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