activation is not permanent; as an intrinsic GTPase activity hydrolyzes GTP in the $G\alpha$ subunit, which then binds to the $\beta\gamma$ dimer, returning the G protein to the basal inactive state.

Fungal $G\alpha$ subunits have been classified in three subgroups, I, II, and III [1]. Subgroup I $G\alpha$ subunits are related to the mammalian $G\alpha_i$ group based on sequence similarity and have been implicated in the regulation of several relevant biological processes, including growth [25,37], conidiation [12,21,38], sexual development [19,20,40], pathogenicity [14,19,30], and secondary metabolism [2,13,35,39].

*Corresponding author: J.F. Martín
Instituto de Biotecnología (INBIOTEC)
Parque Científico de León. Av. Real, 1
24006 León, Spain
Tel. +34-987291505. Fax +34-987291506
E-mail: jf.martin@unileon.es

Common control of differentiation and secondary metabolism by G protein signaling in fungi was first reported by Hicks et al. [18], who found that inactivation of the subgroup I G α subunit FadA of *Aspergillus nidulans* was a requisite for both conidiation and sterigmatocystin biosynthesis. Conversely, a GTPase inactivating mutation (*fadA*^{G42R}) that resulted in a constitutively activated FadA subunit blocked both processes. However, other secondary metabolites are regulated differently; penicillin biosynthesis is positively regulated by subgroup I G α subunits in both *A. nidulans* [35] and *Penicillium chrysogenum* [13], and trichothecene production in *Fusarium oxysporium* is increased when the fungus is transformed with the *A. nidulans fadA*^{G42R} allele [35]. In *Trichoderma atroviride*, the G α subunit Tga1 has opposing roles in regulating the biosynthesis of different antifungal substances [28]. These results indicate that G protein signaling differentially controls secondary metabolite production, sometimes in opposite directions [13,35]; therefore, each case must be analyzed on a species-specific basis.

Other physiological effects of G protein signaling are related to stress resistance. As a general rule, the absence of the subgroup I G α subunit results in phenotypes more resistant to stress conditions. This phenomenon was observed in Δ *fga1* strains of *F. oxysporum*, whose conidia are more resistant to thermal stress [22], and in Δ *gna-1* strains of *Neurospora crassa*, which are more tolerant to thermal and oxidative stress [37]. *Cryphonectria parasitica* Δ *cpg-1* strain is more resistant than the parental strain to chronic heat and hyperosmolarity, whereas opposing responses occur in strains expressing a constitutively activated Cpg-1 [30]. However, different results regarding the response to hypertonic stress were obtained in *N. crassa* Δ *gna-1* and *Cochliobolus heterostrophus* Δ *cg1* strains, which are more sensitive to hypertonic conditions than the wild-type strain [19,20].

Spore germination in fungi is determined by environmental factors that vary depending on the species of fungus and its habitat [5]. Signal transduction pathways involved in germination have been analyzed in some detail in few fungal species [6,8]. The GTPase RasA regulates conidial germination in *A. nidulans*, with RasA and cyclic AMP apparently playing separate roles [8]. Heterotrimeric G proteins are also involved in the regulation of conidial germination. In this process, G α subunits from subgroup III are key elements whose absence in *A. nidulans* [3] and *P. marneffei* [42] causes major germination defects.

G-protein signaling in the genus *Penicillium* has been studied in detail only in the human pathogen *P. marneffei* [41,42] and recently in the penicillin producer *P. chrysogenum* [11–13]. In a previous study, we have cloned the *P. chrysogenum pgal* gene, encoding a G α subunit from subgroup I

[11]. Pgal represses conidiation and stimulates secondary metabolite production [12,13].

Penicillium roqueforti is the fungal ripening agent of blue cheeses and produces different mycotoxins, including roquefortine and PR toxin. In spite of its commercial relevance, little is known about the physiology of this fungal species. In an effort to understand the signal transduction pathways controlling development and secondary metabolite production in *P. roqueforti*, we have obtained transformants of the fungus that incorporate a dominant activating allele of the *P. chrysogenum pgal* gene (*pgaI*^{G42R}) [11]. Constitutive Pgal signaling reduces the rate of apical extension and dramatically reduces conidiation in *P. roqueforti* [11,12]. In the present work, we analyze the effect of constitutive Pgal signaling in *P. roqueforti*, specifically, in conidial germination, the stress response, and mycotoxin production. These three physiological processes are of utmost importance in the handling and control of this microorganism of commercial importance.

