Microbiological Research 212–213 (2018) 67–74

Contents lists available at ScienceDirect

Microbiological Research

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

The developmental regulator Pcz1 affects the production of secondary metabolites in the filamentous fungus *Penicillium roqueforti*

Juan F. Rojas-Aedo[a,1], Carlos Gil-Durán[a,1], Alejandra Goity[b], Inmaculada Vaca[c], Gloria Levicán[a], Luis F. Larrondo[b], Renato Chávez[a,*]

[a] Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile (USACH), Chile
[b] Millennium Institute for Integrative Systems and Synthetic Biology (MIISSB), Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[c] Departamento de Química, Facultad de Ciencias, Universidad de Chile, Chile

ARTICLE INFO

Keywords:
$Zn(II)_2Cys_6$ protein
Pcz1
Secondary metabolism
Penicillium roqueforti

ABSTRACT

Penicillium roqueforti is used in the production of several kinds of ripened blue-veined cheeses. In addition, this fungus produces interesting secondary metabolites such as roquefortine C, andrastin A and mycophenolic acid. To date, there is scarce information concerning the regulation of the production of these secondary metabolites. Recently, the gene named *pcz1* (*Penicillium* C6 zinc domain protein 1) was described in *P. roqueforti*, which encodes for a $Zn(II)_2Cys_6$ protein that controls growth and developmental processes in this fungus. However, its effect on secondary metabolism is currently unknown. In this work, we have analyzed how the overexpression and down-regulation of *pcz1* affect the production of roquefortine C, andrastin A and mycophenolic acid in *P. roqueforti*. The three metabolites were drastically reduced in the *pcz1* down-regulated strains. However, when *pcz1* was overexpressed, only mycophenolic acid was overproduced while, on the contrary, levels of roquefortine C and andrastin A were diminished. Importantly, these results match the expression pattern of key genes involved in the biosynthesis of these metabolites. Taken together, our results suggest that Pcz1 plays a key role in regulating secondary metabolism in the fungus *Penicillium roqueforti*.

1. Introduction

Filamentous fungi are eukaryotic microorganisms with several interesting biological properties, among them, a great ability to synthesize secondary metabolites. These chemical compounds have ecological roles in communication, defense and as virulence factors, providing fungi competitive advantages over those microorganisms that cohabit with them in the natural environment (Keller et al., 2005; Brakhage, 2013; Spiteller, 2015; Macheleidt et al., 2016). From the applied point of view, several fungal secondary metabolites have great biotechnological importance as antibiotics, immunosupressors, industrial pigments, etc. However, other fungal secondary metabolites such as mycotoxins are toxic and potentially deleterious for humans and animals (Yu and Keller, 2005; Macheleidt et al., 2016).

Fungi belonging to the genus *Penicillium*, including the species *Penicillium roqueforti*, are among the most important producers of secondary metabolites (Nielsen et al., 2017). *P. roqueforti* is an ascomycete filamentous fungus widely distributed in nature (Filtenborg et al., 1996; Driehuis, 2013) and industrially utilized for the ripening of blue-veined cheeses, contributing to the development of their organoleptic properties. In addition, *P. roqueforti* is an active producer of several toxic and non-toxic secondary metabolites (García-Estrada and Martín, 2016), including roquefortine C, andrastin A, mycophenolic acid, PR-toxin and isofumigaclavines. In recent years, the gene clusters for the biosynthesis of these compounds in *P. roqueforti* have been identified (Kosalková et al., 2015; Del-Cid et al., 2016; Fernández-Bodega et al., 2017; Hidalgo et al., 2017; Rojas-Aedo et al., 2017), granting access to studies focused on their transcriptional regulation.

Roquefortine C is one of the main secondary metabolites of *P. roqueforti* (Kosalková et al., 2015). This alkaloid is a mycotoxin that affects several cellular processes and has been reported to be neurotoxic (Fontaine et al., 2016). On the other hand, mycophenolic acid is a metabolite with medical relevance. It is a meroterpenoid with inhibitory activity on inosine-monophosphate dehydrogenase of T and B lymphocytes, so it is used as immunosuppressant (Usleber et al., 2008). Finally, the meroterpenoid andrastin A has the ability to inhibit Ras

* Corresponding author.
E-mail address: renato.chavez@usach.cl (R. Chávez).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.micres.2018.05.005
Received 5 January 2018; Received in revised form 16 March 2018; Accepted 3 May 2018
Available online 04 May 2018
0944-5013/

Table 1
Primers designed in this work[a].

Primer name	Sequence (5′–- 3′)	Used for:
LEV-GPD-COMUN-FW[b]	GCGGATAACAATTTCACACAGGAAACAGCCTCACCACAAAAGTCAGACG	- Amplification of the promoter PgpdA for *in vivo* recombination in yeast
GPD-COMUN-RV	GGTGATGTCTGCTCAAGCGGG	
GPD-C6-START-FW[b]	AACAGCTACCCCGCTTGAGCAGACATCACCATGTCCAATGTCGATATATC	- Amplification of the *pcz1* gene for *in vivo* recombination in yeast
C6-END-RV	CTAGTTGGCGCGAATGACCA	
C6-Trp-START-FW[b]	TGTGTCGCGACTGGTCATTCGCGCCAACTAGATAGAGTAGATGCCGACCGC	- Amplification of the terminator TrpC for *in vivo* recombination in yeast
TER-Trp-LEV-COMUN-RV[b]	GTAACGCCAGGGTTTTCCCAGTCACGACGTCGAGTGGAGATGTGGAGTG	
Nde-Fle-fw[c]	AGACTCCATATGAGATTGCGACGGCGTATTGC	- Amplification of the phleomycin-resistance cassette
Nde-Fle-rv[c]	AGACTCCATATGCAAGCTTGCAAATTAAAGCC	
RoqD-qpcr-Fw	AAAGGTTGAGGAGCACTGGA	qRT-PCR experiments of *rpt* gene
RoqD-qpcr-Rv	AACTCCACCCACAACTCTCG	
RoqA-qpcr-Fw	ATCTGTGGCACGATTCATCA	qRT-PCR experiments of *rds* gene
RoqA-qpcr-Rv	CTCGACCCTGACCATTGTTT	
RoqR-qpcr-Fw	TATGCCTTCAAGGGTGGTCT	qRT-PCR experiments of *rdh* gene
RoqR-qpcr-Rv	TTGAAGTTAGCCCAGCGAGT	

[a] The primers for qRT-PCR experiments of *pcz1*, *mpaC*, *mpaG*, *mpaH*, *adrD*, *adrH*, *adrI* and β-tubulin gene were described previously by Gil-Durán et al. (2015), Del-Cid et al. (2016) and Rojas-Aedo et al. (2017), so they are not included in this Table.
[b] These primers have a 29–31 nt overlapping sequence (underlined) necessary for the recombination *in vivo* process.
[c] These primers have *Nde*I restriction sites (underlined).

farnesyl transferase, so it is a molecule with promising anticancer properties (Okamoto et al., 2013).

As mentioned above, the gene clusters for the biosynthesis of roquefortine C, mycophenolic acid and andrastin A have already been identified in *P. roqueforti* (Kosalková et al., 2015; Del-Cid et al., 2016; Rojas-Aedo et al., 2017). The biosynthetic gene cluster for roquefortine C consists of four genes and two of them (*rds* and *rpt*) have been demonstrated to participate in the biosynthesis of this mycotoxin (Kosalková et al., 2015). On the other hand, the cluster for the biosynthesis of mycophenolic acid in *P. roqueforti* is composed of seven genes, and according to functional experiments, all they would be involved in the biosynthesis of the compound (Del-Cid et al., 2016). Finally, the cluster for the biosynthesis of andrastin A is composed of ten genes, whose functional analyses suggest that they all participate in the production of this compound (Rojas-Aedo et al., 2017).

