FUNGAL BIOLOGY 121 (2017) 754–762

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Heterotrimeric G protein alpha subunit controls growth, stress response, extracellular protease activity, and cyclopiazonic acid production in *Penicillium camemberti*

Ramón O. GARCÍA-RICO[a,**], *Carlos GIL-DURÁN*[b], *Juan F. ROJAS-AEDO*[b], *Inmaculada VACA*[c], *Luis FIGUEROA*[b], *Gloria LEVICÁN*[b], *Renato CHÁVEZ*[b,*]

[a]*GIMBIO Group, Department of Microbiology, Faculty of Basic Sciences, Universidad de Pamplona, Campus Universitario de Pamplona, Cl. 5 #4-19, Pamplona, Norte de Santander, Colombia*

[b]*Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Alameda 3363, Estación Central, 9170022, Santiago, Chile*

[c]*Departmento de Química, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, 7800003, Santiago, Chile*

ARTICLE INFO

Article history:
Received 29 November 2016
Received in revised form
8 May 2017
Accepted 23 May 2017
Available online 3 June 2017
Corresponding Editor:
Simon Avery

Keywords:
α-Subunit
Micotoxin
Filamentous fungus
Proteases
Stress resistance
Vegetative growth

ABSTRACT

The fungus *Penicillium camemberti* is widely used in the ripening of various bloomy-rind cheeses. Several properties of *P. camemberti* are important in cheese ripening, including conidiation, growth and enzyme production, among others. However, the production of mycotoxins such as cyclopiazonic acid during the ripening process by *P. camemberti* has raised concerns among consumers that demand food with minimal contamination. Here we show that overexpressing an α-subunit from the subgroup I of the heterotrimeric G protein (Gαi) influences several of these processes: it negatively affects growth in a media-dependent manner, triggers conidial germination, reduces the rate of sporulation, affects thermal and osmotic stress resistance, and also extracellular protease and cyclopiazonic acid production. Our results contribute to understanding the biological determinants underlying these biological processes in the economically important fungus *P. camemberti*.

© 2017 British Mycological Society. Published by Elsevier Ltd. All rights reserved.

Introduction

In filamentous fungi, heterotrimeric GTP binding proteins (G proteins) have been implicated in the regulation of several biological processes. G proteins are composed of three subunits (α, β, and γ) and remain inactive when all subunits are together, with GDP (guanosine 5′- diphosphate) bound to the α subunit (Neves *et al.* 2002). When a receptor coupled to the G protein is stimulated, the α subunit exchanges GDP for GTP (guanosine 5′- triphosphate), causing a conformational

* *Corresponding author*. Tel.: +56 (2)27181091; fax: +56 (2)26812108.
** *Corresponding author*. Tel.: +57 (7)5685303x244.
E-mail addresses: rovigar@hotmail.com (R.O. García-Rico), renato.chavez@usach.cl (R. Chávez).
http://dx.doi.org/10.1016/j.funbio.2017.05.007
1878-6146/© 2017 British Mycological Society. Published by Elsevier Ltd. All rights reserved.

change in specific switch regions and the separation of the α subunit from the βγ dimer (Sprang 1997), allowing them to interact with downstream effectors. The α subunit and the βγ dimer are inactivated by the intrinsic GTPase activity of the Gα subunit. Once GTP is hydrolyzed to GDP, the Gα subunit and the βγ dimer re-associate, bringing back the G protein to its heterotrimeric inactive state (McCudden *et al.* 2005).

Fungal Gα subunits have been classified into three subgroups (I, II, and III) (Bölker 1998; Li *et al.* 2007). Gα subunits from subgroup I (hereafter Gαi) are implicated in regulating several biological processes such as conidiation (Yu *et al.* 1996; Ivey *et al.* 2002; García-Rico *et al.* 2008a), conidial germination (Truesdell *et al.* 2000; Eaton *et al.* 2012), vegetative growth (Liu & Dean 1997; Yang & Borkovich 1999), stress resistance (García-Rico *et al.* 2009; García-Rico *et al.* 2011), and the production of proteases (Emri *et al.* 2008; Tan *et al.* 2009) and secondary metabolites (Calvo *et al.* 2002; Yu & Keller 2005; García-Rico *et al.* 2009), among others.

In almost all fungal species analyzed to date, Gαi negatively affects conidiation (Tag *et al.* 2000; Segers & Nuss 2003). However, in the case of conidial germination the effect of Gαi is not so clear. While it has been described that the disruption of Gαi affects conidial germination in some fungi (Truesdell *et al.* 2000; Eaton *et al.* 2012), in other cases Gαi does not seem to be involved in this process (Jain *et al.* 2002; Mukherjee *et al.* 2004). Further, in the fungus *Penicillum roqueforti* and *Penicillum chrysogenum* Gαi stimulates germination in the absence of any carbon source (García-Rico *et al.* 2009; García-Rico *et al.* 2011).

The effects of Gα subunits over apical growth are variable among fungal species. In *Aspergillus nidulans*, *Neurospora crassa* and *Stagonospora nodorum*, Gαi positively affects apical extension of the fungal colony (Yu *et al.* 1996; Ivey *et al.* 2002; Gummer *et al.* 2012), while in species from the genera *Fusarium* and *Penicillium* the Gαi subunit negatively affects the apical growth rate (Tag *et al.* 2000; García-Rico *et al.* 2007; García-Rico *et al.* 2009; Studt *et al.* 2013). These data suggest that Gαi subunits play a key role in fungal development, although they can have opposing (positive or negative) effects on apical extension rates of hyphae.

Gαi subunits are also related to thermal and hypertonic stress resistance. While Gαi subunits increase the thermal sensitivity of conidia in several fungi (Yang & Borkovich 1999; García-Rico *et al.* 2009; García-Rico *et al.* 2011), their role in hypertonic stress is variable. In *Cryphonectria parasitica*, *P. roqueforti* and *P. chrysogenum*, Gαi negatively affects hypertonic stress resistance (Segers & Nuss 2003; García-Rico *et al.* 2009; García-Rico *et al.* 2011), while in *N. crassa*, *S. nodorum*, *Cochliobolus heterostrophus* and *Alternaria alternata*, Gαi positively affects the rate of apical extension under hypertonic conditions (Ivey *et al.* 1996; Horwitz *et al.* 1999; Yang & Borkovich 1999; Solomon *et al.* 2004; Wang *et al.* 2010).

Gαi subunits also play a role in the production of extracellular proteases and secondary metabolites (mycotoxins, antibiotics or pigments), and in both cases Gαi can have a positive or negative effect, depending on the fungus analyzed (Hicks *et al.* 1997; Calvo *et al.* 2002; Emri *et al.* 2008; García-Rico *et al.* 2008b; García-Rico *et al.* 2009; Tan *et al.* 2009). Therefore, Gαi signaling differentially regulates protease and secondary metabolite production and in each case this must be analyzed individually.