Materials and methods

Fungal strains. *Penicillium roqueforti* CECT 2905 (ATCC 10110) is a wild-type strain producing roquefortine C and PR toxin [10]. *P. roqueforti* transformants PRG42-3 and PRG42-7 were obtained by introducing plasmid pPgaG42R, containing the dominant activating *pgaI*^{G42R} allele from *P. chrysogenum*, by protoplast transformation [11].

Conidial germination kinetics measurements. Fifty ml of Czapek medium either lacking a carbon source or containing 3% glucose was inoculated with 5×10^8 spores, and the cultures incubated at 28°C for 14–16 h. Samples of 100 μ l were taken at regular intervals every 1–2 h, and 10 μ l were observed under the microscope. The numbers of conidia and of germinated conidia were counted in ten randomly chosen fields (in duplicate). Conidia were considered as germinated when the germinative tube was of the same length as or longer than the diameter of the spore. Data were plotted as percentages of germination vs. time, using a non-linear (sigmoidal) regression for the statistical analysis ($P < 0.0001$).

Conidial resistance to heat-shock. Five hundred μ l of a conidial suspension (2×10^5 conidia/ml) were placed in centrifuge microtubes, and the cultures incubated at 50, 45, and 25°C (control) for 1 h. From each tube, 100 μ l of suspension were plated onto Czapek agar to determine the number of remaining viable cells, and plates were incubated at 28°C for 5 days. The number of colonies obtained in each plate was counted, and the percentage of surviving colonies was calculated using the control (treatment at 25°C) to define 100% survival. For each condition, four replicates were averaged.

Hypertonic stress assay. The effect of high osmotic pressure was determined as described in [30]. From a spore suspension containing 1×10^6 conidia/ml, 0.2 μ l were withdrawn and placed onto the center of a Petri dish containing Czapek minimal medium or Power (PW) rich medium [7], supplemented with 1.5 M NaCl or 1.5 M KCl. The plates were incubated at 28°C for 7 days, and the diameter of each colony (taking the white external edge of the colony as reference) was measured in duplicate every 24 h, starting at 48 h of growth. Growth rates were obtained as a linear regression of colony diameter over time. Statistical parameters were analyzed using the SigmaPlot 2001 program.

Roquefortine C production. Fifteen flasks containing YES medium (2% yeast extract, 15% sucrose) were inoculated with $5-6 \times 10^7$ spores, and incubated at 28°C in an orbital shaker at 250 rpm. Every 48 h, one flask was processed as follows: 10-ml samples (in duplicate) were centrifuged at 4000 rcf (relative centrifugal force) for 10 min. The supernatant was discarded and the mycelium was washed twice with NaCl 0.9%, dried with filter paper, and stored at -80°C. When all samples had been collected, mycelia were resuspended with 4 ml of chloroform:methanol (2:1). This mixture was sonicated for 30 min and filtered through a Nylal membrane (30- μ m pore). The dry weight of the mycelia was determined (see below), whereas filtrates were passed through a 0.45- μ m nylon filter (Millex, Millipore) and solvent was evaporated in a Speed-Vac SC-110 (Savant Instruments). The dry samples were then resuspended in 100 μ l of methanol overnight at 4°C. Prior to HPLC injection, samples were centrifuged for 5 min at 16,000 rcf.

HPLC determination of roquefortine C. Roquefortine C was separated by high performance liquid chromatography (HPLC) using a 1525 Binary HPLC Pump (Waters) with a Lichrospher 100-RP18 column 300 \times 4 mm (Merck). Elution was carried out using an isocratic gradient of solvent A (TFA 0.025% diluted in water) and solvent B (TFA 0.025% diluted in acetonitrile) at a flow of 1.2 ml/min. Absorbance was measured at 225 nm. Pure roquefortine C eluted at 15 min. For quantification, dilutions of a standard of pure roquefortine C (Sigma-Aldrich Inc.) were used.

Mycelia dry weight determination. Mycelia from each sample were washed with 1M HCl and 0.9% NaCl. Washed mycelia were dried in an oven at 65°C for 72-84 h and weighed.

Results

Triggering of conidial germination in *Penicillium roqueforti* in the absence of a carbon source.

