Biosynthesis of fusarubins accounts for pigmentation of *Fusarium fujikuroi* perithecia

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Abstract

*Fusarium fujikuroi* produces a variety of secondary metabolites, of which polyketides form the most diverse group. Among these are the highly pigmented naphthoquinones that were shown to possess different functional properties for the fungus. A group of naphthoquinones, polyketides related to fusarubin, were identified in *Fusarium* spp. more than 60 years ago, but neither the genes responsible for their formation nor their biological function have been discovered to date. In addition, although it is known that the sexual fruiting bodies in which the fungus' progeny develops are darkly colored by a polyketide synthase-derived pigment, the structure of this pigment has never been elucidated. Here we present data that link the fusarubin-type polyketides to a defined gene cluster, subsequently designated as *fsr* and demonstrate that the fusarubins are the pigments responsible for coloration of the perithecia. We studied their regulation and the function of the single genes within the cluster by a combination of gene replacements and overexpression of the PKS-encoding gene and present a model for the biosynthetic pathway of the fusarubins based on this data.
Introduction

The rice pathogenic ascomycete *Fusarium fujikuroi* (teleomorph *Gibberella fujikuroi* MP-C), belonging to the *Gibberella fujikuroi* species complex, was first isolated from infected rice plants in 1890 (33), and identified as the causative agent of the “bakanae” or foolish seedling disease due to its ability to produce gibberellic acids (GAs) (44, 34). Typical symptoms of this disease are chlorotic, slender and hyperelongated internodes as well as empty or sterile grains (69). Besides GAs, the fungus produces a variety of other secondary metabolites, such as the red polyketide bikaverin accompanying production of terpenoid GAs in liquid culture (10). Secondary metabolite genes are often organized in gene clusters (32), as found in *F. fujikuroi* for the seven GA biosynthetic genes (7), and the six genes involved in the bikaverin biosynthesis (48, 80). It is assumed that spatial proximity facilitates co-regulation of secondary metabolite genes within the cluster by e.g. epigenetic processes, transcriptional regulation by global regulators or pathway-specific transcription factors located within a gene cluster (53).

Most of the secondary metabolite gene clusters are silent under laboratory conditions (8). Different approaches were undertaken for activation of those gene clusters, e.g. by optimization of culture conditions (pH, nutrient availability) (6, 82), overexpression of pathway-specific transcription factors (9) or global transcriptional regulators such as LaeA (53), and by histone modifications affecting epigenetic control (68).

Secondary metabolites can be grouped into four different classes depending on their structural properties: polyketides, terpenes, non-ribosomal peptides and amino acid-derived compounds. Among secondary metabolites, polyketides form the most abundant group (37), including most of the green and red fungal pigments, all belonging to the group of naphthoquinones.
These polyketides are synthesized by multi-domain non-reducing polyketide synthases (NR-PKSs). NR-PKSs are the minimal fungal PKSs containing β-ketoacyl synthase (KS), malonyl-CoA:ACP transacylase (MAT) and one or two acyl carrier protein (ACP) domains resulting in a defined carbon skeleton through several rounds of elongation (14). Recently two new domains were identified by Crawford et al., the starter unit:ACP transacylase (SAT) domain, responsible for selecting the starter unit, and the product template (PT) domain. The SAT domain which has high similarity to the MAT domain was shown to accept acyl-units other than malonyl-CoA as starter units and is widespread among fungal NR-PKSs (15, 16, 17). The PT domain, located between the AT and the ACP domain, is responsible for specific aldol cyclization and aromatization of poly-β-keto species, thereby determining chain length and specific cyclization patterns of the polyketide precursors in fungal NR-PKSs (17, 18). Finally the respective polyketide is released through various release mechanisms (24), of which the TE-mediated product release by a canonical thioesterase (TE) domain, is the most common. This domain often extends to a domain capable of C-C Claisen cyclization (TE/CLC) in fungal NR-PKSs, e.g. in PksA of A. parasiticus (42). In some cases, NR-PKSs contain a reductase releasing (R) domain or no releasing domain at all, e.g. the PKS involved in asperthecin biosynthesis in A. nidulans (14).

So far, the structure of more than 100 naphthoquinone metabolites has been elucidated (52), indicating the structural diversity of this group. The ability to produce naphthoquinones is widespread among fungal organisms, especially among the genus Fusarium. Only few of these compounds could be linked to PKS-encoding gene clusters so far, among them the red pigments bikaverin in F. fujikuroi (48, 79), aurofusarin in F.
graminearum (28, 39, 51), and the perithecial pigments in F. solani (30), F. graminearum (28) and F. verticillioides (60), whose structure has not been identified yet.

In F. fujikuroi, the only described PKS is the bikaverin-specific, NR-PKS Bik1 (79). In this study, we present the identification and functional characterization of a second NR-PKS in F. fujikuroi with homology to two PKSs from other Fusarium spp., responsible for the pigmentation of perithecia: Pgl1 from F. graminearum (28) and F. verticillioides (60).

Targeted deletion of this PKS-encoding gene in two F. fujikuroi strains of opposite mating types and their subsequent sexual crossing, confirmed the role in perithecia pigmentation also in F. fujikuroi. Detailed molecular analyses revealed the presence of a gene cluster comprising six co-regulated genes. Chemical analyses of the products accumulated by the single deletion mutants of these genes prove this gene cluster to be responsible for biosynthesis of naphthoquinones with structural similarity to fusarubin and allowed to establish the biosynthetic pathway. To our knowledge this is the first report which pinpoints perithecial pigmentation in Fusarium spp. to the gene cluster responsible for the production of fusarubins.

Material and Methods

Fungal Strains and culture conditions: The wild-type strain Fusarium fujikuroi IMI58289 (Commonwealth Mycological Institute, Kew, United Kingdom) was used as a parent strain for all knock-out experiments. As mating partner, F. fujikuroi C-1995 (kindly provided by J.F. Leslie, Kansas Starte University) was used. For all liquid cultivations, the F. fujikuroi strains were pre-incubated for 72 h in 300 ml Erlenmeyer flasks with 100 ml Darken medium (DVK) (22) on a rotary shaker at 28 °C and 180 rpm. A 500 µl aliquot of this culture was used for inoculation of synthetic ICI (Imperial Chemical
Industries Ltd., United Kingdom) medium (29) with either 6 mM glutamine or 6 mM sodium nitrate and incubation for additional 1-12 days. For protoplasting, 500 µl of the pre-incubated culture was taken and transferred into 100 ml ICI media with 10 g/l fructose instead of glucose and 1 g/l (NH₄)₂SO₄ as nitrogen source and incubated on a rotary shaker at 28 °C and 180 rpm no longer than 16 h. 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 the cultures were incubated for additional 30 min. For the identification of the compounds, the fungus was grown for 7 days in ICI media on a rotary shaker at 28 °C and 180 rpm. For pH shift experiments, the strains were grown in ICI media for 4 days on a rotary shaker at 28 °C and 180 rpm and then transferred to ICI media without any nitrogen source adjusted to pH values of either 4 or 8 for additional 2 h (11). For sexual crosses, carrot agar was used according to Klittich and Leslie (40). For DNA isolation, the fungus was grown on cellophane sheets on solidified complete medium (CM) for 3 days at 28 °C. For RNA isolation, the fungus was grown in ICI medium with the desired nitrogen source for 2-7 days at 28 °C and 180 rpm after pre-incubation in DVK.

