The heterotrimeric G α protein Pga1 regulates biosynthesis of penicillin, chrysogenin and roquefortine in *Penicillium chrysogenum*

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We have studied the role of the *pga1* gene of *Penicillium chrysogenum*, encoding the alpha subunit of a heterotrimeric G protein, in secondary metabolite production. The dominant activating *pga1*^{G42R} mutation caused an increase in the production of the three secondary metabolites penicillin, the yellow pigment chrysogenin and the mycotoxin roquefortine, whereas the dominant inactivating *pga1*^{G203R} allele and the deletion of the *pga1* gene resulted in a decrease of the amount of produced penicillin and roquefortine. Chrysogenin is produced in solid medium as a yellow pigment, and its biosynthesis is clearly enhanced by the presence of the dominant activating *pga1*^{G42R} allele. Roquefortine is produced associated with mycelium during the first 3 days in submerged cultures, and is released to the medium afterwards; dominant activating and inactivating *pga1* mutations result in upregulation and downregulation of roquefortine biosynthesis respectively. Pga1 regulates penicillin biosynthesis by controlling expression of the penicillin biosynthetic genes; the three genes *pcbAB*, *pcbC* and *penDE* showed elevated transcript levels in transformants expressing the *pga1*^{G42R} allele, whereas in transformants with the inactivating *pga1*^{G203R} allele and in the *pga1*-deleted mutant their transcript levels were lower than those in the parental strains. Increase of intracellular cAMP levels had no effect on penicillin production. In summary, the dominant activating *pga1*^{G42R} allele upregulates the biosynthesis of three secondary metabolites in *Penicillium chrysogenum* to a different extent.

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INTRODUCTION

In fungi and other eukaryotic organisms, heterotrimeric GTP-binding proteins (G proteins) play an important role as signal transducers of various environmental and nutritional stimuli. They transmit the signals upon activation of different G-protein-coupled membrane receptors to a variety of intracellular effectors that control cell differentiation (Neer, 1995) and secondary metabolism (Calvo *et al.*, 2002).

G proteins consist of three subunits, α , β and γ ; they remain inactive in the heterotrimeric state with GDP bound to the α subunit, but become activated by a guanine nucleotide exchange in response to G-protein-coupled receptor

activation. When GTP is bound to the α subunit, as a result of the nucleotide exchange, this subunit dissociates from the $\beta\gamma$ dimer, and the α subunit and $\beta\gamma$ dimer then interact with downstream effectors in the signal cascade (Hamm, 1998). Certain amino acid changes in the switch region of G α subunits produce mutant subunits unable to separate from the $\beta\gamma$ dimer, resulting in constitutively inactivated G proteins (Bohm *et al.*, 1997), as occurs in the α subunit FadA^{G203R} of *Aspergillus nidulans* (Kurjan, 1992). On the other hand, mutations affecting the endogenous GTP hydrolase (GTPase) activity result in dominant activating α subunits that become permanently bound to GTP, for instance the *A. nidulans* mutant α subunit FadA^{G42R} (Yu *et al.*, 1996).

In fungi, G proteins have been implicated in mediating several processes, including differentiation and virulence (reviewed by Lengeler *et al.*, 2000; Li *et al.*, 2007).

Particularly interesting is the role of G proteins in transduction of environmental signals that control secondary metabolite biosynthesis (Tag *et al.*, 2000; Calvo *et al.*, 2002). Filamentous fungi are well-known producers of

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antibiotics, mycotoxins, pigments and other secondary metabolites (Martín, 2000; Keller *et al.*, 2005). Sequencing of the genome of several fungi, including *Aspergillus niger* (Galagan *et al.*, 2005), *Aspergillus fumigatus* (Nierman *et al.*, 2005), *Aspergillus oryzae* (Machida *et al.*, 2005) and *Penicillium chrysogenum* (M. A. van den Berg and others, unpublished) has revealed an impressive wealth of secondary metabolite gene clusters (Zhang *et al.*, 2004; Hoffmeister & Keller, 2007).

P. chrysogenum is an important filamentous fungus because of its ability to produce large amounts of penicillin (Martín, 1998; Elander, 2003). The biochemistry and molecular genetics of penicillin biosynthesis have been widely studied (reviewed by Aharonowitz *et al.*, 1992; Martín, 2000; Fierro *et al.*, 2002; Liras & Martín, 2006) and the DNA region encoding the penicillin biosynthesis genes has been fully sequenced (Fierro *et al.*, 2006; van den Berg *et al.*, 2007). The regulation of expression of the penicillin biosynthesis genes has been studied (reviewed by Brakhage, 1998; Martín, 2000), but the signal transduction cascade that connects environmental factors to penicillin gene expression remains unknown.

In addition to penicillin, *P. chrysogenum* synthesizes the yellow pigment chrysogenin and the mycotoxin roquefortine. Chrysogenin is produced by the wild-type strain *P. chrysogenum* NRRL 1951 (Asilonu *et al.*, 2000), but its synthesis has been drastically reduced by mutations in the Wis 54-1255 mutant and in all industrial strains, because it is an undesired product that hinders penicillin purification. Although the biosynthetic pathway of this polyketide-derived product is unknown, its easy spectrophotometric detection makes it a valuable marker to study regulation of secondary metabolite production.