The regulation of fungal secondary metabolism is a complex process involving an intricate connection among chemical, cellular and genetics determinants (Lim and Keller, 2014; Calvo and Cary, 2015; Macheleidt et al., 2016). In particular, the regulatory mechanisms governing the biosynthesis of roquefortine C, andrastin A and mycophenolic acid in *P. roqueforti* are not fully understood. An interesting fact to note is that within the genomic clusters for the biosynthesis of these metabolites, there are no genes encoding transcriptional factors that could regulate the expression of the biosynthetic genes (Kosalková et al., 2015; Del-Cid et al., 2016; Rojas-Aedo et al., 2017), so probably their biosynthesis is under the control of wide-domain regulators.

Several aspects of the regulation of roquefortine C biosynthesis have been studied in some detail in *Penicillium* species. These studies indicate that the production of this compound is regulated by an α-subunit from a heterotrimeric G protein, the conidiation-specific protein BrlA, and Sfk1, a transmembrane protein involved in the phosphoinositide second messengers' pathway (García-Rico et al., 2008; García-Rico et al., 2009; Qin et al., 2013; Torrent et al., 2017). Regarding andrastin A and mycophenolic acid, to date only Sfk1 has been involved in the regulation of their production (Torrent et al., 2017).

Fungal development is closely related with secondary metabolites production. In this way, several regulators simultaneously affecting both processes are known. Some examples are LaeA (Bayram et al., 2008; Kosalková et al., 2009; Dagenais et al., 2010; Shaaban et al., 2010; Wiemann et al., 2010), StuA (Twumasi-Boateng et al., 2009; Sigl et al., 2010; Ipcho et al., 2010; Lysoe et al., 2011), MtfA (Ramamoorthy et al., 2013; Smith and Calvo, 2014), CsnE (Zheng et al., 2017a; Zheng et al., 2017b) and McrA (Oakley et al., 2017).

In a previous work, we characterized a novel gene with unknown function in *P. roqueforti*. This gene, named *pcz1* (*Penicillium* C6 zinc domain protein 1), encodes for a $Zn(II)_2Cys_6$ protein (Gil-Durán et al., 2015). Functional studies indicated that Pcz1 is a positive regulator of growth and conidiation, that negatively regulates conidial germination (Gil-Durán et al., 2015). Taking into account that *pcz1* regulates asexual development in *P. roqueforti*, we sought to explore whether this gene may also be regulating secondary metabolism in this fungus.

2. Materials and methods

2.1. Fungal strains

The wild-type strain *P. roqueforti* CECT 2905 (ATCC 10110) was used in this work. *P. roqueforti* strains M9 and M11, derivative from strain CECT 2905, have been previously described (Gil-Durán et al., 2015). In these strains, the *pcz1* gene was silenced using RNAi-silencing technology. *P. roqueforti* strains SE23 and SE24, overexpressing *pcz1*, were constructed in this work (see below).

2.2. Generation of P. roqueforti strains SE23 and SE24 overexpressing pcz1

To overexpress *pcz1* in *P. roqueforti*, the plasmid pSEpcz1 was constructed. In this plasmid, *pcz1* is under the control of the *Aspergillus nidulans* constitutive promoter PgpdA and TtrpA terminator. In addition, pSEpcz1 contains a phleomycin-resistance cassette for selection of *P. roqueforti* transformants. pSEpcz1 was constructed as follows: *pcz1*, the promoter PgpdA and the terminator TtrpA were amplified by PCR using suitable primers (Table 1) and *Pfu* polymerase to avoid undesirable mutations. As template for *pcz1* amplification, genomic DNA from *P. roqueforti* was used, whereas PgpdA and TtrpA were amplified using plasmid pAN7-1 (Punt et al., 1987) as template. Once obtained, the three amplicons were linked to plasmid pRS426 by *in vivo* recombination in *Saccharomyces cerevisiae* BY4741 (*ura3*-), giving rise to plasmid pRS426pcz1. For this purpose, yeasts were transformed with a mix containing the three amplicons and the plasmid pRS426 previously linearized with *Eco*RI and *Xma*I, and selected in suitable medium lacking uracil. Recombinant plasmid pRS426pcz1 was extracted from yeast transformants using standard protocols, propagated into *E. coli* DH5α cells, purified, and sequenced. Sequencing confirmed successful recombination. Finally, a phleomycin-resistance cassette was amplified from plasmid p43gdh (Cardoza et al., 1998) by PCR using suitable primers containing *Nde*I restriction sites (Table 1) and cloned into *Nde*I-digested pRS426pcz1, giving rise to pSEpcz1.

Plasmid pSEpcz1 was introduced into *P. roqueforti* CECT 2905 by

protoplast transformation. For this purpose, protoplasts were obtained, transformed with pSEpcz1and selected on Czapek-sorbitol medium containing phleomycin, exactly as described Gil-Durán et al. (2015). To obtain homokaryotic strains, conidia from colonies were transferred three times onto Czapek medium to stabilize the genotype.

2.3. RT-qPCR experiments

RNA from *P. roqueforti* was extracted as described by Gil-Durán et al. (2015) and used for qRT-PCR assays. The primers and protocols for qRT-PCR analyses of *pcz1*, *mpaC*, *mpaG*, *mpaH*, *adrD*, *adrH*, *adrI* and β-tubulin gene (control of normalization) were described elsewhere (Gil-Durán et al., 2015; Del-Cid et al., 2016; Rojas-Aedo et al., 2017). For qRT-PCR analyses of *rds*, *rpt* and *rdh*, suitable primer sets were designed (Table 1). cDNA synthesis and RT-qPCR reactions were carried out exactly as described by Rojas-Aedo et al. (2017), using a StepOne Real-Time PCR System (Applied Biosystems, USA). The data were analyzed using the ΔΔCt method and normalized to β-tubulin gene expression.

2.4. Phenotypic characterization of transformants, extraction of secondary metabolites and HPLC analyses

Apical extension rates, production of conidia, and conidial germination kinetics were measured exactly as described by Gil-Durán et al. (2015).

The production, extraction and HPLC analyses of roquefortine C, andrastin A and mycophenolic acid from *P. roqueforti* strains were done as described by Torrent et al. (2017), Rojas-Aedo et al. (2017), and Del-Cid et al. (2016), respectively.

3. Results

3.1. Overexpression of pcz1

In a previous work, we performed the RNAi-mediated gene silencing of *pcz1* in *P. roqueforti* (Gil-Durán et al., 2015). In this work, we have generated additional strains overexpressing *pcz1*. For this purpose, plasmid pSEpcz1 was introduced into *P. roqueforti* CECT 2905. After transformation, 70 phleomycin-resistant transformants were obtained. Thirty of them were randomly chosen. DNA was obtained from the thirty transformants and tested for the presence of the *pcz1*-expression cassette by PCR (Fig. S1). Five out the thirty transformants showed the expected PCR bands, so they were selected for RT-qPCR analyses. Two of these transformants (hereafter named SE23 and SE24) showed the highest *pcz1* overexpression levels (three times higher than the wild-type strain; Fig. 1a), and they were selected for further experiments.