Penicillium camemberti is a filamentous fungus that is important in the food industry and is responsible in large measure for the organoleptic properties of bloomy-rind cheese, such as Camembert and Brie. Several studies showed that the biological properties of *P. camemberti* (such as growth, conidial, and protease production) are fundamental for the cheese-ripening process (Nielsen *et al.* 1998; McSweeney & Sousa 2000; Boualem *et al.* 2008; Leclercq-Perlat *et al.* 2013). However, the production of potentially deleterious secondary metabolites (i.e. cyclopiazonic acid) during cheese ripening by *P. camemberti* has raised concerns (Kozlovsky *et al.* 2014). Surprisingly, and despite its biotechnological importance, many of the biological determinants underlying most of the processes carried out by *P. camemberti* remain to be determined. To gains insights into these fundamental processes, we overexpressed a dominant allele of the Gαi protein and analyzed its effect on several physiological processes of the economically important fungus *P. camemberti*.

Materials and methods

Fungal strains

The wild-type strain *Penicillium camemberti* NRRL 877 was kindly provided by Dr Juan F. Martín (Inbiotec, León, Spain). *Penicillium camemberti* transformants Pc-T04, Pc-T06, and Pc-T08 were obtained by introducing plasmid pPgaG42R containing the dominant allele $pga1^{G42R}$ from *Penicillum chrysogenum* (García-Rico *et al.* 2007) into the wild-type strain by protoplast transformation (see below). $pga1^{G42R}$ encodes a Gαi protein where glycine at position 42 was replaced by an arginine. This mutation is expected to disrupt the endogenous GTPase activity of the Gαi subunit, thereby resulting in a dominant active G protein (Yu *et al.* 1996). In addition to these transformants, a *P. camemberti* strain containing the wild-type *pga1*allele was constructed and used as a control.

Transformation of Penicillium camemberti *NRRL 877*

Fungal protoplasts were isolated as described by García-Rico *et al.* (2007), with some modifications. Briefly, approximately 1.5 g of wet mycelium was suspended in 20 ml TPP buffer (potassium phosphate buffer 50 mM pH 5.8, KCl 0.7 M) containing 10 mg ml^{-1} Lysing Enzymes from *Trichoderma harzianum* (Sigma–Aldrich) and 200 U ml^{-1} of β-glucuronidase (Sigma–Aldrich). The suspension was incubated at 28 °C for 2 h with gentle shaking (80 rpm). Protoplasts were obtained by filtering the sample through a nylon filter and transformed as described by Fierro *et al.* (2004). Transformed protoplasts were selected on Czapek-sorbitol medium using phleomycin (20 μg ml^{-1}) as the selection agent. Conidia from these colonies were subsequently transferred three times on the same medium to stabilize the genotype and obtain homokaryotic strains.

DNA and RNA extractions, and RT-PCR assays

Penicillium camemberti DNA and total RNA were extracted as described by Gil-Durán *et al.* (2015).

RT-PCR experiments were performed as described by Cepeda-García et al. (2014) with minor modifications. Briefly, total RNA was treated with RNase-free DNase I (Roche, Germany), and quantified in a MultiSkan GO quantification system using a μDrop plate (Thermo Scientific, Germany) following the manufacturer's instructions. Two hundred ng of treated RNA were used to synthesize *pga1* cDNA using RevertAid Reverse Transcriptase (Thermo Scientific, Germany) and primers 5′-AGTCCCTCGAGCTGCCT-3′ and 5′-CTTCTTGCGCTCGGATCG-3′. Amplification of β-tubulin cDNA was performed with primers 5′-GTAACCAAATCGGTGCTGCTTTC-3′ and 5′-ACCCT CAGTGTAGTGACCCTTGGC-3′ as an internal control. Products of the RT-PCR reactions were resolved by electrophoresis on a 1.5 % agarose gel, stained with ethidium bromide and quantified by densitometric analysis using the 'myImageAnalysis Software' program (Thermo Scientific, Germany).

Values were expressed as the ratio between *pga1*/β-tubulin.

Phenotypic characterization

For phenotypic analyses, the different strains were grown on different solid media: potato dextrose agar (PDA), Czapek minimal medium, and Power rich medium (García-Rico et al. 2007; García-Rico et al. 2008a). Apical extension rates and conidia production were measured according to García-Rico et al. (2007).

Analysis of conidial germination

Conidial germination analysis was conducted as described by García-Rico et al. (2009), with some modifications. Fifty ml of Czapek minimal liquid medium were inoculated with conidia (final concentration 1×10^7 conidia ml^{-1}) and incubated at 25 °C and 120 rpm for 16 h. From the fourth hour of incubation and at regular intervals of 1–2 h, samples were taken and observed under the microscope. The number of germinated and non-germinated conidia was counted from 10 randomly-chosen fields. A conidium was considered as germinated when the length of its germ tube was the same size or longer than its diameter. The percentage of germinated conidia was calculated and plotted against time. The same experiment was performed using Czapek liquid medium without a carbon source and distilled water alone, but samples were taken at 16, 24, and 36 h of incubation.

Analysis of thermal and hypertonic stress resistance

Thermal stress resistance was determined as conidial resistance to heat-shock as described by García-Rico et al. (2009).

The effect of hypertonic stress on vegetative growth was assayed by calculating the relative apical extension rates of the colonies growing in Czapek or Power media supplemented with 1.5 M KCl or 1.5 M NaCl, with respect to the control (standard Czapek and Power). Results were expressed as percentage of apical extension rate as compared to control conditions (Ivey et al. 1996).

Proteolytic activity in culture supernatants

Extracellular proteolytic activity in culture supernatants was determined as described by Chávez et al. (2010). The strains were grown in liquid CAC medium (wheat flour, 30 g l^{-1}; peanut flour, 5 g l^{-1}; sodium citrate, 2 g l^{-1}; citric acid, 2 g l^{-1}; pH 4.5) for 96 h. At this time, aliquots of the different cultures were withdrawn, centrifuged, and the supernatants were assayed for proteolytic activity according to the azocasein method described by Larsen et al. (1998). Enzymatic activity of each strain was normalized by total protein concentration, as determined by the Bradford method.

