Signaling Governed by G Proteins and cAMP Is Crucial for Growth, Secondary Metabolism and Sexual Development in *Fusarium fujikuroi*

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**Abstract**

The plant-pathogenic fungus *Fusarium fujikuroi* is a notorious rice pathogen causing hyper-elongation of infected plants due to the production of gibberellins (GAs). In addition to GAs, *F. fujikuroi* produces a wide range of other secondary metabolites, such as fusarins, fusaric acid and the red polyketides bikaverins and fusarubins. The recent availability of the fungal genome sequence for this species has revealed the potential of many more putative secondary metabolite gene clusters whose products remain to be identified. However, the complex regulation of secondary metabolism is far from being understood. Here we studied the impact of the heterotrimeric G protein and the cAMP-mediated signaling network, including the regulatory subunits of the cAMP-dependent protein kinase (PKA), to study their effect on colony morphology, sexual development and regulation of bikaverins, fusarubins and GAs. We demonstrated that fusarubin biosynthesis is negatively regulated by at least two Gz subunits, FfG1 and FfG3, which both function as stimulators of the adenylyl cyclase FfAC. Surprisingly, the primary downstream target of the adenylyl cyclase, the PKA, is not involved in the regulation of fusarubins, suggesting that additional, yet unidentified, cAMP-binding protein(s) exist. In contrast, bikaverin biosynthesis is significantly reduced in ffg1 and ffg3 deletion mutants and positively regulated by FFAC and FFPKA1, while GA biosynthesis depends on the active FFAC and FFPKA2 in an FfG1- and FfG3-independent manner. In addition, we provide evidence that G Protein-mediated/cAMP signaling is important for growth in *F. fujikuroi* because deletion of ffg3, ffac and ffpka1 resulted in impaired growth on minimal and rich media. Finally, sexual crosses of ffg1 mutants showed the importance of a functional FfG1 protein for development of perithecia in the mating strain that carries the MAT1-1 idiomorph.

**Introduction**

Members of the genus *Fusarium* are known to produce a wide range of secondary metabolites, such as mycotoxins and pigments, under defined environmental conditions. This ability makes them a promising tool to study regulatory aspects of secondary metabolite production at the molecular level. The heterothallic ascomycete *Fusarium fujikuroi* (teleomorph: Gibberella fujikuroi) was first described as the causative agent of the ‘bakanae’ or foolish seedling disease of rice plants due to the production of gibberellins (GAs). In addition to GAs, *F. fujikuroi* is able to produce various other secondary metabolites such as the mycotoxins fusarins C [5], fusaric acid [6] and fumonisins [7], the red polyketide bikaverin [8] or the terpenoid -acorenol [9]. Moreover, the recently sequenced genome of the *F. fujikuroi* wild-type strain IMI58289 revealed the presence of further putative secondary metabolite gene clusters whose products remain to be identified (B. Tudzynski and Co-workers, unpublished data). However, their regulation appears to be quite diverse and little understood.

A prerequisite for the initiation of secondary metabolite biosynthesis under specific conditions is the ability to sense and transduce external signals to downstream targets, e.g. transcription factors, which in turn can activate the expression of genes involved in biosynthesis of a certain secondary metabolite. Prominent components of signal transduction pathways are heterotrimeric G proteins that integrate a variety of signals and transduce them to downstream signaling cascades.

In general, heterotrimeric G proteins are composed of a Gα subunit and a tightly associated dimer of β and γ subunits (Gβγ). The Gα subunit is associated with a seven transmembrane helices-containing G protein-coupled receptor (GPCR) at the plasma-membrane [10]. In the inactive state Gα is bound to GDP. Once the GPCR is activated by its cognate ligand, conformational changes in the receptor induce the exchange of GDP for GTP leading to its activation and subsequent dissociation from the Gβγ dimer. Both the activated Gα subunit and the dissociated Gβγ subunits are now able to bind and regulate downstream effectors [11]. One well known downstream effector of the activated Gα is the adenylyl cyclase that produces the second messenger cyclic AMP (cAMP) which in turn binds to the regulatory subunits of the
cAMP-dependent protein kinase A (PKA). In general, filamentous fungi possess one regulatory subunit and two catalytic subunits of PKA. In the inactive state two regulatory subunits are tightly associated with two catalytic subunits. Upon cAMP-binding to the regulatory subunits the affinity for the catalytic subunits decreases. Subsequently the catalytic subunits of the PKA are released and are now able to phosphorylate downstream targets [12,13]. As G proteins constitute the first components of signaling chains they represent a promising target to study the involvement of such signaling cascades in cellular processes including secondary metabolism.

Most filamentous fungi have three G proteins, each belonging to classes I, II or III [12]. G subunits of class I show high sequence similarity and are known to be involved in the regulation of fungal development, pathogenicity and secondary metabolism. The best studied example with regard to secondary metabolism is FaDA from Aspergillus nidulans. Deletion of faDA resulted in upregulation of sterigmatocystin and aflatoxin biosynthesis, while constitutive activation of faDA resulted in precocious sterigmatocystin production as well as increased penicillin biosynthesis [14–16]. Furthermore, alterations in secondary metabolism have been observed in Ga2 mutants from Botrytis cinerea [17], Cryptocentrum pennisetica [18], F. graminearum [19], F. graminearum [20], Neurospora crassa [20], Penicillium chrysogenum [21], P. marneffei [22] and Trichoderma atrovirens [23].

Contrary to G proteins from class I, G subunits belonging to classes II and III have so far not been linked to secondary metabolism in filamentous fungi. Although G subunits of class III are clearly involved in fungal development and pathogenicity (e.g. A. nidulans, B. cinerea and F. graminearum) [24–26], deletion of class II G proteins had only minor effects on fungal metabolism [27,28].

