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

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Abstract:

Penicilium 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. brevicompactum, 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,* g*sfR1* 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 ß-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 $2^{\Delta\Delta cq}$ 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 Δ*gsfR1* 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.

190

191 *2.6 Characterization of mutants* in vitro

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

200

201 *2.7 Characterization of mutants* in vivo

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

210

211 *2.8 Griseofulvin and patulin extraction*

212 Griseofulvin and patulin produced *in vitro* were extracted simultaneously from the mycelium and the
213 medium by washing the plates with 3 mL of methanol according to Banani et al. (2016) with some
214 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:H_2O (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.

240 Comparison of griseofulvin production on different growth media and *in vivo* was analyzed by liquid
241 chromatography coupled with mass spectrometry. The HPLC-MS/MS system consisted of a binary
242 pump and a vacuum degasser (1260 Agilent Technologies, Santa Clara, CA, USA) connected to a
243 Varian auto-sampler Model 410 Prostar (Hansen Way, CA, USA), equipped with a 20 µL loop and
244 coupled with a Varian 310-MS TQ Mass Spectrometer. The sample was injected (10 µL) onto Luna
245 C18(2) (150mm x 2mm i.d. 3µm, Phenomenex) and eluted under a flow of 200 µL/min. The mobile
246 phase was an isocratic mixture of ACN:HCOOH 0.05% (60:40, v/v) for 5 min.
247 The mass spectrometer was equipped with an electrospray ionization (ESI) source operating in
248 positive ion mode. Griseofulvin detection and quantitation were carried out using multiple reaction
249 monitoring (MRM) mode by selecting three transitions: m/z 353 > 285 CE 18 eV (monitoring), m/z
250 353 > 215 CE 16 eV (quantification) and m/z 353 > 165 CE 16 eV (monitoring). The collision gas
251 (Ar) pressure was set at 2 mbar for all experiments.
252 Quantification was performed by external calibration preparing a calibration curve for each
253 mycotoxin. The calibration curves were prepared by dilution of standard solutions of griseofulvin and
254 patulin.
255
256 *2.10 Statistical analysis*
257 All statistical analyses were performed with one-way ANOVA followed by Duncan test using IBM
258 SPSS statistics software version 24 (SPSS Inc., Chicago, IL, USA), $p < 0.05$ was considered
259 significant. The REST 2009 Software (Qiagen) was used for the statistical analysis of RT-qPCR and
260 qPCR results.
261
262 **3. Results**
263
264 *3.1 Obtainment of knockout mutants for* gsfR1 *and* gsfR2 *and role on griseofulvin biosynthesis*

265 The role of putative transcription factors encoded by *gsfR1* and *gsfR2* was assessed by obtaining
266 deletion mutants and they were compared with the wild-type strain and a deletion mutant for *gsfA*,
267 the gene encoding the PKS.
268 The deletion of genes was mediated by *A. tumefaciens*, which delivered a T-DNA containing a
269 hygromycin resistance cassette flanked by the promoter and terminator of target genes. Due to the
270 presence of these flanking regions, homologous recombination led to replacement of genes with the
271 antibiotic resistance cassette (Figs. 1-2, S1).
272 The knockout mutants were then assessed by amplifying the hygromycin resistance cassette and target
273 gene by PCR (Fig. 1 B, 2 B, S1 B). As expected, the deleted genes were not amplified in knockout
274 mutants, while hygromycin resistance cassette was only absent in the wild type PG3. Ectopic strains
275 had a functional copy of the target gene and the antibiotic resistance cassette, due to non-target
276 integration into the genome of the T-DNA.
277 To confirm the right insertion of the cassette, PCRs with primer pairs designed on the hygromycin
278 resistance cassette and on 3'UTR or 5'UTR of deleted gene were performed (Fig. 1 C, 2 C, S1 C).
279 These primer pairs can only amplify if the T-DNA has been introduced by homologous recombination
280 at the target sites in the 3'UTR and 5'UTR flanking regions. As expected, the PCR fragments were
281 amplified in the knockout mutants, and failed amplification in the wild type PG3 and in the ectopic
282 mutants.
283 The number of integrations in the genome was determined by qPCR by measuring the difference
284 between the quantification cycle (Cq) of target and reference genes in the mutant and in the control
285 wild-type strain (Pfaffl, 2001) (Fig. 1 D, 2 D, S1 D).
286 Three mutants with one single event of integration and two ectopic strains were selected for each
287 knockout event and they were characterized *in vitro* both phenotypically and chemically.
288 Concerning the effect of gene deletion on phenotype, while the deletion of the polyketide synthase
289 did not affect the phenotype (Fig. S2), knockout mutants for *gsfR1* showed the same growth rate as
290 the wild-type strain (Fig. 3A), but exhibited colonies with a markedly less green color, caused by a

291 marked reduction in conidiation *in vitro* (Fig. 3 B, C). On the other hand, knockout *gsfR2* mutants
292 were similar to the wild type in both growth and sporulation (Fig. 5).
293 The ability of knockout mutants to produce griseofulvin was assessed on PDA. Mutants lacking the
294 *gsfA* gene were no longer able to produce griseofulvin *in vitro* (Fig. S2 B), while knockout mutants
295 for the regulator gene *gsfR1* were found to produce higher quantities of griseofulvin compared to
296 wild-type strain, with an average of about 30%, 50% and 110% higher production compared to wild-
297 type strain at 5, 7 and 10 dpi (Fig. 4 A, Fig. 9).
298 The production of patulin was also investigated and deletion of *gsfR1* showed to influence also the
299 regulation of this mycotoxin, especially at 5 dpi, reaching over 3000 µg/plate in all mutants compared
300 to 2300 µg/plate produced by *P. griseofulvum* PG3, which was significantly different according to
301 Duncan's statistical analysis ($p < 0.05$) (Fig. 4 B).
302 The knockout of *gsfR2* did not affect griseofulvin or patulin production, nor conidiation or growth
303 rate, which were comparable between wild type and knockout and ectopic mutants (Fig. 5, 6).
304
305 *3.2 GsfR1 regulates negatively griseofulvin biosynthesis on PDA*
306 The observation that the deletion of *gsfR1* led to a higher griseofulvin production *in vitro* led us to
307 hypothesize that this gene could encode a negative regulator of griseofulvin biosynthesis. To examine
308 this hypothesis, the relative expression of the polyketide synthase gene of three mutants and two
309 ectopic strains was investigated through RT-qPCR. The Δ*gsfR1*deletion mutants showed an increased
310 expression of *gsfA* compared to the wild type, both at 5 and 7 dpi, while ectopic mutants exhibited a
311 similar gene expression to the wild type (Fig. 7).
312
313 *3.3 Virulence and griseofulvin production on apples*
314 To evaluate the effect of griseofulvin on the pathogenicity of *P. griseofulvum*, the virulence of three
315 knockout mutants was compared with that of the wild-type strain and one ectopic mutant on apples
316 (Fig. 8). *ΔgsfA* mutants, which were not able to produce griseofulvin, showed a slightly reduced

317 virulence (Fig. 8 A). In this assay, apples infected with E-A ectopic mutant, which contains 4
318 insertions of the T-DNA, led to a strong reduction of virulence on apples. However, *gsfR2* mutants
319 showed a similar virulence compared to the wild-type strain over a period of 14 days after inoculation
320 (Fig. 8 B). On the contrary, Δ*gsfR1* strains were slightly more virulent (Fig. 8 C) compared to the *P.*
321 *griseofulvum* parental strain. Nevertheless, the ectopic mutant showed an enhanced virulence as well,
322 although it was less virulent than the mutant in the first stages of the infections. For this reason, we
323 cannot exclude that the enhanced virulence of the mutants was at least partly due to the transformation
324 itself.
325 To check if there is a relationship between increased virulence and production of griseofulvin *in situ*,
326 the mycotoxin was extracted from apples inoculated with *gsfR1* mutants and quantified. Results
327 showed a significant increased production of griseofulvin from knockout mutants at 10 dpi (Fig. 8
328 D), while at 14 dpi *gsfR1* mutants produced less griseofulvin compared to wild-type strain.
329
330 *3.4 Global regulators in griseofulvin biosynthesis*
331 By using information on the CIS-BP database, the promoters of *gsfR1* and *gsfA* were scanned in order
332 to find putative binding sites for global regulators. Eighty-one and seventy-five transcription factors
333 were predicted to bind the promoters of *gsfR1* and *gsfA*, respectively. Thirteen transcription factors
334 seem to bind only the promoter of *gsfR1*, while 7 are typical of *gsfA* and 68 were predicted to regulate
335 both genes, ensuing a complex scenario (Tab. S2).
336
337 *3.5 Carbon and nitrogen have a strong effect on* in vitro *conidiation and griseofulvin production*
338 Among the different stimuli, the availability of nutritional sources, especially nitrogen and carbon, is
339 a key factor for the biosynthesis of many SMs and for the growth and differentiation of filamentous
340 fungi. Because of this, it is not surprising that we found many putative TFs involved in carbon or
341 nitrogen consumption, which could bind the promoter of *gsfR1* and *gsfA* (Tab. S2).