3.2. Effect of pcz1 overexpression on the phenotype of P. roqueforti

Previously, Gil-Durán et al. (2015) reported that RNAi-mediated gene silencing of *pcz1* promoted conidial germination, decreased apical growth, and repressed conidiation. Therefore, it would be expected that strains overexpressing *pcz1* show the opposite phenotype. Our results indicate that this prediction was correct. The overexpression of *pcz1* delayed conidial germination as compared with wild-type strain (Fig. 1b). On the other hand, the transformants overexpressing *pcz1* showed increased apical extension rates in all tested media (Fig. 1c). Finally, the overexpressing strains showed a marked increase in conidia production (Fig. 1d). Thus, these results confirm the previous observations about the effects of *pcz1* on *P. roqueforti* phenotypes.

3.3. Effect of pcz1 on mycophenolic acid production in P. roqueforti

To functionally characterize the role of *pcz1* in secondary metabolism in *P. roqueforti*, we used the RNAi-silenced strains M9 and M11 previously obtained by Gil-Durán et al. (2015) and the *pcz1*-overexpressing strains SE23 and SE24 obtained in this work (see above). We measured the production of roquefortine C, andrastin A and mycophenolic acid in these strains, comparing them with the levels produced by the wild-type strain.

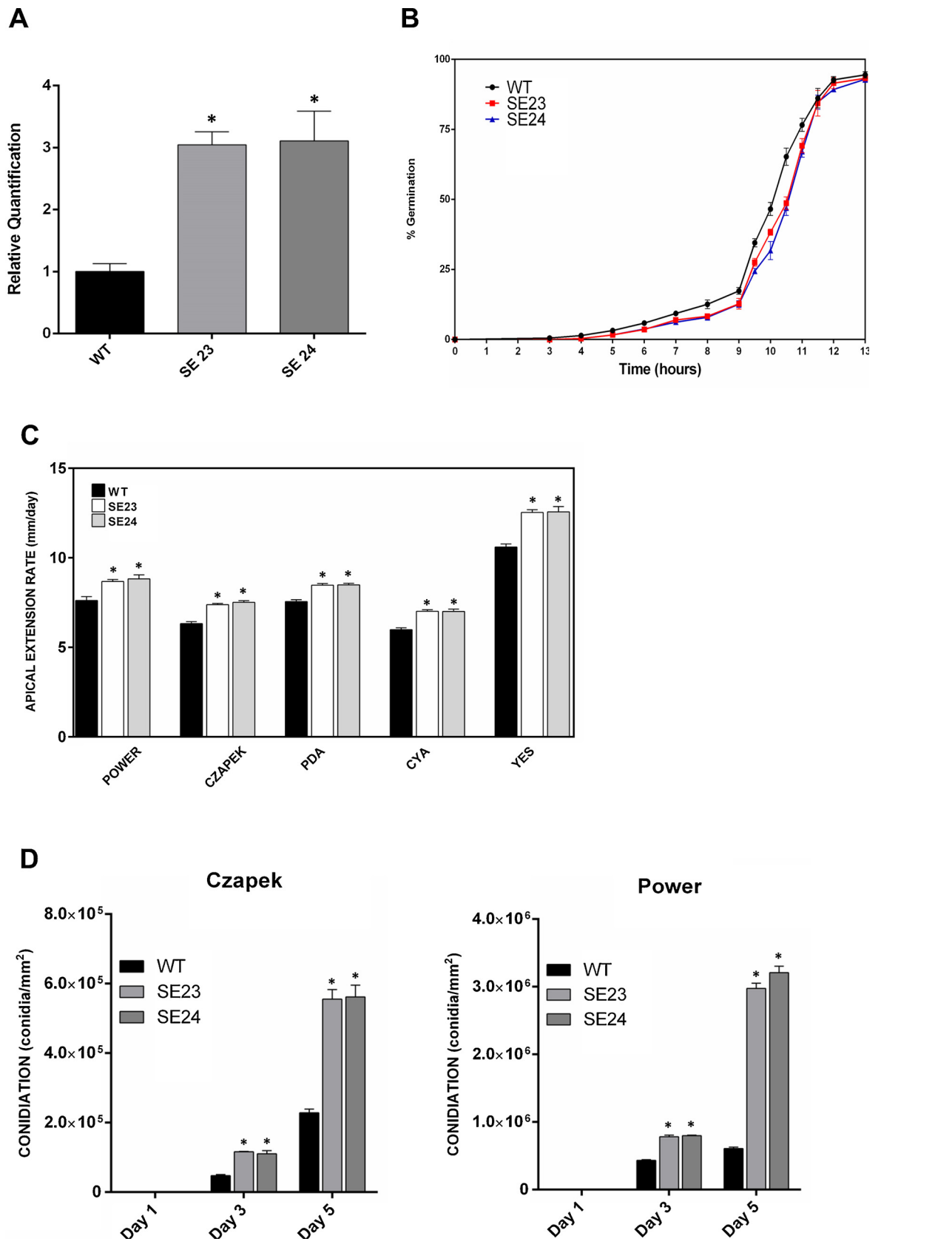
In the case of mycophenolic acid, the RNAi-mediated silencing of *pcz1* drastically decreased its production in *P. roqueforti*. As shown in Fig. 2a, *P. roqueforti* CECT 2905 (wild-type strain) produced an average of 293.5 mg of mycophenolic acid per gram of dry-mycelium, but the compound was undetectable in strains M9 and M11. Conversely, strains SE23 and SE24 overexpressing *pcz1* exhibited increased levels of mycophenolic acid, producing an average of 1323 and 1184 mg of mycophenolic acid per gram of dry-mycelium, respectively (4-4.5 times higher levels as compared to wild-type strain, Fig. 2b). These results clearly indicate that *pcz1* positively affects the production of this secondary metabolite.

To test whether *pcz1* could control the biosynthesis of mycophenolic acid at the transcriptional level, we measured the expression of three genes (namely *mpaC*, *mpaH* and *mpaG*) encoding biosynthetic enzymes from the mycophenolic acid gene cluster from *P. roqueforti*. *mpaC* encodes a polyketide synthase that catalyzes the first step in the biosynthesis of mycophenolic acid (Regueira et al., 2011; Del-Cid et al., 2016). On the other hand, *mpaH* encodes an enzyme that mediated the oxidative cleavage of a farnesylated precursor to give rise to the intermediate demethylmycophenolic acid (Zhang et al., 2015; Del-Cid et al., 2016). Finally, *mpaG* encodes an *O*-methyltransferase that catalyzes the methylation of demethylmycophenolic acid to form mycophenolic acid (Regueira et al., 2011; Del-Cid et al., 2016). As shown in Fig. 2, the levels of production of mycophenolic acid in strains with different *pcz1* backgrounds positively correlated with the expression levels of the analyzed genes. Thus, in the *pcz1* RNAi-silenced strains, the biosynthetic genes *mpaC*, *mpaH* and *mpaG* showed expression levels between 5 and 25% of the wild-type strain (Fig. 2c), while in the SE23 and SE24 strains such genes reached levels 50% higher than in wild-type strain (Fig. 2d). These results indicate that *pcz1* is required for the expression of mycophenolic acid gene cluster.