Roquefortine is a dimethylallyltryptophan-derived metabolite known to be produced in *Penicillium roqueforti* and several other fungi (Rundberget *et al.*, 2004; Frisvad & Filtenborg, 1983; de la Campa *et al.*, 2007), and we have identified this mycotoxin in cultures of different *P. chrysogenum* strains. The gene cluster responsible for the biosynthesis of this mycotoxin is still being elucidated (A. Gómez & J. F. Martín, unpublished results). The roquefortine HPLC assay allows a reliable quantification of this mycotoxin that constitutes another interesting model for studying G-protein-mediated regulation of secondary metabolites in this fungus.

We have previously cloned the *pga1* gene of *P. chrysogenum* encoding a $G\alpha_i$ subunit of a heterotrimeric G protein (García-Rico *et al.*, 2007) and have studied the effect of dominant activating (*pga1*^{G42R}) and dominant inactivating (*pga1*^{G203R}) mutations on growth and differentiation, finding that Pga1 negatively regulates conidiation of *P. chrysogenum* mainly by a cAMP-independent mechanism (García-Rico *et al.*, 2008). It was, therefore, of great interest to study the effect of these opposite mutations of the *pga1* gene on the biosynthesis of different *P. chrysogenum* secondary metabolites (a β -lactam, a polyketide and a

dimethylallyltryptophan derivative), to gain insight into the signal transduction cascade that controls expression of the three different types of secondary metabolites in this fungus.

METHODS

Strains and plasmids. The wild-type *P. chrysogenum* NRRL 1951 and *P. chrysogenum* Wis54-1255 (ATCC 28089), a strain with increased penicillin production, were used for determination of the secondary metabolites penicillin, chrysogenin and roquefortine. Several strains with different *pga1* genetic backgrounds were used to test the function of the Pga1 α subunit in the production of the secondary metabolites; these strains were obtained by genetic manipulation of *P. chrysogenum* NRRL 1951 and Wis54-1255, and are summarized in Table 1.

Strains PgaG42R-B, PgaG42R-C and PgaG42R-D were obtained by transformation of *P. chrysogenum* NRRL 1951 with plasmid pPgaG42R, containing the dominant activating allele *pga1*^{G42R} expressed from its own promoter (García-Rico *et al.*, 2007). Strains PgaG42R-1, PgaG42R-3 and PgaG42R-4 were obtained by transformation of *P. chrysogenum* Wis54-1255 with plasmid pPgaG42R (García-Rico *et al.*, 2007), and strains GpdG42R-1, GpdG42R-3 and GpdG42R-5 were obtained by transformation of *P. chrysogenum* Wis54-1255 with plasmid pGpdG42R, containing the dominant activating allele *pga1*^{G42R} expressed from the *A. nidulans* *gpdA* promoter (García-Rico *et al.*, 2007).

Transformants of *P. chrysogenum* NRRL 1951 and *P. chrysogenum* Wis54-1255 with plasmid pJL43b1 (García-Rico *et al.*, 2007), named NRRL-control and Wis-control respectively, were used as controls in the secondary metabolite production experiments; pJL43b1 was used as vector for constructions with the different *pga1* alleles but contains no *pga1*. Fungal transformation was performed as described previously (García-Rico *et al.*, 2007).

Culture conditions for penicillin production. Cultures in flasks were performed as follows. Conidia of *P. chrysogenum* were collected from plates of Power medium (Fierro *et al.*, 1996) and inoculated at a concentration of 1×10^7 conidia ml⁻¹ in flasks with 50 ml defined inoculum medium (Casqueiro *et al.*, 1999), which were incubated in an orbital shaker at 250 r.p.m., 25 °C for 36 h. Then 8 ml of this seed culture was transferred, in duplicate, to flasks with 100 ml lactose-containing (3 %, w/v) complex production (CP) medium (Kosalkova *et al.*, 2000), and incubated at 250 r.p.m., 25 °C for 144 h. Every 24 h, samples of 5 ml were taken to determine penicillin G, pH and dry weight.

Fermentation in bioreactors at controlled pH was carried out as follows. Seed cultures were developed as indicated above in a total volume of 290 ml. The whole seed culture volume was inoculated into 3100 ml CP medium in 5 l fermenters (Biostat B, Braun), which were maintained at a constant pH of 6.8, 25 °C and stirrer speed of 350 r.p.m. for 144 h. Every 24 h, samples of 10 ml, in duplicate, were taken to determine penicillin G and dry weight, as described previously (García-Rico *et al.*, 2007).

Extraction and quantification of chrysogenin. Chrysogenin was extracted from solid cultures in CYA medium (7 days old) of the different strains of *P. chrysogenum*. The agar cultures of two plates of each strain were collected in 50 ml tubes and extracted with ethyl acetate containing 0.5 % formic acid (Nielsen & Smedsgaard, 2003).

The extraction was performed for 30 min with an ultrasonic treatment (50/60 Hz, P Selecta Ultrasons) to disrupt the agar pieces. After the extraction the organic extract was collected by centrifugation at 4000 r.p.m. for 10 min at 4 °C. The extraction procedure was repeated twice and the mixture of both extracts was dried in a vacuum evaporator

Table 1. Strains used in the present work, derived from *P. chrysogenum* NRRL 1951 or *P. chrysogenum* Wis54-1255