342 We focused our attention on the effect of carbon and nitrogen on griseofulvin production. To do that,
343 two *gsfR1* deletion mutants and one ectopic strain were grown for ten days on three different media
344 (PDA, MEA and MPA); one knockout mutant for the polyketide synthase gene was also included as
345 control (denoted as KO). These three media are characterized by different C/N ratios: PDA is
346 composed of potato extract and dextrose and has the highest carbon content, while on MEA and MPA,
347 peptone was added, increasing the nitrogen content, which is ten times higher on MPA (thanks to the
348 addition of meat extract) compared to MEA (Núñez et al., 2000). As expected, carbon and nitrogen
349 showed a huge impact both on griseofulvin and conidia production (Fig. 9).
350 Conidiation of the wild-type strain was reduced in both media containing peptone, with a stronger
351 reduction on MEA (Fig. 9A), and a significant reduction of griseofulvin production was observed in
352 MPA (23%) compared to PDA or MEA (Fig. 9B).
353 Conidiation of the *gsfA* knockout mutant, lacking the gene encoding the polyketide synthase, was
354 similar to that observed for the wild-type strain in the three media tested. However, the knockout
355 mutant was not able to produce griseofulvin in any condition.
356 When focusing on *gsfR1* mutants, a more complex scenario was observed. Deletion of *gsfR1* gene led
357 to a significant reduction of conidia on MEA and PDA compared to wild type, ectopic strain and *gsfA*
358 mutants, suggesting that this gene could play a role in the direct or indirect regulation of conidia
359 production. Considering griseofulvin production, a significant increase of griseofulvin production
360 was observed on PDA (Fig. 4 and 9B). Instead, on the other two media, richer in nitrogen, there was
361 a significantly reduced production compared to wild type.
362
363 **4. Discussion**
364 *4.1 Role of transcription factors GsfR1 and GsfR2 on griseofulvin biosynthesis*
365 The griseofulvin gene cluster previously identified in *P. aethiopicum* includes two genes, *gsfR1* and
366 *gsfR2*, which encode for putative transcription factors. The *gsfR2* gene in *P. griseofulvum* was located
367 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 *fum21* 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).

394 The interconnection of signals and responses is also testified by the complex pattern of TFs that can
395 putatively bind the promoters of *gsfR1* and *gsfA*, triggering the activation or repression of griseofulvin
396 biosynthesis and regulating at the same time other SM gene clusters and many aspects of the biology
397 of *P. griseofulvum*, such as conidiation and virulence. Surprisingly, in the recently released genome
398 of griseofulvin producer *Xylaria flabelliformis* (Mead et al., 2019) the gene *gsfR1* was missing,
399 pointing to a possible regulation of griseofulvin biosynthesis independently of *gsfR1* action.
400 Considering the second putative transcription factor of the cluster, the deletion of *gsfR2* seems to have
401 no effect on the parameters of *P. griseofulvum* considered, and it is likely that the gene is involved in
402 a different biosynthetic pathway. Indeed, the gene *gsfK* encoding for a putative reductase is located
403 next to *gsfR2*, and its deletion in *P. aethiopicum* did not affect the production of griseofulvin (Cacho
404 et al., 2013). These findings led to the assumption that both *gsfK* and *gsfR2* are probably involved in
405 the biosynthesis of another SM. Moreover, in *X. flabelliformis* (Mead et al., 2019), *gsfJ* and *gsfG*
406 genes were missing, suggesting that these two genes are not necessary for griseofulvin production.
407 Therefore, considering also that the knockout of *gsfR2* does not impair griseofulvin production, we
408 suggest that the core genes for the biosynthesis of this metabolite are only 7 instead of 13.
409
410 *4.2 Role of global regulators in griseofulvin biosynthesis*
411 Secondary metabolism gene clusters are usually regulated by several global regulatory proteins in
412 response to many environmental stimuli, such as light, carbon and nitrogen levels, pH and redox
413 status, iron concentration and signaling from other organisms (Brakhage, 2013; Hoffmeister and
414 Keller, 2007). Many of these global regulators are well characterized and their putative binding sites
415 in the promoter regions of genes could be detected.
416 One of the transcription factors predicted to regulate *gsfR1* and not *gsfA* is KXG54396.1, a *P.*
417 *griseofulvum* gene similar to *mtfA*, whose deletion or overexpression in *A. flavus* cause a reduction of
418 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

444 griseofulvin production, in the presence of difficult carbon sources, but further experiments would be
445 required to check this possibility.
446 Several transcription factors binding the promoters of both *gsfR1* and *gsfA* have not been
447 characterized yet. However, among them, there were homologs of the well-known global regulators
448 AreA and CreA, which are respectively a positive and a negative transcription factor acting in
449 response to nitrogen or carbon (Katz et al., 2008; Wilson and Arst, 1998). Moreover, the binding sites
450 of NirA and the carbon catabolite repressor MIG1 were also found in the promoters of both genes,
451 increasing the possibilities of production of griseofulvin being heavily influenced by carbon and
452 nitrogen availability (Burger et al., 1991; Randhawa et al., 2018). Binding sites for genes regulating
453 the utilization of different carbon and nitrogen sources are also present. These include: *facB*,
454 regulating acetate utilization (Todd et al., 1998), *argR*, regulating arginine metabolism (Dubois et al.,
455 1987), *nirA*, regulating nitrate utilization (Burger et al., 1991), *amdR*, regulating amides, omega
456 amino acids and lactams catabolism (Andrianopoulos and Hynes, 1990), and the quinic acid
457 utilization activator (Wheeler et al., 1996). Other two transcription factors, the cutinase transcription
458 factor 1 beta and SKN7, respectively increase the expression of cutinases and the resistance to ROS
459 respectively, suggesting a role of the griseofulvin cluster in pathogenesis (Cao et al., 2009), as shown
460 in the pathogenicity trials.
461 In addition to those indicated above, other identified transcription factors were related to sexual and
462 asexual development, heat shock, stress resistance, cellobiose response, sulphur catabolism and
463 assimilation, iron homeostasis and adhesion, and hyphal growth (Tab. S2).
464 Both promoters of *gsfR1* and *gsfA* also contain binding sites similar to those recognized by Apa-2
465 (Chang et al., 1993), strengthening the hypothesis that the griseofulvin regulatory system is somewhat
466 similar to the aflatoxins one, regulated by MtfA.
467
468 *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.

4.4 Regulation of griseofulvin biosynthesis and conidiation

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 g*sfR1*. Many TFs have a role in co-regulating

521 spore production and mycotoxin formation. MtfA, RtfA, NsdC, RafA and StuA all regulate aflatoxin
522 production, conidiation and sclerotia development in *A. flavus* (Yao et al., 2018). Furthermore, the
523 regulator of patulin biosynthesis was recently proposed to be connected with the development of *P.*
524 *expansum*, even though the authors suggests that this is only a remote possibility that have to be
525 proved (Snini et al., 2016). Additionally, *gsfR1* is putatively bound by transcription factors involved
526 in asexual and sexual development, that strongly supports this model. Further studies are needed to
527 investigate the role of *gsfR1* in regulation of sporulation or conidiation, together with already known
528 global transcription factors.
529
530 *4.5 Role of griseofulvin on pathogenicity of* P. griseofulvum
531 Considering that SM are not essential for primary metabolism of fungi, along the fact that they confer
532 several advantages to the producer organism, it is reasonable to assume that they could have a role in
533 pathogenicity of fungal pathogens. The relationship between SM production and pathogenicity has
534 been extensively investigated previously, and several examples linking SM and mycotoxins with
535 pathogenesis are well documented (Macheleidt et al., 2016; Scharf et al., 2014). Considering causal
536 agents of blue molds in apples, previous studies have investigated the role of patulin in infection and
537 development of *P. expansum*, but contrasting results emerged. For instance, Ballester et al. (2015)
538 and Li et al. (2015) pointed out that patulin produced by fungal pathogens is not essential for apple
539 infection, as deletion of *patK*, *patL*, and *patN*, genes involved for patulin biosynthesis, did not affect
540 the virulence . Conversely, Sanzani et al. (2012) and Barad et al. (2013) observed a direct correlation
541 with virulence, because mutants which displayed a reduced production of patulin were less virulent
542 on apples. These contrasting results obtained in the same pathosystem can be explained by the use of
543 different strains from different countries and by the great quantity of variable factors involved *in vivo*
544 trials such as temperature, pH, cultivar sensitivity and storage conditions (Barad et al., 2016b).
545 Among these factors, apple variety plays an important role as demonstrated by Snini et al. (2016).
546 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 Δ*gsfR2* strains were comparable to the wild type both in griseofulvin production and in pathogenicity. Considering that Δ*gsfR2* 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 Δ*gsfA* and Δ*gsfR1* 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 Δ*gsfR1* 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, together with high availability of simple sugars, can trigger the activation of SM gene clusters,