3.4. Effect of pcz1 on roquefortine C production in P. roqueforti

The RNA-mediated silencing of *pcz1* drastically decreased the production of roquefortine C (Fig. 3a). On the one hand, *P. roqueforti* CECT 2905 (wild-type strain) produced an average of 551.4 mg of roquefortine C per gram of dry-mycelium, whereas the compound was undetectable in strains M9 and M11. Remarkably, when strains overexpressing *pcz1* were analyzed, they also showed decreased levels of roquefortine C, although the effect was not so drastic as compared to RNAi-silenced transformants. Thus, strains SE23 and SE24 produced an average of 42.7 and 238.9 mg of roquefortine C per gram of dry-mycelium, respectively, representing a decrease between 2.3–12.9 times the production levels observed in the wild-type strain (Fig. 3b).

The results obtained above correlated with the expression of genes from the roquefortine C gene cluster, namely *rds*, *rdh* and *rpt*. The *rds* gene encodes a nonribosomal peptide synthetase that forms tryptophanyl-histidyl diketopiperazine from the precursors L-tryptophan and L-histidine. On the other hand, *rdh* encodes a dehydrogenase that acts on the diketopiperazine or the intermediate roquefortine D. Finally, *rpt* encodes an enzyme that prenylates roquefortine D or the reduced diketopiperazine (Kosalková et al., 2015). As shown in Fig. 3c, the levels of expression of the three biosynthetic genes in the RNAi-silenced transformants were barely detectable (2.7% or lower of the wild-type strain levels), whereas the strains overexpressing *pcz1* showed levels of the expression of the biosynthetic genes between 14 and 38% of the wild-type strain levels (Fig. 3d).



(caption on next page)

3.5. Effect of pcz1 on andrastin A production in P. roqueforti

Finally, we assessed the production of andrastin A in *P. roqueforti* strains with different *pcz1* backgrounds. We evidenced a behavior similar to the one observed for roquefortine C. *P. roqueforti* wild-type produced an average of 924.7 mg of andrastin A per gram of dry-

Fig. 1. Effect of the overexpression of *pcz1* on *P. roqueforti* phenotype. (A) qRT-PCR analysis of the expression of *pcz1* in *P. roqueforti* CECT 2905 (WT) and strains SE23 and SE24. Error bars represent the standard deviation of three replicates in three different experiments. The symbol * indicates that increases in the expression of *pcz1* in transformants SE23 and SE24 were statistically significant ($P < 0.05$ using Student's *t*-test) respect to the wild-type strain. (B) Germination kinetics of *P. roqueforti* CECT 2905 (WT) and transformants SE23 and SE24. Data are expressed as the percentage of germinated conidia vs. hours of incubation. Error bars represent the standard deviation of three replicates in three independent experiments. (C) Apical extension rates (mm/h) of *P. roqueforti* CECT 2905 (WT) and transformants SE23 and SE24. The experiments were performed in five media: Power, Czapek, PDA, CYA and YES. Error bars represent standard deviation of three replicas from three different experiments. The symbol * indicates that increases in the apical extension rates of strains SE23 and SE24 were statistically significant ($P < 0.05$ using Student's *t*-test) as compared to the wild-type strain. (D) Conidial production by *P. roqueforti* CECT 2905 and strains SE23 and SE24. The measurements were performed in poor medium Czapek (left panel) and rich medium Power (right panel) after 1, 3 and 5 days of cultivation. Error bars represent the standard deviation of three replicas from three independent experiments. The symbol * indicates that increases in conidial production of SE23 and SE24 were statistically significant ($P < 0.05$ using Student's *t*-test) as compared to the wild-type strain.

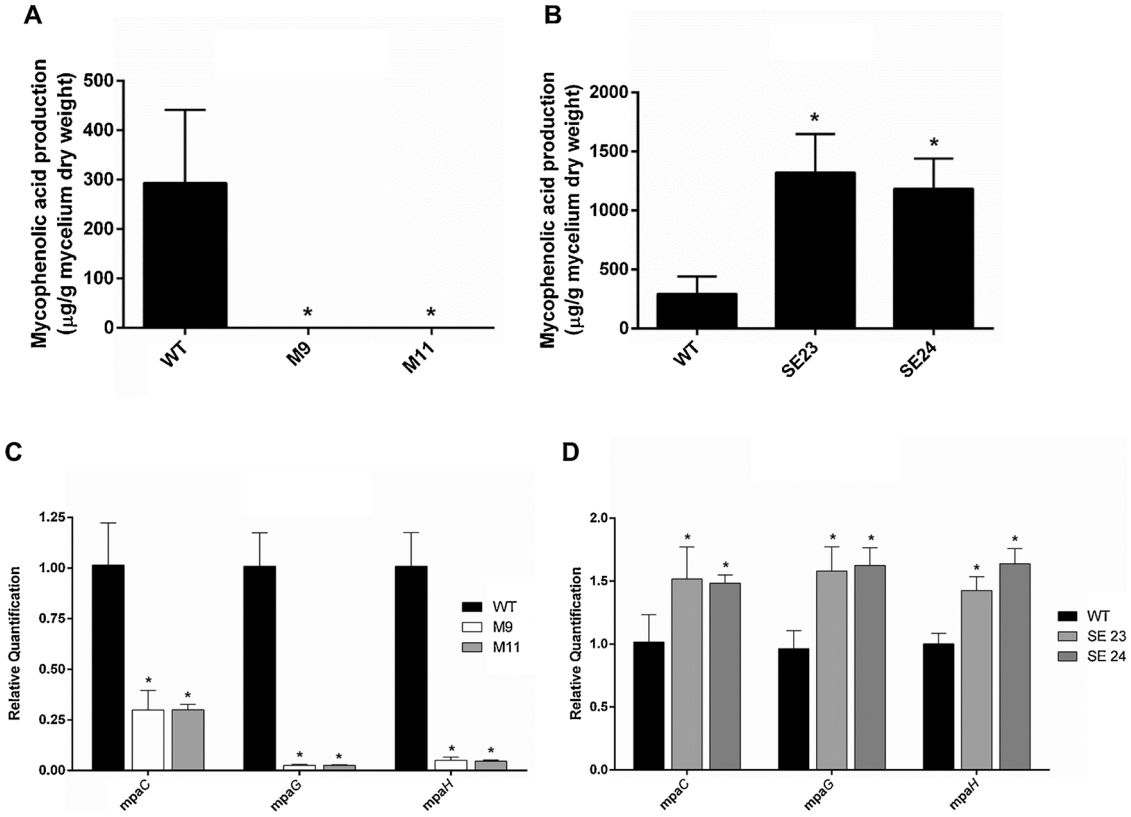


Fig. 2. Production of mycophenolic acid by *P. roqueforti* strains and analysis of the expression of mycophenolic acid biosynthetic genes. (A) Production of mycophenolic acid by *pcz1*-silenced transformants M9 and M11 in comparison with the control wild-type strain (WT). (B) Production of mycophenolic acid by strains SE23 and SE24 overexpressing *pcz1*, in comparison with the control wild-type strain (WT). (C) qRT-PCR analysis of the expression of *mpaC*, *mpaG* and *mpaH* in *pcz1*-silenced transformants M9 and M11 in comparison with the wild-type strain (WT). (D) qRT-PCR analysis of the expression of *mpaC*, *mpaG* and *mpaH* in strains SE23 and SE24 overexpressing *pcz1*, in comparison with the wild-type strain. In all cases, data are average of three replicas from three different experiments. Error bars indicate the standard deviation of the mean value. The symbol * indicates statistically significant differences of transformants ($P < 0.05$ using Student's *t*-test) as compared to the wild-type strain.

