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This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/1770317 since 2021-01-31T22:47:11Z

Published version:
DOI:10.1016/j.ijfoodmicro.2020.108687

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Elaborated regulation of griseofulvin biosynthesis in *Penicillium griseofulvum* and its role on conidiation and virulence

Silvia Valente \(^a\)†, Agnese Cometto \(^a\), Edoardo Piombo \(^a\), Giovanna Roberta Meloni \(^a\), Ana-Rosa Ballester \(^b\), Luis González-Candelas \(^b\)* and Davide Spadaro \(^a\)*

\(^a\) Dept. Agricultural, Forest and Food Sciences (DISAFA), Università degli Studi di Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy.

\(^b\) IATA-CSIC – Instituto de Agroquímica y Tecnología de Alimentos. Calle Catedrático Agustín Escardino 7, Paterna, 46980-Valencia, Spain.

*:Corresponding authors

E-mail addresses: davide.spadaro@unito.it, lgonzalez@iata.csic.es

**Abstract:**

*Penicillium griseofulvum*, the causal agent of apple blue mold, is able to produce in vitro and on apple a broad spectrum of secondary metabolites (SM), including patulin, roquefortine C and griseofulvin. Among them, griseofulvin is known for its antifungal and antiproliferative activity, and has received interest in many sectors, from medicine to agriculture. The biosynthesis of SM is finely regulated by filamentous fungi and can involve global regulators and pathway specific regulators, which are usually encoded by genes present in the same gene cluster as the backbone gene and tailoring enzymes. In the griseofulvin gene cluster, two putative transcription factors were previously identified, encoded by genes *gsfR1* and *gsfR2*, and their role has been investigated in the present work. Analysis of *P. griseofulvum* knockout mutants lacking either gene suggest that *gsfR2* forms part of a different pathway and *gsfR1* exhibits many spectra of action, acting as regulator of griseofulvin and patulin biosynthesis and influencing conidia production and virulence on apple. The analysis of *gsfR1*...
promoter revealed that the regulation of griseofulvin biosynthesis is also controlled by global regulators in response to many environmental stimuli, such as carbon and nitrogen. The influence of carbon and nitrogen on griseofulvin production was further investigated and verified, revealing a complex network of response and confirming the central role of *gsfR1* in many processes in *P. griseofulvum*.

**Key words:**

Transcription factor, knockout, gene cluster, regulation, patulin, apple blue mold
1. Introduction

Blue mold of pome fruit is one of the most important postharvest diseases of apple fruit. The major causal agent is Penicillium expansum, but many other Penicillium spp. can be found together causing blue mold, such as P. griseofulvum, P. aurantiogriseum, P. crustosum, P. verrucosum, P. carneum, P. commune, P. brevicipactum, P. solitum and P. puberulum (Moslem et al., 2010, 2013; Spadaro et al., 2011; Wu et al., 2019).

These species, as other Penicillium spp., can produce a wide range of secondary metabolites (SM), which are so called because they are not essential for primary metabolism and growth, but they play an essential role in the interactions, such as competition or communication with other microorganisms, and in many other cellular processes (Brakhage, 2013). Many of these SM have industrial value, such as antibiotics and antitumor compounds, while other metabolites can be toxic for humans and animals and are defined as mycotoxins.

In apples, the major concern is related to the presence of patulin, which is a mycotoxin with acute and chronic toxicity, characterized by stability in acid environment and during thermal processes, and therefore can be found in apples, apple juices and other derived products (Reddy et al., 2010).

Among causal agents of blue mold, P. griseofulvum is able to produce elevate amount of patulin, even higher compared to P. expansum, up to 4,500 µg/kg in vivo. The European residue limit for patulin is between 10 and 50 µg/kg depending on food commodities (Banani et al., 2016; Moslem et al., 2013; Spadaro et al., 2008).

P. griseofulvum can also produce other well-known mycotoxins such as roquefortine C and cyclopiazonic acid, and a well characteristic secondary metabolite called griseofulvin (Banani et al., 2016). Griseofulvin was initially recognized as an antibiotic, this antifungal compound is classified as a potential carcinogenic for humans by the International Agency on Research on Cancer (World Health Organization, 2001). Nevertheless, griseofulvin is commonly found in many medical and veterinary products to treat cutaneous dermatophyte infections, and it was recognized in 2017 as an
essential medicine by the World Health Organization for its antifungal properties (World Health Organization, 2017). Moreover, its role against cancer cells has been investigated by many researchers (Mauro et al., 2013; Rathinasamy et al., 2010). Because of its antiproliferative activity, griseofulvin and derived compounds have also been investigated for their potential use against fungal pathogens, such as Botrytis cinerea, Sclerotinia sclerotiorum, Magnaporthe grisea, Corticium sasaki, Puccinia recondita, Blumeria graminis f. sp. hordei, Alternaria solani, Fusarium solani and Colletotrichum gloeosporioides, showing efficacy both in vitro and in vivo (Bai et al., 2019; Ribeiro et al., 2018; Stierle and Stierle, 2015; Tang et al., 2015).

Due to the importance of griseofulvin in a wide range of applications, ranging from medicine to agriculture, griseofulvin biosynthetic genes were previously identified in P. aethiopicum (Chooi et al., 2010). The putative griseofulvin gene cluster consists of 13 genes, including a nonreducing polyketide synthase (PKS), tailoring enzymes and two putative transcription factors. The gene cluster was later determined in P. griseofulvum and 10 genes of the cluster were found in the same order, while genes encoding for a reductase (gsfK), an hydrolase (gsfH) and a transcription factor (gsfR2) were not located in the same genomic region (Banani et al., 2016).

The putative genes encoding the backbone enzyme (gsfA), O-methyltransferases (gsfB, gsfC, gsfD) halogenase (gsfI), dehydrogenase /reductase (gsfE) and cytochrome P450 (gsfF) were confirmed by producing deletion mutants (Cacho et al., 2013; Chooi et al., 2010), but the role of the putative transporter as well as the regulatory genes remains to be investigated.

Regulatory genes are an interesting target for mutagenesis because silencing or promoting their expression can provide significant enhancement of production of compounds with industrial value.

In this work, the regulation of griseofulvin biosynthesis was investigated by characterizing knockout mutants for the putative transcription factors encoded by gsfR1 and gsfR2 and comparing them with wild type and deletion mutants for the backbone gene of the cluster, gsfA. However, the regulation of SM biosynthesis is incredibly complex and involves not only pathway-specific regulators, which are commonly encoded by genes located inside the gene cluster, but also global regulators expressed in
response to various environmental stimuli (Brakhage, 2013). A second approach aimed to study the involvement of global regulators of the cluster was therefore investigated, particularly by evaluating the effect of carbon and nitrogen sources on *P. griseofulvum* growth and on griseofulvin biosynthesis. The findings reported here lead to a better comprehension of griseofulvin biosynthesis and its role in the growth and virulence of *P. griseofulvum*.