573 including griseofulvin, which is also enhanced in presence of ROS and cutinases according to the
574 findings of our work. This hypothesis is supported by the fact that *gsfR1* mutants produced less
575 griseofulvin compared to wild type after 14 days, so it is reasonable to assume that positive regulation
576 of griseofulvin biosynthesis is activated at this point. Moreover, if GsfR1 acts as a negative regulator,
577 it is reasonable to observe an earlier production of griseofulvin in the Δg*sfR1* knockout mutants, as it
578 was found in infected apples by day 10, when the production of griseofulvin is higher in the knockout
579 mutants. To our knowledge, the current work presents the first attempt to investigate the role of
580 griseofulvin in virulence of *P. griseofulvum* and further investigation is necessary to prove its
581 involvement.
582
583 **5. Conclusions**
584 This work led to a better understanding of the complex regulation of griseofulvin biosynthesis. The
585 role of putative transcription factors was investigated. Based on our results, *gsfR2* is not involved in
586 this pathway and it is probably part of another gene cluster. The putative reductase *gsfK*, *gsfG* and
587 *gsfJ* are putatively not essential for griseofulvin biosynthesis due to their absence in the genome of
588 the producer species *X. flabelliformis*. Therefore, the griseofulvin gene cluster should be considered
589 composed of 7 genes, instead of 13, as previously described.
590 The gene *gsfR1*, on the other hand, is involved in griseofulvin biosynthesis, acting as a negative
591 regulator of the cluster on PDA and on apples. However, in different culture conditions, characterized
592 by nitrogen and complex sugars richness, *gsfR1* could act as a positive regulator of griseofulvin
593 biosynthesis. These findings suggest that *gsfR1* can trigger different responses depending on external
594 stimuli, especially nitrogen and carbon availability. The deletion of *gsfR1* has a huge impact on many
595 aspects of *P. griseofulvum,* ranging from secondary metabolism to virulence and conidiation. It is
596 remarkable to notice that *gsfR1* seems to be involved in the regulation of patulin biosynthesis, which
597 *P. griseofulvum* can produce in high amounts on apples. The regulation of patulin biosynthesis in *P.*
598 *griseofulvum* was previously suggested to be peculiar because the expression of the specific

599 transcription factor and other biosynthetic genes involved in patulin biosynthesis did not increase
600 with the increased production of the toxin (Banani et al., 2016), differently from what observed in *P.*
601 *expansum* (Ballester et al., 2015). Therefore, these findings support the idea that *P. griseofulvum*
602 regulate patulin biosynthesis in a distinctive manner, highlighting variability of the regulation of
603 secondary metabolism in different fungal species. The knowledge of the mechanism involved in the
604 regulation of patulin could be useful to conceive strategies to limit its production on apples, by
605 modifying its transcriptional activation, through environmental stimuli.
606 Concerning pathogenicity of *P. griseofulvum*, the deletion mutants for *gsfA* were less virulent than
607 the wild-type strain, while the deletion mutants for *gsfR1* were slightly more virulent *in vivo*
608 suggesting a direct role of griseofulvin on virulence of *P. griseofulvum*. Until now, this is the first
609 study on the role of griseofulvin in pathogenicity, and further investigations are needed to confirm
610 this data and to highlight the mechanisms of action involved.
611 *gsfr1* is also involved in the asexual multiplication of *P. griseofulvum*, as a reduced conidiation was
612 observed compared to the wild type for all *in vitro* conditions tested.
613 In conclusion, g*sfR1* represents a good example of how the regulation of SM biosynthesis and fungal
614 development can be complex and extremely interconnected.
615

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622

623 **Declaration of interest**

624 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.

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Figures

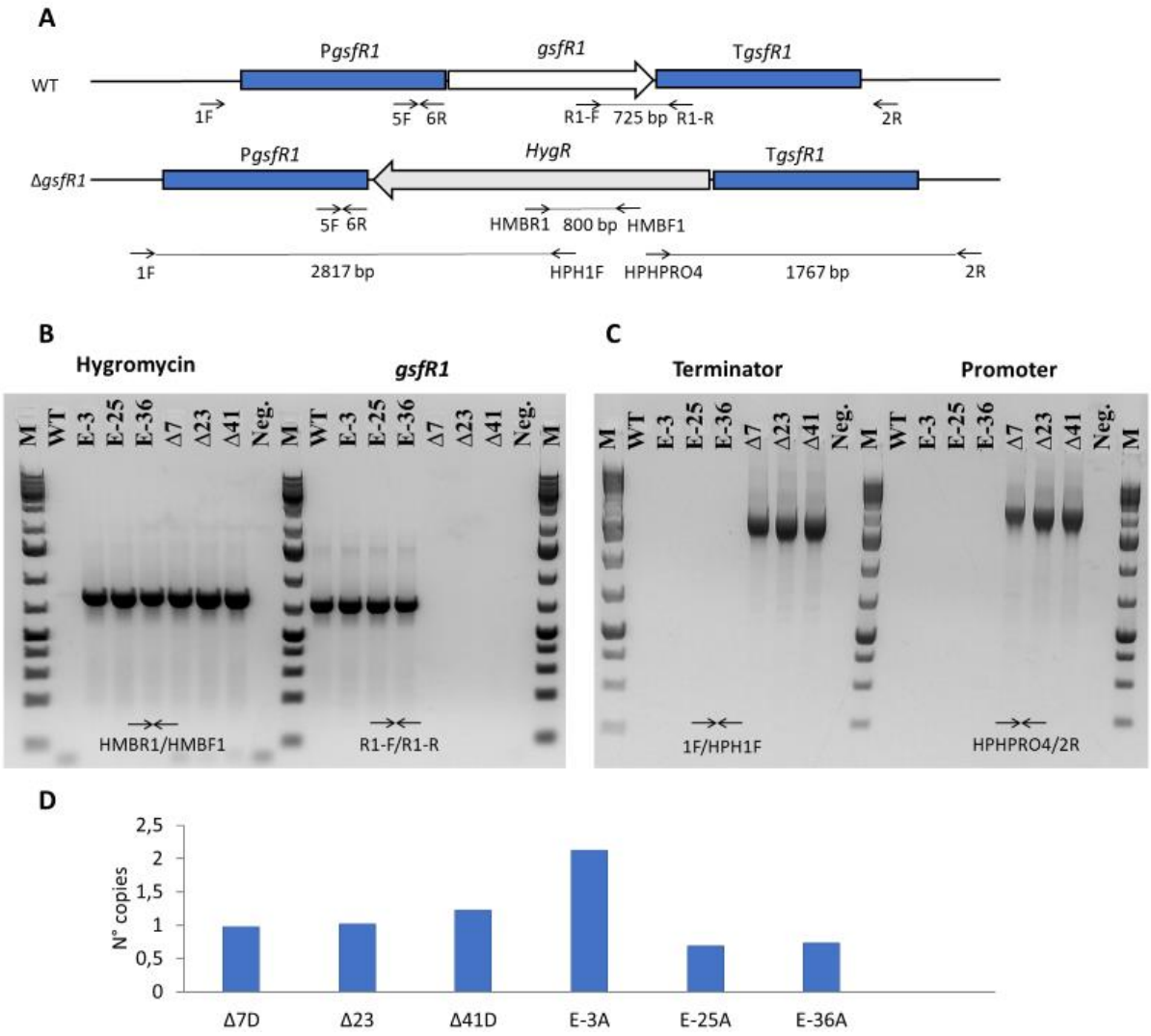


Figure 1 – PCR analysis of *gsfR1* mutants. Schematic presentation of the *gsfR1* locus in the wild type and deletion mutants, primers used are presented as arrows (**A**). Amplification of hygromycin resistance cassette (*HygR*) and *gsfR1* gene (**B**); confirmation of orientation of inserted T-DNA (**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).