**HPLC-DAD analyses of the naphthoquinones:** For HPLC-DAD analysis, culture fluid of 1-12 day old cultures was filtered over a 0.2 µm membrane filter (Millex®, Millipore) and directly used without further preparation. The samples were separated on a 150 x 3.00 mm, 3 micron, Gemini 3u C6-Phenyl 110 Å column (Phenomenex, Aschaffenburg, Germany), using a binary gradient delivered by Hitachi L-7100 (Merck) pump with 1% formic acid as solvent A and ACN as solvent B. The binary gradient started with a linear gradient from 20% B to 50% B in 15 min, followed by an isocratic step for 5 min and an additional linear gradient step to 75% B in 5 min. After each HPLC
run, the column was washed by increasing the gradient to 100% B and equilibrated for 5 min at starting conditions. The flow rate was set to 0.3 ml min⁻¹. The desired compounds were detected using a Hitachi L-2455 Elite LaChrom Diode Array Detector (DAD). To compare chromatograms of different culture filtrates, the wavelength was set to 450 nm.

**HPLC-UV-FTMS analyses of the naphthoquinones:** Liquid media of 1-12 day old cultures were filtered over a 0.2 µm membrane filter and directly analyzed by high-performance liquid chromatography with Fourier transformation mass spectrometry detection (HPLC-UV-FTMS). To this end, an Accela LC 60057-60010 system (Thermo Fisher Scientific, Bremen, Germany) was linked to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific). Data acquisition was performed with Xcalibur 2.07 SP1 (Thermo Scientific). The samples were separated on a 150 x 3.00 mm, 3 micron, Gemini 3u C6-Phenyl 110 Å column (Phenomenex, Aschaffenburg, Germany), using a binary gradient as mentioned above. The flow rate was set to 0.3 ml min⁻¹. Ionization was performed with heated electrospray ionization. Further conditions of the mass spectrometer were as follows: capillary temperature, 225 °C; APCI vaporizer temperature, 250 °C; sheath gas flow, 40; aux gas flow, 20; source voltage, 3.5 kV; capillary voltage, 35 V; tube lens, 110 V; multiple 00 offset, -4.00 V; lens 0 voltage, -4.20; gate lens offset, -35.00 V; multipole 1 offset, -8.00 V; and front lens, -5.25. Scan events were as follows: [1] a total ion scan of a mass range from m/z 250 to 800 with a resolution of 30000 in the positive ion mode (FTMS), [2] Depending on scan event [1] the most intense parent ion of the list noted below was fragmented in the ITMS. If no parent mass was found, the most intense ion was fragmented: activation type: CID, normalized collision energy: 35, activation Q = 0.250, isolation width: 2, [3] Depending on scan event [1] the most intense parent ion of the list was fragmented. If no parent
mass was found, the most intense ion was fragmented in the FTMS, activation type: HCD, normalized collision energy: 50, activation Q =0.250, isolation width: 1.5, resolution: 15000 [4] total ion scan of a mass range from m/z 200 to 800 in the negative ion mode with the ITMS. The exact masses of the compounds were identified if the proton adduct ([M+H]+) and in addition the sodium adduct ([M+Na]+) or the exact mass in the negative ion mode ([M-H]−) was also detected.

**Purification of the naphthoquinoid metabolites:** For purification of the naphthoquinones 1l of 7-day old culture fluid of the Wt (for final products), the deletion mutants Δfsr2 and Δfsr3 (for intermediates) and Δfsr2-5/gpd::fsr1 (for the PKS-derived product) were extracted by solid phase extraction using C18-SPE cartridges (Phenomenex, Aschaffenburg, Germany). Further purification was accomplished by semi-preparative HPLC on a 250 x 10.00 mm, 5 micron, Gemini Gemini 5u C6-Phenyl 110 Å column (Phenomenex, Aschaffenburg, Germany), using an isocratic gradient delivered by a Jasco PU-2087 pump with 1% formic acid as solvent A and ACN as solvent B. The flow rate was set to 3 ml min⁻¹. The desired compounds were detected using a Jasco UV-2075 UV-detector at 450 nm (for final products) and 400 nm (for intermediates and PKS-derived product). The naphthoquinoid metabolites 1-8 were obtained after semi-preparative HPLC in amounts of 10-30 mg for structure elucidation.

**Nuclear magnetic resonance (NMR) measurements:** The ¹H, ¹³C and 2-D NMR spectra were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany) NMR spectrometer. Signals are reported in parts per million referenced to DMSO-d₆. For structural elucidation and NMR signal assignment 2-D NMR experiments, such as (H,H)-correlated spectroscopy (H,H-COSY), heteronuclear multiple-quantum correlation
(HMOC) and heteronuclear multiple bond correlation (HMBC) were performed. Pulse programs for these experiments were taken from the software library.

**Sequence data and phylogenetic analyses:** BLASTP analysis (1) was performed using the predicted protein sequences of Fsr1 to Fsr6 as well as FF_03983 and FF_03990 against NCBI non-redundant protein sequences (Tab. 1). PKS protein sequences were retrieved from the following sources: F. graminearum, F. oxysporum and F. verticillioides from www.broadinstitute.org; F. solani from www.jgi-psf.org; characterized PKS of other fungi (32, 14) from www.ncbi.nlm.nih.gov. Extraction of KS domains used for phylogenetic analyses was performed using the PKS/NRPS Analysis Web-site http://nrps.igs.umaryland.edu/nrps (4). Phylogenetic analysis was performed by comparing the KS domains using the web-based tool at www.phylogeny.fr (23). The nucleotide and protein sequences of the fsr gene cluster were deposited in GenBank under accession number HE613440.

**Plasmid constructions:** The F. fujikuroi knock-out strains were created using yeast recombinational cloning (20). The 5’ and 3’ flanks of the corresponding genes were amplified with appropriate primer pairs (Tab. S1), for the 5’ flank 5F and 5R primers were used and for the 3’ flank primers 3F and 3R were used, based on the genomic sequence available of F. fujikuroi IMI58289 (B. Tudzynski and co-workers). The hygromycin resistance cassette, consisting of the hygromycin B phosphotransferase gene *hph* (31), driven by the trpC promoter, was amplified using the primer pair hph-F/R from the template pCSN44 (66). Alternatively the nourseothricin resistance cassette was used, also driven by the trpC promoter. The nourseothricin resistance cassette was amplified using the plasmid pZPnat1 (GenBank: AY631958.1) as a template with the primer pair hph F/hphR-trpC-T2. All primers used are listed in table S1. The obtained
fragments were cloned into the *Saccharomyces cerevisiae* strain FY834 (82) together with the EcoR1/Xho1 restricted plasmid pRS426 (19). For restoration of pigment biosynthesis in Δfsr1, the respective gene was amplified with the corresponding terminator sequence in four PCR reactions. All four amplified products were cloned into *Saccharomyces cerevisiae* FY834 together with a HindIII restricted modified plasmid pRS426, which additionally contained a nourseothricin resistance cassette driven by an oliC promoter amplified from pNR1(50) using the primer pair nat-OE-prom/nat-OE-term. The gene of interest itself is driven by the gpd promoter which was amplified from pveAgfp (80) using the primer pair gpd-yeast-for/gpd-yeast-rev.