2. Materials and Methods

2.1. Fungal strain

*Penicillium griseofulvum* Dierckx strain PG3 was previously isolated from rotten apples in Piedmont (Spadaro et al., 2011) and grown on Potato Dextrose Agar (PDA, Merck KGaA, Darmstadt, Germany) with 50 μg/mL streptomycin (Merck) in the dark at 25 °C. *P. griseofulvum* transformed strains were grown on PDA containing 500 μg/mL of hygromycin B (ThermoFischer Scientific, Waltham, MA USA) in the same conditions. Conidial suspensions were obtained by adding 5 mL of sterile water with 0.01 % (w/v) Tween-20 and gently scraping the surface of fungal cultures grown on Petri plates. The final conidia concentration was measured using a hemocytometer and adjusted to the required concentration, depending on the type of experiment. Conidial suspension of wild-type and transformed strains were maintained in glycerol (40%) at -80 °C.

2.2 Bioinformatic analysis

The promoter of the genes *gsfR1* and *gsfA* were extracted from the genome of *P. griseofulvum* PG3 (Genbank: GCA_001561935.1), and they were scanned for transcription factor (TF) binding using the CIS-BP database (http://cisbp.ccbr.utoronto.ca/TFTools.php) with default parameters. *P. chrysogenum* was used as model species when performing the analysis. Identified *P. chrysogenum* TFs were blasted against PG3 proteome to check for their presence in *P. griseofulvum*, and they were then identified by blasting against NCBI non-redundant protein database.
2.3 Obtainment of knockout mutants

Knockout mutants were obtained as previously described (Ballester et al., 2015), by amplifying 5’ (promoter) and 3’ (terminator) flanking regions of gsfA, gsfR1 and gsfR2 genes from the genomic DNA of P. griseofulvum PG3 with specific primers pairs (O1/O2 and A3/A4, Tab. S1) containing a tail for USER cloning (Frandsen et al., 2008). Amplified fragments were mixed with pRFHU2 vector pre-digested with PacI and NtBbVCI (New England Biolabs, Ipswich, MA, USA) and treated with USER enzyme (New England Biolabs). Five µL of the USER mixture was directly used to transform chemically competent cells of Escherichia coli strain DH5α, with heat shock protocol, then bacterial cells were recovered and plated on Luria Bertani Agar (LB, Miller, Merck) supplemented with 25 µg/mL of kanamycin (ThermoFischer Scientific). Resistant transformants were screened by colony PCR and plasmid DNA from positive colonies containing both promoter and terminator was obtained using NucleoSpin® Plasmid EasyPure kit (Macherey-Nagel, Düren, Germany) following the manufacturer’s instructions. The correct integration of promoter and terminator of genes was confirmed by sequencing the amplified fragments with primers pairs RF1/RF6 and RF2/RF5 (Tab. S1). Then, 10 ng of plasmid were introduced by electroporation into electro-competent A. tumefaciens AGL1 cells and A. tumefaciens mediated transformation of P. griseofulvum was conducted as described by Buron-Moles et al. (2012). Transformants were maintained on PDA with 500 µg/mL of hygromycin B, according to the results of a sensitivity assay performed on PDA for P. griseofulvum.

2.4 DNA and RNA extraction

DNA of P. griseofulvum PG3 and knockout mutants was obtained using E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) or using the slightly modified protocol for isolation of high molecular weight DNA from fungal mycelium (Möller et al., 1992). Briefly, a conidial suspension (10^5 conidia/mL) was inoculated in 50 mL of Glucose Yeast Peptone medium (GPY; 4% w/v glucose, 0.5% w/v yeast extract and 0.5% w/v peptone) and incubated on a rotary shaker (200 rpm) at 24 °C for 2 days. Fungal mass was collected, dried and frozen in liquid nitrogen. DNA
extraction from 0.5 g of frozen mycelium was performed in 50 mL tubes containing 5 mL TES (100 mM Tris HCl, pH 8.0, 10 mM EDTA, 2% SDS), 25 μL proteinase K, and 50 μL β-mercaptoethanol. DNA concentration and purity were checked by spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, USA).

RNA extraction was performed according to Ballester and collaborators (2015) with some adjustments. First, 5 μL of a conidial suspension (10^6 conidia/mL) were inoculated on PDA with a cellophane membrane and incubated at 24 °C in the dark. One hundred mg of mycelium were collected in 2 mL tube at 5, 7 and 10 dpi and frozen in liquid nitrogen. Two tungsten beads (diameter: 2.7 mm) were added to the mycelium that was crushed using TissueLyser (Qiagen, Hilden, Germany) for 1 min at 20.00 Hz speed. Then, 750 μL of extraction buffer (100 mM Tris-HCl pH 8.0; 100 mM lithium chloride, 10 mM EDTA pH 8.0; 1% SDS; 1% PVP-40; 1% β-mercaptoethanol) and 375 μL of phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) were added, then the mixture was vortexed and incubated at 65 °C for 10 min. After cooling, 375 μL of phenol:chloroform:isoamyl alcohol were added and the extract was vortexed. The homogenate was centrifuged at 13,800 x g for 10 min and the aqueous phase was re-extracted with 750 μL of phenol:chloroform:isoamyl alcohol. Nucleic acids were precipitated by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 2 volumes of cold 100% ethanol and incubating at -20 °C for 60 min. The precipitated RNA was pelleted by centrifugation for 15 min at 13,800 x g. After washing with 500 μL of 70% ethanol, pellet was dissolved in 600 μL of TES buffer (10 mM Tris–HCl pH 8; 5 mM EDTA pH 8; 0.1% SDS) and precipitated overnight at −20 °C with 200 μL of 12 M lithium chloride. After centrifugation at 13,800 x g for 60 min, the pellet was washed with 500 μL of 70% ethanol, suspended with 250 μL of 3 M sodium acetate, pH 6.0, to remove residual polysaccharides and washed with 70% ethanol. Finally, RNA extract was dissolved in 50 μL of DEPC-water and its concentration was measured spectrophotometrically and verified by gel electrophoresis.

DNase treatment was performed using TURBO DNA-free™ Kit (Thermo Fischer Scientific). The samples were diluted 1:2 in a final volume of 50 μL and rigorous DNase treatment followed. First-
strand cDNA was conducted with High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) using 1 μg of total RNA.

2.5 PCR and qPCR

Upstream and downstream fragments of \( gsfR1 \), \( gsfR2 \) and \( gsfA \) genes were amplified by PCR with Top-Taq DNA polymerase (Bioron Diagnostics GmbH, Römerberg, Germany) and a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The cycling conditions of the PCR were 5 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 20 s at 58 °C, 90 s at 72 °C and a final extension of 5 min at 72 °C. The PCR mixture contained 1X PCR Buffer, 0.2 mM dNTPs, 0.4 μM of each primer, 0.5 U of polymerase and 10 ng of genomic DNA of \( P. griseofulvum \). Taq DNA Polymerase (Qiagen) was used to perform the other PCRs. To confirm the correct integration of T-DNA in the genome, several PCRs were conducted to amplify the hygromycin cassette and \( gsfA \), \( gsfR1 \) and \( gsfR2 \) genes. The cycling conditions of the PCRs were 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 58 °C, 90 s at 72 °C and 5 min at 72 °C.