Cyclopiazonic acid (CPA) production

Cyclopiazonic acid (CPA) production was determined as described by Rodríguez et al. (2012) with some modifications. Briefly, each strain was grown on malt extract agar plates (malt extract, 30 g l^{-1}; peptone, 5 g l^{-1}; agar, 15 g l^{-1}; pH 5.4) for 15 d at 25 °C. Mycelia from three Petri dishes was scraped off and extracted with 5 ml of chloroform. After centrifugation, the solution was filtered through a 0.45 μm pore size nylon membrane, and samples were dried in a rotary evaporator. The extracts were suspended in 400 μl of acetonitrile just before HPLC analysis. HPLC analysis was performed on a Waters 1525 HPLC system (Waters, Ireland) by injecting 20 μl of each sample into a 4.6 × 250 mm (5 μm) RPC18 SunFire column (Waters, Ireland). The column was held at 35 °C. A solvent gradient with water and acetonitrile (both acidified with 0.02 % trifluoroacetic acid) was used. The gradient was 15 % acetonitrile for 25 min, an increase to 68 % in 2 min, and then 100 % for 5 min. The flow was 1.2 ml min^{-1}. CPA showed a retention time of 25.4 min. Pure CPA (Calbiochem, San Diego, CA) was used as standard.

Results and discussion

Expression of pga1^{G42R} decreases apical extension and conidiation in Penicillium camemberti

The subunit Gαi is central for fungal growth, but its role in development is variable among fungal species. To functionally characterize the effect of Gαi in *P. camemberti*, wild-type strain NRRL 877 was transformed with plasmid pPgaG42R expressing the dominant *pga1*^{G42R} allele (García-Rico et al. 2007). After transformation, 40 phleomycin-resistant transformants were obtained, of which 11 were randomly selected and subjected to RT-PCR (see Materials and Methods). Three of these transformants (named Pc-T04, Pc-T06, and Pc-T08) expressed the *pga1*^{G42R} allele to levels between 29 and 67 % higher that the wild-type strain (Fig 1), confirming the successful overexpression of *pga1*^{G42R}. The presence of plasmid pPgaG42R in all strains was confirmed by PCR (data not shown).

Overexpressing the *pga1*^{G42R} allele altered the normal phenotype of *P. camemberti*, affecting growth and sporulation (Fig 2). As expected, the rate of apical growth was faster in the more nutritive Power medium than in PDA and Czapek (minimal medium). The *pga1*^{G42R} allele negatively affected hyphal extension, resulting in smaller thalli than in the wild-type strain in all tested media. We did not observe changes in thalli aspect (data not shown). In Czapek medium, the apical growth rate of strains overexpressing *pga1*^{G42R} ranged between 0.035 and 0.055 mm h^{-1}, *versus* 0.088 mm h^{-1} in the wild-type strain

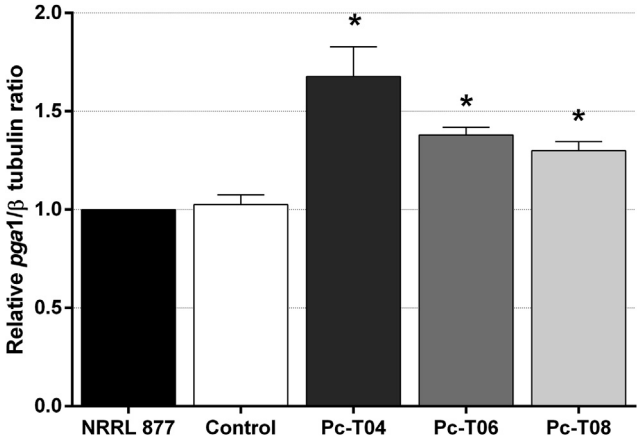

Fig 1 – Expression of *pga1*^{G42R} in transformants Pc-T04, Pc-T06 and Pc-T08. Transcript levels of *pga1* and β-tubulin in *P. camemberti* NRRL 877 (WT) were quantified by densitometric analysis using the 'myImageAnalysis Software' program (Thermo Scientific, Germany), and the *pga1*/β-tubulin ratio was normalized as a ratio of 1.0. The same experiment was performed using transformants Pc-T04, Pc-T06, and Pc-T08, and normalized with respect to the *pga1*/β-tubulin ratio from the wild-type strain. A strain containing the wild-type *pga1* allele was used as a control. Error bars represent standard deviation of three replicates from three different experiments. The symbol * indicates that overexpression of the *pga1*^{G42R} allele in transformants Pc-T04, Pc-T06, and Pc-T08 was statistically significant ($p < 0.05$ using Student's t-test) as compared to the wild-type and control strains.

(Fig 3), indicating that the transformants grew at a rate between 40 and 62 % of wild-type strain levels. A similar result was observed in PDA medium (transformants grew at a rate between 38 and 55 % of wild-type strain levels). In Power medium the effect of overexpressing *pga1*^{G42R} was less evident and transformants showed apical growth rates ranging between 73 and 84 % of wild-type strain levels (Fig 3). Together, these results indicate that expressing the *pga1*^{G42R} allele in *P. camemberti* caused a reduction in the apical extension rate, which seems to be dependent on the culture medium. It is known that changes in the nutrient source affect fungal growth (Riquelme *et al.* 2011). For example, in *P. camemberti* growth of the mycelium is affected by nutrient availability (Adour *et al.* 2002; Aziza *et al.* 2005). In this context, G protein-mediated signaling plays a key role in detecting nutrient availability in fungi (Li *et al.* 2007). Thus, and although the role of additional signaling pathways cannot be ruled out at this point, our results suggest that in *P. camemberti*, changes in nutrient availability affect growth through the Gαi signaling pathway.

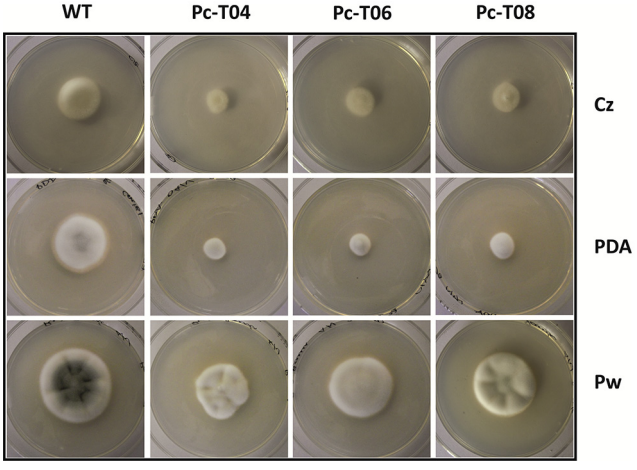

Fig 2 – Phenotypes of *P. camemberti* NRRL 877 (WT) and transformants Pc-T04, Pc-T06, and Pc-T08. Colonies were grown for 7 d at 28 °C in Czapek (Cz), PDA, and Power (Pw) media. Note the marked reduction in colony diameter in strains Pc-T04, Pc-T06, and Pc-T08 as compared to the wild-type strain, especially in Czapek and PDA media.