**Standard molecular methods:** For DNA isolation, lyophilized mycelium was ground to a fine powder in liquid nitrogen and dispersed in extraction buffer after Cenis (12) and afterwards used for PCR amplification and Southern blot analyses. PCR reactions contained 25 ng genomic DNA, 5 pmol of each primer, 200 nM deoxynucleoside triphosphates, and 1 unit BioTherm™ DNA polymerase (GeneCraft GmbH, Lüdinghausen, Germany) in case of diagnostic PCR and amplification of the flanks for yeast recombinational cloning. PCR reactions were performed with an initial denaturing step at 94 °C for 3 min followed by 35 cycles of 1 min at 94 °C, 1 min at 56-60 °C, 1-2 min at 70 °C and a final elongation step at 70 °C for 10 min. For the amplification of the knock-out constructs, the TAKARA polymerase kit was used as indicated.

For restoration of fusarubin biosynthesis in Δfsr1 as well as for the identification of the PKS-derived product, *fsr1* was amplified in 4 fragments using a proof-reading polymerase. Under these conditions, PCR reactions contained 25 ng genomic DNA, 5
pmol of each primer, and 1 unit of Phusion® polymerase (Finnzymes, Thermo Fisher Scientific, Finland).

For Southern blot analysis, genomic DNA was completely digested using appropriate enzymes, separated on a 1% (w/v) agarose gel and transferred onto nylon membranes (Nytran® SPC, Whatman) by downward blotting (3). ³²P-labelled probes were generated using the random oligomer-primer method and hybridized to the membranes overnight 65 °C according to the protocol of Sambrock et al. (64). After hybridization the membrane was washed with 1x SSPE (0.18 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA, pH 7), 0.1% SDS at the same temperature.

For RNA isolation, lyophilized mycelium was ground with a mortar and pestle and the powder was extracted using the RNAagents total RNA isolation kit (Promega, Germany) according to the manufacturer’s instructions. An amount of 20 µg per sample was loaded on a 1% agarose gel and run under denaturing conditions (64). The separated RNA was transferred onto nylon membranes (Nytran® SPC, Whatman). ³²P-labelled probes were created as mentioned above and were hybridized to the membrane over night at 65 °C. Plasmid DNA from Saccharomyces cerevisiae was extracted using the yeast plasmid isolation kit (SpeedPrep, DualsystemsBioTech) and directly used for PCR reaction.

**Fungal transformations:** Preparation of protoplasts from F. fujikuroi IMI58289 was carried out as previously described (74). About 10⁷ protoplasts were transformed with 10 µg of the amplified replacement cassettes for the knock-outs of the putative gene cluster (fsr1-fsr6) and one gene upstream (FF_03983) and downstream of the cluster (FF_03990). For restoration of fsr1 and for the identification of the PKS-derived product,
the \( \text{fsr1} \) deletion mutant as well as the \( \Delta \text{fsr2-5} \) deletion mutant was transformed with 10µg of the vector pRS426 containing \( \text{fsr1} \) driven by the gpd promoter.

For sexual crosses, the mating partner MRC1995 of \( \text{F. fujikuroi} \) IMI 58289 was also transformed with the knock-out cassette for \( \text{fsr1} \). Transformed protoplasts were regenerated as described by Tudzynski (75). The medium contained the appropriate resistance marker. Single conidial cultures were established from either hygromycin B- or nourseothricin-resistant transformants and used for DNA isolation and Southern analysis.

**Results**

Modification of culture condition reveals a second red polyketide in \( \text{Fusarium fujikuroi} \). In \( \text{F. fujikuroi} \), the biosyntheses of both GAs and bikaverin is strongly repressed by high levels of glutamine. In synthetic ICI medium with low levels of glutamine (6 mM), the fungus starts to produce these metabolites after depletion of nitrogen (10, 79). However, when the fungus was grown with 6 mM of sodium nitrate as sole nitrogen source, only gibberellins were produced, while accumulation of bikaverin was no longer detected due to the alkaline \( \text{pH} \) (Fig. 1B). Surprisingly, the liquid culture still got deeply red pigmented after approximately five days of cultivation (Fig. 1A). High-performance liquid chromatography (HPLC) and HPLC coupled with Fourier transformation mass spectrometry detection (HPLC-UV-FTMS) (data not shown) confirmed that the red pigment was not bikaverin (Fig. 1B). Measurements of the \( \text{pH} \) values of the different growth media in a time course showed significant differences depending on the nitrogen source. While the \( \text{pH} \) was maintained at acidic \( \text{pH} \) values between 5 and 6 with glutamine, the \( \text{pH} \) increased to values of 6.5 to 7.5 in cultures with...
sodium nitrate (Fig. 1C). The ambient alkaline conditions explain the lack of bikaverin biosynthesis as shown before (79). The second red pigment, in contrast, was only produced if the ambient pH of the liquid culture immediately increased to a neutral or alkaline value. Thus, pH conditions in the first 24 h after inoculation seem to determine which of the pigments is produced.

Cloning, disruption and characterization of the responsible polyketide synthase-encoding gene (fsr1). Due to the intensive red color of the cryptic compound, we assumed structural similarity to aromatic pigments synthesized by NR-PKSs, and hence hypothesized the involvement of a non-reducing PKS in its biosynthesis. A BLASTP analysis (1) with the Bik1 sequence against the recently sequenced genome of *F. fujikuroi* IMI58289 (B. Tudzynski and co-workers) revealed a second NR-PKS with 40% identity (e-value = 0) to Bik1. This PKS (Fsr1) consists of 2286 amino acids (aa), encoded by an open reading frame (ORF) of 7069 base pairs (bp) which is interrupted by four putative introns. ClustalW alignment of Fsr1 with the well characterized NR-PKSs responsible for aflatoxin production in *A. parasiticus* (PksA), perithecia pigment in *F. solani* (PksN) and bikaverin production in *F. fujikuroi* (Bik1) revealed the presence of the following domains. 1. In the N-terminus, the recently identified SAT domain harboring the active GXCXG site can be found (Fig. 2A, Fig. S1). 2. The SAT domain is followed by the KS and MAT domains (Fig. 2A, Fig. S1). 3. Next, we identified a domain with high similarity to the newly identified PT domain presenting the catalytic histidine and aspartate residues (Fig. 2A, Fig. S1). 4. Adjacent to the PT domain, Fsr1 possesses two ACP domains (Fig. 2A, Fig. S1). 5. At the C-terminus, the enzyme exhibits a reductase (R) domain (Fig. 2A) instead of the canonical TE domain. In contrast to TE and TE/CLC domains, R domains show sequence similarities to the
short chain dehydrogenase/reductase (SDR) superfamily exhibiting Rossman-fold
des and nucleotide binding motifs (36). ClustalW alignment of Fsr1 R domain with
characterized R domains from bacterial and fungal PKSs (38, 5, 45, 13) and the R
domain of the yeast α-amino adipate reductase Lys2p (25) shows a high amino acid
conservation (Fig. S2).

Phylogenetic analysis of the KS domains of characterized fungal NR-PKSs revealed a
close relatedness of Fsr1 to Pgl1 from F. verticillioides and F. graminearum, which were
both shown to be responsible for perithecia pigmentation (28, 60). However, the
chemical structures of these perithecial pigments have not been elucidated yet.