First indication for the involvement of G protein signaling in secondary metabolism in F. fujikuroi was the observation that deletion of ffpka1 resulted in the secretion of a red pigment into the surrounding medium when grown on agar plates. To our surprise the red pigment could not be linked to bikaverin biosynthesis because expression of the bikaverin genes was not elevated in this mutant. Recently a second group of red pigments, the fusarubins, was the observation that F. fujikuroi [28]. These results encouraged us to study the involvement of G protein-mediated signaling and further downstream targets with the main focus on secondary metabolism. In this paper we show that FfG1 and FfAC, but also other components of secondary metabolism.

Materials and Methods

Fungal strains and culture conditions

For all knock-out experiments either the wild-type strain Fusarium fujikuroi IMI58289 (Commonwealth Mycological Institute, Kew, United Kingdom) or F. fujikuroi MRC-1995 (kindly provided by J.F. Leslie, Kansas State University) was used. For cultivation in subculture F. fujikuroi strains were pre-incubated in 300 ml Erlenmeyer flasks with 100 ml Darken medium (DVK) [29] on a rotary shaker for 72 h at 28°C and 180 rpm. For RNA isolation and secondary metabolite analysis a 500 µl aliquot of this culture was used for inoculation of synthetic ICI (Imperial Chemical Industries Ltd., United Kingdom) medium [30] with the desired nitrogen source and cultivated for an additional 3–7 days. Protoplasts were produced in 100 ml ICI medium with 10 g/l fructose instead of glucose and 1 g/l (NH₄)₂SO₄ using 500 µl of the pre-incubated culture and additional cultivation for more than 16 h on a rotary shaker at 28°C and 180 rpm. For the nitrogen shift-experiments the mycelia were grown in ICI media on a rotary shaker at 28°C and 180 rpm. After 4 days either 33 mM glutamine, 33 mM sodium nitrate or no nitrogen was added and cultures were incubated for additional 30 min. Plate assays were performed on complete medium (CM) as well as on Crzpeck Dox (CD) medium for up to 7 days [31,32]. For sexual crosses the fungal strains were grown on carrot agar according to Klinkitch and Leslie [33]. For DNA isolation, the fungus was grown on solid CM with Cellophane overlays for 3 days at 28°C. For RNA isolation, the fungus was grown in ICI medium with the specified nitrogen source for 3–4 days at 28°C and 180 rpm upon pre-incubation in DVK.

Plasmid constructions

Plasmid construction for deletion of ffpka1 was accomplished by amplification of about 1 kb upstream and downstream sequences of ffpka1 using the primer pairs ffpka1_5_SacII//ffpka1_5_HphI (upstream) and ffpka1_3_SalI//ffpka1_3_XhoI (downstream) containing the following restriction sites: 5′ SacII//XbaI, 3′ SalI//XhoI. Subsequently the fragments were cloned into pNR1 which contains the nourseothricin resistance cassette [34]. Finally the knock-out fragment was restricted using SacII/Xhol and used for F. fujikuroi transformation. All other knock-out fragments subsequently used for F. fujikuroi transformation were created using yeast recombinational cloning [35]. The 5′ and 3′ regions of the corresponding genes were amplified with appropriate primer pairs, for the 5′ region, 5F and 5R primers were used, and for the 3′ region, primers 3F and 3R were used, work based on the genomic sequence available for F. fujikuroi IMI58299 (B. Tuzdynski and co-workers, unpublished data). Hygromycin and nourseothricin were used as resistance markers. The hygromycin resistance cassette, consisting of the hygromycin B phosphotransferase gene hph [36], driven by the tetrC promoter, was amplified using the primer pair hph-F/R from the template pCSN44. All primers used for PCR were obtained from Eurofins GmbH (Ebersberg, Germany) (table S1). The obtained fragments were cloned into the Saccharomyces cerevisiae strain FY834 together with the EcoRI/Xhol-restricted plasmid pRS426. For complementation of Δffg1 and constitutive activation of FIG1 the gene ffg1, including ~2-kb promoter and ~200-bp terminator sequence, was amplification in two fragments with appropriate primer pairs, work based on the genomic sequence available for the pre-incubated culture and additional cultivation for more than 16 h on a rotary shaker at 28°C and 180 rpm. For the nitrogen shift-experiments the mycelia were grown in ICI media on a rotary shaker at 28°C and 180 rpm. After 4 days either 33 mM glutamine, 33 mM sodium nitrate or no nitrogen was added and cultures were incubated for additional 30 min. Plate assays were performed on complete medium (CM) as well as on Crzpeck Dox (CD) medium for up to 7 days [31,32]. For sexual crosses the fungal strains were grown on carrot agar according to Klinkitch and Leslie [33]. For DNA isolation, the fungus was grown on solid CM with Cellophane overlays for 3 days at 28°C. For RNA isolation, the fungus was grown in ICI medium with the specified nitrogen source for 3–4 days at 28°C and 180 rpm upon pre-incubation in DVK.

Standard molecular methods

For DNA isolation, lyophilized mycelium was ground to a fine powder in liquid N₂ and prepared according to Cenis [30] and afterwards used for PCR amplification. For PCR, 25 ng genomic DNA, 5 pmol of each primer, 200 nM deoxynucleoside triphosphates, and 1 unit BioTherm™ DNA polymerase (GeneCraft GmbH, Ludwigshafen, Germany) was used for diagnostic PCR and amplification of upstream and downstream regions in yeast-
The mycelium was ground in liquid N$_2$ and RNA was extracted using the RNAagents total RNA isolation kit (Promega, USA). DNA and protein were extracted using the yeast plasmid isolation kit (SpeedPrep, DualsystemsBioTech) and directly used for PCR reaction. In case of complementation, the obtained vectors were cloned into E. coli and subsequently extracted using the GenJetTM Plasmid Miniprep Kit (Fermentas GmbH, St. Leon-Rot, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNA and protein sequence alignments were done with DNA STAR (Madison, WI, USA).