Figure 1 shows the percentage of germination of the transformant strains PRG42-3 and PRG42-7, carrying the dominant

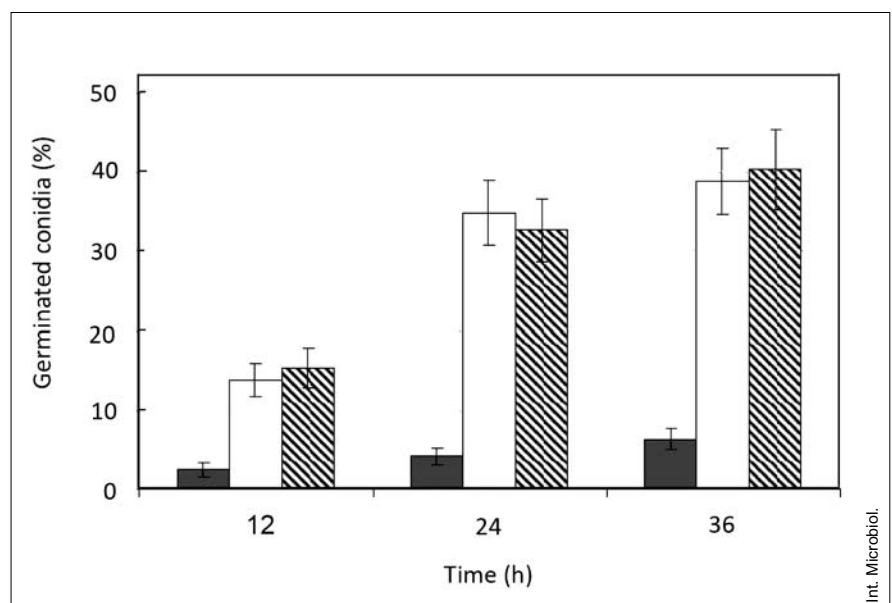
activating *pgaI*^{G42R} allele from *P. chrysogenum*, and of the parental strain *P. roqueforti* CECT 2905 in Czapek minimal medium lacking a carbon source. At 12 h of incubation, the germination ability of the parental strain was very low (2.4% of the total number of conidia), whereas the germination percentage of the transformant strains was about 15%. These differences were maintained throughout the observation period (6 vs. 36% at 36 h).

Decrease in stress tolerance of *Penicillium roqueforti* strains carrying the *pgaI*^{G42R} allele.

Figure 2 shows the effect on conidial viability of wild-type strain CECT 2905 and of the transformant strains PRG42-3 and PRG42-7 when subjected to thermal treatments at 45 and 50°C. The parental strain CECT 2905 was more resistant than the transformants at both temperatures, with a percentage of conidial survival at 45°C of 48% vs. only 20%. Likewise, 15% of the conidia from strain CECT 2905 survived at the more stressful temperature of 50°C, whereas the percentage dropped to 6-7% in the *pgaI*^{G42R}-allele-carrying transformants.

Similar results were obtained regarding osmotic stress. As expected, the apical growth rate was faster in PW rich medium than in Czapek minimal medium (Fig. 3). NaCl seems to be a more powerful stress-inducing agent than KCl. Independently of the culture media or salt used, transformants carrying the dominant activating *pgaI*^{G42R} allele were less tolerant to stress than the parental CECT 2905 strain, with growth rates of the transformants averaging about 60% of those of the parental strain.

Fig. 1. Germination of the *Penicillium roqueforti* parental strain CECT 2905 (gray bars) and transformants PRG42-3 (white bars) and PRG42-7 (dashed bars) growing on Czapek minimal medium without a carbon source. Data are the percentage of germination with respect to Czapek containing 3% glucose (100% germination), calculated by counting germinated conidia against total conidia in 10 randomly chosen microscopy fields.