In order to investigate whether fsr1 is involved in production of the cryptic pigment
observed in axenic culture (Fig. 1B), the ORF was deleted by targeted gene
replacement. Three independent knock-out mutants were obtained and designated
Δfsr1-T4, -T6 and -T8. Diagnostic PCR (data not shown) and Southern blot analysis
(Fig. S3) confirmed both the absence of the wild-type gene and of additional ectopic
integrations of the replacement fragment. When grown in medium with 6 mM sodium
nitrate, none of the mutants exhibited red pigmentation, indicating that Fsr1 is
responsible for the biosynthesis of the novel red pigment. Since all Δfsr1 mutants
behaved similarly (Fig. S4), only Δfsr1-T4 was arbitrarily chosen for subsequent work in
this study. Re-introduction of the complete ORF into Δfsr1 resulted in full restoration of
pigment biosynthesis confirming that Fsr1 is indeed responsible for the formation of the
new red pigments (Fig. 3C).

Identification and characterization of the corresponding gene cluster. Since the
biosynthetic genes for many fungal secondary metabolites are organized in gene
clusters (32), we analyzed the genes in the proximity of fsr1. The five genes downstream
of *fsr1* (Fig. 2B) show homologies to genes known to be typically involved in the biosynthesis of secondary metabolites (Tab. 1). To determine whether these genes adjacent to *fsr1* are involved in pigment biosynthesis, we studied their expression profiles under biosynthesis-favoring (6 mM sodium nitrate) and non-favoring (6 mM glutamine) conditions. Northern blot analyses revealed co-regulation of six genes (*fsr1* to *fsr6*), while *FF_03983*, upstream of *fsr1*, and *FF_03990*, downstream of *fsr6*, are not expressed and therefore probably not involved in the biosynthesis (Fig. 4).

**Regulation of the *fsr* gene cluster.** To get a deeper insight into regulation of *fsr* gene expression, a time-course growth experiment was performed under pigment-favoring conditions. Northern blot analyses revealed a time-dependent expression of the *fsr* genes (shown for *fsr1*-3): transcripts are detectable at the third day, their intensity peaks at the fourth day and then continuously decreases until the signals are almost non-detectable at day six past inoculation (Fig. 5A). To further unravel the pH-dependent expression, the wild type was grown under biosynthesis-favoring conditions for five days before the mycelium was shifted into synthetic ICI medium without any nitrogen source with pH values set either to 4 or 8. The results clearly show that *fsr2* and *fsr3* gene expression is repressed at acidic conditions, while the genes are expressed under alkaline conditions (Fig. 5B).

To study the impact of nitrogen quality as well as quantity on gene expression in more detail, the wild type was grown for five days under pigment production conditions. Then either 33 mM glutamine, 33 mM sodium nitrate or water (no nitrogen) were added to the cultures, respectively, and the effect on *fsr* gene expression was studied after additional 30 min. Northern blot analyses show that glutamine strictly represses pigment
biosynthesis, while the genes are still expressed if sodium nitrate or water were added (Fig. 5C).

Next, we wanted to show whether single deletions of each of the fsr genes affect expression of the other cluster genes, as it has been shown for bikaverin genes (79). To do so, deletion mutants of all six genes were generated. Surprisingly, deletion of the PKS-encoding gene fsr1 resulted in a complete down-regulation of the other 5 fsr genes, while single deletions of the genes fsr2 to fsr5 did not affect the expression of any other pathway gene. The deletion of fsr6, encoding a putative Zn(II)2Cys6 transcription factor, resulted in the expected down-regulation of all fsr genes, including fsr1 (Fig. S6A).

**Chemical identification of intermediates and final products.** Cultivation of the wild type in ICI medium with sodium nitrate as sole nitrogen source resulted in a complex spectrum of different pathway-specific compounds (Fig. 6A), that are all missing in the deletion mutant Δfsr1 (Fig. 6B). The structure of the main compounds was extensively elucidated using their exact masses, characteristic UV spectra and nuclear magnetic resonance (NMR) data, except for compound 2 due to insufficient accumulation in the liquid culture. By using these methods we identified the previously described naphthoquinones 8-O-methylfusarubin 1, 8-O-methylnectriafurone 2, 8-O-methyl-13-hydroxynorjavanicin 3 and 13-hydroxynorjavanicin 5 along with one new compound, which was subsequently designated as 8-O-methylanhydrofusarubinlactol 4 (67, 72, 70, 71). The identified structures were in agreement with published data. Additionally, we are presenting for the first time $^{13}$C NMR data for the compounds 2-5 (for detailed spectroscopic data see supporting information). These compounds and structurally related metabolites are known to be produced by different *Fusarium* spp.. The first naphthoquinones with a similar structure were isolated and characterized by Arnstein and...
Cook from *F. javanicum* and therefore designated as javanicin and oxyjavanicin (2), later re-named fusarubin (63). O-demethylanhydrofusarubin was the first and, until now, the only naphthoquinone pigment of the fusarubin family that has been described in *F. fujikuroi* (21). To our knowledge, none of the other compounds presented here have been described in *F. fujikuroi* before.

To confirm that the six co-regulated *fsr* genes are indeed involved in biosynthesis or regulation of pigment production, the product spectra of the Δ*fsr1*-Δ*fsr6* mutants and deletion mutants of the two border genes, *FF_03983* and *FF_03990* (see Fig. 2B) were analyzed. The fact that *FF_03983* and *FF_03990* deletion strains showed no alteration in the product spectra strengthened our suggestion made by co-regulation studies (Fig. 4) that these genes are not involved in biosynthesis or regulation of the pigments (data not shown).

The pathway-related products synthesized by the deletion mutants Δ*fsr1*-Δ*fsr6* were analyzed in detail to demonstrate their involvement in the biosynthesis of fusarubins (Fig. 6B). Δ*fsr1*, Δ*fsr2* and Δ*fsr3* are the only mutants showing an alteration in the product spectrum, indicating their role in the formation of the final products.

Under pigment-favoring conditions, three major peaks were accumulated by the Δ*fsr2* and Δ*fsr3* mutants, labeled as 6 and 7 in the chromatogram of Δ*fsr2*, and 8 in the chromatogram of Δ*fsr3*. Compound 6 showed a similar NMR spectrum as 6-O-demethyl-5-deoxyfusarubin previously identified from *Nectria hamatococca* (55), but with slightly different but distinct chemical shifts resulting from the position of the hydroxyl group at C10 instead of C5. Hence compound 6 was designated as 6-O-demethyl-10-deoxyfusarubin. Compounds 7 and 8 gave similar NMR spectra of the polyketide backbone, without the hydroxyl groups at position C5 and C10, where the A- and B-rings
are already formed, but not the C-ring. The structures show characteristic chemical shifts indicating the presence of an aldehyde, which was confirmed by 2D-NMR analyses. The only difference is the presence of an additional methyl group at position C6 in compound 8. The two intermediates were therefore designated as 6-O-demethylfusarubinaldehyde and fusarubinaldehyde, respectively (for detailed spectroscopic data see supporting information). All three compounds have not been described before.