To verify the efficiency of the DNase treatment, a PCR was performed using ITS1 and ITS4 universal primers. The cycling conditions of the PCR were 3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and 5 min at 72 °C.

RT-qPCRs were performed with StepOne™ and StepOnePlus™ Real-Time PCR System with Power SYBR™ Green PCR Master Mix (ThermoFischer Scientific); cycling conditions were 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. In order to determine the number of insertions in the genome of the transformants, the \( 2^{ΔΔCt} \) method (Pfaffl, 2001) was used using genomic DNA of samples and comparing the amplification of β-tubulin gene or histone H3, with the amplification of the promoter of the corresponding gene target of deletion.

To determine the expression of \( gsfA \) and \( gsfR1 \) genes in \( ΔgsfR1 \) mutants, cDNA was added in the reaction instead of gDNA.

All primer sequences used in the PCR and qPCR reactions are listed in Tab. S1.
2.6 Characterization of mutants in vitro

To check growth rate, conidiation and griseofulvin production, deletion and ectopic mutants were compared with wild type *P. griseofulvum* PG3 by inoculating 5 µl of spore suspension (1*10^6 conidia/mL) on PDA and incubating the plates at 25 °C in the dark. Some mutants were also inoculated on Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, 2% agar) and Meat Peptone Agar (MPA, 2.5% meat extract, 0.25% peptone, 2% agar) under the same conditions. Colony diameter (cm), number of asexual spores (conidia/plate) and griseofulvin production (µg/plate) were measured up to 10 days post inoculation (dpi). At least 5 plates were inoculated for each strain and every assay was performed three times.

2.7 Characterization of mutants in vivo

Apples ‘Golden Delicious’ were purchased from a local supermarket and stored at 4 °C until use. Fruits were surface disinfected with sodium hypochlorite (1% as chlorine), washed with deionized water and allowed to air dry before inoculation, then 21 fruits for each treatment were inoculated by wounding in three points at the equatorial region of the fruits with a sterile needle (3 mm depth, 3-4 mm wide) (Spadaro et al., 2013). Ten µl of conidial suspension (1*10^8 conidia/mL) of each strain were pipetted into each wound, while controls were inoculated with deionized water. Wounded apples were placed in plastic trays, covered with a transparent polyethylene film and stored at room temperature for 3-14 days.

2.8 Griseofulvin and patulin extraction

Griseofulvin and patulin produced *in vitro* were extracted simultaneously from the mycelium and the medium by washing the plates with 3 mL of methanol according to Banani et al. (2016) with some modifications. The plates were scraped and then placed in rotary shaken at 450 rpm for 15 min. The
liquid was then collected and centrifuged (13000 x g) at room temperature for 5 min. After that, the supernatant was filtered through a 0.45 μm syringe filter and analysed by HPLC.

Griseofulvin was extracted from rotten apples as previously described by Banani et al. (2016) with some changes. Briefly, the portion around the inoculation sites from 7 apples was collected and combined. Ten g of each sample were weighed and then placed in a centrifuge tube with 10 drops of pectinase enzyme solution (Sigma Chemical Co., St Louis, USA; 5 U/g juice) and 10 mL of water were added. The mixture was left at 38 °C for 2 hours and then centrifuged at 3,600 x g for 5 min. Five mL of clear liquid were placed into a clean tube and griseofulvin was extracted with 5 mL of ethyl acetate (three times). The organic phase was evaporated to dryness using rotary evaporator and the residual dissolved in 500 μL of MeOH:H2O (1:1, v/v) transferred into a HPLC vial for HPLC-MS/MS analysis.

2.9 Chemical analyses

To assess the production of griseofulvin and patulin on PDA, the analyses were carried out using the method previously described by Banani et al. (2016) with few modifications. A Waters HPLC system equipped with a 600 Consolider pump, a 717 plus Autosampler and a 2996 photodiode array detector were used. The analytical column used was a Kinetex 5 μm Biphenyl 100Å column (150 x 4.6 mm; Phenomenex, Torrance, CA, USA) coupled to a SecurityGuard ULTRA Cartridges UHPLC Biphenyl guard column (4.6 mm; Phenomenex). The chromatography conditions were: flow rate of 1 mL/min on a linear gradient of 5 to 95 % solvent B in 30 min followed by isocratic 95 % solvent B for another 10 min (solvent A: acid water pH 4.0 with acetic acid; solvent B: acetonitrile); the injection volume was 20 μL. The spectra were acquired between 200 nm and 600 nm wavelength, and patulin and griseofulvin quantifications was performed at 294 nm and 304 nm, respectively.

Patulin and griseofulvin were identified in the samples by comparing the retention time and UV-vis spectra with those of chemical standards.
Comparison of griseofulvin production on different growth media and *in vivo* was analyzed by liquid chromatography coupled with mass spectrometry. The HPLC-MS/MS system consisted of a binary pump and a vacuum degasser (1260 Agilent Technologies, Santa Clara, CA, USA) connected to a Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 μL loop and coupled with a Varian 310-MS TQ Mass Spectrometer. The sample was injected (10 μL) onto Luna C18(2) (150mm x 2mm i.d. 3μm, Phenomenex) and eluted under a flow of 200 μL/min. The mobile phase was an isocratic mixture of ACN:HCOOH 0.05% (60:40, v/v) for 5 min.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in positive ion mode. Griseofulvin detection and quantitation were carried out using multiple reaction monitoring (MRM) mode by selecting three transitions: m/z 353 > 285 CE 18 eV (monitoring), m/z 353 > 215 CE 16 eV (quantification) and m/z 353 > 165 CE 16 eV (monitoring). The collision gas (Ar) pressure was set at 2 mbar for all experiments.

Quantification was performed by external calibration preparing a calibration curve for each mycotoxin. The calibration curves were prepared by dilution of standard solutions of griseofulvin and patulin.

2.10 Statistical analysis

All statistical analyses were performed with one-way ANOVA followed by Duncan test using IBM SPSS statistics software version 24 (SPSS Inc., Chicago, IL, USA), *p* < 0.05 was considered significant. The REST 2009 Software (Qiagen) was used for the statistical analysis of RT-qPCR and qPCR results.

3. Results

3.1 Obtainment of knockout mutants for gsfR1 and gsfR2 and role on griseofulvin biosynthesis
The role of putative transcription factors encoded by *gsfR1* and *gsfR2* was assessed by obtaining deletion mutants and they were compared with the wild-type strain and a deletion mutant for *gsfA*, the gene encoding the PKS.

The deletion of genes was mediated by *A. tumefaciens*, which delivered a T-DNA containing a hygromycin resistance cassette flanked by the promoter and terminator of target genes. Due to the presence of these flanking regions, homologous recombination led to replacement of genes with the antibiotic resistance cassette (Figs. 1-2, S1).