mycelium, whereas the compound was undetectable in strains M9 and M11 (Fig. 4a). In the case of strains SE23 and SE24 overexpressing *pcz1*, they produced an average of 513.9 and 531.8 mg of andrastin A per gram of dry-mycelium, respectively, representing a decrease between 1.7–1.8 times the production levels observed in the wild-type strain (Fig. 4b). These results correlated with the expression level of genes *adrD*, *adrH* and *adrI* from the andrastin A gene cluster. *adrD* encodes a polyketide synthase that synthesizes 3,5-dimethylorsellinic acid (DMOA). On the other hand, *adrH* encodes a FAD-dependent monooxygenase that forms epoxyfarnesyl-DMOA methyl ester from the precursor farnesyl-DMOA methyl ester. Finally, *adrI* encodes a terpene cyclase that catalyzes the cyclization of epoxyfarnesyl-DMOA methyl ester to produce andrastin D, the first andrastin of the pathway (Matsuda et al., 2013; Rojas-Aedo et al., 2017). As shown in Fig. 4c, *adrD*, *adrH* and *adrI* transcripts were barely detected in the RNAi-silenced transformants M9 and M11 (between 1 and 3% of wild-type strain levels). In the case of strains SE23 and SE24, the biosynthetic genes *adrD*, *adrH* and *adrI* were expressed at 43–75% the levels observed in wild-type strain (Fig. 4d).

4. Discussion

In our previous report, we analyzed the effects of down-regulating *pcz1* in *P. roqueforti* using RNAi-silencing technology (Gil-Durán et al., 2015). As a result, a decrease in apical growth rate, the promotion of conidial germination, and a strong repression of conidiation were observed. In this work and using the opposite strategy (overexpression of *pcz1*), we confirmed an important role of this *P. roqueforti* regulator in these processes. Thus, overexpression of *pcz1* produced increased apical growth, delayed conidial germination, and a marked increase in conidia formation. Taken together, these results give strong support to the key role of *pcz1* in developmental processes in *P. roqueforti*.

In addition to such developmental role, the results summarized in this work strongly support that *pcz1* has an important role in the control

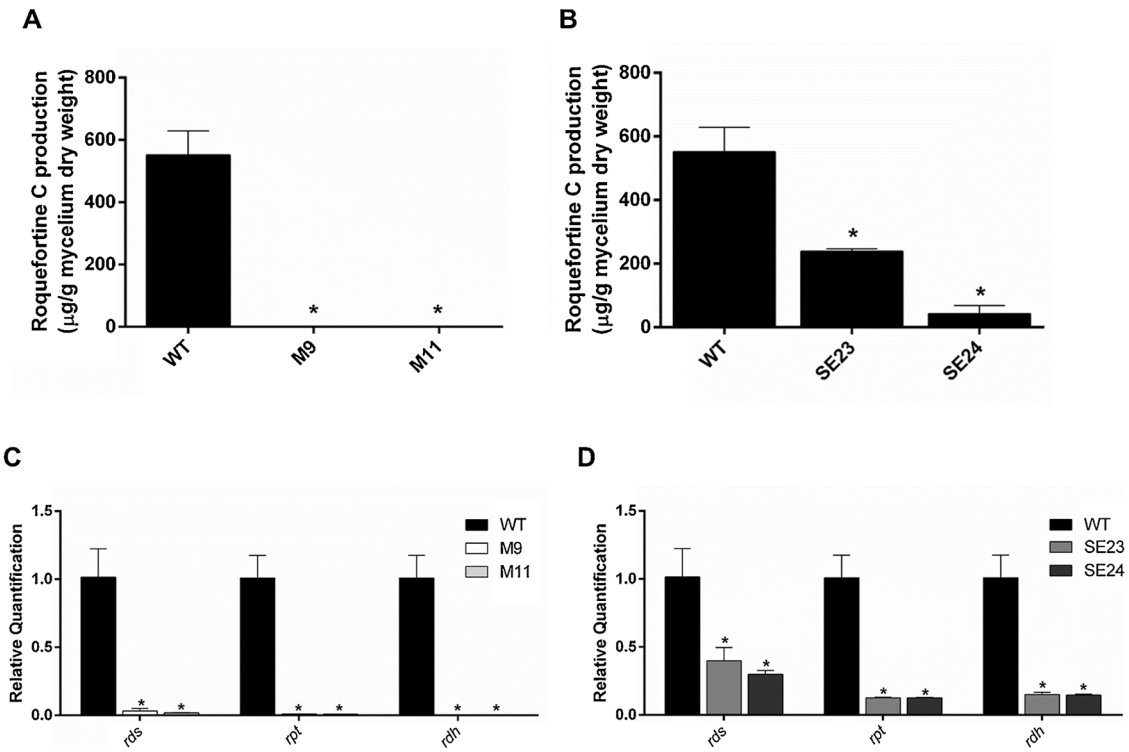


Fig. 3. Production of roquefortine C by *P. roqueforti* strains and analysis of the expression of roquefortine C biosynthetic genes. (A) Production of roquefortine C by *pcz1*-silenced transformants M9 and M11 in comparison with the control wild-type strain (WT). (B) Production of roquefortine C by strains SE23 and SE24 overexpressing *pcz1*, in comparison with the control wild-type strain (WT). (C) qRT-PCR analysis of the expression of *rds*, *rpt* and *rdh* in *pcz1*-silenced transformants M9 and M11 in comparison with the wild-type strain (WT). (D) qRT-PCR analysis of the expression of *rds*, *rpt* and *rdh* in strains SE23 and SE24 overexpressing *pcz1*, in comparison with the wild-type strain. In all cases, data are average of three replicas from three different experiments. Error bars indicate the standard deviation of the mean value. The symbol * indicates statistically significant differences of transformants ($P < 0.05$ using Student's *t*-test) as compared to the wild-type strain.

of secondary metabolism in *P. roqueforti*. In the case of mycophenolic acid, overexpression of *pcz1* increased the production of this secondary metabolite. Conversely, the RNAi mediated silencing of *pcz1* reduced mycophenolic acid production. These changes in mycophenolic acid production positively correlated with the expression levels of genes encoding enzymes involved in the biosynthesis of the compound. All together, these results support that *pcz1* is a positive regulator of the biosynthesis of mycophenolic acid in *P. roqueforti*. To the best of our knowledge, Pcz1 would be the first zinc-finger protein involved in the regulation of mycophenolic acid, so in the future, it would be very interesting to test if this regulation may involve the direct binding of Pcz1 to promoter of genes from the mycophenolic acid gene cluster, or whether there are intermediate transcriptional regulators involved.