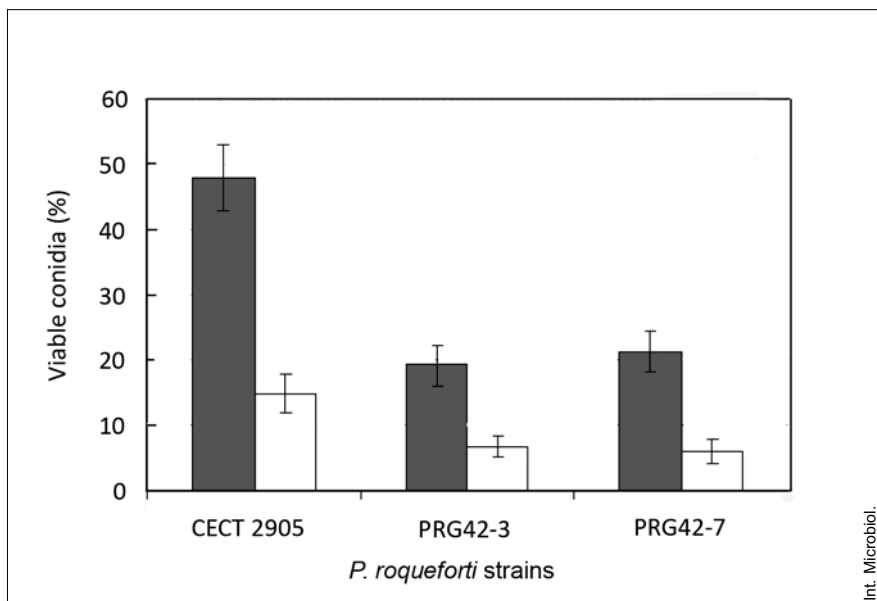
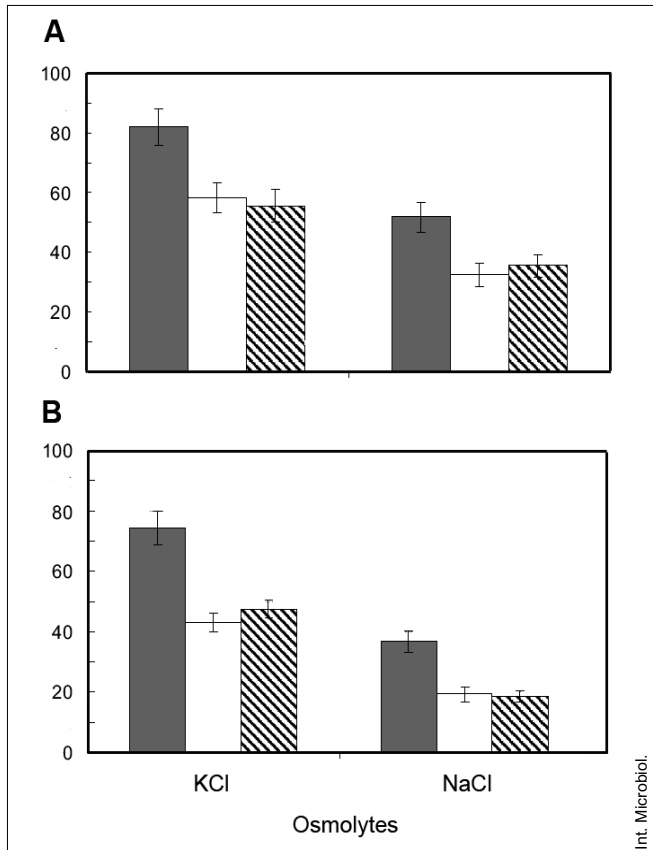


Fig. 2. Conidia viability of *Penicillium roqueforti* parental strain CECT 2905 and transformants PRG42-3 and PRG42-7 after 1 h of heat shock at 45°C (gray bars) and 50°C (white bars), expressed as percentage of viable conidia (able to germinate and form colonies) with respect to a non-treated control defined as having 100% viability.

Effect of the constitutively active Pga1 α subunit in the production of roquefortine C.

In the parental strain, the highest levels of roquefortine C production occurred between day 16 and day 21 of culture, with a peak of 0.4 $\mu\text{g}/\text{mg}$ of dry mycelium at day 18 (Fig. 4).

Afterwards, roquefortine C levels quickly fell to initial levels. In the transformant PRG42-7, carrying the dominant activating *pga1*^{G42R} allele, the production of roquefortine C increased, reaching 0.7 $\mu\text{g}/\text{mg}$ of dry mycelium at day 18. Moreover, in contrast to the parental strain, the transformant maintained high levels of roquefortine C (always >0.5 $\mu\text{g}/\text{mg}$ of dry mycelium) after day 18. This result indicates that the constitutively active Pga1^{G42R} α subunit should stimulate the production of roquefortine C, confirming the importance of G-protein-mediated signaling in regulating the production of this secondary metabolite. In addition to the increase in mycotoxin production, differences in color and aroma of the liquid cultures were observed.