The knockout mutants were then assessed by amplifying the hygromycin resistance cassette and target gene by PCR (Fig. 1 B, 2 B, S1 B). As expected, the deleted genes were not amplified in knockout mutants, while hygromycin resistance cassette was only absent in the wild type PG3. Ectopic strains had a functional copy of the target gene and the antibiotic resistance cassette, due to non-target integration into the genome of the T-DNA.

To confirm the right insertion of the cassette, PCRs with primer pairs designed on the hygromycin resistance cassette and on 3’UTR or 5’UTR of deleted gene were performed (Fig. 1 C, 2 C, S1 C). These primer pairs can only amplify if the T-DNA has been introduced by homologous recombination at the target sites in the 3’UTR and 5’UTR flanking regions. As expected, the PCR fragments were amplified in the knockout mutants, and failed amplification in the wild type PG3 and in the ectopic mutants.

The number of integrations in the genome was determined by qPCR by measuring the difference between the quantification cycle (Cq) of target and reference genes in the mutant and in the control wild-type strain (Pfaffl, 2001) (Fig. 1 D, 2 D, S1 D).

Three mutants with one single event of integration and two ectopic strains were selected for each knockout event and they were characterized *in vitro* both phenotypically and chemically.

Concerning the effect of gene deletion on phenotype, while the deletion of the polyketide synthase did not affect the phenotype (Fig. S2), knockout mutants for *gsfR1* showed the same growth rate as the wild-type strain (Fig. 3A), but exhibited colonies with a markedly less green color, caused by a
marked reduction in conidiation in vitro (Fig. 3 B, C). On the other hand, knockout gsfR2 mutants were similar to the wild type in both growth and sporulation (Fig. 5).

The ability of knockout mutants to produce griseofulvin was assessed on PDA. Mutants lacking the gsfA gene were no longer able to produce griseofulvin in vitro (Fig. S2 B), while knockout mutants for the regulator gene gsfR1 were found to produce higher quantities of griseofulvin compared to wild-type strain, with an average of about 30%, 50% and 110% higher production compared to wild-type strain at 5, 7 and 10 dpi (Fig. 4 A, Fig. 9).

The production of patulin was also investigated and deletion of gsfR1 showed to influence also the regulation of this mycotoxin, especially at 5 dpi, reaching over 3000 µg/plate in all mutants compared to 2300 µg/plate produced by P. griseofulvum PG3, which was significantly different according to Duncan’s statistical analysis (p < 0.05) (Fig. 4 B).

The knockout of gsfR2 did not affect griseofulvin or patulin production, nor conidiation or growth rate, which were comparable between wild type and knockout and ectopic mutants (Fig. 5, 6).

3.2 GsfR1 regulates negatively griseofulvin biosynthesis on PDA

The observation that the deletion of gsfR1 led to a higher griseofulvin production in vitro led us to hypothesize that this gene could encode a negative regulator of griseofulvin biosynthesis. To examine this hypothesis, the relative expression of the polyketide synthase gene of three mutants and two ectopic strains was investigated through RT-qPCR. The ΔgsfR1 deletion mutants showed an increased expression of gsfA compared to the wild type, both at 5 and 7 dpi, while ectopic mutants exhibited a similar gene expression to the wild type (Fig. 7).

3.3 Virulence and griseofulvin production on apples

To evaluate the effect of griseofulvin on the pathogenicity of P. griseofulvum, the virulence of three knockout mutants was compared with that of the wild-type strain and one ectopic mutant on apples (Fig. 8). ΔgsfA mutants, which were not able to produce griseofulvin, showed a slightly reduced
virulence (Fig. 8 A). In this assay, apples infected with E-A ectopic mutant, which contains 4 insertions of the T-DNA, led to a strong reduction of virulence on apples. However, gsfR2 mutants showed a similar virulence compared to the wild-type strain over a period of 14 days after inoculation (Fig. 8 B). On the contrary, ∆gsfR1 strains were slightly more virulent (Fig. 8 C) compared to the P. griseofulvum parental strain. Nevertheless, the ectopic mutant showed an enhanced virulence as well, although it was less virulent than the mutant in the first stages of the infections. For this reason, we cannot exclude that the enhanced virulence of the mutants was at least partly due to the transformation itself.

To check if there is a relationship between increased virulence and production of griseofulvin in situ, the mycotoxin was extracted from apples inoculated with gsfR1 mutants and quantified. Results showed a significant increased production of griseofulvin from knockout mutants at 10 dpi (Fig. 8 D), while at 14 dpi gsfR1 mutants produced less griseofulvin compared to wild-type strain.

3.4 Global regulators in griseofulvin biosynthesis

By using information on the CIS-BP database, the promoters of gsfR1 and gsfA were scanned in order to find putative binding sites for global regulators. Eighty-one and seventy-five transcription factors were predicted to bind the promoters of gsfR1 and gsfA, respectively. Thirteen transcription factors seem to bind only the promoter of gsfR1, while 7 are typical of gsfA and 68 were predicted to regulate both genes, ensuing a complex scenario (Tab. S2).

3.5 Carbon and nitrogen have a strong effect on in vitro conidiation and griseofulvin production

Among the different stimuli, the availability of nutritional sources, especially nitrogen and carbon, is a key factor for the biosynthesis of many SMs and for the growth and differentiation of filamentous fungi. Because of this, it is not surprising that we found many putative TFs involved in carbon or nitrogen consumption, which could bind the promoter of gsfR1 and gsfA (Tab. S2).
We focused our attention on the effect of carbon and nitrogen on griseofulvin production. To do that, two *gsfR1* deletion mutants and one ectopic strain were grown for ten days on three different media (PDA, MEA and MPA); one knockout mutant for the polyketide synthase gene was also included as control (denoted as KO). These three media are characterized by different C/N ratios: PDA is composed of potato extract and dextrose and has the highest carbon content, while on MEA and MPA, peptone was added, increasing the nitrogen content, which is ten times higher on MPA (thanks to the addition of meat extract) compared to MEA (Núñez et al., 2000). As expected, carbon and nitrogen showed a huge impact both on griseofulvin and conidia production (Fig. 9).

Conidiation of the wild-type strain was reduced in both media containing peptone, with a stronger reduction on MEA (Fig. 9A), and a significant reduction of griseofulvin production was observed in MPA (23%) compared to PDA or MEA (Fig. 9B).

Conidiation of the *gsfA* knockout mutant, lacking the gene encoding the polyketide synthase, was similar to that observed for the wild-type strain in the three media tested. However, the knockout mutant was not able to produce griseofulvin in any condition.

When focusing on *gsfR1* mutants, a more complex scenario was observed. Deletion of *gsfR1* gene led to a significant reduction of conidia on MEA and PDA compared to wild type, ectopic strain and *gsfA* mutants, suggesting that this gene could play a role in the direct or indirect regulation of conidia production. Considering griseofulvin production, a significant increase of griseofulvin production was observed on PDA (Fig. 4 and 9B). Instead, on the other two media, richer in nitrogen, there was a significantly reduced production compared to wild type.