In contrast, deletions of fsr4 and fsr5 did not alter the product spectrum of the metabolites, indicating that they are not involved in the modification of the PKS-derived precursor under these specific conditions. Interestingly, product formation was enriched in Δfsr4 compared to the wild type, suggesting, that fsr4 might possess a regulatory function in biosynthesis of fusarubins. Deletion of fsr6, a putative Zn(II)2Cys6 transcription factor, resulted in the total loss of the respective products (Fig. 6B), indicating its function as a pathway-specific transcription factor.

In order to identify the Fsr1-derived product, a mutant (Δfsr2-5) was generated where all genes are deleted except for fsr1 and fsr6. Surprisingly, this deletion resulted not only in down-regulation of the deleted genes, fsr2 to fsr5, but also of the two remaining genes, fsr1 and fsr6 (Fig. S6B). To overcome this effect, an fsr1 overexpression vector (gpd_priz;fsr1) was transformed into the Δfsr2-5 deletion mutant, resulting in Δfsr2-5/OE:fsr1 mutants overexpressing fsr1 by the strong A. nidulans gpd (glucose-6-phosphatase dehydrogenase) promoter. Using this approach, one major compound accumulated and was found to be identical with 6-O-demethylfusarubinaldehyde (compound 7) found in Δfsr2 (Fig. 6C, for detailed information see supporting information). This heptaketide, lacking the methyl group as well as the two hydroxyl
groups at C5 and C10 is therefore the earliest intermediate in the biosynthetic pathway of the fusarubins.

The fusarubins are responsible for the pigmentation of the perithecia. Due to the close relatedness of Fsr1 to Pgl1 from *F. graminearum* and *F. verticillioides*, respectively (28, 60) (Fig. 2A), we wanted to investigate whether the fusarubin-type compounds are identical with the yet unknown perithecia pigments in *F. fujikuroi*. Therefore sexual crosses were performed between the wild type IMI58289 (Mat-1) and strain *F. fujikuroi* C1995 with the opposite mating type (Mat-2) on the one hand, and between the deletion mutants IMI58289/Δfsr1 and C1995/Δfsr1 on the other hand. To do so, the fsr1 knockout fragment was also transformed into C1995 and deletion mutants were identified by diagnostic PCR (data not shown). Microscopic analysis of the fruiting bodies of both sexual crosses clearly showed that the loss of fsr1 in both mating partners resulted in colorless perithecia that lack the normal purple pigmentation (Fig. 7).

Discussion

Polyketides represent a highly diverse group of natural products within fungal secondary metabolites. They are generated by large multi-domain enzymes, the PKSs (14). Among them are naphthoquinones, a class of polyketides formed by NR-PKSs. They are often deeply colored, and many of them show phytotoxic, insecticidal, antibacterial and fungicidal activities (52). Due to their structural properties they are also thought to be involved in protection against extreme environmental conditions, such as UV irradiation and desiccation (30).

Up to now, only the red polyketides bikaverin and nor-bikaverin (40) as well as O-demethylanhydrofusarubin (21) were identified as naphthoquinone pigments of *F. fujikuroi*.
In this study we describe the identification of new secondary metabolites with naphthoquinoid structure as well as the corresponding biosynthetic genes organized in a gene cluster in the genome of *F. fujikuroi*. These metabolites are similarly red pigmented as bikaverin, but their production is differently regulated by external factors such as pH and nitrogen sources. The new red pigments were identified as members of the fusarubin family (Fig. 6D). Although most of these structures have been described before in different *Fusarium* spp. (52), their biological function and the biosynthetic genes remained mysterious until now.

**Fusarubin-type naphthoquinones are responsible for coloration of the fruiting bodies.** In this study we show for the first time that the fusarubin-type naphthoquinones (Fig. 6D) are identical with the perithecial pigments (Fig. 7). Perithecia are the sexual fruiting bodies in which the fungus’ progeny, the ascospores, are formed. Here naphthoquinones might function as protectants, as suggested for their role in *F. solani* (30), against harmful environmental conditions such as, reactive oxygen species (ROS), UV irradiation, desiccation and fungivorous insects. In *A. fumigatus* conidia of mutants defective in PKS-derived pigment biosynthesis are more susceptible to killing by macrophages than pigmented wild-type conidia (35). Furthermore, studies in *A. nidulans* have shown that certain insects preferentially feed on fungal mutants exhibiting a restricted secondary metabolite profile indicating that a fungus capable of the production of its full secondary metabolite spectrum has an enhanced survival rate (61). The colorless perithecia of *fsr1* mutants exceeded the colored ones produced by the wild type in their size (Fig. 7), suggesting that the red pigments are also important for the cell wall integrity of the perithecia. A similar correlation was found for *A. fumigatus*, where PKS-derived melanin is required for the correct assembly of the cell wall layers in the
conidia (59). Interestingly, in *F. fujikuroi* the fusarubin-type pigments are not restricted to
the fruiting bodies, but occur also in the culture broth when grown in liquid culture. This
raises the question if these secondary metabolites have further functions besides
protection of the perithecia. One possibility would be a functional redundancy between
the red pigments, as fusarubins and bikaverin are not produced under the same culture
conditions in *F. fujikuroi*. A similar situation was found for *F. graminearum*, where
deletion of the aurofusarin-specific PKS gene *aur1* affected the expression of *pgl1*
responsible for formation of pigmented perithecia (28).

**The biosynthetic pathway of the fusarubins.** We identified the main compounds
produced by the wild type as the naphthoquinones 8-O-methylfusarubin 1, 8-O-
methylnectriafurone 2, 8-O-methyl-13-hydroxynorjavanicin 3, 8-O-methyl-
anhydrofusarubinlactol 4, and 13-hydroxynorjavanicin 5 (Fig. 6D) based on their exact
masses, characteristic UV fingerprints and by NMR data (67, 72, 70 71). All of these
compounds, despite 8-O-methylanhydrofusarubinlactol 4, which has never been
described before, are known metabolites of other *Fusarium* spp. (52). However, this is
the first report on identification of these naphthoquinones in *F. fujikuroi*. The finding that
these structurally related but distinctly different naphthoquinones are all products of the
same NR-PKS might indicate that most of these structures identified in *Fusarium* spp.
are probably intermediates or final products of one and the same biosynthetic pathway.

To analyze this pathway in detail, it is important to identify the first intermediate
released by the responsible PKS. Fsr1 shows the typical domain organization of a non-
reducing PKS consisting of a SAT, KS, MAT and two ACP domains, with one exception:
the often found TE or TE/CLC domain is replaced by a R domain (Tab. 1). This finding
led to the hypothesis that the PKS-derived heptaketide is most likely released as an
aldehyde. By overexpressing fsr1 in the Δfsr2-5 mutant in a Fsr6-independent manner, we were able to identify the first intermediate of the pathway as 6-O-demethylfusarubinaldehyde 7 (Fig. 6C, Fig. 6D). This reductive release mechanism resulting in the formation of an aldehyde intermediate was previously shown for the 3-methylorcinaldehyde synthase Pks1 in Acremonium strictum (5), and AfoE encoding the NR-PKS of the asperfuranone pathway (13) that also harbor a R domain. This mechanism has also been shown in bacteria, e.g. for Lgr, the non-ribosomal peptide synthase (NRPS) of the gramicidin pathway in Bacillus brevis (38), SmfC, one NRPS of the SFM-A gene cluster in Streptomyces lavendulae (45), and MxcG, the NRPS responsible for the formation of the aldehyde intermediate in the Myxochelin biosynthesis of Stigmatella aurantiaca (46). Furthermore, the α-aminoadipate reductase Lys2p that also harbors an R domain was shown to be responsible for formation of an aldehyde intermediate in the lysine biosynthetic pathway of Saccharomyces cerevisiae (25). In the biosynthetic pathway of fusarubins, the A and B ring of 7 are most likely formed by a specific C4/C9-type aldol cyclization and aromatization catalyzed by the identified PT domain of Fsr1. This is in accordance to the mechanism the PT domain of A. fumigatus PksA catalyzes during Aflatoxin formation (15, 18, 47). As phylogenetic analysis of the Fsr1 PT domain shows close relatedness to PksA (Fig S5), this C4/C9-type cyclization is very likely to occur in F. fujikuroi but needs experimental proof in the future. Parisot et al. described a similar metabolite, 6-O-demethylnectriachrysone, isolated from F. solani mutants created by random UV-mutagenesis (57). This slightly different structure most likely results from rearrangement of the released aldehyde to a more stable intermediate during product isolation procedure, which was different from our method. This finding indicates that 6-O-demethylfusarubinaldehyde 7 is also the first
intermediate in *F. solani* instead of the proposed fusarubinic acid (54) similarly as it was shown for *F. fujikuroi* in this paper. This is the first report of a NR-PKS harboring a domain showing C4/C9 cyclization.