Different to mycophenolic acid, the effect of *pcz1* on roquefortine C and andrastin A is rather complex. The RNAi mediated silencing of *pcz1* led to a drastic reduction in the production of both metabolites in *P. roqueforti*. However, the overexpression of *pcz1* did not increase the production of the metabolites and instead, it led to decreased levels of roquefortine C and andrastin A. Importantly, the expression of the biosynthetic genes was in agreement with the levels of production observed for each metabolite in the overexpressing and silenced strains. This kind of contrasting role of a regulator over fungal secondary metabolism, particularly the negative effect of the overexpression over the synthesis of secondary metabolites, has been observed before. For example, in a comparative global analysis of the secondary metabolites produced by *Trichoderma reesei* strains, Derntl et al. (2017) found a group of metabolites that tended to be less abundant in strains either lacking or overexpressing the transcription factor Xpp1. In other study, Ramamoorthy et al. (2013) analyzed the effect of the transcription factor MtfA on secondary metabolism in *A. nidulans*. The authors found that the deletion of *mtf*A blocked the production of sterigmatocystin, terrequinone A, and penicillin, as well as the expression of genes from their biosynthetic clusters. However, when *mtfA* was overexpressed, the expected opposite effect (that is, increased production) was observed only for penicillin, but not for sterimatocystin and terrequinone A, whose production decreased again. A similar result was found when the orthologue of *mtfA* in *A. flavus* was analyzed. In this case, peanuts seeds infected with *A. flavus* strains either lacking or overexpressing *mtfA*, showed a decrease in aflatoxin B1 content with respect to control strains (Zhuang et al., 2016).

The inhibitory effect of the overexpression of Pcz1 on the synthesis of roquefortine C and andrastin A in *P. roqueforti* raises an interesting question: why is the production of these secondary metabolites reduced when Pcz1 is overexpressed? A plausible hypothesis is an indirect negative control by Pcz1 of the expression of these secondary metabolite gene clusters. This mechanism has been previously observed for the regulator *mtfA* in *A. nidulans* and *A. flavus*. In these fungi, the overexpression of *mtfA* causes a decrease of *aflR* expression. AflR is the positive regulator that activates the transcription of aflatoxin and sterigmatocystin pathway genes in *Aspergillus*, so reduced levels of *aflR* transcripts result in decreased production of aflatoxin and sterigmatocystin in *A. flavus* and *A. nidulans*, respectively (Ramamoorthy et al., 2013; Zhuang et al., 2016). Since the absence of *mtfA* also produces reduced levels of aflatoxin and sterigmatocystin, it has been proposed that wild-type levels of MtfA, in a balanced stoichiometry with other present factors, are required to normal biosynthesis of secondary metabolites in *A. flavus* and *A. nidulans* (Ramamoorthy et al., 2013; Zhuang et al., 2016). According to this proposal, in our case we can hypothesize that wild-type levels of Pcz1 are required to normal production of roquefortine C and andrastin A in *P. roqueforti*. Overexpression of Pcz1 would alter normal balance of putative regulators of roquefortine C and andrastin A, resulting in decreased levels of these metabolites. It should be highlighted that to date, specific regulators (either positive or negative) of roquefortine C and andrastin A pathways

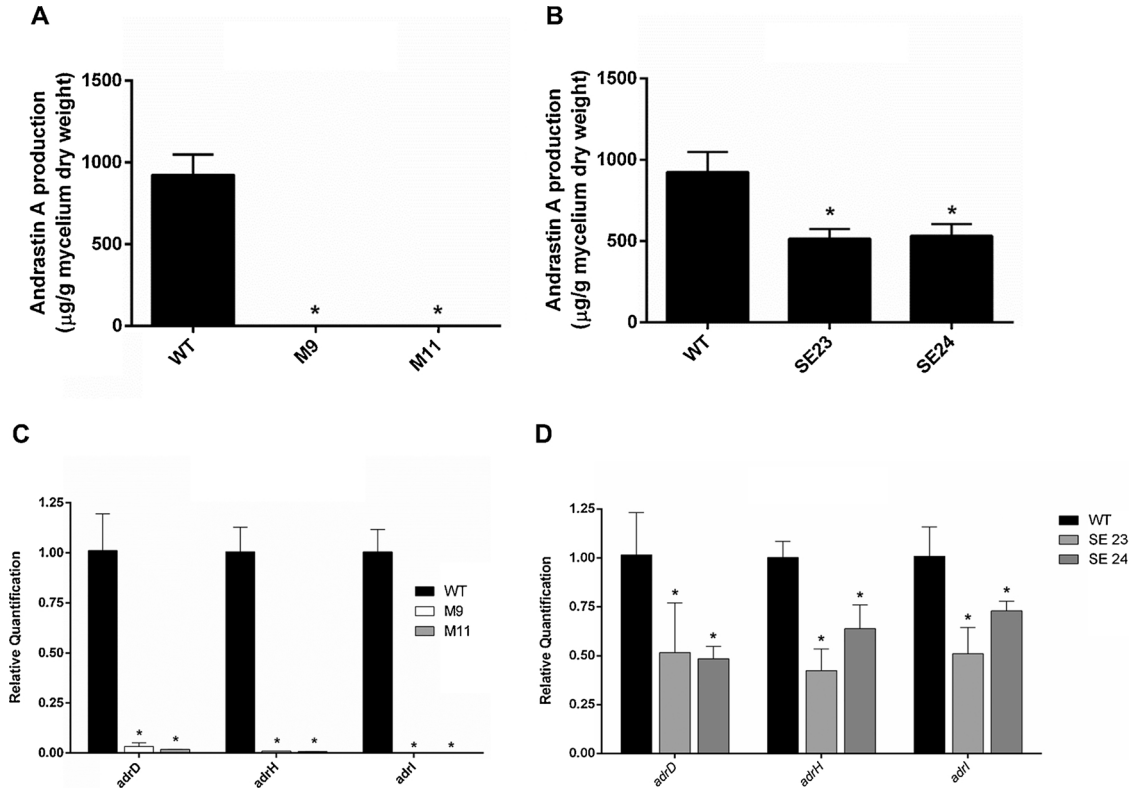


Fig. 4. Production of andrastin A by *P. roqueforti* strains and analysis of the expression of andrastin A biosynthetic genes. (A) Production of andrastin A by *pcz1*-silenced transformants M9 and M11 in comparison with the control wild-type strain (WT). (B) Production of andrastin A by strains SE23 and SE24 overexpressing *pcz1*, in comparison with the control wild-type strain (WT). (C) qRT-PCR analysis of the expression of *adrD*, *adrH* and *adrI* in *pcz1*-silenced transformants M9 and M11 in comparison with the wild-type strain (WT). (D) qRT-PCR analysis of the expression of *adrD*, *adrH* and *adrI* in strains SE23 and SE24 overexpressing *pcz1*, in comparison with the wild-type strain. In all cases, data are average of three replicas from three different experiments. Error bars indicate the standard deviation of the mean value. The symbol * indicates statistically significant differences of transformants ($P < 0.05$ using Student's *t*-test) as compared to the wild-type strain.

have not been identified in any fungus, so this hypothesis remains to be verified.

The different effects of RNAi-silencing and overexpression of *pcz1* on the production of secondary metabolites in *P. roqueforti* may also be explained by other mechanisms, such as the "competition" for the use of the sharing precursors or intermediates. It should be noticed that mycophenolic acid and andrastin A are synthesized from the same pool the precursors, namely acetyl-CoA, malonyl-CoA, and S-adenosyl methionine (Regueira et al., 2011; Matsuda et al., 2013), whereas the biosynthesis of roquefortine C requires dimethylallyl diphosphate (Kosalková et al., 2015), an isoprenoid synthesized in the mevalonate pathway using acetyl-CoA as the sole carbon source (Albermann et al., 2013). Therefore, roquefortine C, mycophenolic acid and andrastin A share acetyl-CoA as a precursor. Taking into account these facts, we can hypothesize that in *pcz1* RNAi-silenced strains, mycophenolic acid, andrastin A and roquefortine C would be reduced because their biosynthetic genes would not be fully expressed. But when *pcz1* is overexpressed, the mycophenolic acid gene cluster would be more efficiently activated. As a consequence, the common precursors would be efficiently used to produce mycophenolic acid in detriment of andrastin A and roquefortine C. The lack of precursors for andrastin A and roquefortine C makes unnecessary the full expression of their gene clusters, resulting in a low production of these compounds.