4. Discussion

4.1 Role of transcription factors *GsfR1* and *GsfR2* on griseofulvin biosynthesis

The griseofulvin gene cluster previously identified in *P. aethiopicum* includes two genes, *gsfR1* and *gsfR2*, which encode for putative transcription factors. The *gsfR2* gene in *P. griseofulvum* was located in a separated genomic region of the biosynthetic gene cluster, differently from *P. aethiopicum*. 
Previous reports demonstrated the mode in which some TFs regulate the expression of a specific gene cluster even when they are located outside the cluster itself. This is the case of both *fum2l* and *zfr1*, that are located inside and outside the gene cluster respectively, and regulate fumonisin biosynthesis in *Fusarium verticilloides* (Brown et al., 2007; Flaherty and Woloshuk, 2004). In this work, our aim was to elucidate the role of putative transcription factors in griseofulvin biosynthesis by generating deletion mutants. Deletion mutants for the *gsfA* core biosynthetic gene encoding a polyketide synthase lacking the ability to produce griseofulvin were obtained and used as control (Chooi et al., 2010).

Our results show that the *gsfR1* gene encodes a putative transcription factor that not only acts on griseofulvin biosynthesis but also plays an essential role as an important regulator of *P. griseofulvum* development and secondary metabolism. GsfR1 seems to regulate griseofulvin biosynthesis as a negative regulator of the cluster when tested *in vitro* on PDA medium and on apples. Indeed, the deletion of this regulatory gene led to an increase of griseofulvin production in these conditions and a higher expression of the *gsfA* gene *in vitro*. Conversely, on media supplemented with peptone, *gsfR1* gene positively regulates the griseofulvin biosynthesis, as all mutant strains were able to produce significantly less amount of the compound compared to wild type. Similar results were obtained studying the *mtfA* gene encoding the TF of aflatoxin in *Aspergillus flavus* and *A. parasiticus*, revealing that the regulatory activity of this class of enzymes is largely dependent on environmental conditions (Zhuang et al., 2016).

A differential regulation of patulin production was also revealed in knockout mutants of *gsfR1*, which displayed increased patulin production at 5 dpi *in vitro*. This result suggests that *gsfR1* could be involved in regulation of other SMs produced by *P. griseofulvum*, demonstrating a multiplicity of downstream pathways that are regulated by the same transcription factor.

A crosstalk between different SM gene clusters has been largely recognized. For instance, in *A. nidulans* the putative transcription factor encoded by *scpR* controls the expression of *inpA* and *inpB*, two genes involved in the biosynthesis of a NRP-like compound, and the same TF is also involved in regulation of asperfuranone biosynthesis, a polyketide compound (Bergmann et al., 2010).
The interconnection of signals and responses is also testified by the complex pattern of TFs that can putatively bind the promoters of gsfR1 and gsfA, triggering the activation or repression of griseofulvin biosynthesis and regulating at the same time other SM gene clusters and many aspects of the biology of *P. griseofulvum*, such as conidiation and virulence. Surprisingly, in the recently released genome of griseofulvin producer *Xylaria flabelliformis* (Mead et al., 2019) the gene gsfR1 was missing, pointing to a possible regulation of griseofulvin biosynthesis independently of gsfR1 action.

Considering the second putative transcription factor of the cluster, the deletion of gsfR2 seems to have no effect on the parameters of *P. griseofulvum* considered, and it is likely that the gene is involved in a different biosynthetic pathway. Indeed, the gene gsfK encoding for a putative reductase is located next to gsfR2, and its deletion in *P. aethiopicum* did not affect the production of griseofulvin (Cacho et al., 2013). These findings led to the assumption that both gsfK and gsfR2 are probably involved in the biosynthesis of another SM. Moreover, in *X. flabelliformis* (Mead et al., 2019), gsfJ and gsfG genes were missing, suggesting that these two genes are not necessary for griseofulvin production.

Therefore, considering also that the knockout of gsfR2 does not impair griseofulvin production, we suggest that the core genes for the biosynthesis of this metabolite are only 7 instead of 13.

### 4.2 Role of global regulators in griseofulvin biosynthesis

Secondary metabolism gene clusters are usually regulated by several global regulatory proteins in response to many environmental stimuli, such as light, carbon and nitrogen levels, pH and redox status, iron concentration and signaling from other organisms (Brakhage, 2013; Hoffmeister and Keller, 2007). Many of these global regulators are well characterized and their putative binding sites in the promoter regions of genes could be detected.

One of the transcription factors predicted to regulate gsfR1 and not gsfA is KXG54396.1, a *P. griseofulvum* gene similar to mtfA, whose deletion or overexpression in *A. flavus* cause a reduction of aflatoxins production (Zhuang et al., 2016). Environmental conditions influence the regulatory
activity of the gene in *A. flavus*, where the deletion increases conidiation *in vitro* and reduces it *in vivo* (Zhuang et al., 2016).

Other transcription factors putatively binding *gsfR1* and not *gsfA* included NsdD, which is necessary for sexual development and represses asexual one (Han et al., 2001), a quinic acid utilization activator, an homolog of the fluconazole resistance protein and *stuA*, a gene able to regulate cell pattern formation (Miller et al., 1992), carbon metabolism, effector expression and the synthesis of penicillin and several mycotoxins (IpCho et al., 2010; Sigl et al., 2011; Yang et al., 2018). Another transcription factor, KXG45700.1, was similar to LreA, a protein involved in conidiation and response to light (Igbalajobi et al., 2019), while KXG46600.1 was similar to Res2, necessary for meiosis (Zhu et al., 1997). KXG47124.1 has 60% identity with the transcription factor Msn2 of *A. parasiticus*, involved in conidiation, hyphal growth and on occasionally stress response in several fungi (Liu et al., 2013; Song et al., 2018; Tian et al., 2017). KXG48399.1 is homologous to another transcription factor *flbC* related to asexual development, whose knock-out causes delayed conidiation in *A. nidulans* (Kwon et al., 2010). The *gsfR1* promoter was predicted to be bound also by homologs of PAP1, SebA and NapA, transcription factors involved in oxidative stress response (Asano et al., 2007; Dinamarco et al., 2012; Ikner and Shiozaki, 2005). The presence of the binding sites for this group of transcription factors suggest a complex regulation of *gsfR1*, that is markedly influenced by sexual and asexual development and environmental conditions.

The promoter of *gsfA* had only 7 unique binding sites for transcription factor, including SreP, a repressor of siderophore biosynthesis in presence of high iron concentrations (Haas et al., 1997). Other transcription factors putatively regulating *gsfA* included two regulators of filamentous growth, a transcriptional activator of gluconeogenesis, a pathway necessary in *A. nidulans* for growing with certain carbon sources metabolized via Acetyl-CoA (Hynes et al., 2007), and a homolog of *gal4*, necessary in *Saccharomyces cerevisiae* for growth using galactose or lactose as only carbon sources (Riley et al., 1987). This data seems to suggest an increase in *gsfA* expression, and therefore
griseofulvin production, in the presence of difficult carbon sources, but further experiments would be required to check this possibility.