Although a SAT domain was identified in Fsr1 and 7 is most likely built by a C14 alkyl-chain, it remains unclear whether this alkyl chain consists of malonyl-derived building blocks only, or if the SAT domains accepts a starter unit of different chain length. Investigation of the SAT domain of *A. fumigatus* PksA provides experimental proof that this SAT domain accepts a hexanoyl starter unit (15). However, this hexanoyl starter is assembled by a fatty acid synthase (FAS) α-subunit (HexA) and a FAS β-subunit (HexB) that are encoded by genes in close proximity to *pksA* in *A. fumigatus* (78). Other examples from NR-PKS harboring a SAT domain that are known to accept starter units other than malonyl-CoA are found in *F. graminearum* trichothecene and in *A. nidulans* asperfuranone biosynthesis. In both cases the NR-PKS accepts the product of a HR-PKS as starter unit. Interestingly, the genes encoding the NR- and the HR-PKS belong to the same gene cluster (39, 13), resembling the situation of the aflatoxin gene cluster in *A. fumigatus*. In *F. fujikuroi* there are no HR-PKS- and no FAS-encoding genes found in close proximity to the fusarubin gene cluster. However, the nature of the building blocks assembled by Fsr1 needs experimental proof. This would provide valuable information that might help answering the question whether the protein involved in starter unit formation needs to be encoded in one gene cluster as the NR-PKS-encoding gene in case the starter unit is not malonyl-CoA.

The presence of a tandem ACP motif in Fsr1, was also observed in WA of *A. nidulans*, where the two ACP domains were shown to be redundant (26), suggesting a similar case for Fsr1 in *F. fujikuroi*. 
To identify further intermediates of this pathway, single deletion mutants of all six pathway genes were generated and the resulting metabolites analyzed by HPLC-DAD (Fig. 6B) and HPLC-UV-FTMS (data not shown). Of all mutants, only Δfsr2 and Δfsr3 showed an altered metabolite spectrum compared to the wild type. The structure of the accumulated intermediates was additionally elucidated by performing NMR experiments. By that 6-O-demethylfusarubinaldehyde 7 and 6-O-demethyl-10-deoxyfusarubin 6 were identified in the fsr2 deletion mutant, while fusarubinaldehyde 8 was the main product of the fsr3 deletion mutant. These results confirmed the hypothesized functions of those two enzymes (Tab. 1) as an O-methyltransferase (Fsr2) and a monooxygenase (Fsr3) as previously proposed for mutants created by UV-mutagenesis in F. solani (57, 56).

The intermediates found in Δfsr2 and Δfsr3 and putative intermediates described in the literature indicate that Fsr2 and Fsr3 function independently from each other. Nevertheless, the fact that the second hydroxyl group is not present at position C10 in Δfsr2 (compound 6), indicates that the methylation of the hydroxyl group at position C6 is necessary for this step. Furthermore, methylation at C8 only takes place if both hydroxyl groups at C5 and C10 are present and does not seem to be an essential biosynthetic step as indicated by the accumulation of 13-hydroxynorjavanicin 5 in the wild type. A similar flexible pathway was shown for the biosynthesis of bikaverin, where the mono-methylated form, nor-bikaverin, co-exists with the di-methylated bikaverin in liquid culture (79). The accumulation of only one intermediate in Δfsr3 instead of the five structurally related but distinct metabolites found in the wild type suggests that Fsr3 is not only responsible for the hydroxylation steps at position C5 and C10, but also for the reduction of the released aldehyde or alternatively the oxidation and subsequent 13-hydroxylation after the cleavage of CO₂. This series of possible biosynthetic steps...
indicates its role as a multifunctional monooxygenase. So far, multiple oxidization reactions by a single enzyme have only been described for Cytochrome-P450 monooxygenases as for three of the four P450 monooxygenases in the gibberellin biosynthesis of *F. fujikuroi* (76, 78, 62) and Tri4 in the trichothecene biosynthesis of *F. graminearum* (73). In *A. fumigatus* a chain-shortening of YWA1 was shown to be catalyzed by Ayg1 resulting in the formation 1,3,6,8-tetrahydroxynaphthalene (T4HN) (27). Since no protein with significant homology to Ayg1 was identified to be encoded in the *fsr* gene cluster a similar mechanism is very unlikely. However, if Fsr3 is capable of multiple oxidation steps including the cleavage of CO₂ awaits experimental proof.

The proteins encoded by *fsr4* and *fsr5* show homologies to proteins harboring an oxidoreductase and a short-chain dehydrogenase domain, respectively. Surprisingly, no enzymatic function could be determined for either of the two, as deletion of their encoding genes did not alter the product spectrum (Fig. 6B). Remarkably, deletion of *fsr4*, but not *fsr5*, seems to enhance the biosynthesis of the respective products (Fig. 6B) although *fsr1*-*fsr3* genes were not up-regulated in the *fsr4* deletion mutant at the time point investigated (Fig. S6A). Proteins harboring a short-chain dehydrogenase domain were previously shown to possess regulatory functions in filamentous fungi. Thus, the protein Nmr1, also having a predicted short-chain reductase domain, was linked to the nitrogen-dependent regulation of secondary metabolism in *F. fujikuroi*. Yeast two hybrid analyses showed that Nmr1 interacts with the global nitrogen regulator AreA thereby inactivating AreA under nitrogen sufficient conditions leading to a subsequent down-regulation of genes responsible for gibberellin biosynthesis (65). Additionally, an *nmr*-like gene, *bik4*, was identified in the bikaverin gene cluster, and the encoded protein, Bik4, was suggested to be a co-regulator of the Zn(II)₂Cys₆
transcription factor Bik5 (79). However, since fusarubin-derivatives are produced in the perithecia under natural conditions, it cannot be ruled out that Fsr4 and Fsr5 catalyze specific enzymatic steps during perithecia development.