For northern blot analysis RNA was isolated from lyophilized mycelium. The mycelium was ground in liquid N$_2$ and RNA was extracted using the RNAagents total RNA isolation kit (Promega, Mannheim, Germany) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNA and protein sequence alignments were done with DNA STAR (Madison, WI, USA).

**Fungal transformations**

Protoplasts were prepared either from F. fujikuroi IMI58289, for deletion of ffg1, ffg3, ffa, ffpka1 and ffpka2, or from F. fujikuroi MRC-1993, for deletion of ffg1. Transformation was carried out as previously described [42]. About 10$^7$ protoplasts were transformed with $\sim$10 mg of the amplified replacement cassettes for generating the knock-out. For complementation of A$_{ffg1}$ the deletion mutant A$_{ffg1-T9}$ was transformed with 10 mg of the plasmid pNDO-OGG-ffg1$,C$, containing ffg1 driven by its native promoter. According to this A$_{ffg3-T6}$ and A$_{ffpc-T7}$ were complemented with 10 mg of the plasmids NDN-OGG-ffg3$,C$ and NDN-OGG-ffpc$,C$, respectively. For constitutive activation of FfG1 the F. fujikuroi wild-type strain IMI58289 was transformed with 10 mg of the plasmid NDN-OGG-ffg1$^{G42R}$. Transformed protoplasts were regenerated as described by Tuzdynski et al. [43]. The medium contained the appropriate resistance marker. Single conidial cultures were established from either hygromycin B- or nourseothricin-resistant transformants and used for subsequent DNA isolation.

**Analysis of secondary metabolites**

For analysis if bikaverin and fusarubins, culture fluids from 3-to 7-day old cultures were filtered through a 0.2 $\mu$m membrane filter (Millipore, Milipore) and directly used for analysis without further preparation, as described in Studt et al. [28]. For gibberellic acid analysis, aliquots (20 ml) of culture fluid from 7–day-old cultures were extracted over a C18-cartridge and measured using HPLC-UV as described by Wiemann et al. [44]. GA accumulation was quantified relative to the dry weight of the respective trains to exclude the possibility that merely impaired growth of an isolate was responsible for the observed effects on GA biosynthesis.

**Results**

**Identification of Gz subunits in Fusarium fujikuroi**

Like most other filamentous fungi F. fujikuroi possesses three Gz subunits, designated as FfG1 (AJ315470), FfG2 (HF563536) and FfG3 (HF563557). These three proteins were identified in the genome of the F. fujikuroi wild-type strain IMI58289 by a BlastP search [45] using the protein sequences of BCG1 (GenBank: CCD53386.1), BCG2 (GenBank: CCD44342.1) and BCG3 (GenBank: CCD41756.1) from Botrytis cinerea as a reference [17,26]. Gz proteins from different fungi, including F. fujikuroi, can be divided into three main classes based on amino acid sequence similarities: FfG1 groups together with homologues in other fungi into class I, e.g. FadA from A. nidulans [46], MAGB from M. oryzae [27], GNA-1 from N. crassa [47], FGA1 from F. oxysporum [48] and GPA1 from F. graminearum [19]. This class of Gz proteins share sequence similarities with the mammalian Gz, proteins that are thought to inhibit adenylyl cyclase activity [12]. FfG3 grouped together with homologues in class III, e.g. GanB from A. nidulans [24], MAGA from M. oryzae [27], NGA-3 from N. crassa [49] and GPA2 from F. graminearum [50] all showing high similarity with mammalian Gz, which stimulate adenylyl cyclase activity [12]. Finally, FfG2 belongs to the fungal Gz class II which has no mammalian counterpart [12] (fig. 1).

**FfG1 functions as a negative regulator for fusarubin biosynthesis**

In order to study the functions of FfG1 in F. fujikuroi the gene was deleted by homologous integration of a hygromycin resistance cassette into the wild-type strain IMI58289, hereafter called wild type (WT). Homologous integration of the resistance cassette was verified by diagnostic PCR (fig. S1A). Two independent deletion mutants, A$_{ffg1-T9}$ and A$_{ffg1-T13}$, were obtained that show identical phenotypes. Hence, A$_{ffg1-T9}$ was arbitrarily chosen for further investigations. In addition to ffg1 deletion mutants, we generated transformants expressing a constitutively active version of the Gz subunit FfG1 with an exchange of glycine for arginine at position 42 [FfG1$^{G42R}$] in the WT background. This mutation was previously shown to prevent the intracellular GTPase activity of Gz and thereby the inactivation of the heterotrimeric G protein [46]. The transformation and subsequent characterization of nourseothricin-resistant transformants yielded three independent transformants [ffg1$^{G42R}$] (fig. S2). Integration of the vector and presence of point mutation was verified by diagnostic PCR and subsequent sequencing of the obtained PCR fragments (data not shown).