Overexpressing the *pga1*^{G42R} allele drastically reduced conidia formation in all tested media (Fig 4). In Czapek and PDA, conidia formation was between 3.6 % and 3.5 % of wild-type strain levels, while in Power medium (a medium optimized for sporulation) it was 4.2 %. A similar result was observed through the entire sporulation cycle (after 3, 5, and 7 d of growth) in all tested media (Fig 4). These results clearly indicate that the *pga1*^{G42R} allele strongly inhibits conidiation. In almost all fungal species analyzed to date, Gαi negatively affects conidiation, either by decreasing conidia production (e.g. Tag *et al.* 2000) or completely abolishing it (e.g. Segers & Nuss 2003). In fungi, conidiation is regulated by a central pathway composed of three genes (*brlA*, *abaA*, and *wetA*; Mirabito *et al.* 1989) and this pathway can be activated via Gαi-cAMP (Zuber *et al.* 2002; García-Rico *et al.* 2008a). Further, Boualem *et al.* (2008) showed that the expression of *brlA* and *wetA* increased in *P. camemberti* grown on solid medium. Thus, although further experimental confirmation is needed, it seems plausible that in *P. camemberti* the Gαi subunit reduces conidial production by affecting genes of the conidiation central pathway through the modulation of cAMP levels, as shown to occur in other fungi.

Expression of pga1^{G42R} *induces conidial germination in absence of carbon source in* Penicillium camemberti

In general, no significant differences in conidial germination were observed between any of the tested strains in Czapek medium (data not shown). However, in Czapek medium without a carbon source (sucrose), approximately 50 % of conidia from transformants expressing *pga1*^{G42R} germinated after 36 h (Fig 5A). A similar result was observed in distilled water alone (Fig 5B). As expected, the wild-type strain was unable to germinate in the absence of a carbon source. These results indicate that transformants expressing *pga1*^{G42R} can germinate in the absence of any organic molecules, suggesting that Gαi signalling participates in sensing carbon sources, which is the signal for conidial germination (d'Enfert 1997). Truesdell *et al.* (2000) and Eaton *et al.* (2012) described that the disruption of Gαi negatively affects conidial germination in *Colletotrichum trifolii* and *Neurospora crassa*, respectively. Further, in *Penicillum roqueforti* and *Penicillum chrysogenum* the Gαi protein triggers germination in the absence of carbon sources (García-Rico *et al.* 2009; García-Rico *et al.* 2011). In filamentous fungi, germination depends on several abiotic external factors and is triggered by the presence

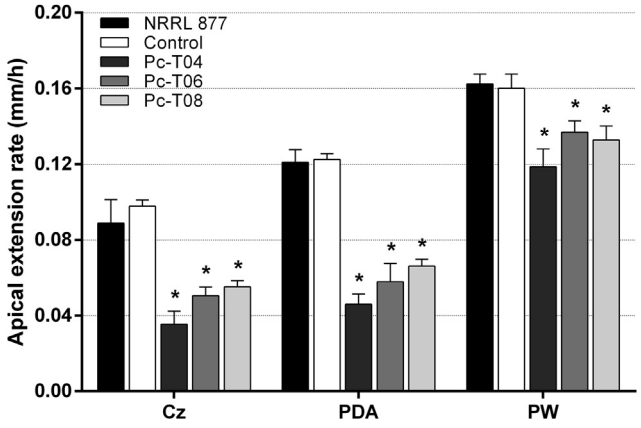

Fig 3 – Comparison of the apical extension rates (mm h^{-1}) of *P. camemberti* NRRL 877 and transformants Pc-T04, Pc-T06, and Pc-T08. Three different media (Czapek (Cz), PDA and Power (PW)) were used. A strain containing the wild-type *pga1* allele was used as control. Error bars represent standard deviation of three replicas from three different experiments. The symbol * indicates that reductions in the apical extension rates of strains Pc-T04, Pc-T06, and Pc-T08 were statistically significant ($p < 0.05$ using Student's t-test) as compared to the wild-type and control strains.

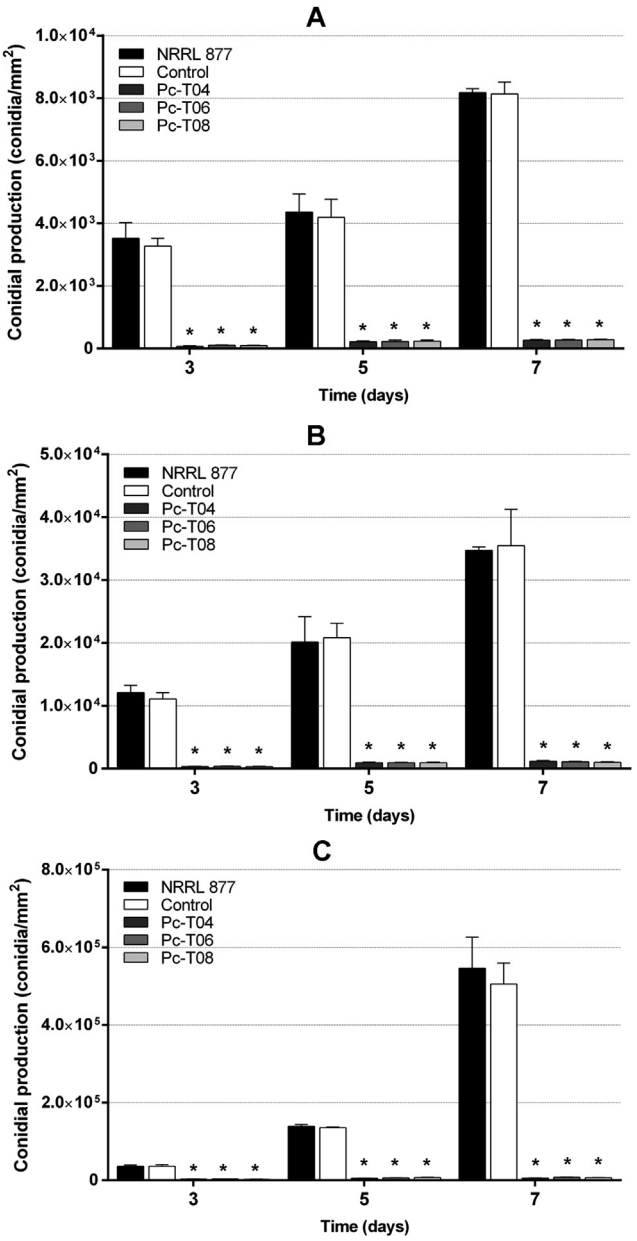

Fig 4 – Conidial production by *P. camemberti* NRRL 877 and transformants Pc-T04, Pc-T06, and Pc-T08. Measurements were performed in Czapek (A), PDA (B), and Power (C) media after 3, 5, and 7 d of cultivation. A strain containing the wild-type *pga1* allele was used as a control. Error bars represent the standard deviation of three replicas from three independent experiments. The symbol * indicates that reductions in conidial production of strains Pc-T04, Pc-T06, and Pc-T08 were statistically significant ($p < 0.05$ using Student's t-test) as compared to the wild-type and control strains.

of organic compounds (d'Enfert 1997), while in submerged cultures germination is independent of the carbon source (Xue *et al.* 2004). In summary, Gαi signalling would participate in sensing carbon sources in *P. camemberti*.