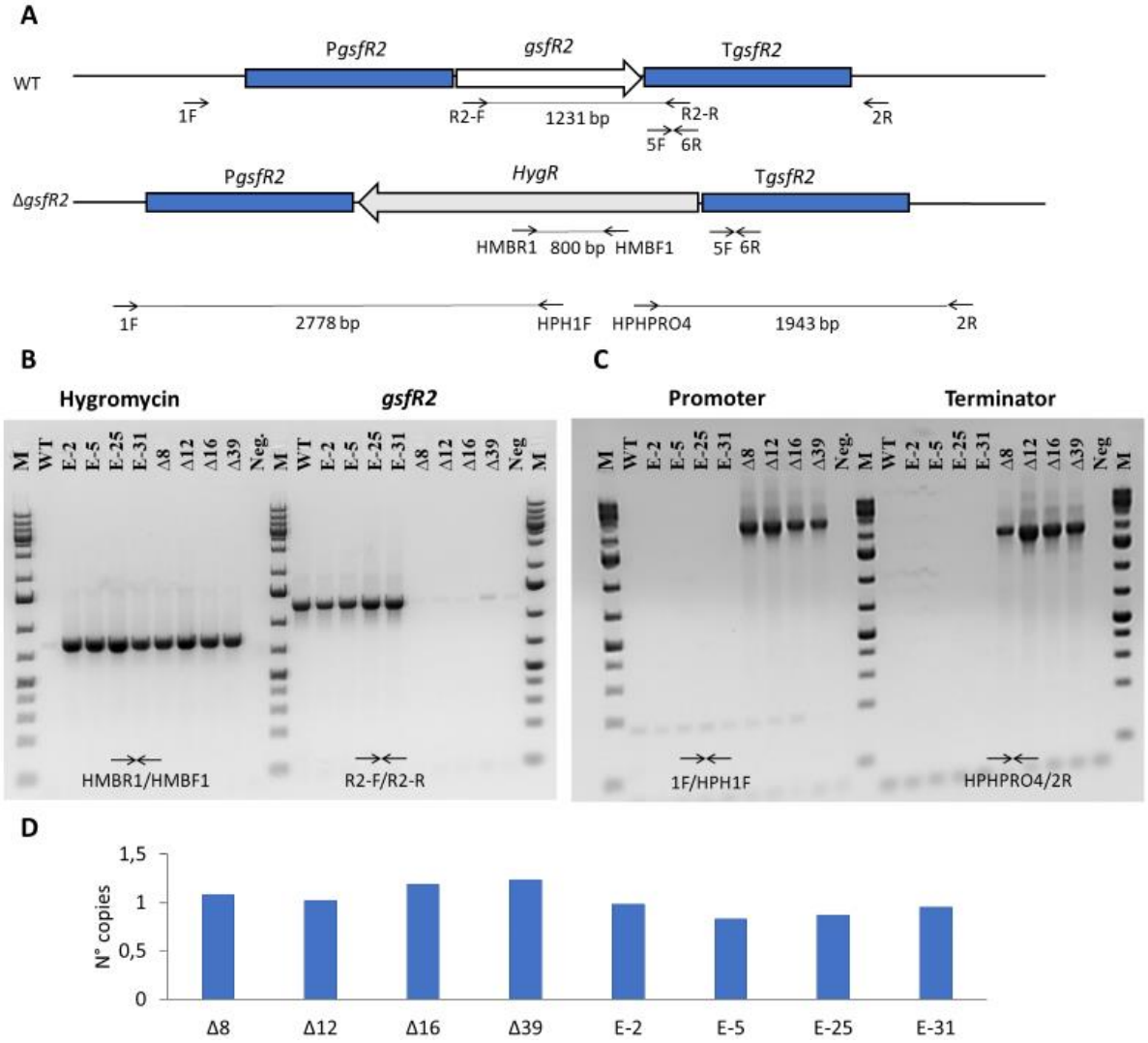
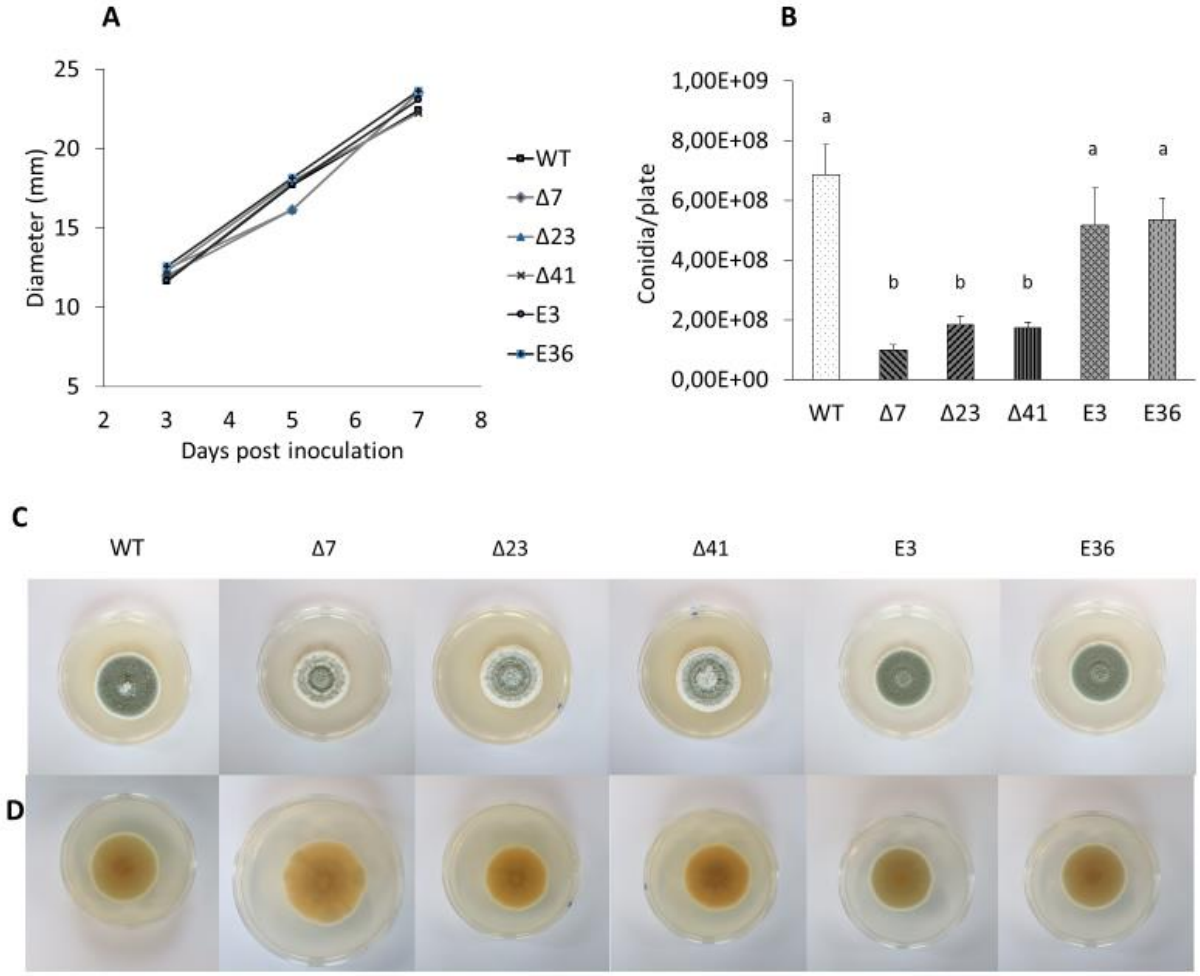


Figure 2 – PCR analysis of *gsfR2* mutants. Schematic presentation of the *gsfR2* locus in the wild type and deletion mutants, primers used are presented as arrows (**A**). Amplification of hygromycin resistance cassette (*HygR*) and *gsfR2* gene (**B**); confirmation of orientation of inserted T-DNA (**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).



931

932 **Figure 3** – Effect of *gsfR1* deletion on *P. griseofulvum* growth *in vitro.* Colony diameter (**A**) and
933 conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT =
934 wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains. Values followed
935 by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

