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Interplay between pathway-specific and global regulation of the fumonisin gene cluster in the rice pathogen *Fusarium fujikuroi*

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Abstract The rice pathogenic fungus *Fusarium fujikuroi* is known to produce a large variety of secondary metabolites. Besides the gibberellins, causing the *bakanae* effect in infected rice seedlings, the fungus produces several mycotoxins and pigments. Among the 47 putative secondary metabolite gene clusters identified in the genome of *F. fujikuroi*, the fumonisin gene cluster (*FUM*) shows very high homology to the *FUM* cluster of the main fumonisin producer *Fusarium verticillioides*, a pathogen of maize. Despite the high level of cluster gene conservation, total fumonisin FB_1 and FB_2 levels (FB_x) produced by *F. fujikuroi* were only 1–10 % compared to *F. verticillioides* under inducing conditions. Nitrogen repression was found to be relevant for wild-type strains of both species. However, addition of germinated maize kernels activated the FB_x production only in *F. verticillioides*, reflecting the different host specificity of both wild-type strains. Overexpression of the pathway-specific transcription factor Fum21 in *F. fujikuroi* strongly activated the *FUM* cluster genes leading to 1000-fold elevated FB_x levels. To gain further insights into the nitrogen metabolite repression of FB_x biosynthesis, we studied the impact of the global nitrogen regulators AreA and AreB and demonstrated that both GATA-type transcription factors are essential for full activation of the *FUM* gene cluster. Loss of one of them obstructs the pathway-specific transcription factor Fum21 to fully activate expression of *FUM* cluster genes.

Keywords *Fusarium fujikuroi* · Fumonisins · Biosynthesis · Regulation · Over-expression

Introduction

The rice pathogenic ascomycete *Fusarium fujikuroi* is a member of the Asian clade of the *Gibberella fujikuroi* species complex closely related to *Fusarium proliferatum* (Nirenberg and O'Donnell 1998). It is the causative agent of the *bakanae* ("foolish seedling") disease of rice. The most characteristic symptoms of this disease are excessively elongated seedlings with chlorotic stems and leaves due to the ability to produce and secrete gibberellins, a family of plant hormones (Bömke and Tudzynski 2009).

Besides the gibberellins, the fungus produces several other secondary metabolites (SM) including pigments, such as bikaverin and fusarubins (Wiemann et al. 2009; Studt et al. 2012) as well as harmful mycotoxins such as fusarins, fusaric acid, apicidin F, and fujikurins (Niehaus et al. 2013, 2014a, b; von Bargen et al. 2015). The recently sequenced genome comprises many more, so far uncharacterized, gene clusters showing a huge potential of the fungus to produce further secondary metabolites (Wiemann et al. 2013).