Expressing the pga1^{G42R} allele decreases heat shock and hypertonic stress resistance in Penicillium camemberti

Since Gαi exerts its action through cAMP and several stress-response regulators and genes are controlled by the cAMP–PKA pathway in fungi (Bahn *et al.* 2007; Conrad *et al.* 2014), we aimed to determine if Gαi played a role in stress tolerance in *P. camemberti*.

Expression of *pga1*^{G42R} decreased thermal stress resistance approximately 1.7 fold at 45 °C and 8.8 fold at 50 °C (Fig 6A). Further, expression of *pga1*^{G42R} decreased osmotic stress resistance to 1.5 M NaCl or KCl. The relative apical extension rate of transformants expressing the dominant allele was approximately 72 % and 52 % of wild-type levels in the presence of KCl and NaCl in Power medium, and 65 % and 62 % in Czapek medium (Fig 6B). Our results indicate that upregulating of Gαi signalling by expressing *pga1*^{G42R} impaired the ability of *P. camemberti* to efficiently respond to thermal and osmotic stress. Similar results were obtained in *Neurospora crassa*, *Fusarium oxysporum*, *Cryphonectria parasitica*, *Penicillium roqueforti*, and *Penicillium chrysogenum* (Yang & Borkovich 1999; Jain *et al.* 2002; Segers & Nuss 2003; García-Rico *et al.* 2009; García-Rico *et al.* 2011).

Expression of pga1^{G42R} decreases extracellular proteolytic activity in Penicillium camemberti

Proteases produced by *P. camemberti* play a key role in developing the organoleptic properties of cheese (McSweeney & Sousa 2000). Highlighting their role in this process, up to 52 different mRNAs linked to extracellular proteases are expressed in *P. camemberti* during cheese-ripening (Lessard *et al.* 2014). To determine if Gαi was implicated in regulating the production of extracellular proteases, we compared the extracellular

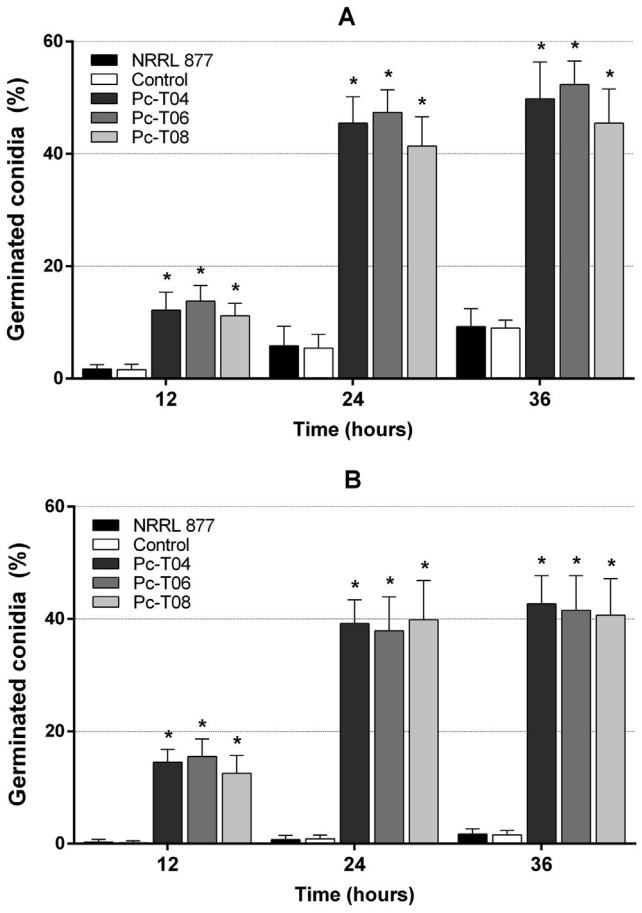

Fig 5 – Percentage of germinated conidia of *P. camemberti* NRRL 877 and transformants Pc-T04, Pc-T06, and Pc-T08 in Czapek minimal medium lacking a carbon source (A), and in distilled water alone (B) after 12, 24, and 36 h. Incubations were carried out at 25 °C and 120 rpm. A strain containing the wild-type *pga1* allele was used as a control. Error bars represent the standard deviation of three replicas from three independent experiments. The symbol * indicates that increases in the percentage of germinated conidia of strains Pc-T04, Pc-T06, and Pc-T08 were statistically significant ($p < 0.05$ using Student's t-test) as compared to the wild-type and control strains.

proteolytic activity of strains expressing $pga1^{G42R}$ and the wild-type strain. All the tested transformants showed a significantly lower proteolytic activity than the wild-type strain (Table 1). The proteolytic activity of transformants Pc-T04, Pc-T06, and Pc-T08 was 26.3, 18.4, and 51.3 % of the wild-type levels, indicating that expressing the $pga1^{G42R}$ allele in *P. camemberti* importantly reduces the production of extracellular proteases. To date, opposing results about the role of Gαi in the production of extracellular proteases have been reported. Transcriptomic and proteomic studies showed that inactivating Gαi decreased the production of extracellular proteases in phytopathogenic fungi (Dawe *et al.* 2004; Schulze-Gronover *et al.* 2004; Tan *et al.* 2009). Interestingly, downregulating the production of extracellular proteases in these fungi correlated with decreased virulence (Gao & Nuss 1996; Schulze-Gronover *et al.* 2001; Solomon *et al.* 2004), suggesting that proteases (and their control by Gαi) are important for phytopathogenicity. Conversely, inactivating Gαi in the non-phytopathogenic fungus *Aspergillus nidulans* increased proteolytic activity (Emri *et al.* 2008). In that study, the authors suggest that increasing the production of extracellular proteases would allow *A. nidulans* to use minor energy sources, allowing fungi lacking the Gαi to survive under carbon starvation (Emri *et al.* 2008). *Penicillium camemberti* is also a non-phytopathogenic fungus, and the $pga1^{G42R}$ allele produces a dominant active G protein signaling (Yu *et al.* 1996), which could be considered the opposite of inactivating Gαi. Hence, our results are in agreement with those reported for *A. nidulans*, as activating Gαi downregulates the production of extracellular proteases in *P. camemberti* (the opposite effect of inactivating Gαi in *A. nidulans*).