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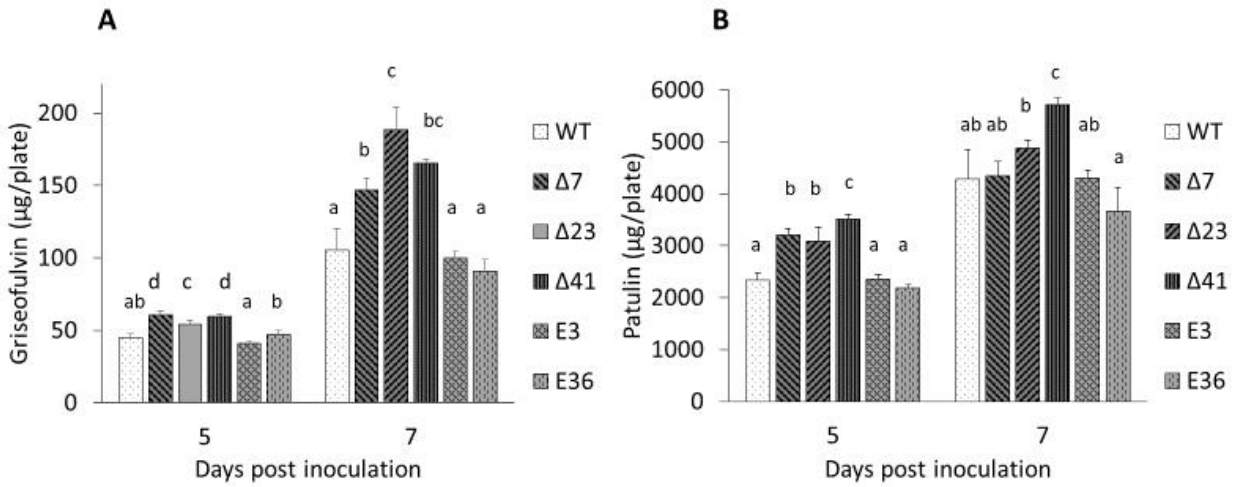
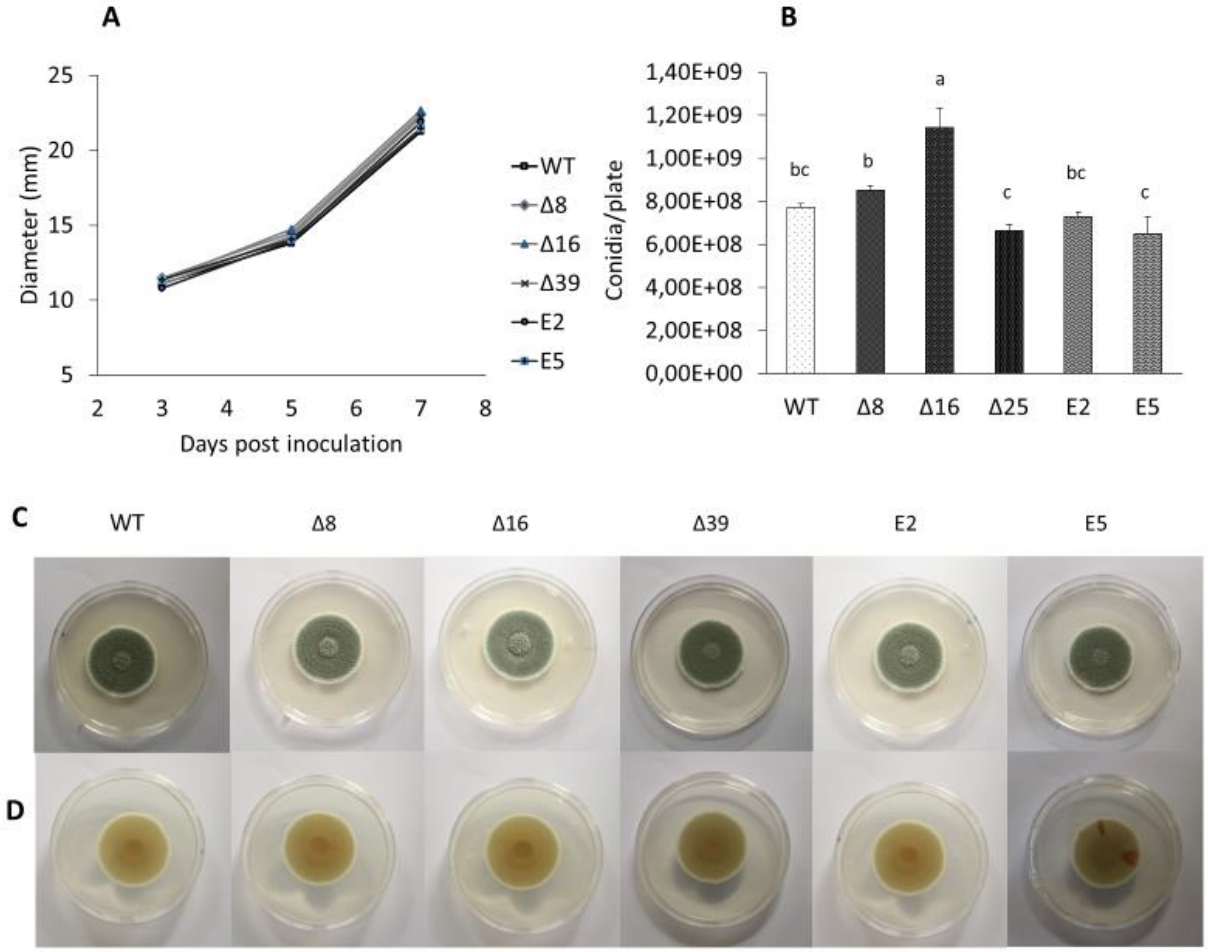


Figure 4 - Effect of *gsfR1* deletion on griseofulvin and patulin production. Griseofulvin (**A**) and patulin (**B**) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).



943

944 **Figure 5** – Effect of *gsfR2* deletion on *P. griseofulvum* growth *in vitro.* Colony diameter (**A**) and
945 conidia production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT =
946 wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR2* and E = ectopic strains. Values followed
947 by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

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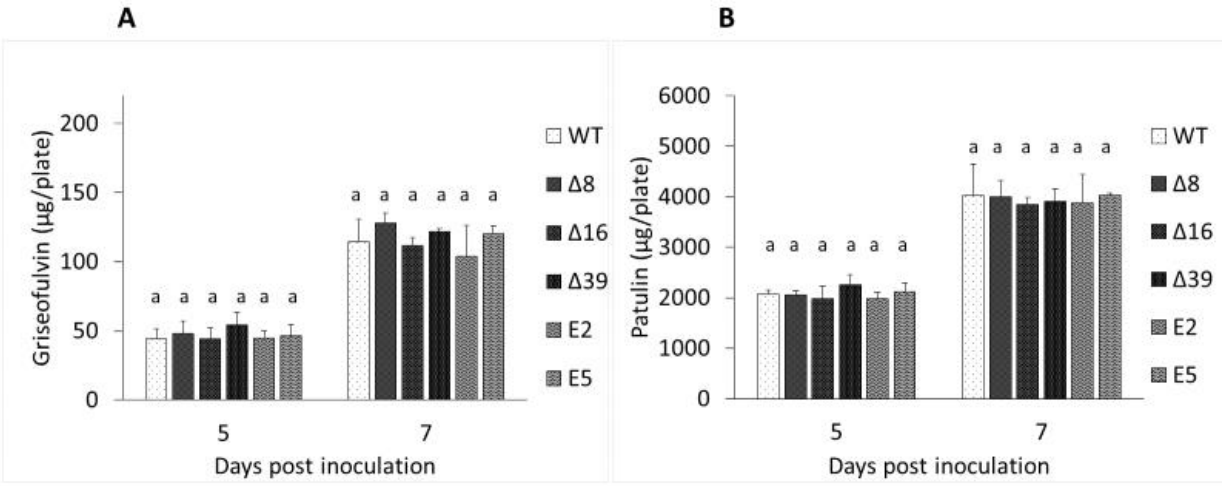


Figure 6 - Effect of *gsfR2* deletion on griseofulvin and patulin production. Griseofulvin (**A**) and patulin (**B**) production were measured at 5 and 7 dpi. WT = wild type *P. griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are not statistically different by Duncan's multiple range test ($p < 0.05$).

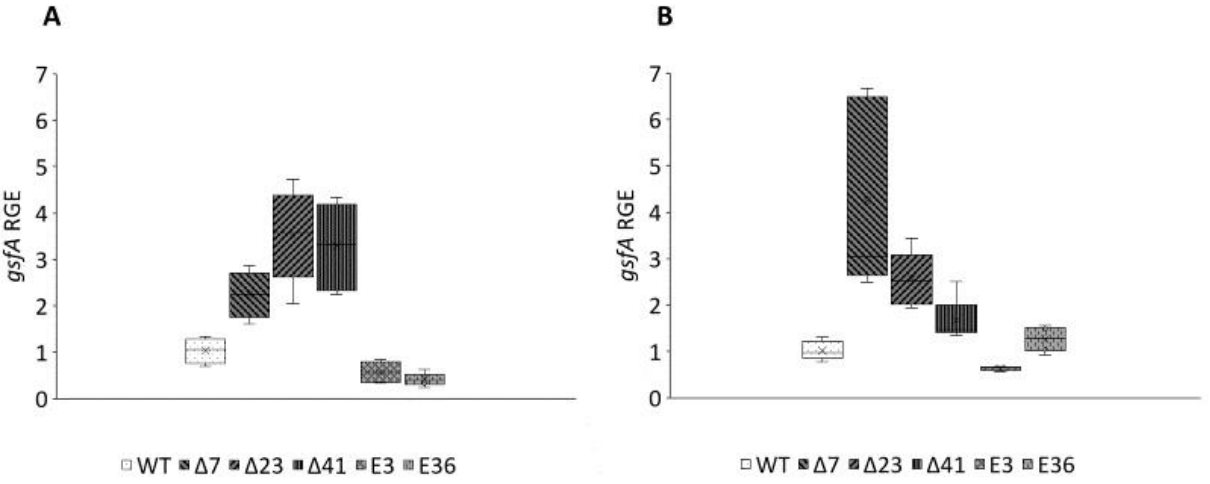
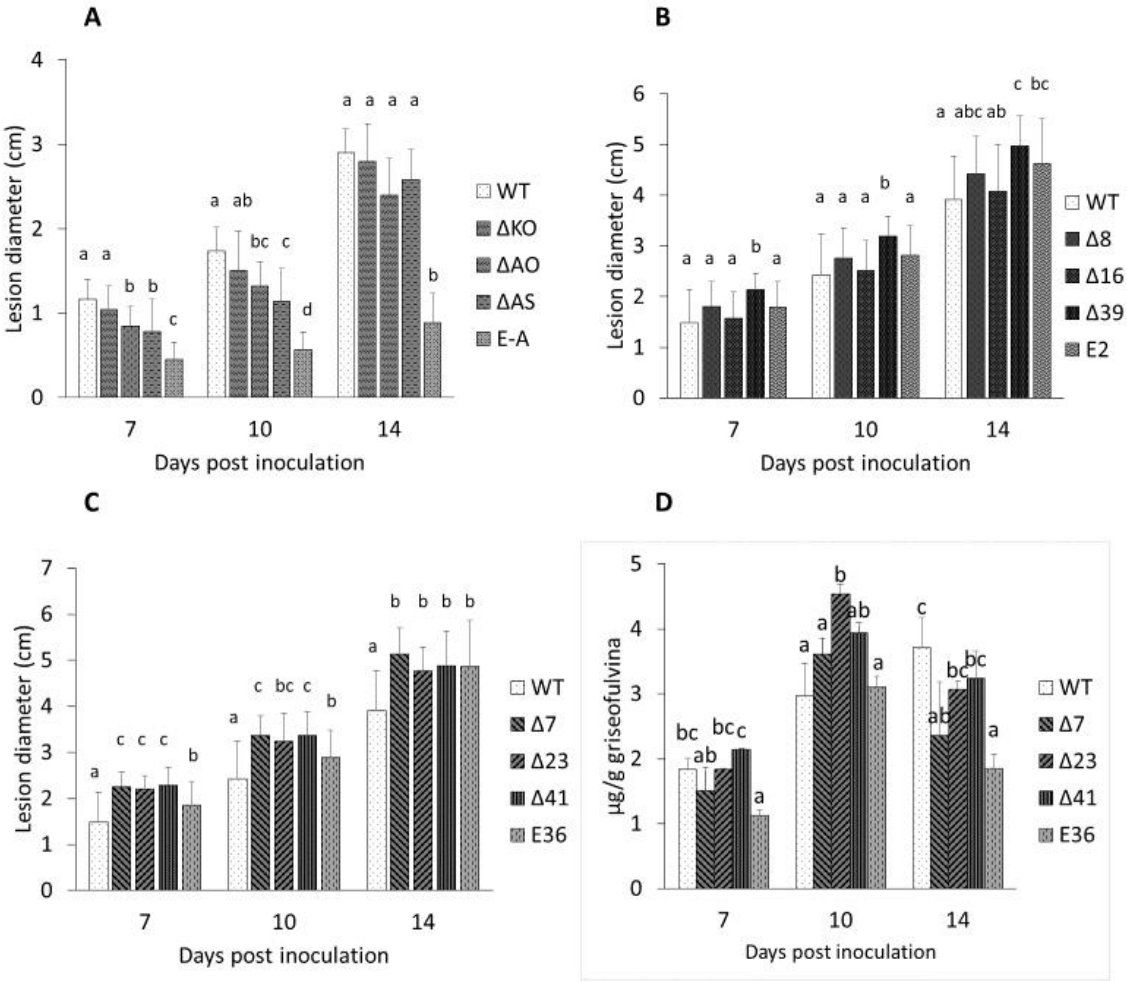