To study the impact of ffg1 mutations on secondary metabolism we grew the wild type and the A$_{ffg1}$ and the ffg1$^{G42R}$ mutants in synthetic ICI medium with either 6 mM glutamine or 6 mM sodium nitrate, representing optimal culture conditions for the production of the two red polyketides, bikaverins and fusarubins, respectively [51,52,28]. Using sodium nitrate as sole nitrogen source both the wild type and the deletion mutant showed the typical dark red pigmentation characteristic for the accumulation of fusarubins (fig. 2A). Using glutamine, the coloration of the culture broth of A$_{ffg1}$ differed markedly from that of the wild type. Subsequent analysis using high performance liquid chromatography (HPLC) coupled to a diode array detector (HPLC-DAD) revealed that the A$_{ffg1}$ mutant produced only trace amounts of bikaverin in contrast to the wild type. However, high concentra-
are up-regulated under nitrogen starvation as shown previously for changes in gene expression. Fusarubin biosynthetic genes (under nitrogen limiting conditions (6 mM sodium nitrate). nitrate was added to cultures which were grown for four days glutamine (33 mM), sodium nitrate (33 mM) or water (no regulation of fusarubin biosynthesis under nitrogen excess, ffg1 genes were not expressed (fig. 2B). Neither deletion nor bikaverin under bikaverin-favoring conditions, and, accordingly, fusarubin production has been detected under both conditions doi:10.1371/journal.pone.0058185.g001
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Figure 1. Phylogenetic relationship between Fusarium fujikuroi FFG1, FFG2, FFG3 and G proteins from other fungi. The phylogram was generated based on multiple sequence alignments [41] using the following G proteins: Fada, GanA and GanB from Aspergillus nidulans [15,24]; BcG1, BcG2 and BcG3 from Botrytis cinerea [17,26]; FFG1, FFG2, FFG3 from Fusarium fujikuroi (this paper); GPA1, GPA2 from F. graminearum [19]; FGA1, FGA2 from F. oxysporum [47,49]; MAGB, MAGC and MAGA from Magnaporthe grisea [27]; GNA-1, GNA-2 and GNA-3 from Neurospora crassa [46,48]; GPA1 and GPA2 from Saccharomyces cerevisiae [86] and GPA1, GPA2, GPA3 and GPA4 from Ustilago maydis [87]. The Gα protein class IV GPA2 that is unique for U. maydis was used out-group. The bar presents 0.6 character change. Organisms: An Aspergillus nidulans, Bc Botrytis cinerea, F F. fujikuroi, Fg F. graminearum, Fo F. oxysporum, Mo Magnaporthe grisea, Nc Neurospora crassa, Sc Saccharomyces cerevisiae and Um U. maydis. doi:10.1371/journal.pone.0058185.g001

tions of fusarubins, not present in the wild type under these conditions, were produced in Δffg1 (fig. 2A). Northern blot analysis verified the HPLC data as fusarubin biosynthetic genes (fos1 encoding the polyketide synthase; fos2 encoding an O-methyltransferase) were up-regulated in Δffg1 under both fusarubin-favoring (6 mM sodium nitrate) and bikaverin-favoring (6 mM glutamine) conditions (fig. 2B).

In the Δffg1412R mutant, no expression of fusarubin genes and no fusarubin production has been detected under both conditions (fig. 2A-B). Surprisingly, the Δffg1412R mutant produced also less bikaverin under bikaverin-favoring conditions, and, accordingly, bikaverin biosynthetic genes were significantly down-regulated. Under repressing conditions (6 mM sodium nitrate) the bikaverin genes were not expressed (fig. 2B). Neither deletion nor constitutive activation of Δffg1412R showed any alterations with regard to GA biosynthesis or colony morphology (data not shown).

To investigate whether deletion of Δffg1 also results in deregulation of fusarubin biosynthesis under nitrogen excess, glutamine (33 mM), sodium nitrate (33 mM) or water (no nitrogen) was added to cultures which were grown for four days under nitrogen limiting conditions (6 mM sodium nitrate). Cultures were incubated for an additional 30 min and analyzed for changes in gene expression. Fusarubin biosynthetic genes (fos1) are up-regulated under nitrogen starvation as shown previously and upon addition of sodium nitrate (fig. 2B). However, repression of fusarubin biosynthetic genes by glutamine excess could not be overruled in the ffg1 deletion mutant compared to the wild type (fig. 2C). Complementation of Δffg1 deletion mutants by homologous reintroduction of the respective gene, driven by its native promoter, rescued the wild-type phenotype with regard to fusarubin and bikaverin biosynthesis (fig. S3/S4).

Functional characterization of further components of the cAMP pathway
A prominent downstream target of heterotrimeric G proteins is the adenyl cyclase. The activated enzyme produces cAMP which in turn activates the PKA via binding to its regulatory subunit [11]. In order to find out if FIG1 regulates the fusarubin biosynthesis via the cAMP/PKA signaling pathway, we studied the single components of this pathway in more detail. The adenyl cyclase sequence as well as the two catalytic subunits of the PKA were identified by BlastP analyses [43] in the genome database of F. fujikuroi IMI58289 (B. Dudzynski and co-workers, unpublished data) using the corresponding protein sequences of B. cinerea BAC [53], BcPKA1 and BcPKA2 [32] as query. Accordingly, the F. fujikuroi proteins were designated as FflAC (HF563555), FfPKA1 (HF563558) and FfPKA2 (HF563559). All genes were deleted in the F. fujikuroi wild type. Three independent deletion mutants of Δflac were obtained Δffg1412R, Δffg1412R and Δffg1412R (fig. S1C). As all three mutants showed the same phenotype Δffg1412R was arbitrarily chosen for further studies. Deletion of Δffg1412R yielded three independent deletion mutants: Δffg1412R, Δffg1412R, and Δffg1412R resulted in four independent mutants: Δffg1412R, Δffg1412R, Δffg1412R and Δffg1412R. As all Δffg1412R mutants on the one hand, and all Δffg1412R on the other hand, showed a similar phenotype, Δffg1412R and Δffg1412R were arbitrarily chosen for further investigation (fig. S1D-C).