Strain	<i>pga1</i> genotype*	Source/reference
NRRL 1951	Wild-type <i>pga1</i>	NRRL
PgaG42R-B	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
PgaG42R-C	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
PgaG42R-D	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
GpdG203R-T	<i>pga1</i> ^{G203R} (<i>gpdA</i> promoter)	García-Rico <i>et al.</i> (2007)
AS1	<i>pga1</i> antisense RNA	García-Rico <i>et al.</i> (2007)
Wis54-1255	Wild-type <i>pga1</i>	ATCC 28089
PgaG42R-1	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
PgaG42R-3	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
PgaG42R-4	<i>pga1</i> ^{G42R} (<i>pga1</i> promoter)	This work
GpdG42R-1	<i>pga1</i> ^{G42R} (<i>gpdA</i> promoter)	This work
GpdG42R-3	<i>pga1</i> ^{G42R} (<i>gpdA</i> promoter)	This work
GpdG42R-5	<i>pga1</i> ^{G42R} (<i>gpdA</i> promoter)	This work
G203R-T	<i>pga1</i> ^{G203R} (<i>gpdA</i> promoter)	García-Rico <i>et al.</i> (2008)
Δ <i>pga1</i>	<i>pga1</i> deleted	García-Rico <i>et al.</i> (2008)

*The endogenous wild-type *pga1* gene is present along with the mutant alleles *pga1*^{G42R} (dominant activating) and *pga1*^{G203R} (dominant inactivating).

(Rota-Vapour 210, Buchi). The dry solid residue was redissolved in 1 ml methanol, centrifuged and used for spectrophotometric determination at 280 nm and 400 nm, corresponding to the UV and visible light absorbance peaks of chrysogenin (Asilonu *et al.*, 2000).

Quantification of roquefortine. Roquefortine C in liquid cultures of the different *P. chrysogenum* strains in YES medium (Scott *et al.*, 1970) was quantified by HPLC. Samples (10 ml) were taken from duplicate flasks at 24, 48, 72, 96 and 144 h of incubation, and centrifuged at 4500 g for 10 min to separate the soluble roquefortine in the supernatant from the mycelium-associated mycotoxin. The mycelium was washed twice with 0.9% NaCl, placed into Falcon tubes and extracted with 20 ml dichloromethane for 30 min with ultrasound treatment to disrupt the mycelial pellets.

The extract was evaporated under vacuum in a rotary concentrator (Rota-Vapour, Buchi). The dry residue was redissolved in 120 μ l methanol, centrifuged and used for HPLC analyses.

Chromatographic determinations of roquefortine in the mycelial extracts or in the aqueous culture supernatant were performed in a Shimadzu HPLC system consisting of an SPD-M10Avp photodiode array detector, a solvent delivery module, a system controller and an autoinjector. All analyses were carried out in a Symmetry (Waters) reverse-phase C₁₈ column (150 mm $\times$ 3.9 mm) of particle size 5 μ m. Elution of roquefortine was performed at room temperature with a mobile phase of acetonitrile/water containing 0.04% trifluoroacetic acid (flow rate 0.7 ml min⁻¹). A linear gradient, from 70:30 to 20:80 (v/v) water/acetonitrile in 25 min, was used for separation. After 5 min elution with the 20:80 mixture the eluent composition was changed to the starting conditions. A standard curve of pure roquefortine (provided by Institute Biomar, León, Spain) was made as control for the quantitative determinations.

Induction of high intracellular cAMP concentrations. High intracellular cAMP concentrations were induced in strain *P. chrysogenum* Wis54-1255 by addition of theophylline (Sigma-Aldrich) as described previously (García-Rico *et al.*, 2008). Intracellular cAMP concentrations were determined as described previously (García-Rico *et al.*, 2008).

RESULTS

Effect of the constitutive activation and constitutive inactivation of Pga1 on penicillin biosynthesis by the wild-type strain *P. chrysogenum* NRRL 1951

The production of penicillin was first compared between strains derived from *P. chrysogenum* NRRL 1951 with different *pga1* genetic backgrounds: strains PgaG42R-B, PgaG42R-C and PgaG42R-D carry the *pga1*^{G42R} (dominant activating) allele expressed from its own *pga1* promoter; strain GpdG203R-T carries the *pga1*^{G203R} (dominant inactivating) allele expressed from the *gpdA* promoter; and strain AS-1 expresses a *pga1* antisense RNA for attenuation of *pga1* transcription (García-Rico *et al.*, 2007). As controls, the wild-type strain NRRL 1951 and a transformant with the plasmid vector without *pga1* alleles (NRRL-control) were used.

All transformants expressing the constitutively activated Pga1^{G42R} α subunit produced clearly higher levels of penicillin (200–260% with respect to the control); the increased production was observed from 72 h to 120 h of fermentation (Fig. 1a). Transformants with the constitutively inactivated Pga1^{G203R} α subunit showed production levels similar to those of the wild-type strain, and the same result was observed in strain AS-1 expressing a *pga1* antisense RNA. These two strains cause an increase in the pH of the medium (Fig. 1a lower-right panel), an effect also observed in *pga1*-inactivated derivatives of strain Wis54-1255 (Fig. 2c), whose implications will be analysed in the following sections.