Figure 7 – Relative gene expression (RGE) of *gsfA* gene, at 5 (**A**) and 7 (**B**) dpi on PDA. WT = wild type *P. griseofulvum*, Δ = deletion mutants for *gsfR1* and E = ectopic strains. The expression is relative to the expression of the β-tubulin gene.



960

961 **Figure 8 -** Effect of gene deletions on virulence and griseofulvin production *in vivo*. Lesion diameter
962 caused by the wild type strain was compared with rot diameter of knockout ad ectopic strains for *gsfA*
963 (**A**) *gsfR2* (**B**) and *gsfR1* (**C**). Griseofulvin production (µg/g of apple) was compared between wild
964 type and Δ*gsfR1* strains (**D**). Measurements were taken at 7, 10 and 14 dpi. WT = wild type *P.*
965 *griseofulvum*, Δ = deletion mutants and E = ectopic strains. Values followed by the same letter are
966 not statistically different by Duncan's multiple range test ($p < 0.05$).

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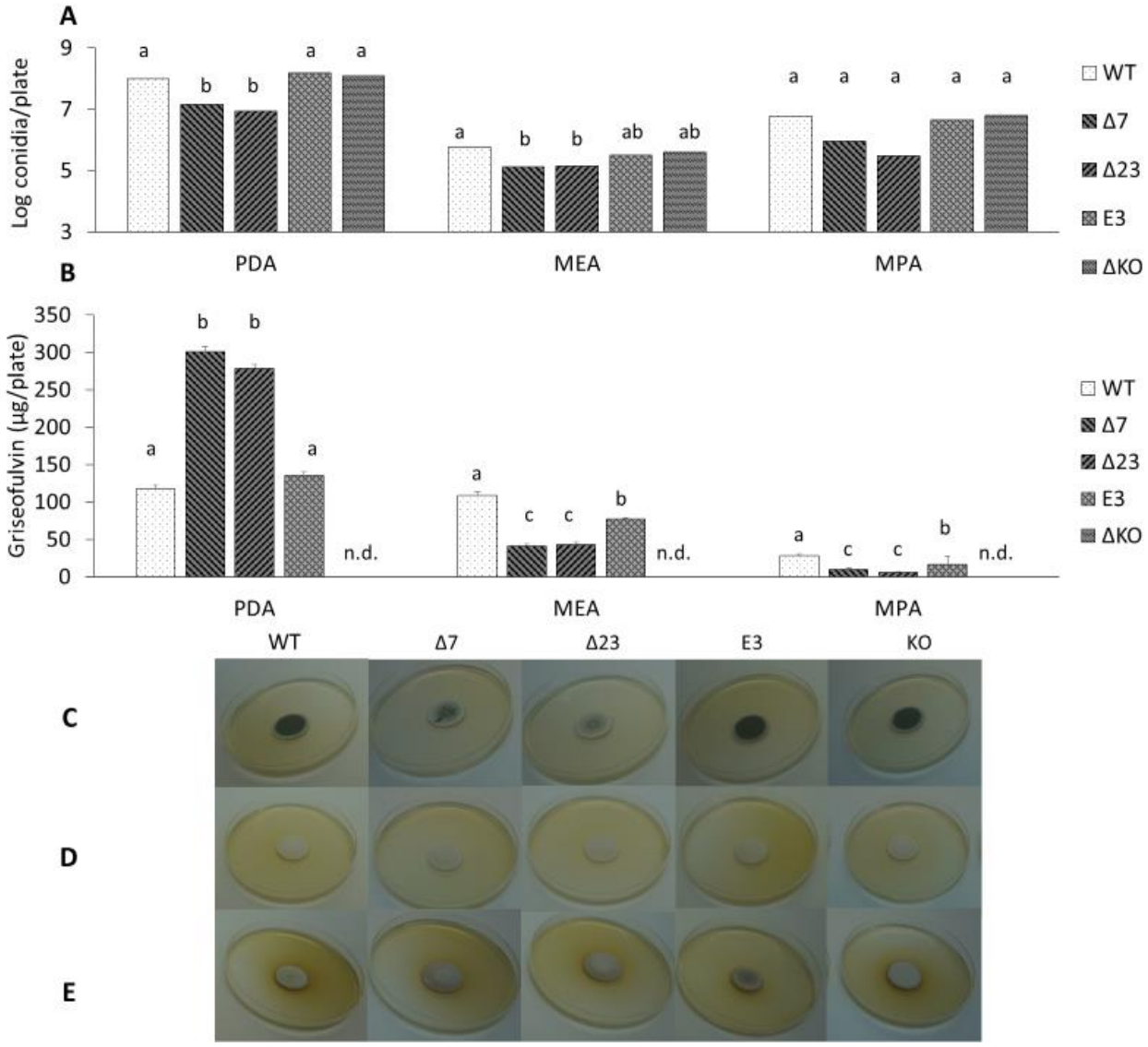

968

969 **Figure 9** – Effect of carbon and nitrogen on conidiation and on griseofulvin production *in vitro*.
970 Logarithm of conidia per plate (**A**) and griseofulvin produced (**B**) were measured at 10 dpi in three
971 solid media (PDA, MEA and MPA). Colony view on PDA (**C**), MEA (**D**) and MPA (**E**). WT = wild
972 type *P. griseofulvum*, Δ7, Δ23 = deletion mutants for *gsfR1*, E3 = ectopic strain and ΔKO = deletion
973 mutant for *gsfA*. Values followed by the same letter are not statistically different by Duncan's
974 multiple range test ($p < 0.05$); n.d. = non detectable.