Finally, a regulatory cross-talk mechanism could also explain the differences in the production of secondary metabolites in our *P. roqueforti* strains. In fungi, cross-talk between secondary metabolism gene clusters was originally described in *A. nidulans* (Bergmann et al., 2010), and belongs to a complex process of communication among (apparently) non-related gene clusters. This process is generally observed when a secondary metabolism gene cluster is subjected to genetic manipulation and as a result, a non-related gene cluster changes its gene expression pattern. Regulatory cross-talk between secondary metabolites has also been observed in *Penicillium* species. In *P. chrysogenum*, a strain deleted in the penicillin cluster and therefore, unable to synthesize penicillin, produced increased amounts of PR-toxin (Hidalgo et al., 2014; Martín, 2017). Remarkably, previous evidence suggests that mycophenolic acid would be subjected to regulatory cross-talk in *P. roqueforti* (Hidalgo et al., 2014). In this fungus, the silencing of four genes of the unrelated PR-toxin gene cluster resulted in the overproduction of large amounts of mycophenolic acid (Hidalgo et al., 2014). Accordingly, changes in the expression levels of *pcz1* could trigger a complex network of regulatory cross-talk between the gene clusters of mycophenolic acid, andrastin A and roquefortine C and depending the case, the cross-talk would produce the overproduction or reduction in the levels of these compounds. To date, cross-talk between the biosynthetic gene clusters for mycophenolic acid, andrastin A and roquefortine C has not been demonstrated, so further experimental work is needed to determine if Pcz1 could be affecting cross-talk between these clusters in *P. roqueforti*.

Our previous work highlighted the existence of *pcz1* orthologues in a wide range of species of filamentous fungi from the phylum Ascomycota (Gil-Durán et al., 2015). In the light of the roles described herein for *P. roqueforti pcz1*, it will be interesting to address, in future research, the role of such orthologues in other fungi, exploring the conservation of transcriptional mechanisms regulating secondary metabolism and development.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by project Fondecyt 1120833 and "Proyecto DICYT, Código 021743CR, Vicerrectoría de Investigación, Desarrollo e Innovación, Universidad de Santiago de Chile", and MIISSB Iniciativa Científica Milenio-MINECON. JFR-A and CG-D have received doctoral fellowships CONICYT-PFCHA/Doctorado Nacional/2013-21130251 and CONICYT-PFCHA/Doctorado Nacional/2014-63140056, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.micres.2018.05.005.

References

Albermann, S., Linnemannstöns, P., Tudzynski, B., 2013. Strategies for strain improvement in *Fusarium fujikuroi*: overexpression and localization of key enzymes of the isoprenoid pathway and their impact on gibberellin biosynthesis. Appl. Microbiol. Biotechnol. 97, 2979–2995.

Bayram, O., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.J., Keller, N.P., Yu, J.H., Braus, G.H., 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320, 1504–1506.

Bergmann, S., Funk, A.N., Scherlach, K., Schroeckh, V., Shelest, E., Horn, U., Hertweck, C., Brakhage, A.A., 2010. Activation of a silent fungal polyketide biosynthesis pathway through regulatory cross talk with a cryptic nonribosomal peptide synthetase gene cluster. Appl. Environ. Microbiol. 76, 8143–8149.

Brakhage, A.A., 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. 11, 21–32.

Calvo, A.M., Cary, J.W., 2015. Association of fungal secondary metabolism and sclerotial biology. Front. Microbiol. 6, 62.

Cardoza, R.E., Moralejo, F.J., Gutiérrez, S., Casqueiro, J., Fierro, F., Martín, J.F., 1998. Characterization and nitrogen-source regulation at the transcriptional level of the *gdhA* gene of *Aspergillus awamori* encoding an NADP-dependent glutamate dehydrogenase. Curr. Genet. 34, 50–59.

Dagenais, T.R., Giles, S.S., Aimanianda, V., Latgé, J.P., Hull, C.M., Keller, N.P., 2010. *Aspergillus fumigatus* LaeA-mediated phagocytosis is associated with a decreased hydrophobin layer. Infect. Immun. 78, 823–829.

Del-Cid, A., Gil-Durán, C., Vaca, I., Rojas-Aedo, J.F., García-Rico, R.O., Levicán, G., Chávez, R., 2016. Identification and functional analysis of the mycophenolic acid gene cluster of *Penicillium roqueforti*. PLoS One 11, e0147047.

Derntl, C., Kluger, B., Bueschl, C., Schuhmacher, R., Mach, R.L., Mach-Aigner, A.R., 2017. Transcription factor Xpp1 is a switch between primary and secondary fungal metabolism. Proc. Natl. Acad. Sci. U. S. A. 114, E560–E569.

Driehuis, F., 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci. 22, 16–34.

Fernández-Bodega, Á., Álvarez-Álvarez, R., Liras, P., Martín, J.F., 2017. Silencing of a second dimethylallyltryptophan synthase of *Penicillium roqueforti*reveals a novel clavine alkaloid gene cluster. Appl. Microbiol. Biotechnol. 101, 6111–6121.

Filtenborg, O., Frisvad, J.C., Thrane, U., 1996. Moulds in food spoilage. Int. J. Food Microbiol. 33, 85–102.

Fontaine, K., Mounier, J., Coton, E., Hymery, N., 2016. Individual and combined effects of roquefortine C and mycophenolic acid on human monocytic and intestinal cells. World Mycotoxin J. 9, 51–61.

García-Estrada, C., Martín, J.F., 2016. Biosynthetic gene clusters for relevant secondary metabolites produced by *Penicillium roqueforti* in blue cheeses. Appl. Microbiol. Biotechnol. 100, 8303–8313.

García-Rico, R.O., Fierro, F., Mauriz, E., Gómez, A., Fernández-Bodega, M.A., Martín, J.F., 2008. The heterotrimeric Gα protein *pga1* regulates biosynthesis of penicillin, chrysogenin and roquefortine in *Penicillium chrysogenum*. Microbiology 154, 3567–3578.

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

Gil-Durán, C., Rojas-Aedo, J.F., Medina, E., Vaca, I., García-Rico, R.O., 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.

Hidalgo, P.I., Ullán, R.V., Albillos, S.M., Montero, O., Fernández-Bodega, M.Á., García-Estrada, C., Fernández-Aguado, M., Martín, J.F., 2014. Molecular characterization of the PR-toxin gene cluster in *Penicillium roqueforti* and *Penicillium chrysogenum*: cross talk of secondary metabolite pathways. Fungal Genet. Biol. 62, 11–24.

Hidalgo, P.I., Poirier, E., Ullán, R.V., Piqueras, J., Meslet-Cladière, L., Coton, E., Coton, M., 2017. *Penicillium roqueforti* PR toxin gene cluster characterization. Appl. Microbiol. Biotechnol. 101, 2043–2056.

Ipcho, S.V., Tan, K.C., Koh, G., Gummer, J., Oliver, R.P., Trengove, R.D., Solomon, P.S., 2010. The transcription factor StuA regulates central carbon metabolism, mycotoxin production, and effector gene expression in the wheat pathogen *Stagonospora nodorum*. Eukaryot. Cell 9, 1100–1108.