Expression of $pga1^{G42R}$ decreases the production of the secondary metabolite cyclopiazonic acid (CPA) in Penicillium camemberti

CPA is one of the main mycotoxins produced by *P. camemberti*. To study if Gαi affects this process, CPA production was analysed in strains expressing $pga1^{G42R}$ and in the wild-type strain. Transformants expressing $pga1^{G42R}$ produced between 29.7 and 32.1 % of wild-type CPA levels after 15 d of culture (Table 1), indicating that $pga1^{G42R}$ negatively affects the production of this mycotoxin. In fungi, the role of Gαi subunits in the production of secondary metabolites is variable. For example, in *Aspergillus* spp. Gαi negatively regulates the production of several secondary metabolites (Calvo *et al.* 2002), while in other fungi (i.e. *Penicillum chrysogenum*, *Penicillum roqueforti*) Gαi stimulates their production (García-Rico *et al.* 2008b; García-Rico *et al.* 2009). Further studies are warranted to fully understand the role of Gαi in secondary metabolism. One possibility is that Gαi triggers complex signal transduction networks that culminate in the activation or repression of specific transcription factors which, in turn, control the expression of genes implicated in synthesizing specific secondary metabolites. Our results suggest that in *P. camemberti*, Gαi negatively regulates CPA production. To the best of our knowledge, only two genes have been implicated in controlling CPA synthesis, namely *veA* and *laeA* (Duran *et al.* 2007; Hong *et al.* 2015). Inactivating *veA* decreases CPA production in *Aspergillus flavus* (Duran *et al.* 2007), while overexpressing *laeA* increases CPA production in *Aspergillus fumisynnematus* (Hong *et al.* 2015). Interestingly, *veA* and *laeA* encode the VeA and LaeA proteins, which are part of the velvet complex, a regulatory unit that regulates secondary metabolism and development in fungi (Bayram & Braus 2012). Although the functional connection between G proteins and the velvet complex is unknown (Bayram & Braus 2012), there are some cases where both pathways seem to control the same biological processes in fungi (Jeong *et al.* 2003; Han *et al.* 2008; Lee *et al.* 2012). Therefore, determining if the velvet complex and GαI act together in regulating CPA production in *P. camemberti* should be investigated.

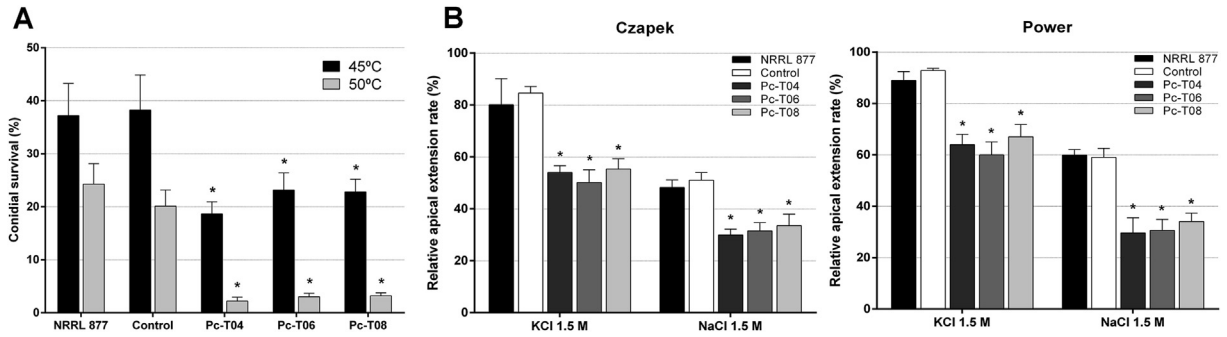

Fig 6 – Comparative analysis of thermal and hypertonic stress resistance of *P. camemberti* NRRL 877 and transformants Pc-T04, Pc-T06, and Pc-T08. (A) Conidial survival after 1 h of heat shock treatments at 45 °C and 50 °C, expressed as percentage of viable conidia (able to germinate and form colonies) against a non-treated control which was set to 100 % viability. (B) Relative apical growth rates in Czapek minimal medium or Power medium containing 1.5 M KCl or 1.5 M NaCl. Data are expressed as the percentage of apical growth rates for each strain grown in the medium supplemented with the indicated salt *versus* growth without salt. In (A) and (B), a strain containing the wild-type *pga1* allele was used as control. Error bars represent the standard deviation of three replicas from three independent experiments. The symbol * indicates that reductions in stress resistance of strains Pc-T04, Pc-T06, and Pc-T08 were statistically significant ($p < 0.05$ using Student's t-test) as compared to the wild-type and control strains.

Table 1 – Extracellular proteolytic activity and cyclopiazonic acid (CPA) production by *P. camemberti* NRRL 877 and transformants Pc-T04, Pc-T06, and Pc-T08.

Strain	Proteolytic activity (U mg^{-1} of protein)	CPA (μg g^{-1} dry weight[a])
NRRL 877 (wild-type)	1574.76 ± 93.72	69.47 ± 13.13
Control	1617.92 ± 26.06	67.42 ± 11.37
Pc-T04	415.59 ± 59.30[b]	20.62 ± 10.80[b]
Pc-T06	290.75 ± 24.88[b]	19.87 ± 1.60[b]
Pc-T08	808.44 ± 42.50[b]	22.33 ± 4.28[b]

a CPA production was normalized by dry weight of fungal mycelia. For this purpose, the mycelium from each sample was dried as described by García-Rico *et al.* (2009).
b Significant differences as compared to the wild-type and control strains ($p \leq 0.001$, Student-t test).

Conclusions

Despite that several biological properties of *Penicillium camemberti* are important and have biotechnological applications, its physiology remains poorly understood. In this work, we show that expressing a dominant allele of a Gαi protein in *P. camemberti* decreases apical extension, conidiation, thermal and hypertonic stress resistance, and extracellular protease and CPA production. In addition, the dominant $pga1^{G42R}$ allele triggered conidial germination in the absence of a carbon source. Our results shed light into the regulation of these biological processes in the economically important fungus *P. camemberti*.

Acknowledgements

This work received funds from "Programa de Cooperación Científica Internacional CONICYT-COLCIENCIAS" under the project "Folio 2009-096". R.C. acknowledges the support of DICYT-USACH. R.O. G.-R. thanks the University of Pamplona and the Microbiology and Biotechnology Research Group (GIMBIO) for their support. C.G.-D. and JFRA have received doctoral fellowships CONICYT-PFCHA/Doctorado Nacional/2014-63140056 and CONICYT-PFCHA/DoctoradoNacional/2013-21130251, respectively. We are grateful to Dr. Francisco Fierro (UAM-Iztapalapa, Mexico D.F.) for his useful suggestions.

REFERENCES

Adour L, Couriol C, Amrane A, Prigent Y, 2002. Growth of *Geotrichum candidum* and *Penicillium camembertii* in liquid media in relation with the consumption of carbon and nitrogen sources and the release of ammonia and carbon dioxide. *Enzyme Microbiology and Technology* **31**: 533–542.