The stimulatory effect of the *pga1*^{G42R} mutation on penicillin production correlated with increased expression

Fig. 1. (a) Specific production of penicillin and pH changes during shaking flask cultures of strains derived from *P. chrysogenum* NRRL 1951 with different *pga1* genetic backgrounds. Left panels: cultures of the parental strain NRRL 1951, NRRL-control (transformed with the vector without insert; see Methods) and three different transformants carrying the dominant activating mutation *pga1*^{G42R}. Right panels, cultures of the parental and control strains, the transformant carrying the dominant inactivating *pga1*^{G203R} allele (GpdG203R-T) and the *pga1* antisense attenuated AS-1 strain. Note the large increase in penicillin yield of the three *pga1*^{G42R} transformants. (b) Effect of changes in the *pga1* gene on expression of the penicillin biosynthesis genes *pcbC* and *penDE*. The blots were hybridized with a 0.98 kb *NcoI* probe containing the ORF of the *pcbC* gene, a 1.0 kb *XmnI*-*EcoRI* probe internal to the *penDE* gene, and, as control, a 0.5 kb probe from the *P. chrysogenum* γ -actin gene. The same amount (15 µg) of RNA was loaded in each lane. Lanes: 1, wild-type *P. chrysogenum* NRRL 1951; 2, PgaG42R-B (expressing the dominant activating *pga1*^{G42R} allele); 3, GpdG203R-T (expressing the dominant inactivating *pga1*^{G203R} allele). Note that both *pcbC* and *penDE* show increased expression in the PgaG42R-B strain at 48 h and 72 h of incubation. See Table S1 for the quantitative densitometric data.

of the *pcbC* and *penDE* genes (the second and third genes of the penicillin pathway) in the *pga1*^{G42R} transformants (Fig. 1b) (see also Supplementary Table S1, available with the online version of this paper). This increased expression was already observed in cultures after 48 h of cultivation, and was higher (about fourfold in both genes) at 72 h, coinciding with the phase of high penicillin production. In contrast, the transformant expressing the constitutively inactivated Pga1^{G203R} α subunit showed no significant differences in the expression levels of either of the genes at any time, as compared to the wild-type NRRL 1951.

Stimulatory effect of the *pga1*^{G42R} allele expressed from different promoters on penicillin biosynthesis by the improved penicillin producer strain Wis54-1255

P. chrysogenum Wis54-1255 produces significant higher levels of penicillin than the wild-type strain NRRL1951,

although it still contains a single copy of the penicillin gene cluster (Fierro *et al.*, 1995). Studies on the effect of the mutations of the G α subunit on penicillin biosynthesis were initially performed in shake flasks and later in fermenters under pH-controlled conditions, to avoid the effect of pH changes on penicillin gene expression (Gutiérrez *et al.*, 1999; Suárez & Peñalva, 1996).

Two different constructions to express the *pga1*^{G42R} allele were used in this study: transformants PgaG42R, which contain the *pga1*^{G42R} allele under the control of its own promoter, and transformants GpdG42R, in which the *pga1*^{G42R} allele was expressed from the strong constitutive promoter of the *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) gene. Three transformants with each of these promoters were studied in comparison with the parental strain Wis54-1255 and a control transformant containing the vector without *pga1* insert (Wis-control).

Fig. 2. Specific production of penicillin and pH changes during shaking flask cultures of the improved strain *P. chrysogenum* Wis54-1255 and transformants with different mutations in the *pga1* gene. The parental Wis54-1255 strain and a transformant with the pJL43b1 vector without insert (Wis-control) were used as controls. (a) Strains PgaG42R-1, PgaG42R-3 and PgaG42R-4, expressing the *pga1*^{G42R} allele from its own promoter. (b) Strains GpdG42R-1, GpdG42R-3 and GpdG42R-5, expressing the *pga1*^{G42R} allele from the constitutive *gpdA* promoter. (c) Strains Δ *pga1* (*pga1* deleted) and G203R-T, expressing the *pga1*^{G203R} allele from the *gpdA* promoter. Note that all strains bearing the *pga1*^{G42R} mutant allele expressed from either the *pga1* or the *gpdA* promoters overproduce penicillin.

Results from shaking flask cultures showed that the six transformants containing the *pga1*^{G42R} allele produced increased levels of penicillin at 72–120 h of incubation, 50–60 % higher than the untransformed Wis 54-1255 strain (Fig. 2a, b). The stimulatory effect was similar in both constructions with the *gpdA* or *pga1* promoters, although the accumulation of penicillin was faster at early times (24–48 h) in the constructions with the *gpdA* promoter (Fig. 2b).

The lower relative increase in penicillin production in the *P. chrysogenum* Wis54-1255-derived transformants as compared with transformants of the wild-type NRRL 1951 (200–260 %; Fig. 1a) suggests that the increase in production in the improved Wis54-1255 strain may be due to mutations introduced during the strain improvement programme that result in a positive effect in the activity of the G-protein-controlled cascade; therefore, the influence of exogenous copies of *pga1*^{G42R} is lower in the higher penicillin producer Wis 54-1255.

Transcriptional studies of the *pcbC* and *penDE* genes in the Wis54-1255 transformants showed that there is a clear

increase in the expression of both *pcbC* and *penDE* in the transformants carrying the *pga1*^{G42R} allele expressed from either the *pga1* or the *gpdA* promoters (transformant PgaG42R-1 is shown in Fig. 3, lanes 2), ranging from about 1.6-fold at 24 h to about 2.2-fold at 72 h of culture in both genes, as observed by densitometric analysis of the films (Supplementary Table S2). These results support the conclusions on the positive effect of constitutively activated G protein on penicillin gene expression obtained with the wild-type strain NRRL 1951.

The *pga1*^{G42R} allele increases penicillin production in pH-controlled fermenter cultures

Differences greater than 0.7 units in the pH values occurred in shake flask cultures between the parental Wis54-1255 and control strains on the one side (pH values below 6.5) and strains G203R-T and Δ *pga1* on the other side (pH values above 7.0) during the first 72 h of fermentation in shake flask cultures (Fig. 2c, lower panel).