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976

Supplementary material

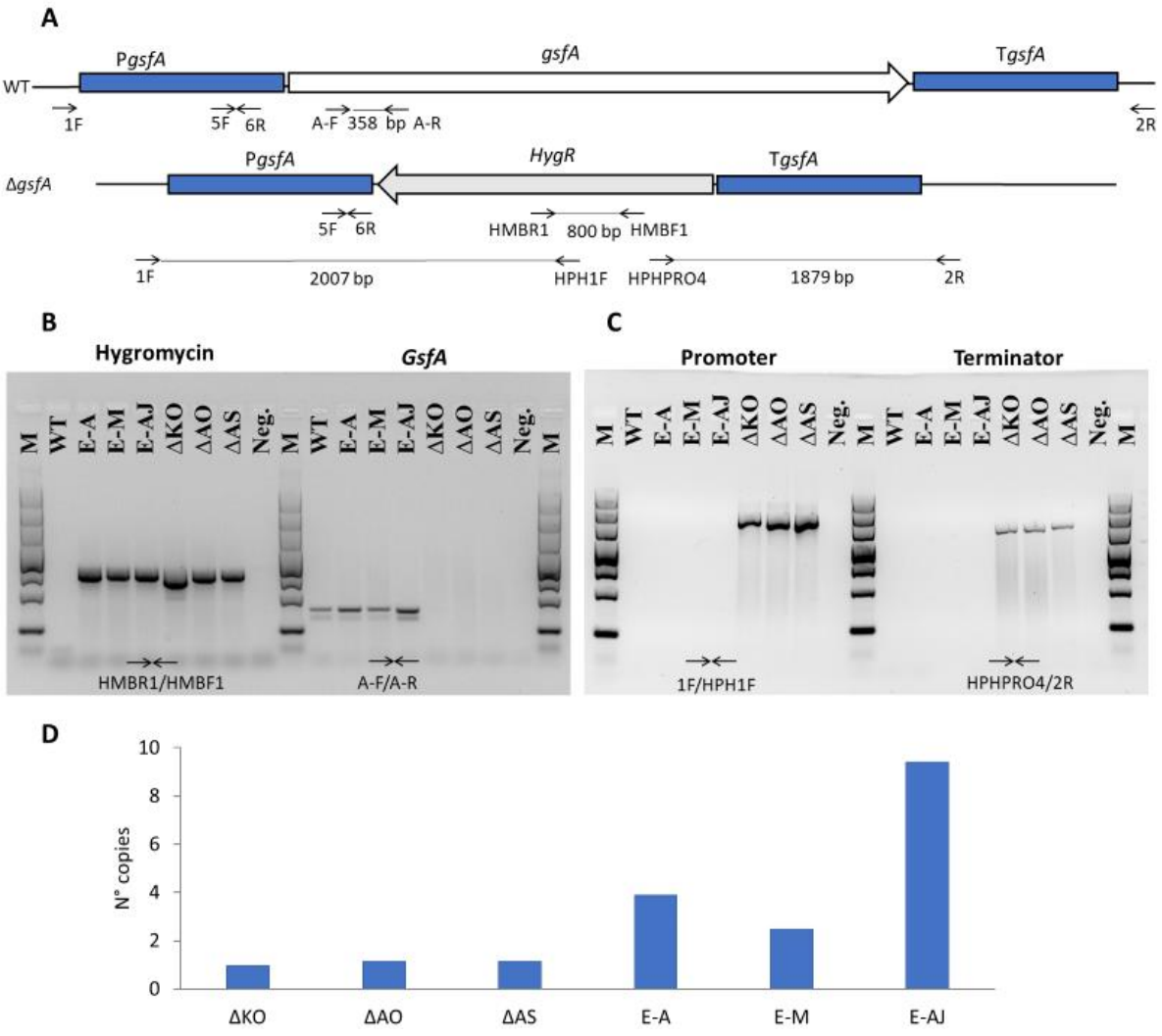
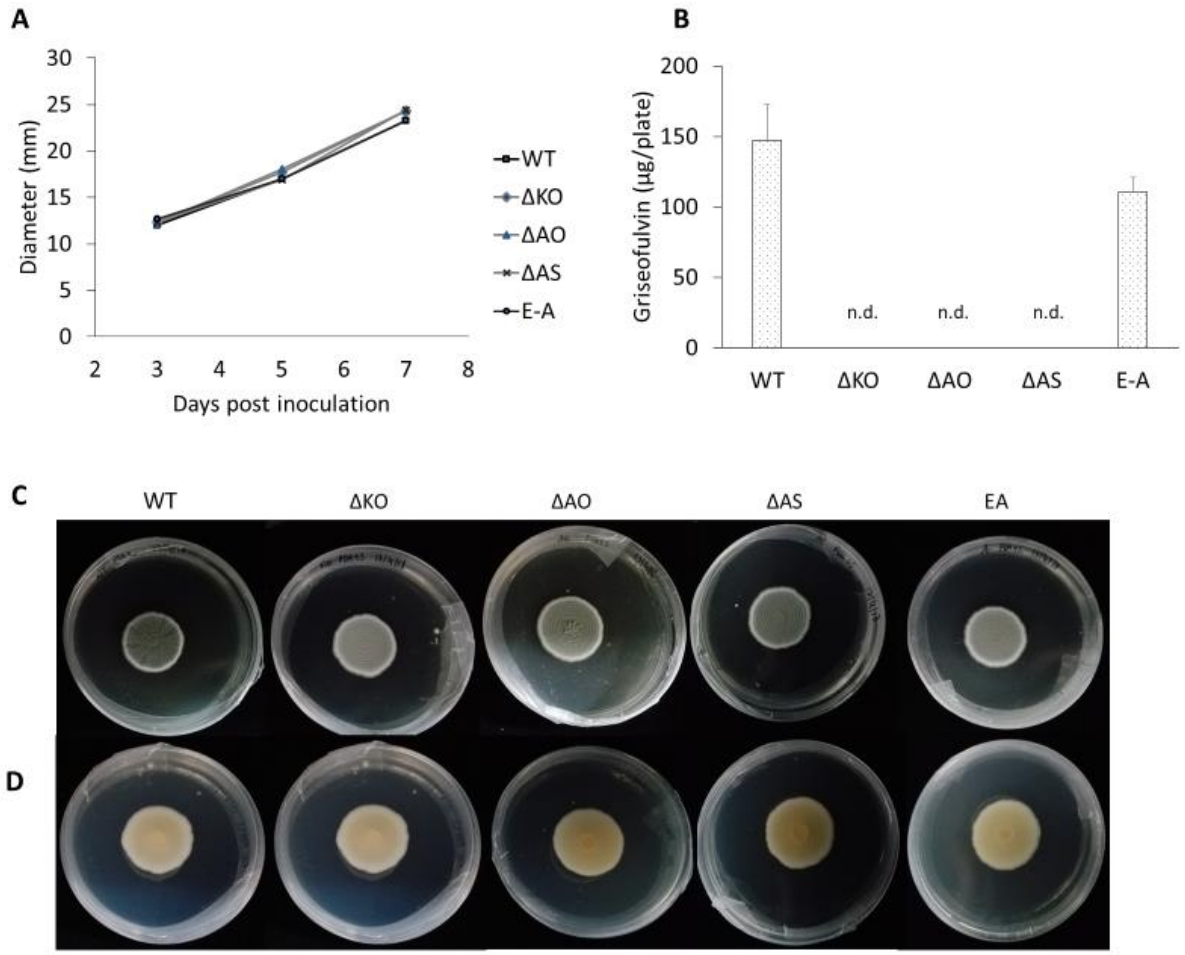


Figure S1 – PCR analysis of *gsfA* mutants. Schematic presentation of the *gsfA* locus in the wild type and deletion mutants, primers used are presented as arrows (A). Amplification of hygromycin resistance cassette (*HygR*) and *gsfA* gene (B); confirmation of orientation of inserted T-DNA (C); copies 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).



988

989 **Figure S2** – Effect of *gsfA* deletion on *P. griseofulvum.* Colony diameter (**A**) and griseofulvin
990 production (**B**) from 3 to 7 dpi. Plate view (front **C** and reverse **D**) at 7 dpi on PDA. WT = wild type
991 *P. griseofulvum*, Δ = deletion mutants for *gsfA* and E = ectopic strains, n.d. = non detectable.

992

993 **Table S1** – List of primers used in this work.

994

Primer name	Primer sequence (5'→3')	Description
Construction and analysis of knockout mutants		
O1- *gsfR1*	GGTCTTAAUGTCGGCATCGGACGAATTATAG	Insertion of *gsfR1* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfR1*	GGCATTAAUCATCTTTCTGAGGAGGGAGAGA	
A3- *gsfR1*	GGACTTAAUGCGCGAAACGGTATTTCTAT	Insertion of *gsfR1* terminator in pRFHU2. Tail for USER reaction in red.

A4- *gsfR1*	GGGTTTAAUGTGTGAGCGTGAATGGTAATG	
O1- *gsfR2*	GGTCTTAAUTCGGTGTCCACGTTTCTATTC	Insertion of *gsfR2* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfR2*	GGCATTAAUTTGCTTGCCACGGTTATCT	
A3- *gsfR2*	GGACTTAAUGACGAGCCAGTTCTCCTAATG	Insertion of *gsfR2* terminator in pRFHU2. Tail for USER reaction in red.
A4- *gsfR2*	GGGTTTAAUCTCCTTGCCCGATGTCTTATC	
O1- *gsfA*	GGTCTTAAUGCACACCTGAGCAAGATCAA	Insertion of *gsfA* promoter in pRFHU2. Tail for USER reaction in red.
O2- *gsfA*	GGCATTAAUTATTGCAGCTGCCGAGAGTA	
A3- *gsfA*	GGACTTAAUGGTCAATTCGGCGCACTTAA	Insertion of *gsfA* terminator in pRFHU2. Tail for USER reaction in red.
A4-*gsfA*	GGGTTTAAUTCGAGAGGGCAAGAATGTGT	
RF2	TCTCCTTGCATGCACCATTCCTTG	Confirmation of terminator fusion into pRFHU2 plasmid
RF5	GTTTGCAGGGCCATAGAC	
RF1	AAATTTTGTGCTCACCGCCTGGAC	Confirmation of promoter fusion into pRFHU2 plasmid
RF6	ACGCCAGGGTTTTCCCAGTC	
1F-*gsfR1*	CCCAACATGAGTGAAAGCATAAC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfR1*
1F-*gsfR2*	TCGCACCAGAGCAAACATAC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfR2*
1F-*gsfA*	TGATAGAGCATTCGCGGTCC	Confirmation of orientation of inserted T-DNA. Upstream region of *gsfA*
HPH1F	ACGAGGTCGCCAACATCTTCTTCT	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
2R-*gsfR1*	AGTGCAGTCGGTCAACAATAC	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfR1*
2R-*gsfR2*	GCTAGACGATGCGACACTACTA	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfR2*
2R-*gsfA*	TTGGGAAACTCGTCGACCTG	Confirmation of orientation of inserted T-DNA. Downstream region of *gsfA*