Aziza C, Couriol C, Amrane A, Boutrou R, 2005. Evidences for synergistic effects of *Geotrichum candidum* on *Penicillium camembertii* growing on cheese juice. *Enzyme Microbiology and Technology* **37**: 218–224.

Bahn Y-S, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME, 2007. Sensing the environment: lessons from fungi. *Nature Reviews* **5**: 57–69.

Bayram Ö, Braus GH, 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews* **36**: 1–24.

Bölker M, 1998. Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. *Fungal Genetics and Biology* **25**: 143–156.

Boualem K, Waché Y, Garmyn D, Karbowiak T, Durand A, Gervais P, Cavin JF, 2008. Cloning and expression of genes involved in conidiation and surface properties of *Penicillium camemberti* grown in liquid and solid cultures. *Research in Microbiology* **159**: 110–117.

Calvo AM, Wilson RA, Bok JW, Keller NP, 2002. Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**: 447–459.

Cepeda-García C, Domínguez-Santos R, García-Rico RO, García-Estrada C, Cajiao A, Fierro F, Martín JF, 2014. Direct involvement of the CreA transcription factor in penicillin biosynthesis and expression of the *pcbAB* gene in *Penicillium chrysogenum*. *Applied Microbiology and Biotechnology* **98**: 7113–7124.

Chávez R, Roa A, Navarrete K, Trebotich J, Espinosa J, Vaca I, 2010. Evaluation of properties of several cheese-ripening fungi for potential biotechnological applications. *Mycoscience* **51**: 84–87.

Conrad M, Schothorst J, Kankipati HN, Van Zeebroeck G, Rubio-Texeira M, Thevelein JM, 2014. Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **38**: 254–299.

Dawe AL, Segers GC, Allen TD, McMains VC, Nuss DL, 2004. Microarray analysis of *Cryphonectria parasitica* Gα- and Gβγ-signalling pathways reveals extensive modulation by hypovirus infection. *Microbiology* **150**: 4033–4043.

d'Enfert C, 1997. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* **21**: 163–172.

Duran RM, Cary JW, Calvo AM, 2007. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. *Applied Microbiology and Biotechnology* **73**: 1158–1168.

Eaton CJ, Cabrera IE, Servin JA, Wright SJ, Cox MP, Borkovich KA, 2012. The guanine nucleotide exchange factor RIC8 regulates conidial germination through Gα proteins in *Neurospora crassa*. *PLoS ONE* **7**: e48026.

Emri T, Szilágyi M, Justyák A, Pócsi I, 2008. Heterotrimeric G protein mediated regulation of proteinase production in *Aspergillus nidulans*. *Acta Microbiologica et Immunologica Hungarica* **55**: 111–117.

Fierro F, Laich F, García-Rico RO, Martín JF, 2004. High efficiency transformation of *Penicillium nalgiovense* with integrative and autonomously replicating plasmids. *International Journal of Food Microbiology* **90**: 237–248.

Gao S, Nuss DL, 1996. Distinct roles for two G protein α subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proceedings of the National Academy of Science of the USA* **93**: 14122–14127.

García-Rico RO, Martín JF, Fierro F, 2007. The *pga1* gene of *Penicillium chrysogenum* NRRL 1951 encodes a heterotrimeric G protein alpha subunit that controls growth and development. *Research in Microbiology* **158**: 437–446.

García-Rico RO, Fierro F, Martín JF, 2008a. Heterotrimeric Gα protein Pga1 of *Penicillium chrysogenum* controls conidiation mainly by a cAMP-independent mechanism. *Biochemistry and Cell Biology* **86**: 57–69.

García-Rico RO, Fierro F, Mauriz E, Gómez A, Fernández-Bodega MA, Martín JF, 2008b. The heterotrimeric Gα protein Pga1 regulates biosynthesis of penicillin, chrysogenin and roquefortine in *Penicillium chrysogenum*. *Microbiology* **154**: 3567–3578.

García-Rico RO, Chávez R, Martín JF, Fierro F, 2009. Effect of a heterotrimeric G protein α subunit on conidia germination, stress response, and roquefortine C production in *Penicillium roqueforti*. *International Microbiology* **12**: 123–129.

García-Rico RO, Martín JF, Fierro F, 2011. Heterotrimeric Gα protein Pga1 from *Penicillium chrysogenum* triggers germination in response to carbon sources and affects negatively resistance to different stress conditions. *Fungal Genetics and Biology* **48**: 641–649.

Gil-Durán C, Rojas-Aedo JF, Medina E, Vaca I, García-Rico RO, Villagrán S, Levicán G, Chávez R, 2015. The *pcz1* gene, which encodes a $Zn(II)_2Cys_6$ protein, is involved in the control of growth, conidiation, and conidial germination in the filamentous fungus *Penicillium roqueforti*. *PLoS ONE* **10**: e0120740.

Gummer JPA, Trengove RD, Oliver RP, Solomon PS, 2012. A comparative analysis of the heterotrimeric G-protein Gα, Gβ and Gγ subunits in the wheat pathogen *Stagonospora nodorum*. *BMC Microbiology* **12**: 131.

Han KH, Kim JH, Moon H, Kim S, Lee SS, Han DM, Jahng KY, Chae KS, 2008. The *Aspergillus nidulans esdC* (early sexual development) gene is necessary for sexual development and is controlled by *veA* and a heterotrimeric G protein. *Fungal Genetics and Biology* **45**: 310–318.

Hicks JK, Yu JH, Keller NP, Adams TH, 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway. *EMBO Journal* **16**: 4916–4923.

Hong EJ, Kim NK, Lee D, Kim WG, Lee I, 2015. Overexpression of the *laeA* gene leads to increased production of cyclopiazonic acid in *Aspergillus fumisynnematus*. *Fungal Biology* **119**: 973–983.

Horwitz BA, Sharon A, Lu SW, Ritter V, Sandrock T, Yoder OC, Turgeon BG, 1999. A G protein α subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fungal Genetics and Biology* **26**: 19–32.

Ivey FD, Hodge PN, Turner GE, Borkovich KA, 1996. The $G\alpha_i$ homologue *gna-1* controls multiple differentiation pathways in *Neurospora crassa*. *Molecular Biology of the Cell* **7**: 1283–1297.

Ivey FD, Kays AM, Borkovich KA, 2002. Shared and independent roles for a Gαi protein and adenylyl cyclase in regulating development and stress responses in *Neurospora crassa*. *Eukaryotic Cell* **1**: 634–642.

Jain S, Akiyama K, Mae K, Ohguchi T, Takata R, 2002. Targeted disruption of a G protein α subunit gene results in reduced pathogenicity in *Fusarium oxysporum*. *Current Genetics* **41**: 407–413.