Discussion

In this work, *P. roqueforti* transformants expressing the constitutively active Pga1 α subunit from *P. chrysogenum* were able to germinate in minimal medium lacking a carbon source at a rate six-fold higher than the wild-type strain CECT 2905. Similar observations have been made in *A. nidulans* strains expressing a constitutively active GanB α subunit (sub-

Fig. 3. Relative apical growth rates of *Penicillium roqueforti* parental strain CECT 2905 (gray bars) and transformants PRG42-3 (white bars) and PRG42-7 (dashed bars) in PW rich medium (A) and Czapek minimal medium (B) supplemented with 1.5 M NaCl or 1.5 M KCl. Data are expressed as the percentage of apical growth rates for each strain on each medium containing salt, with respect to the same strain growing in the same medium without salt. Apical growth rates were obtained as a linear regression of colony diameter vs. time.

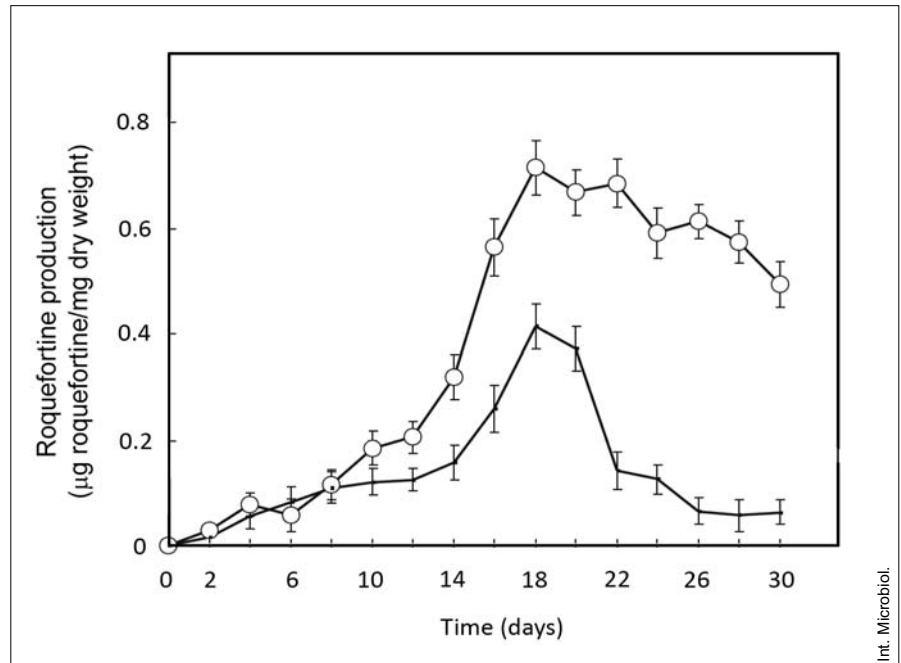


Fig. 4. Specific roquefortine C production by the *Penicillium roqueforti* parental strain CECT 2905 (line) and the transformant PRG42-7 (circles) when cultured in YES medium.

group III) [3] or a constitutively active RasA protein [27]. The participation of G α subunits from subgroup III in the regulation of conidial germination has been well-established in *P. marneffei* [42], *A. nidulans* [3], and *Botrytis cinerea* [6]. However, the role of G α subunits from subgroup I in germination seems to be species-specific. In fact, in *B. cinerea* [14], *F. oxysporum* [22], and *T. vires* [26] the lack of involvement of these subunits in germination has been proven. However, in *tgaA* mutants of *T. vires*, the morphology of the germ tubes differed from that of wild-type germ tubes. In contrast, in the phytopathogenic fungus *Colletotrichum trifolii*, disruption of the G α -subunit-encoding gene *ctg-1* drastically reduced the conidial germination rate from 82% to 1–2%, in accordance with the accumulation of *ctg-1* transcript in the germinating conidia [36]. In *C. heterostrophus*, *CGAI*-deleted mutants have a reduced rate of conidial germination (down to 7%, from 98% in the wild-type) and of appressorium formation [19].

In yeasts, the cAMP/PKA pathway has been related to control of the germination process as well as to vegetative growth [17,33]. In *A. nidulans*, Fillinger et al. [8] have demonstrated that cAMP-mediated signaling plays an important role in conidial germination. In *P. roqueforti* strains expressing a constitutively active Pga1 α subunit cAMP levels are about 40% higher than in the parental CECT 2905 strain [12]; this result suggests that regulation of conidiation by Pga1 in *P. roqueforti* may be mediated by cAMP.