Fig. 3. Effect of different mutations of *pga1* on expression of the *pcbC* and *penDE* genes in shaking flask cultures. Lanes: 1, parental strain Wis54-1255; 2, transformant PgaG42R-1 containing the *pga1*^{G42R} allele; 3, *P. chrysogenum* Δ *pga1*. The same amount of RNA (15 μ g) was loaded in all lanes. Hybridizations were performed with the same probes as in Fig. 1(b). Expression of both *pcbC* and *penDE* genes is clearly higher in transformants with the *pga1*^{G42R} allele (lanes 2), particularly at 48 and 72 h of incubation. See Table S2 for the quantitative densitometric data.

To exclude a possible effect of pH changes on penicillin gene expression and production (Gutiérrez *et al.*, 1999; Suárez & Peñalva, 1996), experiments were performed in four identical 5 l Braun Biostat B fermenters under constant pH values (pH adjusted to 6.8).

The kinetics of penicillin biosynthesis in the fermenters showed a consistent increase in penicillin production by the PgaG42R-1 transformant as compared to the parental Wis54-1255 strain (Fig. 4a); the increase was evident after 48 h of cultivation and reached about 50 % at 96 and 120 h.

In contrast, both strain G203R-T, expressing a constitutively inactivated Pga1^{G203R} α subunit, and the Δ *pga1* strain showed a decrease of penicillin production as compared to the parental Wis54-1255 strain; the two strains produced about 65 % (48 h) and 85 % (96 h) of the amount of penicillin in the control strain Wis54-1255.

This result demonstrates that the Pga1 α subunit positively regulates penicillin production in *P. chrysogenum*, and also shows the influence of pH on the production. The fact that strains G203R-T and Δ *pga1* produce similar amounts of the antibiotic to the parental Wis54-1255 strain in shake flask cultures (Fig. 2c, upper panel) is due to the increase in the pH of the medium caused by these mutations. When pH values are kept at 6.8, the penicillin production falls to 65–85 % of the amount produced by Wis54-1255, and then the negative effect of inactivating the Pga1 α subunit becomes evident.

Pga1 controls penicillin production by regulating expression of the penicillin biosynthetic genes

Expression of the *pcbC* and *penDE* genes in the pH-controlled fermenters was clearly enhanced in the

Fig. 4. (a) Specific production of penicillin in fermenter cultures under pH-controlled conditions of strains of *P. chrysogenum* with different *pga1* genetic backgrounds. Note that the strain PgaG42R-1 containing the *pga1*^{G42R} allele overproduces penicillin, whereas the strain containing the *pga1*^{G203R} allele and the deletion mutant Δ *pga1* yielded lower penicillin concentrations. (b) Effect of modifications of *pga1* on expression of the penicillin biosynthesis genes *pcbC* and *penDE* in fermenter cultures of *P. chrysogenum* under controlled pH conditions. Lanes: 1, G203R-T; 2, Wis54-1255 parental strain; 3, PgaG42R-1; 4, Δ *pga1*. Hybridizations were performed with the same probes as in Fig. 1(b). Note the increased expression of the two genes in the Wis 54-1255 *pga1*^{G42R} transformant (lanes 3), whereas the deletion mutant Δ *pga1* (lanes 4) and the *pga1*^{G203R} transformant (lanes 1) showed reduced expression. See Table 2 for the quantitative densitometric data.

PgaG42R-1 strain (Fig. 4b, lanes 3) as compared to the parental Wis54-1255 strain throughout the fermentation, especially in the first 24–48 h (4-fold and 3.5-fold respectively at 24 h) (Table 2). Expression of the first gene (*pcbAB*) of the penicillin pathway was also clearly stimulated in the PgaG42R-1 strain (Supplementary Fig. S1), but the degradation of the large (11.5 kb) *pcbAB* transcript prevented its quantification.

In contrast, Pga1 constitutive inactivation and deletion of the *pga1* gene cause a reduction in the expression of the three penicillin biosynthetic genes, as observed in the intensity of the corresponding signals in strain G203R-T (Fig. 4b, lanes 1) and strain Δ *pga1* (Fig. 4b, lanes 4), which was confirmed by densitometric analysis of the Northern hybridization (Table 2). This decreased expression correlated with the reduction in penicillin production observed in these strains (Fig. 4a). These results show that the Pga1 α

Table 2. Quantitative densitometric analysis of the Northern blot hybridization signals of the *pcbC* and *penDE* genes in Fig. 4(b)

The data represent the quotient between the signal (absorbance units mm^{-2}) of each of the genes (*pcbC* and *penDE*) and the γ -actin gene signals, and indicate the level of expression of the genes at each time with respect to the level of γ -actin in each lane. The analysis was performed with ImageQuant TL version 2005 (Amersham Biosciences). The data are the means $\pm$ SD of three densitometric determinations.

Gene	24 h				48 h			
	G203R-T	Wis54-1255	PgaG42R-1	Δ pga1	G203R-T	Wis54-1255	PgaG42R-1	Δ pga1
<i>pcbC</i>	0.18 $\pm$ 0.04	0.33 $\pm$ 0.06	1.33 $\pm$ 0.10	0.28 $\pm$ 0.05	0.19 $\pm$ 0.03	0.43 $\pm$ 0.07	1.39 $\pm$ 0.11	0.31 $\pm$ 0.06
<i>penDE</i>	0.21 $\pm$ 0.04	0.35 $\pm$ 0.06	1.23 $\pm$ 0.12	0.19 $\pm$ 0.06	0.23 $\pm$ 0.04	0.38 $\pm$ 0.05	1.47 $\pm$ 0.13	0.24 $\pm$ 0.05
	72 h				120 h			
	G203R-T	Wis54-1255	PgaG42R-1	Δ pga1	G203R-T	Wis54-1255	PgaG42R-1	Δ pga1
<i>pcbC</i>	0.62 $\pm$ 0.08	1.11 $\pm$ 0.06	2.09 $\pm$ 0.10	0.64 $\pm$ 0.07	0.95 $\pm$ 0.08	1.40 $\pm$ 0.11	2.29 $\pm$ 0.13	1.02 $\pm$ 0.10
<i>penDE</i>	0.41 $\pm$ 0.05	1.03 $\pm$ 0.06	1.74 $\pm$ 0.08	0.38 $\pm$ 0.05	1.04 $\pm$ 0.07	1.33 $\pm$ 0.09	2.12 $\pm$ 0.12	0.97 $\pm$ 0.07

subunit controls penicillin production by regulating expression of the penicillin biosynthetic genes.