Jeong HY, Kim H, Han DM, Jahng KY, Chae KS, 2003. Expression of the *mnpA* gene that encodes the mannoprotein of *Aspergillus nidulans* is dependent on *fadA* and *flbA* as well as veA. *Fungal Genetics and Biology* **38**: 228–236.

Kozlovsky AG, Zhelifonova VP, Antipova TV, Baskunov BP, Ivanushkina NE, Ozerskaya SM, 2014. Exo-metabolites of mycelial fungi isolated in production premises of cheese-making and meat-processing plants. *Food Additives and Contaminants Part A* **31**: 300–306.

Larsen MD, Kristiansen KR, Hansen TK, 1998. Characterization of the proteolytic activity of starter cultures of *Penicillium roqueforti* for production of blue veined cheeses. *International Journal of Food Microbiology* **43**: 215–221.

Leclercq-Perlat MN, Picque D, Martin Del Campo Barba ST, Monnet C, 2013. Dynamics of *Penicillium camemberti* growth quantified by real-time PCR on Camembert-type cheeses under different conditions of temperature and relative humidity. *Journal of Dairy Science* **96**: 4031–4040.

Lee J, Myong K, Kim JE, Kim HK, Yun SH, Lee YW, 2012. FgVelB globally regulates sexual reproduction, mycotoxin production and pathogenicity in the cereal pathogen *Fusarium graminearum*. *Microbiology* **158**: 1723–1733.

Lessard MH, Viel C, Boyle B, St-Gelais D, Labrie S, 2014. Metatranscriptome analysis of fungal strains *Penicillium camemberti* and *Geotrichum candidum* reveal cheese matrix breakdown and potential development of sensory properties of ripened Camembert-type cheese. *BMC Genomics* **15**: 235.

Li L, Wright SJ, Krystofova S, Park G, Borkovich KA, 2007. Heterotrimeric G protein signaling in filamentous fungi. *Annual Review of Microbiology* **61**: 423–452.

Liu S, Dean RA, 1997. G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **10**: 1075–1086.

McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS, 2005. G-protein signaling: back to the future. *Cellular and Molecular Life Sciences* **62**: 551–577.

McSweeney PLH, Sousa MJ, 2000. Biochemical pathways for the production of flavour compounds in cheese during ripening: a review. *Lait* **80**: 293–324.

Mirabito PM, Adams TH, Timberlake WE, 1989. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. *Cell* **57**: 859–868.

Mukherjee PK, Latha J, Hadar R, Horwitz BA, 2004. Role of two G-protein alpha subunits, TgaA and TgaB, in the antagonism of plant pathogens by *Trichoderma virens*. *Applied and Environmental Microbiology* **70**: 542–549.

Neves SR, Ram PT, Iyengar R, 2002. G protein pathways. *Science* **296**: 1636–1639.

Nielsen MS, Frisvad JC, Nielsen PV, 1998. Colony interaction and secondary metabolite production of cheese-related fungi in dual culture. *Journal of Food Protection* **61**: 1023–1029.

Riquelme M, Yarden O, Bartnicki-Garcia S, Bowman B, Castro-Longoria E, Free SJ, Fleissner A, Freitag M, Lew RR, Mouriño-Pérez R, Plamann M, Rasmussen C, Richthammer C, Roberson RW, Sanchez-Leon E, Seiler S, Watters MK, 2011. Architecture and development of the *Neurospora crassa* hypha – a model cell for polarized growth. *Fungal Biology* **115**: 446–474.

Rodríguez A, Werning ML, Rodríguez M, Bermúdez E, Córdoba JJ, 2012. Quantitative real-time PCR method with internal amplification control to quantify cyclopiazonic acid producing molds in foods. *Food Microbiology* **32**: 397–405.

Schulze-Gronover C, Kasulke D, Tudzynski P, Tudzynski B, 2001. The role of G protein α subunit in the infection process of grey mould fungus *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **14**: 1293–1302.

Schulze-Gronover C, Schorn C, Tudzynski B, 2004. Identification of *Botrytis cinerea* genes up-regulated during infection and controlled by the Gα subunit BCG1 using suppression subtractive hybridization (SSH). *Molecular Plant-Microbe Interactions* **17**: 537–546.

Segers GC, Nuss DL, 2003. Constitutively activated Gα negatively regulates virulence, reproduction and hydrophobin gene expression in the chestnut blight fungus *Cryphonectria parasitica*. *Fungal Genetics and Biology* **38**: 198–208.

Solomon PS, Tan KC, Sanchez P, Cooper RM, Oliver RP, 2004. The disruption of a Gα subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular Plant-Microbe Interactions* **17**: 456–466.

Sprang SR, 1997. G protein mechanisms: insights from structural analysis. *Annual Review of Biochemistry* **66**: 639–678.

Studt L, Humpf H-U, Tudzynski B, 2013. Signaling governed by G proteins and cAMP is crucial for growth, secondary metabolism and sexual development in *Fusarium fujikuroi*. *PLoS ONE* **8**: e58185.

Tan KC, Heazlewood JL, Millar AH, Oliver RP, Solomon PS, 2009. Proteomic identification of extracellular proteins regulated by the Gna1 Gα subunit in *Stagonospora nodorum*. *Mycological Research* **113**: 523–531.

Tag A, Hicks J, Garifullina G, Ake Jr C, Phillips TD, Beremand M, Keller N, 2000. G protein signalling mediates differential production of toxic secondary metabolites. *Molecular Microbiology* **38**: 658–665.

Truesdell GM, Yang Z, Dickman MB, 2000. A Gα subunit gene from the phytopathogenic fungus *Colletotrichum trifolii* is required for conidial germination. *Physiological and Molecular Plant Pathology* **56**: 131–140.

Xue T, Nguyen CK, Romans A, May GS, 2004. A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in *Aspergillus fumigatus*. *Eukaryotic Cell* **3**: 557–560.

Wang NY, Lin CH, Chung KR, 2010. A G α subunit gene is essential for conidiation and potassium efflux but dispensable for pathogenicity of *Alternaria alternata* on citrus. *Current Genetics* **56**: 43–51.

Yang Q, Borkovich KA, 1999. Mutational activation of a Gαi causes uncontrolled proliferation of aerial hyphae and increased sensitivity to heat and oxidative stress in *Neurospora crassa*. *Genetics* **151**: 107–117.

Yu JH, Keller N, 2005. Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437–458.

Yu JH, Wieser J, Adams TH, 1996. The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. *EMBO Journal* **15**: 5184–5190.

Zuber S, Hynes MJ, Andrianopoulos A, 2002. G-protein signaling mediates asexual development at 25 degrees C but has no effect on yeast-like growth at 37 degrees C in the dimorphic fungus *Penicillium marneffei*. *Eukaryotic Cell* **1**: 440–447.