The cAMP/PKA pathway has impact on secondary metabolism
Similarly to Δffg1, deletion of Δflac also caused significant up-regulation of fusarubin biosynthesis under favoring (6 mM sodium nitrate) (data not shown) as well as under non-favoring conditions (6 mM glutamine), while bikaverin biosynthesis is decreased in both conditions (fig. 3A). Surprisingly, neither deletion of Δflac nor Δflac induced the formation of fusarubin. However, bikaverin production was reduced in the Δflac but not the Δflac mutant (fig. 3A). It is notable that deletion of Δflac resulted in an about three times higher accumulation of fusarubins compared to the Δflac mutants under both fusarubin-favoring (data not shown) and non-favoring conditions (fig. 3A). Expression of fusarubin-biosynthetic genes in Δflac mutants started earlier, already after 3 days of incubation, while expression in Δflac mutants was detectable only after 4 days (fig. 3C). The much stronger effect of Flac on fusarubin biosynthesis, compared to that of the upstream-acting FIG1, indicates the existence of an additional upstream activator of Flac besides FIG1 exist. In several filamentous fungi two of the three Gα subunits (class I and III) were shown to stimulate the AC activity [11,12]. For example, in B. cinerea addition of exogenous cAMP can partially restore the defects of the respective knockout mutants [17,53,26]. To test whether FIG3, a Gα protein belonging to class III, is also involved in fusarubin regulation we analyzed the Δflac deletion mutants in more detail. Indeed, we found elevated fusarubin gene expression and a strong accumulation of these pigments under non-favoring conditions indicating the involvement of FIG3 in fusarubin biosynthesis, as it was shown for FIG1 and Flac (fig. 3). Complementation of Δflac and Δflac deletion mutants by homologous reintroduction of the respective

Figure 1. Phylogenetic relationship between Fusarium fujikuroi FFG1, FFG2, FFG3 and Gα proteins from other fungi. The phylogram was generated based on multiple sequence alignments [41] using the following G proteins: Fada, GanA and GanB from Aspergillus nidulans [15,24]; BcG1, BcG2 and BcG3 from Botrytis cinerea [17,26]; FFG1, FFG2, FFG3 from Fusarium fujikuroi (this paper); GPA1, GPA2 from F. graminearum [19]; FGA1, FGA2 from F. oxysporum [47,49]; MAGB, MAGC and MAGA from Magnaporthe grisea [27]; GNA-1, GNA-2 and GNA-3 from Neurospora crassa [46,48]; GPA1 and GPA2 from Saccharomyces cerevisiae [86] and GPA1, GPA2, GPA3 and GPA4 from Ustilago maydis [87]. The Gα protein class IV GPA2 that is unique for U. maydis was used out-group. The bar presents 0.6 character change. Organisms: An Aspergillus nidulans, Bc Botrytis cinerea, F F. fujikuroi, Fg F. graminearum, Fo F. oxysporum, Mo Magnaporthe grisea, Nc Neurospora crassa, Sc Saccharomyces cerevisiae and Um U. maydis. doi:10.1371/journal.pone.0058185.g001
gene, driven by its native promoter, restored the wild-type phenotype with regard to fusarubin biosynthesis (fig. S3).

**FFVEL1 and FFG1 regulate pigment biosynthesis in an opposite manner**

Previously we have shown that the global regulator Velvet negatively regulates bikaverin production in *F. fujikuroi*: bikaverin biosynthetic genes are dramatically upregulated in the Δffvel1 mutant under inducing conditions [54]. Even under conditions unfavorable for bikaverin production small amounts of bikaverin can be detected in the liquid medium. Studying the impact of the Δffvel1 deletion on fusarubin biosynthesis, we observed a strong inhibition under both conditions (fig. 4). Therefore, deletion of Δffg1 caused opposing effects compared to the deletion of Δffvel1: less bikaverin, but increased formation of fusarubins under inducing,
as well as repressing, conditions in contrast to the elevated bikaverin and reduced fusarubin production in the Δffvel1 mutant (fig. 4).

**GA biosynthesis is regulated by FfAC and FfPKA2 and does not involve FfG1 and FfG3.**

GA biosynthesis is the ‘trade mark’ of *F. fujikuroi*, as only a few fungal species are able to produce these isoprenoid phytohormones. Therefore, we wanted to know if biosynthesis of GAs is also regulated via the G protein/cAMP pathway. We cultivated the wild type and mutant strains under GA biosynthesis-inducing conditions (6 mM glutamine) for 7 days. The quantity of produced GAs was analyzed by HPLC-UV. Interestingly, neither of the two Ga subunits, FfG1 and FfG3, was involved in regulation of GA biosynthesis. However, deletions of ffac and ffpka2 resulted in a significant reduction of GA biosynthesis of about 70% (fig. 5), while deletion of ffpka1 had no effect. These data indicate that FfAC can be activated by additional components other than the two Gα subunits, and that the two catalytic subunits of the PKA have distinct functions.

**Figure 3. Deletion of ffg1, ffac and ffg3 results in fusarubin biosynthesis under unfavorable conditions.** The WT and cAMP pathway mutants (Δffg1, Δffac, Δffpka1, Δffpka2, Δffg3) were grown for 3, 4 (for Northern blot analysis) or 7 days (for chemical analysis) in synthetic ICI medium (6 mM glutamine (Gln)). A) Liquid cultures were directly used for HPLC-DAD analysis (for details see material and methods). Fusarubins (FSR) are highlighted by the red box, bikaverins (BIK) by the purple box. Flasks containing culture broth of the WT and cAMP pathway mutants (Δffg1, Δffac, Δffpka1, Δffpka2) after 7 days of cultivation are shown on the left, corresponding HPLC-DAD chromatograms at 450 nm on the right. The scale for Δffac was set to 340 mAU due to a higher accumulation of compounds compared to the other strains. B) Photograph of the culture broth and corresponding HPLC-DAD chromatogram at 450 nm of Δffg3. C) Northern blot of WT and cAMP pathway mutants after 3 and 4 days of growth under fusarubin unfavorable conditions. The fusarubin biosynthetic genes, *fsr1* and *fsr2*, and the bikaverin biosynthetic genes, *bik1* and *bik2*, were used for probing.

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Involvement of G protein-mediated cAMP/PKA signaling in fungal growth

To study the involvement of the analyzed signaling components on fungal growth in *F. fujikuroi*, we grew the wild type and the different mutants for 7 days on both complete medium (CM) and minimal Czapek-Dox (CD) medium and determined the radial growth rates. When grown on CM, \( \Delta ffg3 \), \( \Delta ffac \) and \( \Delta ffpka1 \) showed reduced growth rates, while no growth defects were observed for \( \Delta ffg1 \) and \( \Delta ffpka2 \) (fig 6A–B). The same tendency was observed when the indicated strains were grown on CD medium. Although \( \Delta ffg1 \) grows in a wild type manner, \( \Delta ffg3 \), \( \Delta ffac \) and \( \Delta ffpka1 \) mutants showed impaired growth. Surprisingly, while \( \Delta ffpka2 \) showed normal growth on complete medium, radial growth was reduced about 25% on minimal medium (fig. 6A–B).