It is interesting to note that the decrease in the expression of the penicillin genes observed in strain Δ pga1 does not take place in shake flask cultures (Fig. 3, lanes 3), which is explained by the activation of penicillin gene expression caused by the increase in the pH values in this strain (Gutiérrez *et al.*, 1999; Suárez & Peñalva, 1996).

Increase of intracellular cAMP levels has no effect on penicillin production

We have previously reported that cAMP is a secondary messenger in the Pga1-mediated signalling pathway (García-Rico *et al.*, 2008). Pga1 positively regulates cAMP intracellular levels, and the conidiation process is partially regulated via cAMP. To test whether cAMP has a role in the regulation of penicillin production by Pga1, we used the phosphodiesterase inhibitor theophylline to increase intracellular cAMP concentrations.

Shake flask fermentations of strain Wis54-1255 were carried out in 10 mM theophylline-supplemented and non-supplemented CP medium. The presence of 10 mM theophylline caused an increase in the intracellular cAMP concentration of 52–64 % (Table 3). However, this increase in the cAMP concentration had no effect on penicillin production throughout the fermentation (Supplementary Fig. S2). This result suggests that the regulation of penicillin biosynthesis by Pga1 may not be mediated by cAMP.

Transformants with the *pga1*^{G42R} allele overproduce large amounts of chrysogenin

The wild-type strain *P. chrysogenum* NRRL 1951 produces the yellow pigment chrysogenin (Fig. 5a), particularly in solid cultures in CYA and Power media. The production of this pigment was higher in the strains containing the *pga1*^{G42R} allele as compared to the wild-type NRRL 1951 (Fig. 5b). These results were confirmed by extracting the pigment from solid cultures in CYA medium. The

transformant expressing the constitutively activated Pga1^{G42R} α subunit showed a twofold increase in chrysogenin content as compared to the wild-type strain. Transformants with the constitutively inactivated Pga1^{G203R} α subunit produced an amount of chrysogenin slightly higher than that of the wild-type strain (Fig. 5c). Therefore, the biosynthesis of chrysogenin is stimulated by a constitutively activated G protein, as occurs with penicillin.

P. chrysogenum Wis 54-1255 produced only a small amount of chrysogenin, as expected, and deletion of the *pga1* gene did not result in significant differences of production (Fig. 5c).

The *pga1*^{G42R} mutation stimulates roquefortine production in *P. chrysogenum*

P. chrysogenum Wis 54-1255 produced low levels of roquefortine in liquid cultures in YES medium (optimal for roquefortine biosynthesis) as compared to *P. roqueforti* (A. Gómez & J. F. Martín, unpublished results). The roquefortine produced by *P. chrysogenum* Wis 54-1255 remained mycelium-associated for 72 h and then was released into the culture medium at 96 h, coinciding with a

Table 3. Relative amount of cAMP in flask cultures of strain Wis54-1255 in the presence and absence of 10 mM theophylline

Cultures were grown on non-supplemented CP medium (Control) and on CP medium supplemented with 10 mM theophylline (Theo-1, 2 and 3). cAMP contents are expressed as a percentage of the amount in the non-supplemented culture. Standard deviations correspond to three replicates in two independent experiments.

	cAMP relative amount (%)	
	24 h	72 h
Control	100.0	100.0
Theo-1	152.3 $\pm$ 11.3	155.4 $\pm$ 9.14
Theo-2	161.1 $\pm$ 18.1	157.6 $\pm$ 14.14
Theo-3	163.5 $\pm$ 12.2	153.5 $\pm$ 13.6

Fig. 5. Effect of modification of the *pga1* gene on production of the yellow pigment chrysogenin. (a) Structure of chrysogenin. (b) Photographs of the parental *P. chrysogenum* NRRL 1951 and the PgaG42R-B and GpdG203R-T transformants on CYA medium (bottom view) after 4 days of cultivation at 28 °C. Note the yellow colour of the PgaG42R-B transformant; (c) Pigment production by *P. chrysogenum* NRRL 1951 (wild-type), PgaG42R-B and GpdG203R-T. For comparative purposes, results for strains Wis 54-1255 and its deletion mutant $\Delta pga1$ are also included; note that these two strains produce little pigment as compared to the wild-type. The formation of chrysogenin is notably increased in the PgaG42R-B transformant, expressing the *pga1*^{G42R} dominant activating allele, as compared to the parental strain. The absorbance was measured at 280 and 400 nm, corresponding to the absorbance peaks of chrysogenin (see Methods).

change in pH and other fermentation parameters. The mycelium-associated form does not appear to be intracellular, but remains adhered to the fungal cell wall.