Several transcription factors binding the promoters of both gsfRI and gsfA have not been characterized yet. However, among them, there were homologs of the well-known global regulators AreA and CreA, which are respectively a positive and a negative transcription factor acting in response to nitrogen or carbon (Katz et al., 2008; Wilson and Arst, 1998). Moreover, the binding sites of NirA and the carbon catabolite repressor MIG1 were also found in the promoters of both genes, increasing the possibilities of production of griseofulvin being heavily influenced by carbon and nitrogen availability (Burger et al., 1991; Randhawa et al., 2018). Binding sites for genes regulating the utilization of different carbon and nitrogen sources are also present. These include: facB, regulating acetate utilization (Todd et al., 1998), argR, regulating arginine metabolism (Dubois et al., 1987), nirA, regulating nitrate utilization (Burger et al., 1991), amdR, regulating amides, omega amino acids and lactams catabolism (Andrianopoulos and Hynes, 1990), and the quinic acid utilization activator (Wheeler et al., 1996). Other two transcription factors, the cutinase transcription factor 1 beta and SKN7, respectively increase the expression of cutinases and the resistance to ROS respectively, suggesting a role of the griseofulvin cluster in pathogenesis (Cao et al., 2009), as shown in the pathogenicity trials.

In addition to those indicated above, other identified transcription factors were related to sexual and asexual development, heat shock, stress resistance, cellobiose response, sulphur catabolism and assimilation, iron homeostasis and adhesion, and hyphal growth (Tab. S2).

Both promoters of gsfRI and gsfA also contain binding sites similar to those recognized by Apa-2 (Chang et al., 1993), strengthening the hypothesis that the griseofulvin regulatory system is somewhat similar to the aflatoxins one, regulated by MtfA.

4.3 Global regulation of griseofulvin biosynthesis is triggered by carbon and nitrogen
Nitrogen is a central element for the life of living organisms, and many specific regulatory genes are expressed when nitrogen sources such as ammonium, glutamate or glutamine are scarce. These genes encode transcription factors that repress the utilizations nitrogen sources that are easy to assimilate, in the so-called mechanism “Nitrogen Metabolism Repression” (NMR). All these genes are responsible for the activation of pathways required for the uptake of alternative nitrogen sources (Marzluf, 1997; Tudzynski, 2014). AreA is one of the main enzymes involved in NMR, and putatively regulate griseofulvin gene cluster, acting both on gsfR1 and gsfA. Similarly, the global repressor in response to carbon, CreA, was found to be putatively involved in the regulation of griseofulvin biosynthesis, together with transcription factors that modulate the utilization of different carbon sources.

The effect of carbon and nitrogen sources on growth, conidiation and griseofulvin biosynthesis was investigated in vitro, inoculating the strains in three different media, characterized by different C/N ratios. Núñez and collaborators (2000) used MEA and MPA to evaluate the effect of carbon and nitrogen on the production of verrucosidin, showing that MEA induce a higher production of this compound and that the type of growth medium had significant influence on mycotoxin production. The wild type showed a similar pattern of griseofulvin production on PDA and MEA, while in MPA a significant reduction was observed. This result is reasonable because carbon is essential for building the polyketide structure.

On the other hand, the deletion of gsfR1 seems to have a differential effect on MEA and MPA. In contrast to what observed on PDA plates, the production of griseofulvin drops drastically in deletants compared to wild type. The high nitrogen concentration of these media likely triggered the NMR, resulting in the activation of SM gene clusters. This could explain the higher production of griseofulvin in the wild type compared to gsfR1 deletion mutants. This result also indicates that GsfR1 can regulate griseofulvin biosynthesis both in a positive and negative manner, depending on external stimuli.
Fungal development and secondary metabolism are deeply connected and many examples have elucidated that some compounds (especially mycotoxins) can be secreted at the time of conidiation, while other SM such as linoleic-acid in 
*A. nidulans* or zearalenone in *Fusarium graminearum* can induce sporulation, and some of them are required for formation of sexual ad asexual spores and for their survival (e.g. pigments) (Calvo et al., 2002). Knockout mutants for *gsfR1* produce less conidia compared to the parental strain, but this behavior is not surprising since the deletion of regulatory genes is often associated with a reduced conidiation, such as for deletion of *aflR* TF for aflatoxin biosynthesis (Wilkinson et al., 2004). The reduced conidiation at first was thought to be related with the antifungal properties of griseofulvin, which is highly produced on PDA from knockout mutants. From this perspective, the role of *gsfR1* is to avoid an excessive production of griseofulvin, which is known to have an antifungal activity and could have toxic effects on the producer. Nevertheless, a strongly reduced production of conidia was observed in MEA and MPA, where the mutants exhibit a similar and lower griseofulvin production, respectively, compared to production on PDA. Consequently, the reduced conidiation of *gsfR1* mutants cannot be linked with the antifungal activity of griseofulvin, but instead seems to be the result of both the influence of the culture media and the effect of deletion of *gsfR1* itself.

Concerning the culture media, MEA and MPA media were supplemented with peptone. The malt extract added in MEA provides carbon and nitrogen content, while in MPA the meat extract contributes to richness in nitrogen. In previous studies on aflatoxin biosynthesis it was shown that simple sugars support fungal growth, sporulation and aflatoxin production, unlike complex sugars and peptone, which strongly repress aflatoxin production (Calvo et al., 2002; Yao et al., 2018).

The reduced number of conidia produced by all tested strains in MEA and MPA appears therefore to be related with the nutrient composition of these media. Instead, the reduced conidiation of knockout mutants in all tested media compared to wild type could be explained as a consequence of a direct or indirect positive regulation of conidiation driven by *gsfR1*. Many TFs have a role in co-regulating...
spore production and mycotoxin formation. MtfA, RtfA, NsdC, RafA and StuA all regulate aflatoxin production, conidiation and sclerotia development in *A. flavus* (Yao et al., 2018). Furthermore, the regulator of patulin biosynthesis was recently proposed to be connected with the development of *P. expansum*, even though the authors suggest that this is only a remote possibility that have to be proved (Snini et al., 2016). Additionally, *gsfR1* is putatively bound by transcription factors involved in asexual and sexual development, that strongly supports this model. Further studies are needed to investigate the role of *gsfR1* in regulation of sporulation or conidiation, together with already known global transcription factors.