Organization of the perithecia pigment gene cluster in *F. fujikuroi* and related species. The gene cluster for the perithecial pigment in *F. solani* differs from the gene clusters found in *F. verticillioides*, *F. graminearum* (60) and *F. fujikuroi* (this paper). Remarkably, a core gene cluster consisting of genes encoding the PKS (Pgl1, Fsr1), the methyltransferase (Omt1, Fsr2) and the monooxygenase (Fdm1, Fsr3) is syntenic in all four *Fusaria*, whereas the entire genomic region from gene FF_03983 to FF_03990 including the cluster genes fsr1-fsr6, is syntenic in *F. verticillioides* only (60). In *F. graminearum* and in *F. solani*, the synteny is abrogated between fsr3 and fsr4 compared to *F. fujikuroi*, suggesting that in *F. graminearum* and *F. solani* the respective perithecial pigment gene cluster may consist of genes different from the ones in *F. fujikuroi*. This constitutional difference of the respective gene cluster in *F. solani* and *F. fujikuroi* could account for the different production conditions and the slightly different metabolite spectrum described by Kurobane *et al.* for *F. solani* compared to *F. fujikuroi* in this study (43).

Interestingly, Ma *et al.* described seven genes that were co-regulated with pgl1 in *F. verticillioides* by using a microarray approach (49). However, in *F. fujikuroi* only five of the homologous genes, fsr2-5 adjacent to the PKS-encoding fsr1 were found to be strictly co-regulated (Fig. 4), possibly due to different culture conditions used. Restriction of the fusarubin gene cluster in *F. fujikuroi* to the genes fsr1-6 is fortified by the fact that deletion of the two border genes of fsr1 and fsr6 did not show any impact on biosynthesis and were not expressed under all conditions tested in this study (Fig. 4).
The two groups of red pigments in *F. fujikuroi* show contrasting regulation. Interestingly, the two groups of pigments in *F. fujikuroi*, bikaverins and fusarubins, are found to be expressed under contrary pH conditions. The most remarkable difference is that bikaverin is only produced at acidic conditions, while the fusarubin pigments are only formed, when the pH of the culture medium is neutral or alkaline (Fig. 1). Due to the strict response of *fsr* gene expression to changing pH conditions, the involvement of the pH regulator PacC is currently investigated. In *A. nidulans*, this C2H2 zinc finger transcription factor is known to preferentially activate target genes at alkaline pH and represses genes that are expressed at acidic pH (58). For the bikaverin genes, a non-canonical pH regulation has been demonstrated where the genes are repressed under alkaline conditions in a PacC-dependent manner (79).

Another interesting yet unknown mechanism for regulating the expression of bikaverin genes is their strict interdependency. If any of the biosynthesis genes *bik1*-*bik3* is deleted, the remaining genes are also not expressed due to a yet unknown mechanism (79). This is not the case for the *fsr* genes, where only the deletion of the PKS-encoding gene, *fsr1*, and the gene coding for a Zn(II)2Cys6 transcription factor, *fsr6*, results in a total loss of gene expression, while deletion of any other gene does not affect the expression of the remaining *fsr* genes (Fig. S6A). In addition to these contrasting regulation pattern, there is also a common regulation mechanism: high amounts of nitrogen repress the expression of bikaverin (79) as well as fusarubin (described here) biosynthetic genes. We clearly demonstrated that this repression is specifically triggered by glutamine (Fig. 5C). How glutamine sensing and subsequent signal transduction works in *F. fujikuroi* is currently under investigation.
In conclusion, we describe the identification of 5 new red pigments in *F. fujikuroi* which are produced under specific culture conditions (alkaline pH; nitrogen limitation) different from those for bikaverins. By a combination of HPLC, MS and NMR analyses, we identified 8-O-methylfusarubin 1, 8-O-methylnectria furone 2, 8-O-methyl-13-hydroxynorjavanicin 3, 8-O-methylnanhydro-fusarubin lactol 4, and 8-O-demethyl-13-hydroxynorjavanicin 5 as true metabolites of the pigment pathway in *F. fujikuroi* (67, 72, 70, 71). Based on the newly identified structures we were able to establish a biosynthetic pathway for fusarubin-like metabolites: The heptaketide backbone of the polyketide results from the condensation of 7 acetyl-CoA subunits and is subsequently released by the PKS Fsr1 as 6-O-demethylfusarubinaldehyde 7. After several methylation and hydroxylation steps by Fsr2 and Fsr3, respectively, the final products 1-5 accumulate in the liquid culture of the wild type *F. fujikuroi*, with 8-O-methylfusarubin 1 as the main product (Fig. 8). In addition, we pinpointed these pigments for the first time to a concrete gene cluster in the genus *Fusarium* and demonstrated that the fusarubins described here are the so far unknown perithecial pigments.

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Katharina W. von Bargen for helping with the acquisition of NMR data as well as Kathleen Plamper for excellent technical support.

References


Tab. 1: Gene information about the putative fsr cluster.

<table>
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<tr>
<th>Gene</th>
<th>bp</th>
<th>Intron</th>
<th>Amino Acid</th>
<th>Domains and motifs</th>
<th>Predicted function</th>
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</thead>
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<tr>
<td>FF_03983</td>
<td>1788</td>
<td>0</td>
<td>595</td>
<td>IPR002937 Amine oxidase</td>
<td>Unknown function</td>
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<tr>
<td>fsr1 (FF_03984)</td>
<td>7069</td>
<td>4</td>
<td>2286</td>
<td>IPR014031 Beta-ketoacyl synthase IPR014043 Acyl transferase domain IPR009081 Acyl carrier protein-like</td>
<td>Non-reducing PKS responsible for the condensation of 7 acetyl-CoA units</td>
</tr>
<tr>
<td>fsr2 (FF_03985)</td>
<td>1209</td>
<td>1</td>
<td>387</td>
<td>IPR001077 O-methyltransferase, family 2</td>
<td>Methyltransferase responsible for the methylation of hydroxy groups at C6 and C8</td>
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<tr>
<td>fsr3 (FF_03986)</td>
<td>1660</td>
<td>2</td>
<td>522</td>
<td>IPR002938 Monooxygenase, FAD-binding IPR003042 Aromatic-ring hydroxylase-like</td>
<td>Monooxygenase responsible for reduction / oxidation of the PKS-derived aldehyde as well as for incorporation of hydroxyl groups at C5, C10 and C13</td>
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<td>fsr4 (FF_03987)</td>
<td>1117</td>
<td>2</td>
<td>338</td>
<td>IPR002085 Alcohol dehydrogenase superfamily, zinc-containing IPR016040 NAD(P)-binding domain</td>
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<td>fsr5 (FF_03988)</td>
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<td>1</td>
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<td>IPR002198 Short-chain dehydrogenase/reductase SDR</td>
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Fig. 1: Identification of a new group of red pigments by changing standard growth conditions. The wild type (Wt) *F. fujikuroi* was grown in ICI medium with either 6 mM glutamine (Gln) or 6 mM sodium nitrate (NO₃⁻) for five days. Samples were taken and used for HPLC-DAD analyses as described in Material and Methods.

A: Pictures of the flasks with the Wt after five days of inoculation. B: HPLC-chromatogram (detected at 450 nm). Peaks for norbikaverin 1 and bikaverin 2 are labeled in the upper chromatogram. New unknown compounds in the lower chromatogram are labeled with a question mark. C: pH values over the time of five days. The pH was measured every 12 hours.