HPHPRO4	GCACCAAGCAGCAGATGATA	Confirmation of orientation of inserted T-DNA. Hygromycin cassette.
HMBF1	CTGTCGAGAAGTTTCTGATCG	Amplification of hygromycin resistance cassette
HMBR1	CTGATAGAGTTGGTCAAGACC	
R1-F	CTTGCGGGTCTCGAAGTAAA	Amplification of *gsfR1* gene
R1-R	CCGTCATCCGAATGTCTATCTG	
R2-F	TGCGAATACAACGGCCGATA	Amplification of *gsfR2* gene
R2-R	CATGAGAACCTCACGGAGAAA	
A-F	GCTTTGGTTTGGTTGTCGAT	Amplification of *gsfA* gene
A-R	CATCCCCTGGATCTTTCTCA	
Determination of number of integration and qPCR		
5F-*gsfR1*	GGATCTATCCATGTCGGAACAC	Determine number of integrations of T-DNA in *gsfR1* mutants and calculate relative expression of *gsfR1*
6R-*gsfR1*	CCTCACAGCCTGTTTGGTTA	
5F-*gsfR2*	GAAGCCGGATGATTTAGAGGAG	Determine number of integrations of T-DNA in *gsfR2* mutants
6R-*gsfR2*	CATGAGAACCTCACGGAGAAA	
5F-*gsfA*	TGCTAGAGTGCGTGACAGT	Determine number of integrations of T-DNA in *gsfA* mutants
6R-*gsfA*	AGGACGCTCTCAAAAGGTGG	
PeHis3_F2	TCTCCGCTTCCAGTCCTCTG	Amplification of histone H3 to determine number of integrations of T-DNA in *gsfR1* and *gsfR2* mutants
PeHis3_R2	TTGGTGTCCTCGAAGAGAGAGAC	
Pgr_tub_1F	CGAGTTGACCCAGCAGATGT	Amplification of β-tubuline to determine number of integrations of T-DNA in *gsfA* mutants and to calculate relative expression of *gsfR1* and *gsfA*.
Pgr_tub_2R	GTCTGGACGTTGTTGGGGAT	
gsfA-F	AAAAGACGGACAGTGACAGC	Calculate relative expression of *gsfA*.
gsfA-R	TATCCGCGATTTCACACTCG	

995

996 **Table S2** – Transcription factors putatively binding the promoter of *gsfR1* and *gsfA*. It is reported the
997 name of homolog and the regulated processes in other organisms with references when available.

Protein	**Homolog**	**Regulated processes**	**Reference**
Transcription factors binding only the promoter of *gsfR1*			
KXG45700.1	LreA	Conidiation, response to light	(Igbalajobi et al., 2019)
KXG46329.1	Fluconazole resistance protein	resistance to fluconazole	

KXG46595.1	NsdD	activation of sexual development	(Han et al., 2001)
KXG46600.1	res2	Meiosis	(Zhu et al., 1997)
KXG47124.1	Msn2	Conidiation, hyphal growth and stress response	(Liu et al., 2013; Song et al., 2018; Tian et al., 2017)
KXG47905.1	quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG48399.1	FlbC	conidiation	(Kwon et al., 2010)
KXG48752.1	Fluconazole resistance protein 3	Fluconazole resistance	
KXG48995.1	hypothetical protein		
KXG49526.1	Transcription factor PAP1		
KXG53661.1	Transcription factor		
KXG54396.1	MtfA (ID 50%)	regulation of aflatoxin production	(Zhuang et al., 2016)
KXG54691.1	StuA	Regulation of cell pattern formation	(Miller et al., 1992)
Transcription factors binding only the promoter of *gsfA*			
KXG46092.1	gal4	Using galactose or lactose as only carbon sources	(Riley et al., 1987)
KXG47012.1	Hypothetical protein		
KXG47549.1	SREP	repressor of siderophore biosynthesis in presence of high iron concentrations	(Haas et al., 1997)
KXG47976.1	Zinc finger, C2H2-type/integrase, DNA-binding		
KXG51025.1	transcriptional activator of gluconeogenesis	transcriptional activator of gluconeogenesis	(Hynes et al., 2007)
KXG51359.1	Filamentous growth regulator	Filamentous growth	
KXG52025.1	Achaete-scute transcription factor related		
Transcription factors putatively binding the promoters of both *gsfR1* and *gsfA*			

KXG45164.1	Basic-leucine zipper (bZIP) transcription factor		
KXG45306.1	Zinc finger, C2H2		
KXG45454.1	Transcription factor		
KXG45545.1	Hypothetical protein		
KXG46064.1	RlmA	response to cell wall stress, asexual development	(Damveld et al., 2005; Kovács et al., 2013)
KXG46347.1	RosA	represses sexual development	(Vienken et al., 2005)
KXG46776.1	Homeodomain-like		
KXG46828.1	Quinic acid utilization activator	Quinic acid utilization	(Wheeler et al., 1996)
KXG46909.1	Non-histone chromosomal protein 6		
KXG46994.1	Aflatoxin biosynthesis regulatory protein		(Chang et al., 1993)
KXG47056.1	High mobility group, HMG-I/HMG-Y		
KXG47172.1	Winged helix-turn-helix transcription repressor		
KXG47315.1	Protein of unknown function DUF3468		
KXG47412.1	NirA	Nitrate induction	(Burger et al., 1991)
KXG47486.1	Tbf1	Coordinated ribosomal protein (RP) gene expression	(Hogues et al., 2008)
KXG47556.1	Protein of unknown function DUF3468		
KXG47599.1	CRZ1	Calcineurin pathway	(Cramer et al., 2008)
KXG47656.1	C6 transcription factor		
KXG47688.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG47847.1	AmdA	acetate induction	(Lints et al., 1995)
KXG47936.1	Hypothetical protein		
KXG47959.1	Hypothetical protein (RfeB)		

KXG48139.1	PrnA		
KXG48212.1	Homeodomain-like		
KXG48341.1	Activator of stress genes		
KXG48355.1	GATA transcription factor nreB		
KXG48533.1	Putative transcription factor kapC		
KXG48617.1	Protein of unknown function DUF3468		
KXG48682.1	Biofilm and cell wall regulator 1	Biofilm and cell wall	(Nobile and Mitchell, 2005)
KXG48765.1	Homeodomain		
KXG48781.1	Activator of stress genes 1	stress response	
KXG48784.1	Hypothetical protein		
KXG48884.1	AtfA	Spore germination	(Wolfers et al., 2015)
KXG48955.1	Hypothetical protein		
KXG48983.1	TATA-box binding protein		
KXG49138.1	Hypothetical protein		
KXG49157.1	facB	Regulation of genes involved in acetate utilization	(Todd et al., 1998)
KXG49345.1	acu-15	Positive regulator of acetate induction	(Bibbins et al., 2002)
KXG50499.1	carbon catabolite repressor		
KXG50601.1	Hypothetical protein (Sfp1)		
KXG50650.1	ARO80	catabolism of aromatic amino acids	(Lee et al., 2013)
KXG50787.1	transcription factor Prf		
KXG50810.1	HMG box protein		
KXG50911.1	Hypothetical protein		
KXG51030.1	cef1	pre-mRNA splicing and cell cycle control	(Kellner et al., 2014)
KXG51076.1	C6 transcription factor		

KXG51406.1	cellobiose response regulator 2	cellobiose response	
KXG51472.1	Heat shock factor (HSF)-type	Heat response	
KXG51622.1	Fungal transcriptional regulatory protein		
KXG51830.1	Hypothetical protein		
KXG52055.1	CCAAT-binding transcription factor, subunit B		
KXG52059.1	bZIP transcription factor FlbB		
KXG52166.1	C6 transcription factor		
KXG52228.1	Zinc finger, C2H2-like		
KXG52264.1	Basic-leucine zipper (bZIP) transcription factor		
KXG52366.1	Forkhead transcription factor (Sep1)		
KXG52406.1	Hypothetical protein		
KXG52464.1	DNA damage and replication checkpoint protein Rfx1		
KXG52761.1	Respiration factor		
KXG52955.1	Hypothetical protein		
KXG53008.1	stress response transcription factor SrrA/Skn7	response to oxidative stress	(Cao et al., 2009)
KXG53655.1	Acetamidase regulatory protein	catabolism of certain amides, omega amino acids and lactams	(Andrianopoulos and Hynes, 1990)
KXG53834.1	Homeodomain-like		
KXG53935.1	Cutinase transcription factor 1 beta		
KXG54406.1	MCM1	Induces mating, growth rate, pathogenesis and appressorium formation	(Zhou et al., 2011)

KXG54419.1	AreA	nitrogen response	(Katz et al., 2008)
KXG54528.1	Zinc finger, C2H2-like		
KXG54658.1	Transcription factor		

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