When the production of both mycelium-associated and extracellular roquefortine was quantified in the strains containing the different *pga1* mutations (Fig. 6), it was found that the transformant expressing the *pga1*^{G42R} allele produced consistently higher levels of roquefortine

Fig. 6. Specific production of roquefortine C by strains of *P. chrysogenum* with different *pga1* genetic backgrounds: *P. chrysogenum* Wis54-1255, GpdG42R-1, G203R-T and the deletion mutant $\Delta pga1$. Mycelium-associated roquefortine is shown in the upper panel and extracellular roquefortine in the lower panel (see Methods). Note the increased extracellular roquefortine production by the GpdG42R-1 strain containing the dominant activating *pga1*^{G42R} allele, and the lower yield of the G203R-T and the $\Delta pga1$ strains (see text).

throughout the fermentation as compared to the parental strain Wis 54-1255, except at day 3, where the sum of mycelium-associated and released roquefortine showed no significant differences between the two strains. The mycelium-associated roquefortine reached a peak at 72 h and then at 96 h was released into the supernatant, achieving levels of 440 μg extracellular roquefortine per g dry weight in the *P. chrysogenum* *pga1*^{G42R} transformant as compared to 350 μg per g dry weight for the parental strain. The transformant expressing the dominant activating *pga1*^{G203R} allele and the deletion mutant $\Delta pga1$ accumulated less mycelium-associated roquefortine at 72 h than the parental strain; and the released roquefortine at 96 h and thereafter was also lower in these two strains. These results indicate that expression of the constitutively activated Pga1^{G42R} α subunit increases production and secretion of roquefortine in *P. chrysogenum*, whereas formation of the constitutively inactivated Pga1^{G203R} α subunit or deletion of the *pga1* gene leads to lower accumulation of this mycotoxin. The stimulating effect of Pga1^{G42R} on roquefortine production was lower than the

effect exerted by this mutant allele on the biosynthesis of penicillin and chrysogenin.

DISCUSSION

G α subunits homologous to Pga1 of *P. chrysogenum* have been described in several filamentous fungi (García-Rico *et al.*, 2007), but the effect of alteration of G α subunits on the biosynthesis of secondary metabolites has been described only in a few cases (Hicks *et al.*, 1997; Calvo *et al.*, 2002; Yu & Keller, 2005). The results known so far indicate that G α subunits differentially regulate secondary metabolite biosynthesis; in some cases it appears to be a positive and in others a negative regulation. In *Neurospora crassa* the expression of a constitutive active Gna-1 G α subunit caused a reduced secretion of carotenoid pigments (Yang & Borkovich, 1999), in contrast to *Penicillium marneffei*, where transformants expressing the dominant activating gasA^{G42R} allele showed an increased production of the red pigment characteristic of this species (Zuber *et al.*, 2002). Even within the same species there are examples of differential regulation of secondary metabolites by G α subunits, as happens in *A. nidulans* (Tag *et al.*, 2000) and in *Trichoderma atroviride*, in which the G α subunit Tga1 has opposite roles in regulation of the biosynthesis of different antifungal substances (Reithner *et al.*, 2005).

Some fungi produce a variety of secondary metabolites, including antibiotics, mycotoxins and pigments (Zhang *et al.*, 2004; Hoffmeister & Keller, 2007), and in most cases it is unknown if all secondary metabolites respond in the same way (positively or negatively) to regulation by G α subunits.

As shown in this article, dominant activating mutations (G^{42R}) of the *P. chrysogenum* pga1 gene led to increased production of penicillin, roquefortine and the yellow pigment chrysogenin. On the other hand, the dominant inactivating mutation pga1^{G203R} and the deletion of the pga1 gene resulted in a reduction of penicillin production and a decrease of roquefortine accumulation after the third day of fermentation, but these mutations did not abolish production of the secondary metabolites and expression of their genes. Therefore, although the dominant activating Pga1 G α subunit increases the expression of secondary metabolism genes, Pga1 is not strictly required for secondary metabolite production. Since the dominant inactivating G^{203R} mutation does not cause a reduction of chrysogenin levels, this pigment seems to be less sensitive to Pga1 regulation. Alternatively, cycling of Pga1 between GTP- and GDP-bound forms may be required to regulate chrysogenin accumulation properly. A model of the network of regulatory effects of Pga1 on conidiation (García-Rico *et al.*, 2008) and secondary metabolite biosynthesis is depicted in Fig. 7.

It is interesting that Pga1 plays a role in regulation of expression of diverse secondary metabolites such as β -lactams, polyketides and dimethylallyltryptophan-derived

Fig. 7. Network of regulatory effects of Pga1 on conidiation and secondary metabolite biosynthesis. The dissociation of the heterotrimeric G protein (α , β , γ) and the activation of the α subunit (Pga1) in response to input signals is shown in the upper part of the figure. —|, Repression; +, activation. Pga1 negatively regulates conidiation by repressing expression of genes *brlA* and *wetA*, which belong to the conidiation central regulatory pathway (García-Rico *et al.*, 2008) (PkaA, protein kinase A). On the other hand, Pga1 positively regulates penicillin production by inducing expression of *pcbAB*, *pcbC* and *penDE*. A high level of Pga1 activity (expression of the dominant activating *pga1*^{G42R} allele) also stimulates production of two other secondary metabolites: chrysogenin and roquefortine. Solid lines indicate effects demonstrated by experimental data, and dashed lines effects suggested from the results. In the *pga1*G42R dominant activating transformants the heterotrimeric G protein is always dissociated and the α subunit (Pga1) is constitutively activated. In the *pga1*^{G203R} transformants the heterotrimeric G protein remains always undissociated and inactive (no separate Pga1 is present).

metabolites. Early studies in *A. parasiticus* and *A. nidulans* indicated that FadA (homologous to Pga1) has a negative effect on sterigmatocystin formation (Hicks *et al.*, 1997). Later studies showed that FadA has a positive effect on penicillin production in *A. nidulans*, i.e. the effect of the dominant activating *fada*^{G42R} allele is opposite on aflatoxin production and penicillin biosynthesis in this fungus (Tag *et al.*, 2000). The same mutant allele in *Aspergillus flavus* caused a decrease in the production of aflatoxin and cyclopiazonic acid (Calvo *et al.*, 2002), but an increase of trichothecene production in *Fusarium sporotrichioides* (Tag *et al.*, 2000).