When grown on synthetic ICI medium, \( \Delta ffg1 \), \( \Delta ffg3 \) and \( \Delta ffvel1 \) mutants secreted fusarubin into the medium, emphasizing our findings that the two G\(_a\) subunits and the adenylyl cyclase negatively affect fusarubin biosynthesis (fig. 6C). No significant differences were observed in liquid ICI medium regarding biomass accumulation of the single deletion mutants.

FGF1 affects sexual development

Deletion of several class I G\(_a\) subunits have been shown to affect the sexual development, resulting e.g. in female sterility in *Cyphonectria parasitica*, *N. crassa*, and *F. graminearum*, and loss of
perithecial formation in *M. grisea* [55,56,18,57,19,27]. Recently we have shown that fusarubins are the pigments in perithecia in *F. fujikuroi* [28]. As biosynthesis of these perithecial pigments is upregulated in *Dfg1*, we wished to determine whether *FfG1* is also involved in the regulation of sexual development in *F. fujikuroi*.

Therefore, *ffg1* was also deleted in the mating partner of strain IMI58289, *F. fujikuroi* MRC-1995, carrying the MAT1-1 idiomorph, using the same targeted deletion strategy. Two independent deletion mutants were obtained, designated MRC-1995/Δffg1_T44 and MRC-1995/Δffg1_T49 (fig. S1F). Crosses were made between both wild types, one of either wild-type strains and the Δffg1 mutant of the respective mating partner as well as between the two Δffg1 mutant strains. When Δffg1 is only deleted in *F. fujikuroi* IMI58289 that carries the MAT1-2 idiomorph, perithecia were normally produced in a cross with MRC-1995, similarly to the cross between the two wild-type strains. However, Δffg1 deletion in *F. fujikuroi* MRC-1995 carrying the MAT1-1 idiomorph resulted in total loss of perithecial formation in a cross with IMI58289, as it was the case when *ffg1* was deleted in both mating partners. (fig. 7).

**Discussion**

The Gα subunits FfG1 and FfG3 negatively regulate fusarubin biosynthesis

Deletion of *ffg1* resulted in an increased accumulation of fusarubin under both fusarubin-inducing and -repressing conditions, while constitutive activation of FfG1 (FfG1<sup>ΔG42R</sup>) completely

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**Figure 6. Analysis of growth behavior of the cAMP pathway mutants on different media compared to the WT.** A) WT and cAMP pathway mutants (Δffg1, Δffg3, Δffac, Δffpka1, Δffpka2) were grown on solidified ICI medium with 6 mM sodium nitrate as sole nitrogen source. B) WT and cAMP pathway mutants on solidified complete medium (CM) and Czapek dox (CD, minimal medium). After 7 days radial growth rates of the respective mutants were determined. Experiments were done in triplicate. C) Radial growth of the respective mutants grown on CM or CD medium was measured [mm]. Mean values and standard deviations are shown in the diagram.

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abolished fusarubin production under either of the investigated conditions. Bikaverin biosynthesis was decreased under both activating and repressing conditions (fig. 2 A–B), indicating that both pigments are regulated in opposing ways, and that fusarubin biosynthesis requires inactivation of the FfG1-dependent signaling pathway.

The involvement of class I Gα proteins in secondary metabolism has been studied in a variety of filamentous fungi. Interestingly, Gα proteins of class I share sequence similarities with mammalian Gα that are thought to inhibit adenylyl cyclase activity [12]. However, an increasing number of reports indicate that this class of Gα subunits has an activating effect on the adenylyl cyclase activity in filamentous fungi (e.g. in M. oryzae, N. crassa or B. cinerea) [27,57,17,53,26]. In general, there are several examples demonstrating that members of class I Gα subunits are involved in the regulation of secondary metabolism. For example, constitutive activation of PGA1 in P. chrysogenum resulted in increased production of three secondary metabolites: penicillin, the yellow pigment chrysogenin and the mycotoxin roquefortine [21]. Constitutive activation of FadA in F. sporotrichioides led to elevated T2-toxin levels [16], and constitutive active GasA G42R in P. marneffei transformants showed an increased pigment production [22]. In contrast, constitutive activation of GNA-1 in N. crassa resulted in decreased carotenoid biosynthesis [20]. Deletion of Gα resulted in reduced pigmentation in C. parasitica [18] and increased deoxynivalenol and zearalenone production in F. graminearum [19]. Clearly, there are examples for activation as well as repression of secondary metabolite biosynthesis through G protein-mediated signaling. Even within the same species distinct secondary metabolites might be differentially regulated by Gα subunits. For example, in A. nidulans inhibition of fadA is necessary for the activation of sterigmatocystin and aflatoxin biosynthesis [14,15] while penicillin biosynthesis requires the activation of FadA [16]. The same contradictory relationship has been found in T. atroviride in which the Gα subunit Tga1 has opposite roles regarding the regulation of the biosynthesis of different antifungal substances [23] indicating the complexity of G protein-mediated regulation of fungal secondary metabolism.

