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

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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*.

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

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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).