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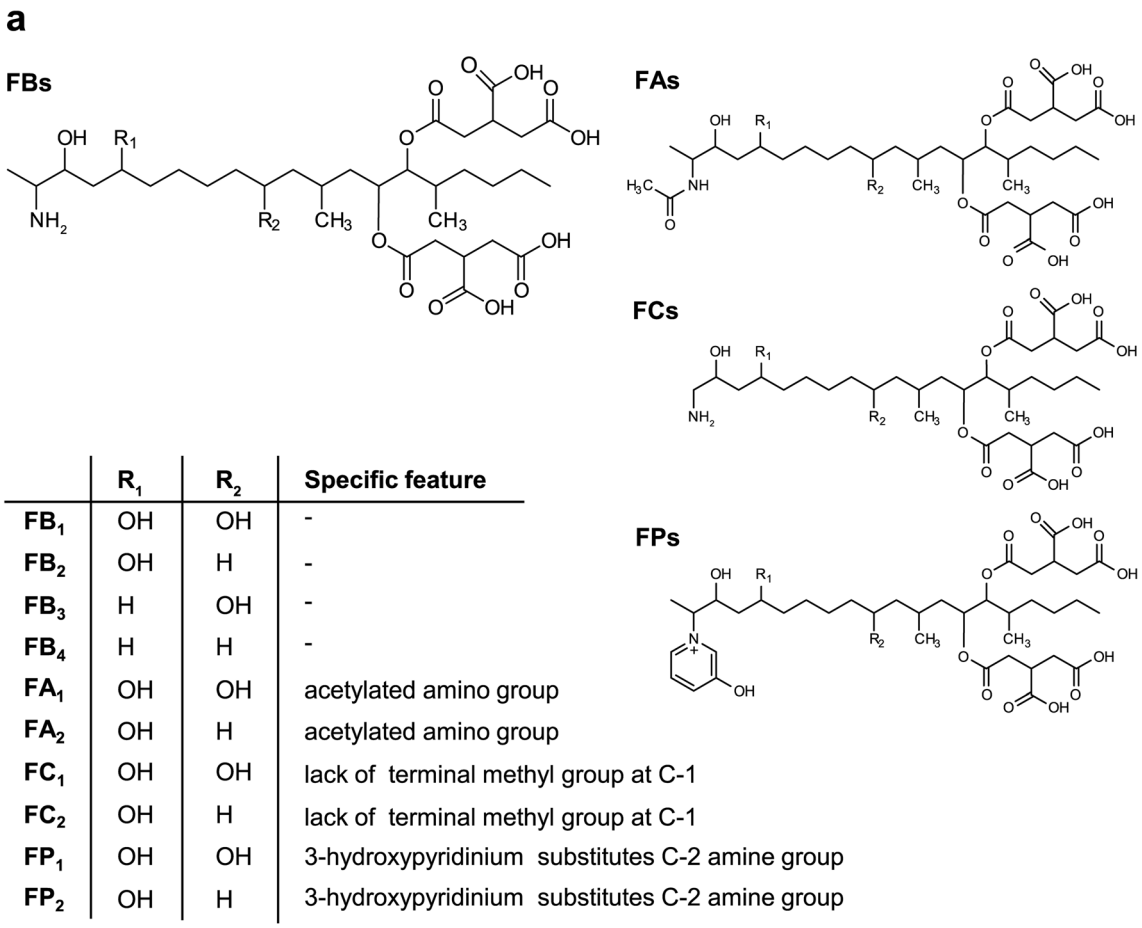
One of the gene clusters identified in *F. fujikuroi* revealed almost perfect homology to the fumonisin gene cluster (*FUM*) in the maize pathogens *Fusarium verticillioides* and *F. proliferatum* (Wiemann et al. 2013). The polyketide metabolites fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) were first isolated and identified in 1988 (Gelderblom et al. 1988). Their chemical structure is characterized by an aminopentol backbone with two tricarballylic acid side chains and one or two hydroxy groups (Bezuidenhout et al. 1988). Besides the fumonisins of the B series ($FB_{1–4}$), several analogs have been found belonging to the three minor series of A fumonisins (*N*-acetyl, FAs), C fumonisins (dimethyl, FCs), and P fumonisins (*N*-3-hydroxypiridinium, FPs) in several *Fusarium* spp. (Gelderblom et al. 1988; Branham and Plattner 1993; Musser et al. 1996; Lazzaro et al. 2013) (Fig. 1a).

Fumonisins, and particularly fumonisin B_1 cause porcine pulmonary edema and equine leukoencephalomalacia in swine and horses, respectively, and are suggested to be potential risk factors for human esophageal cancer and even birth defects (Harrison et al. 1990; Kellerman et al. 1990; Desai et al. 2002; Marasas et al. 2004; Sun et al. 2007). Because of their structural similarity to sphingoid bases and fatty acyl-CoA, fumonisins of the B series inhibit the ceramide synthase leading to perturbation in sphingolipid metabolism which is



	R_1	R_2	Specific feature
FB_1	OH	OH	-
FB_2	OH	H	-
FB_3	H	OH	-
FB_4	H	H	-
FA_1	OH	OH	acetylated amino group
FA_2	OH	H	acetylated amino group
FC_1	OH	OH	lack of terminal methyl group at C-1
FC_2	OH	H	lack of terminal methyl group at C-1
FP_1	OH	OH	3-hydroxypyridinium substitutes C-2 amine group
FP_2	OH	H	3-hydroxypyridinium substitutes C-2 amine group

Fig. 1 Chemical structure of different fumonisins and fumonisin gene cluster comparison. Produced fumonisins are divided into the B, A, C, and P series referring to different residues added to or missing at the basic aminopentol backbone (**a**). The fumonisin gene clusters of *F. fujikuroi* and *F. verticillioides* are highly conserved (**b**) (modified after Wiemann et al. 2013)

important for the maintenance of membrane structures and the prevention of apoptosis (Schmelz et al. 1998; Merrill et al. 2001; Desai et al. 2002).