Interestingly, deletion of ffac resulted in a more severe deregulation of fusarubin biosynthesis than deletion of ffg1 indicating the involvement of at least one further upstream activator of ffac. In B. cinerea, two Gα subunits, BcG1 (class I) and BcG3 (class III), are involved in the activation of the adenylyl cyclase BAC. Deletion of bbg1 and bbg3 resulted in reduced cAMP levels; and respective phenotypes i.e. colony morphology and conidial germination could be restored by exogenous cAMP [17,53,26]. Gα proteins of class III have been shown to play crucial roles in fungal development and pathogenesis in various fungi.
with the constitutive active FIG1G42R, indicating that bikaverin is not a direct target of FIG1 in F. fujikuroi (fig. 2 A–B). Furthermore, deletion of ffg3 and pka1 also resulted in transcriptional downregulation of bikaverin genes and reduced bikaverin production. A recently generated ffpka1 overexpression mutant revealed an elevated expression of bik1 (Y. Eilert and B. Tudzynski, unpublished data). These results indicate that bikaverin biosynthesis in F. fujikuroi is directly regulated by FIAC and FIPKA1, and indirectly by the Gα subunits FIG1 and FIG3 due to their stimulating effects on the adenylyl cyclase.

**GA biosynthesis depends on cAMP and PKA, but does not involve FIG1 or FIG3**

GA biosynthesis was found to be positively affected by FIAC [66; this paper], but neither deletion of ffg1 nor ffg3 resulted in a similar phenotype (fig. 5). These data strongly suggest that an additional upstream activator of FIAC is involved in the regulation of GA biosynthesis in F. fujikuroi. Small GTPases have been shown to stimulate the cAMP pathway in *S. cerevisiae* and *B. cinerea* [72,32] and might therefore also be involved in activation of FIAC and subsequently in regulation of GA biosynthesis in F. fujikuroi. Furthermore, in *Candida albicans* and *Cryptococcus neoformans* the adenylyl cyclase is also activated by bicarbonate, thus combining the functionality of both mammalian adenylyl cyclase families [73–75]. Therefore, stimulation of FIAC due to changing CO2 levels might also be possible in F. fujikuroi. However, involvement of FIG2 cannot be excluded, although deletion of class II Gα proteins showed only minor effects on growth and development in other fungi (e.g. *B. cinerea*, *M. oryzae* [17,27].)

Deletion of ffpka2, but not of ffpka1, resulted in reduced GA yields in a similar manner to the Δfgc mutant indicating that FIPKA2 specifically functions as a positive regulator of GA biosynthesis since FIPKA1 cannot compensate for the function of the missing FIPKA2 (fig. 5). So far investigations on homologues of FIPKA2 in other fungi revealed only a minor role in fungal development and virulence. For example, in *A. fumigatus* PKAC2 is involved in the regulation of germination, cell wall homeostasis and carbohydrate metabolism [76], while deletion of PkaB in *A. nidulans* and *B. cinerea* did not result in any phenotypic alterations [77,32]. However, deletion of both catalytic subunits is lethal in *A. nidulans* [77], indicating that the catalytic subunits can at least partially complement each other. The significant reduction of GA formation in both the Δfgc and Δfpka2 mutants indicates specific functions of both PKA complexes. To our knowledge this is the first example of an independent role for PKA2 in secondary metabolism.

**Growth is regulated by FIG3, FFAC and FFPKA1 in F. fujikuroi**

G proteins have been shown to be involved in several developmental processes, and deletion of several fungal class I Gα subunits have been shown to result in impaired growth (e.g. *A. nidulans*, *M. oryzae* and *N. crassa*) [45,27,57]. Furthermore, deletion of downstream targets of Gα showed similar phenotypes indicating the involvement of the adenylyl cyclase and PKA in the regulation of fungal growth in several fungal species, e.g. *A. nidulans* [60,78], *A. fumigatus* [58,79], *N. crassa* [80], *M. grisea* [69], *B. cinerea* [53,32] and *F. verticillioides* [63]. In *A. nidulans*, constitutive activation of Gα (class I) also propagates the vegetative growth signal through the cAMP/PKA signaling pathway [81,82,60,45].

In *F. fujikuroi*, deletion of ffgl did not result in an altered growth behavior, but deletion of ffg3 caused reduced growth rates on minimal as well as on complete medium (fig. 6). Defects in colony...
Morphology upon deletion Gα subunit class III have also been shown for A. fumigatus, T. atroviride and C. parasitica [58,83,18]. Radial growth was even more impaired in Dffac and Dffpka1 compared to Dffg3 indicating further upstream stimulators of FfAC. Notably, deletion of ffpka2 showed normal growth rates on complete medium, while radial growth was impaired on minimal medium indicating a growth defect of Dffpka2 likely due to a nutritional component that is included in the complete but missing in the minimal medium.

Deletion of the class I Gα subunit FfG1 results in loss of fertility in the MRC-1995 genomic background

In heterothallic ascomycetes such as F. fujikuroi a single mating type locus, MAT, with two alternative forms (MAT1-1 and MAT1-2) called mating types or idiomorphs, controls mating [84]. Sexual reproduction is initiated by the binding of pheromone peptides to the respective pheromone receptors, which are both regulated by MAT loci. By physical interaction of pheromone receptors, each coupled to a heterotrimeric G protein complex, G protein-mediated signaling is involved in fungal sexual reproduction in various organisms (e.g. C. parasitica, N. crassa or F. graminearum) [55,56,18,57,19,11].

It was therefore anticipated that deletion of ffg1 in F. fujikuroi would result in impaired sexual reproduction, but to our surprise only if deletion of ffg1 was performed in the F. fujikuroi wild-type strain MRC-1995, carrying the MAT1-1 idiomorph. Deletion of ffg1 in strain IMI58289, carrying the MAT1-2 idiomorph, showed no alterations in mating as purple perithecia were still developed (fig. 7). Recently, we have shown that the 4’phosphopantetheiny transferase (PPTase), FfPpt1, is essentially involved in production of PKS- and NRPS-derived secondary metabolites, conidiation and sexual development. When the FfPpt1 gene was missing in strain MRC-1995 carrying the MAT1-1 idiomorph, no recogni-
tion took place when contiguously grown with strain IMI58289 of the opposite mating type while deletion of Fppel in IMI58289 resulted in wild-type like formation of perithecia [44]. The reasons for mating type-dependent differences are not yet understood, but these examples provide strong evidence that the MAT1-1 locus specifically regulates components which are essential for sexual reproduction. In the case of fppel deletion in the MAT1-1 background, sexual recognition of both partners was abolished, disclosing the possibility that the MAT1-1 idiomorph specifically controls a PKS- and/or NRPS-derived metabolite or its receptor. However, recognition of the opposite mating partners still took place when fppel was deleted in strain MRC-1995 as hyphae of opposite mating partners grew together (fig. 7A). This strengthens the assumption that G protein-mediated signaling is important for the middle and late stages of sexual reproduction but not for the early events, such as recognition of the respective mating partner as described earlier for F. gamoidea [85].