Keller, N.P., Turner, G., Bennett, J.W., 2005. Fungal secondary metabolism − from biochemistry to genomics. Nat. Rev. Microbiol. 3, 937–947.

Kosalková, K., García-Estrada, C., Ullán, R.V., Godio, R.P., Feltrer, R., Teijeira, F., Mauriz, E., Martín, J.F., 2009. The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie 91, 214–225.

Kosalková, K., Domínguez-Santos, R., Coton, M., Coton, E., García-Estrada, C., Liras, P., Martín, J.F., 2015. A natural short pathway synthesizes roquefortine C but not meleagrin in three different *Penicillium roqueforti* strains. Appl. Microbiol. Biotechnol. 99, 7601–7612.

Lim, F.Y., Keller, N.P., 2014. Spatial and temporal control of fungal natural product synthesis. Nat. Prod. Rep. 31, 1277–1286.

Lysoe, E., Pasquali, M., Breakspear, A., Kistler, H.C., 2011. The transcription factor FgStuAp influences spore development, pathogenicity, and secondary metabolism in *Fusarium graminearum*. Mol. Plant Microbe Interact. 24, 54–67.

Macheleidt, J., Mattern, D.J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V., Brakhage, A.A., 2016. Regulation and role of fungal secondary metabolites. Annu. Rev. Genet. 50, 371–392.

Martín, J.F., 2017. Key role of LaeA and velvet complex proteins on expression of β-lactam and PR-toxin genes in *Penicillium chrysogenum*: cross-talk regulation of secondary metabolite pathways. J. Ind. Microbiol. Biotechnol. 44, 525–535.

Matsuda, Y., Awakawa, T., Abe, I., 2013. Reconstituted biosynthesis of fungal meroterpenoid andrastin A. Tetrahedron 69, 8199–8204.

Nielsen, J.C., Grijseels, S., Prigent, S., Ji, B., Dainat, J., Nielsen, K.F., Frisvad, J.C., Workman, M., Nielsen, J., 2017. Global analysis of biosynthetic gene clusters reveals vast potential of secondary metabolite production in *Penicillium* species. Nat. Microbiol. 2, 17044.

Oakley, C.E., Ahuja, M., Sun, W.W., Entwistle, R., Akashi, T., Yaegashi, J., Guo, C.J., Cerqueira, G.C., Russo Wortman, J., Wang, C.C.C., Chiang, Y.M., Oakley, B.R., 2017. Discovery of McrA, a master regulator of *Aspergillus* secondary metabolism. Mol. Microbiol. 103, 347–365.

Okamoto, R., Takeda, K., Tokuyama, H., Ihara, M., Toyota, M., 2013. Toward the total synthesis of (±)-andrastin C. J. Org. Chem. 78, 93–103.

Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H., van den Hondel, C.A., 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56, 117–124.

Qin, Y., Bao, L., Gao, M., Chen, M., Lei, Y., Liu, G., Qu, Y., 2013. *Penicillium decumbens* BrlA extensively regulates secondary metabolism and functionally associates with the expression of cellulase genes. Appl. Microbiol. Biotechnol. 97, 10453–10467.

Ramamoorthy, V., Dhingra, S., Kincaid, A., Shantappa, S., Feng, X., Calvo, A.M., 2013. The putative C_2H_2 transcription factor MtfA is a novel regulator of secondary metabolism and morphogenesis in *Aspergillus nidulans*. PLoS One 8, e74122.

Regueira, T.B., Kildegaard, K.R., Hansen, B.G., Mortensen, U.H., Hertweck, C., Nielsen, J., 2011. Molecular basis for mycophenolic acid biosynthesis in *Penicillium brevicompactum*. Appl. Environ. Microbiol. 77, 3035–3043.

Rojas-Aedo, J.F., Gil-Durán, C., Del-Cid, A., Valdés, N., Álamos, P., Vaca, I., García-Rico, R.O., Levicán, G., Tello, M., Chávez, R., 2017. The biosynthetic gene cluster for andrastin A in *Penicillium roqueforti*. Front. Microbiol. 8, 813.

Shaaban, M.I., Bok, J.W., Lauer, C., Keller, N.P., 2010. Suppressor mutagenesis identifies a velvet complex remediator of *Aspergillus nidulans* secondary metabolism. Eukaryot. Cell. 9, 1816–1824.

Sigl, C., Haas, H., Specht, T., Pfaller, K., Kürnsteiner, H., Zadra, I., 2010. Among developmental regulators, StuA but not BrlA is essential for penicillin V production in *Penicillium chrysogenum*. Appl. Environ. Microbiol. 77, 972–982.

Smith, T.D., Calvo, A.M., 2014. The *mtfA* transcription factor gene controls morphogenesis, gliotoxin production, and virulence in the opportunistic human pathogen *Aspergillus fumigatus*. Eukaryot. Cell 13, 766–775.

Spiteller, P., 2015. Chemical ecology of fungi. Nat. Prod. Rep. 32, 971–993.

Torrent, C., Gil-Durán, C., Rojas-Aedo, J.F., Medina, E., Vaca, I., Castro, P., García-Rico, R.O., Cotoras, M., Mendoza, L., Levicán, G., Chávez, R., 2017. Role of *sfk1* gene in the filamentous fungus *Penicillium roqueforti*. Front. Microbiol. 8, 2424.

Twumasi-Boateng, K., Yu, Y., Chen, D., Gravelat, F.N., Nierman, W.C., Sheppard, D.C., 2009. Transcriptional profiling identifies a role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *Aspergillus fumigatus*. Eukaryot. Cell 8, 104–115.

Usleber, E., Dade, M., Schneider, E., Dietrich, R., Bauer, J., Märtlbauer, E., 2008. Enzyme immunoassay for mycophenolic acid in milk and cheese. J. Agric. Food Chem. 56, 6857–6862.

Wiemann, P., Brown, D.W., Kleigrewe, K., Bok, J.W., Keller, N.P., Humpf, H.U., Tudzynski, B., 2010. FfVel1 and FfLae1 components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. Mol. Microbiol. 77, 972–994.

Yu, J.H., Keller, N., 2005. Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol. 43, 437–458.

Zhang, W., Cao, S., Qiu, L., Qi, F., Li, Z., Yang, Y., Huang, S., Bai, F., Liu, C., Wan, X., Li, S., 2015. Functional characterization of MpaG', the *O*-methyltransferase involved in the biosynthesis of mycophenolic acid. Chembiochem 16, 565–569.

Zheng, Y., Wang, X., Zhang, X., Li, W., Liu, G., Wang, S., Yan, X., Zou, H., Yin, W.B., 2017a. COP9 signalosome subunit PfCsnE regulates secondary metabolism and conidial formation in *Pestalotiopsis fici*. Sci. China Life Sci. 60, 656–664.

Zheng, Y., Ma, K., Lyu, H., Huang, Y., Liu, H., Liu, L., Che, Y., Liu, X., Zou, H., Yin, W.B., 2017b. Genetic manipulation of the COP9 signalosome subunit PfCsnE leads to the discovery of pestaloficins in *Pestalotiopsis fici*. Org. Lett. 19, 4700–4703.

Zhuang, Z., Lohmar, J.M., Satterlee, T., Cary, J.W., Calvo, A.M., 2016. The master transcription factor *mtfA* governs aflatoxin production, morphological development and pathogenicity in the fungus *Aspergillus flavus*. Toxins (Basel) 8, E29.