4.5 Role of griseofulvin on pathogenicity of *P. griseofulvum*

Considering that SM are not essential for primary metabolism of fungi, along the fact that they confer several advantages to the producer organism, it is reasonable to assume that they could have a role in pathogenicity of fungal pathogens. The relationship between SM production and pathogenicity has been extensively investigated previously, and several examples linking SM and mycotoxins with pathogenesis are well documented (Macheleidt et al., 2016; Scharf et al., 2014). Considering causal agents of blue molds in apples, previous studies have investigated the role of patulin in infection and development of *P. expansum*, but contrasting results emerged. For instance, Ballester et al. (2015) and Li et al. (2015) pointed out that patulin produced by fungal pathogens is not essential for apple infection, as deletion of *patK*, *patL*, and *patN*, genes involved for patulin biosynthesis, did not affect the virulence. Conversely, Sanzani et al. (2012) and Barad et al. (2013) observed a direct correlation with virulence, because mutants which displayed a reduced production of patulin were less virulent on apples. These contrasting results obtained in the same pathosystem can be explained by the use of different strains from different countries and by the great quantity of variable factors involved *in vivo* trials such as temperature, pH, cultivar sensitivity and storage conditions (Barad et al., 2016b). Among these factors, apple variety plays an important role as demonstrated by Snini et al. (2016).

Taking into account the previous works reported, patulin could be considered a virulence factor
(connected to severity of disease), instead of a pathogenicity one (linked to the ability to cause the
disease), helping to establishment of the disease when the pathogen has already colonized the fruit
(Barad et al., 2016b). Indeed, the addition of patulin directly on the wound completely restored the
wild-type phenotype in less virulent strains (Snini et al., 2016).

According to the presented results, griseofulvin may be involved in virulence and not pathogenicity.
Indeed, the deletion mutants for the gsfa gene are still able to induce the disease, even if they are not
able to produce griseofulvin, but the infected apples exhibit reduced lesions. This supports the idea
that griseofulvin alone should not be considered a pathogenicity factor but can act to enhance
virulence.

Δgsfr1 mutants were slightly more virulent compared to the wild type, while Δgsfr2 strains were
comparable to the wild type both in griseofulvin production and in pathogenicity. Considering that
Δgsfr2 mutants displayed no differences on virulence compared to the wild type, the differences on
development of rot diameter of knockout mutants for gsfa and gsfr1 genes could be related to
griseofulvin production. The differences seen in the development of rot diameter on apples by Δgsfa
and Δgsfr1 mutants could suggest a role of griseofulvin production in virulence of P. griseofulvum.

Nevertheless, the ectopic strain showed increased virulence compared to the wild type and therefore
further studies are necessary to understand the molecular mechanism/s underlying this increased
production.

Concerning the griseofulvin produced in vivo by Δgsfr1 mutants, a higher production was observed
at 10 dpi (similarly to what observed on PDA plates) and a lower production at 14 dpi (similarly to
what observed on MEA and MPA plates).

In establishing the interaction with the host, P. griseofulvum would activate a series of well
characterized responses, such as biosynthesis of gluconic acid and ammonium depletion, in order to
improve the acidification of apples and the biosynthesis of polygalacturonases responsible for cell
wall degradation of fruits (Barad et al., 2016a; Prusky et al., 2004). Reduced nitrogen availability,
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including griseofulvin, which is also enhanced in presence of ROS and cutinases according to the findings of our work. This hypothesis is supported by the fact that $g$s$fr1$ mutants produced less griseofulvin compared to wild type after 14 days, so it is reasonable to assume that positive regulation of griseofulvin biosynthesis is activated at this point. Moreover, if GsfR1 acts as a negative regulator, it is reasonable to observe an earlier production of griseofulvin in the $\Delta g$s$fr1$ knockout mutants, as it was found in infected apples by day 10, when the production of griseofulvin is higher in the knockout mutants. To our knowledge, the current work presents the first attempt to investigate the role of griseofulvin in virulence of $P$. griseofulvum and further investigation is necessary to prove its involvement.

5. Conclusions

This work led to a better understanding of the complex regulation of griseofulvin biosynthesis. The role of putative transcription factors was investigated. Based on our results, $g$s$fr2$ is not involved in this pathway and it is probably part of another gene cluster. The putative reductase $g$s$fK$, $g$s$G$ and $g$s$J$ are putatively not essential for griseofulvin biosynthesis due to their absence in the genome of the producer species $X$. flabelliformis. Therefore, the griseofulvin gene cluster should be considered composed of 7 genes, instead of 13, as previously described.

The gene $g$s$fr1$, on the other hand, is involved in griseofulvin biosynthesis, acting as a negative regulator of the cluster on PDA and on apples. However, in different culture conditions, characterized by nitrogen and complex sugars richness, $g$s$fr1$ could act as a positive regulator of griseofulvin biosynthesis. These findings suggest that $g$s$fr1$ can trigger different responses depending on external stimuli, especially nitrogen and carbon availability. The deletion of $g$s$fr1$ has a huge impact on many aspects of $P$. griseofulvum, ranging from secondary metabolism to virulence and conidiation. It is remarkable to notice that $g$s$fr1$ seems to be involved in the regulation of patulin biosynthesis, which $P$. griseofulvum can produce in high amounts on apples. The regulation of patulin biosynthesis in $P$. griseofulvum was previously suggested to be peculiar because the expression of the specific
transcription factor and other biosynthetic genes involved in patulin biosynthesis did not increase with the increased production of the toxin (Banani et al., 2016), differently from what observed in *P. expansum* (Ballester et al., 2015). Therefore, these findings support the idea that *P. griseofulvum* regulate patulin biosynthesis in a distinctive manner, highlighting variability of the regulation of secondary metabolism in different fungal species. The knowledge of the mechanism involved in the regulation of patulin could be useful to conceive strategies to limit its production on apples, by modifying its transcriptional activation, through environmental stimuli.

Concerning pathogenicity of *P. griseofulvum*, the deletion mutants for *gsfA* were less virulent than the wild-type strain, while the deletion mutants for *gsfR1* were slightly more virulent *in vivo* suggesting a direct role of griseofulvin on virulence of *P. griseofulvum*. Until now, this is the first study on the role of griseofulvin in pathogenicity, and further investigations are needed to confirm this data and to highlight the mechanisms of action involved.

*gsfr1* is also involved in the asexual multiplication of *P. griseofulvum*, as a reduced conidiation was observed compared to the wild type for all *in vitro* conditions tested.

In conclusion, *gsfR1* represents a good example of how the regulation of SM biosynthesis and fungal development can be complex and extremely interconnected.

**Acknowledgments**

We thank Prof. Maria Lodovica Gullino for reviewing and editing of the manuscript and Dr. Houda Banani for setting up the knockout on *P. griseofulvum*. This work was supported by Fondazione Cassa di Risparmio di Cuneo (progetto SMART APPLE – Innovative and SMART technologies for sustainable apple production) and by the Spanish Ministry of Science, Innovation and Universities (AGL2017-88120-R, AEI/FEDER, UE).

**Declaration of interest**

None
Author contributions

Silvia Valente: Conceptualization, Investigation, Formal analysis, Writing - original draft; Agnese Cometto: Investigation, Formal analysis, Writing - original draft; Edoardo Piombo: Conceptualization, Formal analysis, Software, Writing - original draft; Giovanna Roberta Meloni: Investigation, Formal analysis, Writing - original draft; Ana-Rosa Ballester: Conceptualization, Investigation, Formal analysis, Writing - review & editing; Luis González-Candelas: Conceptualization, Resources, Writing - review & editing; Davide Spadaro: Conceptualization, Resources, Writing - review & editing.