The reason for the differential positive or negative effect of the dominant activating G α subunit on different secondary metabolites is largely unknown and requires detailed transcriptional analysis and characterization of the different G α subunit targets. As reported in this article, in *P. chrysogenum* the dominant activating G α subunit increases the expression of the three penicillin biosynthetic genes

pcbAB, *pcbC* and *penDE*. It is unknown how the transcription-enhancing signal is transduced from the G α protein to the promoters of penicillin biosynthesis genes. In the aflatoxin cluster of *A. nidulans*, the constitutively activated FadA^{G42R} G α subunit repressed expression of the *aflR* regulatory gene (encoding a positive transcriptional activator of the aflatoxin gene cluster), thus explaining the reduction of sterigmatocystin production by a cascade mechanism (Tag *et al.*, 2000; Calvo *et al.*, 2002).

The molecular cascade involved in expression of the three penicillin biosynthesis genes reported in this work is unknown since no regulatory gene of the zinc-finger family equivalent to AflR has been found in the penicillin gene cluster. A related gene has been reported in the 56.8 kb amplified region surrounding the penicillin gene cluster (Fierro *et al.*, 2006), but it does not seem to play a relevant role in penicillin gene expression (van den Berg *et al.*, 2007). In *A. nidulans* a zinc-finger regulator is located close to the penicillin gene cluster (Fierro *et al.*, 2006), but its possible role as a signal transducer to regulate penicillin gene expression is unknown.

Pga1 regulates apical extension in solid medium (García-Rico *et al.*, 2007), conidiation (García-Rico *et al.*, 2008) and secondary metabolite production in *P. chrysogenum* (this work). cAMP is a secondary messenger in the Pga1-mediated signal transduction pathway, and its intracellular levels are clearly regulated by Pga1 (García-Rico *et al.*, 2008). However, cAMP has a very different degree of involvement in processes regulated by Pga1. While cAMP has an important role in the regulation of apical extension of *P. chrysogenum* NRRL 1951 (R. O. García-Rico *et al.*, unpublished results), it plays only a minor role in regulation of conidiation (García-Rico *et al.*, 2008), and, as shown in this work, it seems to play no relevant role in penicillin production. The cAMP-dependent protein kinase PkaA is a downstream effector of FadA and has been implicated in repression of both conidiation and sterigmatocystin production in *A. nidulans* (Shimizu & Keller, 2001), but the authors proposed the existence of an alternative cAMP/PkaA-independent pathway for the regulation of conidiation by FadA. Our data suggest that different processes regulated by Pga1 are mediated by distinct cAMP-dependent or cAMP-independent pathways in *P. chrysogenum*.

Participation of cAMP in G protein signalling is complex, and it may act at different points in distinct pathways. Two other genes encoding G protein α subunits are usually present in fungi regulating different processes; for instance in *Gibberella zeae* the GzGPA1 G α subunit, a homologue of Pga1 and FadA and belonging to subgroup I of fungal G α subunits (Bölker, 1998), controls sexual sporulation and represses toxin production, whereas GzGPA2 (belonging to subgroup III) regulates pathogenicity, and GzGPA3 (subgroup II) may participate in regulation of vegetative growth along with other subunits (Yu *et al.*, 2008). cAMP has been proposed to mediate different processes regulated

by G protein signalling; in addition to its role as downstream effector of FadA signalling in *A. nidulans* (Shimizu & Keller, 2001), the cAMP/PKA system is involved downstream of group III G α -protein signalling in processes such as cell proliferation, development, stress response, mating and virulence, and proposed to mediate regulation of germination by GanB signalling in *A. nidulans* (Chang *et al.*, 2004).

Mutations in the Δ *pga1* and G203R-T strains are not equivalent. Whereas in strain Δ *pga1* the $\beta\gamma$ dimer of the heterotrimeric G protein is present in the cell in a free form and therefore active, in strain G203R-T the $\beta\gamma$ dimer is titrated out upon binding to the mutant G α subunit and consequently rendered inactive. The $\beta\gamma$ dimer seems to play some role in conidiation, as the phenotype of these strains regarding conidiation in submerged cultures differs significantly (García-Rico *et al.*, 2008). The fact that the phenotype of both strains regarding secondary metabolite production is very similar suggests that regulation of secondary metabolism is mainly or exclusively mediated by the G α subunit.

Our results raise the possibility of targeting the G protein components for improved production of penicillin. This strategy may also serve to enhance the production of unknown secondary metabolites that are encoded by poorly expressed genes, as is the case for roquefortine in *P. chrysogenum*. Some of the clusters for secondary metabolites are considered to be silent because they are not expressed or are transcribed below detectable levels (Martín, 2000; Metsä-Ketelä *et al.*, 2004). Altering the heterotrimeric G protein signalling may be useful to uncover new bioactive secondary metabolites from fungi.

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