In conclusion, our studies describe the complex and diverse involvement of G protein/cAMP-mediated signaling in the regulation of fungal growth, secondary metabolism and sexual development in F. fujikuroi. We demonstrate that Fg1 (class I), Fg3 (class III) and Fac negatively regulate the biosynthesis of fusarubin, probably by additive stimulating effects of Fac by both Gz subunits. Surprisingly, neither FPKA1 nor FPKA2 have any impact on fusarubin production, indicating that another yet unidentified cAMP-binding protein exists that is involved in the regulation of the perithecial pigment. On the other hand, bikaverin production is regulated in an opposite way: its production is inhibited in the ffs1, ffs3 and ffac deletion mutants. The opposite regulation of the two red bars is also underlined by the contrasting effect of the global regulator Velvet: deletion of ffsel resulted not only in increased bikaverin biosynthesis under both inducing and repressing conditions, but also in complete inhibition of fusarubin formation (fig. 8).

Although all three secondary metabolites, GA, fusarubin and bikaverin, depend on the activity of Fac, only bikaverin and GA formation were shown to be regulated by the FIAC in a PKA-dependent manner. Furthermore, our results indicate that bikaverin biosynthesis is regulated by FPKA1, while GA biosynthesis is regulated by FPKA2. Previously we have demonstrated that GA and bikaverin biosynthesis, though both repressed by nitrogen, are regulated by contrasting nitrogen regulation pathways in an AreA-dependent and independent manner, respectively, and that FIve1 strongly represses bikaverin, but positively controls GAs formation [51]. All these data show the complexity of these regulatory networks, and that each secondary metabolite might be controlled by individual signaling pathways.

Supporting Information

**Figure S1** Diagnostic PCR for *Afpg1*, *Afpg3*, *Affac*, *Affpka1*, *Affpka2* and MRC-1995/*Afpg1*. Diagnostic PCR is shown for 5′, 3′ and for the detection of the wild-type gene (WT). A) Diagnostic PCR of the WT and two independent ffs1 deletion mutants, Δffs1_T9 and Δffs1_T13. B) Diagnostic PCR of the WT and four independent fppka2 deletion mutants: Δffppka2_T6, Δffppka2_T7, Δffpka2_T11 and Δffpka2_T12. C) Diagnostic PCR of the WT and three independent deletion mutants for ffsac, Δffsac_T7, Δffsac_T12 and Δffsac_T14. D) Diagnostic PCR of the WT and three independent *ffpka1* deletion mutants: Δffpka1_T4, Δffpka1_T10 and Δffpka1_T15. E) Diagnostic PCR of the WT and four independent *ffpka2* deletion mutants: Δffpka2_T10, Δffpka2_T12, Δffpka2_T13 and Δffpka2_T16. F) Diagnostic PCR of the WT and two independent *ffg1* deletion mutants in the *F. fujikuroi* wild-type strain MRC-1995: MRC-1995/Δffg1_T44 and MRC-1995/Δffg1_T49.

**Figure S2** Strategy for constitutive expression of *Afpg1*G42R in *F. fujikuroi*. Two fragments (I and II) were amplified from genomic DNA of IMI58289. Primers used for introduction of the point mutations are highlighted by orange bars. Plasmid containing the *ffg1* gene with the nucleotide substitution was transformed into the *F. fujikuroi* wild-type strain IMI58289. Homologous integration of the plasmid was verified by PCR and subsequent sequencing of the amplified fragment.

**Figure S3** Complementation of *Afpg1*, *Afpg3* and *Affac*. A) Complementation strategy. The gene of interest including ~2-kb promoter sequence and ~200-bp terminator sequence was amplified using proof-reading polymerase in two to five fragments. The fragments were transformed together with the SacII/SpeI-restricted plasmid ND-NGG using yeast-recombinational cloning (for details see material and methods). Obtained clones were verified by Sequencing. Complementation was analyzed by plate assays. Therefore, the indicated strains were grown for five days on solidified ICI medium. B) Plasmid for complementation and plate assay for *Afpg1*/Afpg1<sup>*</sup>. C) Plasmid for complementation and plate assay for *Afpg3*/Afpg3<sup>*</sup>. D) Plasmid for complementation and plate assay for *Afpg3*/Afpg3<sup>*-Δ<sub>G42R</sub>*</sup>.

**Figure S4** Complementation of *Afpg1*, *Afpg3* and *Affac* restores the wild-type phenotype. The indicated strains were grown for 4 days under bikaverin-favorable conditions (6 mM glutamine (Gln)). Culture filtrates were used for analysis of bikaverin and fusarubin accumulation by HPLC-DAD. The desired compounds were detected at 450 nm. Accumulation of bikaverin A) in the wild type (WT), B) in the deletion of *ffg1* and complementation thereof, C) in the deletion of *ffg3* and complementation thereof and D) in the deletion of *ffac* and complementation thereof.

**Table S1** Primers used during this study.

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**Author Contributions**

Conceived and designed the experiments: LS BT HUH. Performed the experiments: LS. Analyzed the data: LS. Contributed reagents/materials/analysis tools: BT HUH. Wrote the paper: LS.
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