References


Figure 1 – PCR analysis of gsfR1 mutants. Schematic presentation of the gsfR1 locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (HygR) and gsfR1 gene (B); confirmation of orientation of inserted T-DNA (C); copies number of T-DNA inserted in P. griseofulvum (D) obtained through qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type P. griseofulvum, Δ = deletion mutants for gsfR1 and E = ectopic strains, Neg. = negative control (PCR mix without DNA).
Figure 2 – PCR analysis of gsfR2 mutants. Schematic presentation of the gsfR2 locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (HygR) and gsfR2 gene (B); confirmation of orientation of inserted T-DNA (C); copies number of T-DNA inserted in *P. griseofulvum* (D), obtained through qPCR on gDNA using primers 5F/6R and histone H3 gene as reference gene. M= GeneRuler 1Kb Plus DNA ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for gsfR2 and E = ectopic strains, Neg. = negative control (PCR mix without DNA).
Figure 3 – Effect of gsfR1 deletion on *P. griseofulvum* growth *in vitro*. Colony diameter (A) and conidia production (B) from 3 to 7 dpi. Plate view (front C and reverse D) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for gsfR1 and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan’s multiple range test (*p* < 0.05).
**Figure 4** - Effect of *gsfR1* deletion on griseofulvin and patulin production. Griseofulvin (A) and patulin (B) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, ∆ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan’s multiple range test (*p* < 0.05).
Figure 5 – Effect of gsfR2 deletion on *P. griseofulvum* growth *in vitro*. Colony diameter (A) and conidia production (B) from 3 to 7 dpi. Plate view (front C and reverse D) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for gsfR2 and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan’s multiple range test (*p* < 0.05).
Figure 6 - Effect of gsfR2 deletion on griseofulvin and patulin production. Griseofulvin (A) and patulin (B) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan’s multiple range test (*p* < 0.05).

Figure 7 – Relative gene expression (RGE) of gsfA gene, at 5 (A) and 7 (B) dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for gsfR1 and E = ectopic strains. The expression is relative to the expression of the β-tubulin gene.
Figure 8 - Effect of gene deletions on virulence and griseofulvin production *in vivo*. Lesion diameter caused by the wild type strain was compared with rot diameter of knockout ad ectopic strains for $gsfA$ (A) $gsfR2$ (B) and $gsfR1$ (C). Griseofulvin production ($\mu g/g$ of apple) was compared between wild type and $\Delta gsfR1$ strains (D). Measurements were taken at 7, 10 and 14 dpi. WT = wild type *P. griseofulvum*, $\Delta$ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan’s multiple range test ($p < 0.05$).
Figure 9 – Effect of carbon and nitrogen on conidiation and on griseofulvin production in vitro. Logarithm of conidia per plate (A) and griseofulvin produced (B) were measured at 10 dpi in three solid media (PDA, MEA and MPA). Colony view on PDA (C), MEA (D) and MPA (E). WT = wild type P. griseofulvum, ∆7, ∆23 = deletion mutants for gsfR1, E3 = ectopic strain and ∆KO = deletion mutant for gsfA. Values followed by the same letter are not statistically different by Duncan’s multiple range test ($p < 0.05$); n.d. = non detectable.
**Figure S1** – PCR analysis of *gsfA* mutants. Schematic presentation of the *gsfA* locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (*HtgR*) and *gsfA* gene (B); confirmation of orientation of inserted T-DNA (C); copies number of T-DNA inserted in *P. griseofulvum* (D), obtained through qPCR on gDNA using primers 5F/6R and β-tubulin gene as reference gene. M = GelPilot Wide Range Ladder, WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, Neg. = negative control (PCR mix without DNA).
Figure S2 – Effect of gsfA deletion on *P. griseofulvum*. Colony diameter (A) and griseofulvin production (B) from 3 to 7 dpi. Plate view (front C and reverse D) at 7 dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for gsfA and E = ectopic strains, n.d. = non detectable.

Table S1 – List of primers used in this work.

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<td>Insertion of gsfR1 terminator in pRFHU2. Tail for USER reaction in red.</td>
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<td><strong>AGTGAGTCGTTCAACATAC</strong></td>
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<td><strong>2R-gsfR2</strong></td>
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<td><strong>TTGGGAAACTCCTCGGA</strong></td>
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Table S2 – Transcription factors putatively binding the promoter of gsfR1 and gsfA. It is reported the name of homolog and the regulated processes in other organisms with references when available.

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<th>Protein</th>
<th>Homolog</th>
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<td>Conidiation, response to light</td>
<td>(Igbalajobi et al., 2019)</td>
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<td>Fluconazole resistance protein</td>
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<td>Function</td>
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<td>KXG46595.1</td>
<td>NsdD</td>
<td>activation of sexual development</td>
<td>(Han et al., 2001)</td>
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<td>KXG46600.1</td>
<td>res2</td>
<td>Meiosis</td>
<td>(Zhu et al., 1997)</td>
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<td>KXG47124.1</td>
<td>Msn2</td>
<td>Conidiation, hyphal growth and stress response</td>
<td>(Liu et al., 2013; Song et al., 2018; Tian et al., 2017)</td>
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<tr>
<td>KXG47905.1</td>
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<td>Quinic acid utilization</td>
<td>(Wheeler et al., 1996)</td>
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<td>KXG48399.1</td>
<td>FlbC</td>
<td>conidiation</td>
<td>(Kwon et al., 2010)</td>
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<td>KXG53661.1</td>
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<td>KXG54396.1</td>
<td>MtfA (ID 50%)</td>
<td>regulation of aflatoxin production</td>
<td>(Zhuang et al., 2016)</td>
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<td>KXG54691.1</td>
<td>StuA</td>
<td>Regulation of cell pattern formation</td>
<td>(Miller et al., 1992)</td>
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Transcription factors binding only the promoter of *gsfA*

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<td>Using galactose or lactose as only carbon sources</td>
<td>(Riley et al., 1987)</td>
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<td>KXG47549.1</td>
<td>SREP</td>
<td>repressor of siderophore biosynthesis in presence of high iron concentrations</td>
<td>(Haas et al., 1997)</td>
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<td>KXG47976.1</td>
<td>Zinc finger, C2H2-type/integrase, DNA-binding</td>
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<tr>
<td>KXG51025.1</td>
<td>transcriptional activator of gluconeogenesis</td>
<td>transcriptional activator of gluconeogenesis</td>
<td>(Hynes et al., 2007)</td>
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<tr>
<td>KXG51359.1</td>
<td>Filamentous growth regulator</td>
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<tr>
<td>KXG52025.1</td>
<td>Achaete-scute transcription factor related</td>
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Transcription factors putatively binding the promoters of both *gsfR1* and *gsfA*
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<td>KXG46064.1</td>
<td>RlmA response to cell wall stress, asexual development</td>
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<td>(Damveld et al., 2005; Kovács et al., 2013)</td>
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