In this study, conidia from *P. roqueforti* transformants carrying the dominant activating *pga1*^{G42R} allele were less resistant to thermal treatment than conidia from the parental strain

CECT 2905 (Fig. 2). Our data indicate that constitutive activation of Pga1-mediated signaling increases the thermal sensitivity of conidia, as has been observed in *Neurospora crassa* strains expressing a constitutively active form of the GNA-1 α subunit [37]. Conversely, deletion of the *gna-1* gene causes an increase in thermal resistance [37], as has been also shown in *F. oxysporum* and *C. parasitica* after disruption or deletion of their subgroup I G α -subunit-encoding genes [22,30].

In *S. cerevisiae*, a lower protein kinase A activity, caused by the loss of adenylate cyclase activity, increases thermal resistance, in accordance with the high sensitivity to heat of *S. cerevisiae* strains containing a constitutively active protein kinase A [32]. In *N. crassa*, low cAMP levels were found to have similar effects [21], which raises the possibility that cAMP also mediates Pga1 effects on thermal tolerance in *P. roqueforti*. Whatever the exact mechanism, it is now well established that subgroup I G α subunits are negative regulators of thermal tolerance in fungi, an effect that may be achieved by regulation of the expression of one or more proteins involved in heat-shock tolerance. The differential expression of several heat-shock proteins in *N. crassa* during asexual development supports that idea [9,15].

The constitutively active Pga1 subunit conferred a slightly higher sensitivity to hypertonic stress by *P. roqueforti*. This is in agreement with observations in *C. parasitica*, in which deletion of the subgroup I G α -subunit-encoding gene *cpg-1* has been shown to cause a higher tolerance to hypertonic stress [30]. However, in other fungi, the results have been the opposite. In *N. crassa* and *C. heterostrophus*, the absence of their

G α -subunit-encoding genes produces a decrease in the apical extension rate under hypertonic conditions [19,20,37]. The pathway through which subgroup I G α subunits alter the tolerance to hypertonic stress is not yet clear. In *A. nidulans*, heterotrimeric G protein signaling is involved in cell wall composition, affecting its sensitivity to osmotin and expression of the gene encoding the Mnp manoprotein [4,23].

Subgroup I G α subunits regulate secondary metabolism in different fungi [2,13,18,39]. With the aim of analyzing the possible regulation of roquefortine C biosynthesis by G α -protein-mediated signaling in *P. roqueforti*, we followed the kinetics of roquefortine C production in the parental strain CECT 2905 and in the transformant PRG42-7 for 30 days in YES medium. Introduction of the dominant activating *pga1*^{G42R} allele in *P. roqueforti* produced a noticeable increase in the production of roquefortine C. A similar effect has been observed in *F. sporotrichoides* after transformation with the *fada*^{G42R} allele from *A. nidulans*, which causes an increase in the production of the trichotecene toxin T-2 [35]. Roquefortine production is a response to an abrupt drop of nitrogen availability in the medium, and it is affected by the hypertonic composition of the medium [34]. Several G-protein-coupled receptors with putative nitrogen-sensing function have been identified in the genome of different filamentous fungi [24]. In general, the production of secondary metabolites by fungi is regulated by changes in the fungal environment.

Secondary metabolites are differentially regulated by G α subunits. In *A. nidulans*, constitutive activation of Fada represses expression of the sterigmatocystin regulatory gene *afIR*, thus inhibiting production of the mycotoxin [18]. The introduction of the *fada*^{G42R} allele in *A. parasiticus* produces the same effect on norsolorinic acid (an intermediate of the aflatoxin pathway) accumulation [18]; in *A. flavus*, cyclopiazonic acid and aflatoxin production are likewise inhibited by *fada*^{G42R} [2]. However, the *fada*^{G42R} allele has an opposite effect on penicillin production in *A. nidulans*, activating the expression of the penicillin biosynthetic gene *ipnA* [35]. Penicillin production is also positively regulated by the G α subunit Pga1 in *P. chrysogenum*, which activates the expression of the three penicillin biosynthetic genes [13]. The molecular basis accounting for this differential effect of G-protein-mediated signaling on the regulation of secondary metabolism is unknown. In *A. nidulans* and *A. parasiticus*, cAMP has an important role in the regulation of aflatoxin biosynthesis [29,31]. In *P. roqueforti*, the cellular levels of cAMP are regulated by Pga1 [12]; thus, the role of cAMP in the control of secondary metabolism in this fungus should be further investigated.

The cloning and characterization of the genes involved in G-protein-mediated signaling in *P. roqueforti* would help to

confirm our conclusions. Elucidating the molecular basis underlying mycotoxin production and the growth and developmental programs of this food-related fungus would provide new knowledge with implications for the handling and control of this biotechnologically important microorganism.

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