Fig. 2 Phylogeny, PKS domains, gene cluster. A: KS domains of fungal PKS used for the analysis were extracted and the phylogram was generated as described in Material and Methods. Leaves of the phylogram are denoted as “organism_proteinname_accession number” in the case of characterized PKS. For uncharacterized *Fusarium* PKS, the Broad Institute ID is given in case of *F. graminearum* (FGSG), *F. oxysporum* (FOXG) and *F. verticillioides* (FVEG) and the Joint Genome Institute ID in case of *F. solani*. Furthermore leaves show domain architectures of PKSs and the chemical product produced. Branches with branch support values less than 0.5 were collapsed. KS domain of the highly reducing PKS Fum1 was used as outgroup. Scale bar represents 0.3 character changes. *(Aspergillus fumigatus), An (A. nidulans), Ap (A. parasiticus), As (Acremonium strictum), At (Aspergillus terreus), Cl (Colletotrichum lagenarium), Cn (Cercospora nicotianae), Ds (Dothistroma septosporum), Ed (Exophiala dermatitidis), Fg (F. graminearum), Ff (F. fujikuroi), Fs (F. solani), Fv (F. verticillioides), Gl (Glarea lozoyensis), Mp (Monascus purpureus) and Nsp. (Nodulisporium sp. ATCC74245). SAT (starter unit:ACP transacylase) superscript letters indicate amino acid at third
position of the active site other than C, KS (β-ketoacyl synthase domain), MAT (malonyl-CoA:ACP transacylase domain), PT (product template domain), ACP (acyl carrier protein domain), TE (thioesterase domain), TE/CLC (thioesterase/Claisen cyclase domain), R (reductase domain), DH (dehydratase domain), ER (enoyl reductase domain), KR (keto reductase domain), CMet (C-methyltransferase domain).

B: The fsr gene cluster, fsr1-fsr6, is depicted as black boxes. The first gene upstream (FF_03983) and downstream (FF_03990) are highlighted in grey. Arrows indicate direction of translation, white bars show introns within the gene.

**Fig. 3:** *fsr1* is responsible for the production of fusarubins. Fungal strains were grown in 6 mM sodium nitrate. Culture filtrates were taken after six days of growth, when the wild type (Wt) showed red pigmentation. Samples were directly used for HPLC-DAD measurements, as described in Material and Methods. The figure shows the fungal strains in liquid culture with the corresponding HPLC-Chromatogram (detected at 450 nm). A: wild type, B: Δfsr1 deletion mutant, C: restoration of fsr1 in the deletion mutant (Δfsr1/fsr1c). Peaks associated with Fsr1 are highlighted (blue box).

**Fig 4. Co-regulation of the putative fsr gene cluster.** The wild type (Wt) was grown in ICI medium with either 6 mM glutamine (Gln) or 6 mM sodium nitrate (NO₃⁻). Lyophilized mycelium was used for Northern blot analyses. The putative genes of the fsr gene cluster (fsr1-fsr6) and one gene upstream (FF_03983) as well as one gene downstream (FF_03990) of the gene cluster were used for probing.
**Fig. 5:** Expression of *fsr* genes depends on culture age, pH and nitrogen. A: The wild type (Wt) was grown in ICI medium with 6 mM sodium nitrate. The mycelium was harvested after 2, 3, 4, 5, 6 and 7 days, respectively. Lyophilized mycelium was used for northern blot analyses. For probing *fsr*1-3 were used. B: The Wt was grown in ICI medium with 6 mM sodium nitrate for 4 days and then shifted into liquid medium set to pH 4 or pH 8, without any nitrogen. The mycelium was harvested after additional inoculation for 2 hours and used for northern blot analysis. *Fsr*2 and *fsr*3 were used for probing. C: The Wt was grown in ICI medium with 6 mM sodium nitrate. After 4 days either 33 mM glutamine (+N (Gln)), 33 mM sodium nitrate (+ N (NO$_3^-$)) or water (-N) was added. After additional inoculation of 30 min, the mycelium was harvested and used for northern blot analyses. The genes *fsr*2-*fsr*5 were used for probing.

**Fig. 6:** Chemical analyses of fungal strains. For analyses of compounds the indicated fungal strains were grown under fusarubin biosynthesis-favoring conditions (ICI with 6 mM sodium nitrate). Samples were taken after six days of growth and directly used for HPLC-DAD measurement as described in Material and Methods. Numbers indicate compounds found in the liquid culture (8-O-methylfusarubin 1, 8-O-methylnectriafurone 2, 8-O-methyl-13-hydroxynorjavanicin 3, 8-O-methylanhydrofusarubinlactol 4, 13-hydroxynorjavanicin 5, 6-O-demethyl-10-deoxyfusarubin 6, 6-O-demethylfusarubinaldehyde 7, fusarubinaldehyde 8). A: Chemical analysis of the wild type (Wt). B: Chemical analyses of single deletion mutants Δ*fsr*1-Δ*fsr*6. C: Identification of the PKS-derived product by overexpressing *fsr*1 in the Δ*fsr*2-Δ*fsr*5 mutant. D: Structural formula of identified compounds in the wild type 1-5, in the deletion mutants Δ*fsr*2 6-7 and Δ*fsr*3 8 as well as in Δ*fsr*2-Δ*fsr*5/gpd::*fsr*1 7.
**Fig. 7:** The fusarubins are responsible for the coloration of the fruiting bodies in *F. fujikuroi*. The indicated strains were crossed as described in Material and Methods. Perithecia are indicated by black arrows. Size standard is depicted in the lower left corner of the pictures. A: Crossing of the wild type IMI58289 with its mating partner C1995 results in the formation of darkly pigmented fruiting bodies B: Crossing of IMI58289/Δfsr1 with C1995/Δfsr1 results in the formation of colorless perithecia that exceed the pigmented ones in their size.

**Fig. 8:** Biosynthetic pathway of fusarubins in *F. fujikuroi*. Verified route of naphthoquinone formation in *F. fujikuroi* based on the main compounds identified in the liquid culture. The condensation of seven acetyl-CoA units results in the formation of a heptaketide (E: bound to the ACP domain of the NR-PKS), after ring A and B are formed by aldol type cyclization the PKS-derived product is released as 6-O-demethylfusarubinaldehyde 7. Then two hydroxyl groups are incorporated by Fsr3 and simultaneously hydroxyl groups are methylated by Fsr2. The aldehyde is on the one hand reduced to 8-O-methylfusarubin alcohol by Fsr3 that equilibrates mainly with 8-O-methylfusarubin 1 and only small amounts of 8-O-methylnectriafurone 2, indicated by the grey reaction arrow. On the other hand the aldehyde can be oxidized to form 8-O-methylfusarubinic acid driven by Fsr3 equilibrating with 8-O-methylfusarubinlacton, finally resulting in 8-O-methylnorjavanicin 4 after a further reduction step and loss of water. 8-O-methylfusarubinic acid can also undergo decarboxylation resulting in 8-O-methyl-13-hydroxynorjavanicin 4 after another hydroxylation step at C13. Both steps are most likely also accomplished by Fsr3. Structures highlighted in grey were identified by NMR, MS and UV-data in the liquid culture of the *F. fujikuroi* wild type during the course of this work. The identified...
aldehyde as the first intermediate in this biosynthetic pathway is depicted in the grey box. The main route of naphthoquinone formation is indicated by the dark grey arrow.