In general, fumonisin production, in particular of FB_1 and FB_2, has been mainly reported for the maize pathogens *F. verticillioides* and *F. proliferatum*, but also for some isolates of *Fusarium oxysporum* (Rheeder et al. 2002; Munkvold 2003) as well as *Aspergillus niger* and some related species (Frisvad et al. 2007; Varga et al. 2010). Only traces of *N*-acetylated fumonisins (FAs) were found in cultures of *F. verticillioides* and *F. proliferatum* isolates and contaminated maize samples (Lazzaro et al. 2013; Tamura et al. 2014). *F. fujikuroi* was shown to produce no or only very low amounts of fumonisins (Wiemann et al. 2013; Cruz et al. 2013).

The key enzyme of fumonisin biosynthesis in *F. verticillioides* was found to be the polyketide synthase (PKS) Fum1 (Proctor et al. 1999). Subsequent characterization of the surrounding genes resulted in the identification of a cluster consisting of 16 co-expressed genes (FVEG_00315 to FVEG_00329) encoding, beside the PKS, two fatty acid synthases, several monooxygenases, dehydrogenases, transporter proteins, an aminotransferase, and a dioxygenase (Butchko et al. 2006; Brown et al. 2007). Deletion of the genes encoding Fum1 (Proctor et al. 1999), the P450 monooxygenase Fum6, and the aminotransferase Fum8 (Seo et al. 2001) led to total loss of fumonisin production. In contrast, deletion of the genes *FUM2*, *FUM3*, *FUM7*, *FUM10*, *FUM11*, *FUM13*, *FUM14*, and *FUM19* encoding a P450 monooxygenase, a dioxygenase, a dehydrogenase, a fatty acyl-CoA synthase, a tricarboxylate transporter, a short-chain dehydrogenase, a NRPS-like condensation domain-containing enzyme, and an ABC transporter, respectively, only resulted in a variation of the ratio between the end products FB_1 and FB_2 and their precursors fumonisin B_3 (FB_3) and fumonisin B_4 (FB_4), respectively, or slightly modified forms (Butchko et al. 2003a; Butchko et al. 2003b; Proctor et al. 2003; Proctor et al. 2006; Butchko et al. 2006). The homologs of almost all cluster genes are present and well conserved in *F. fujikuroi* (FFUJ_09240 to FFUJ_09255) except for *FUM20* which is absent and *FUM17* which is only present as a pseudogene. However, both genes are very unlikely to be involved in fumonisin biosynthesis of *F. verticillioides* (Proctor et al. 2003; Brown et al. 2005; Wiemann et al. 2013).

Regulation of fumonisin production has been studied mainly in *F. verticillioides*. Under alkaline conditions, fumonisin biosynthesis is repressed by the pH regulator Pac1 (Flaherty et al. 2003). In general, high nitrogen concentrations inhibit fumonisin production in *F. verticillioides* (Shim and Woloshuk 1999). The nitrogen-responsive regulator AreA was shown to be involved in this regulation and is essential for fumonisin production (Kim and Woloshuk 2008). Deletion of the Zn(II)2Cys6 transcription factor (TF) encoding gene *ZFR1*, which was shown to be an activator of fumonisin biosynthesis, indicated an involvement of sugar supply on fumonisin production (Flaherty and Woloshuk 2004; Bluhm et al. 2008). The gene encoding the putative sugar transporter Fst1 was found to be upregulated in the WT during fumonisin production on maize kernels but downregulated in Δ*zfr1* mutants. An independent deletion of *FST1* also confirmed its role as positive regulator of fumonisin biosynthesis (Bluhm et al. 2008). Recently, the global regulator Sge1 was shown to act as a strong activator of the *FUM* cluster in *F. fujikuroi* (Michielse et al. 2015).

The cluster-specific Zn(II)2Cys6 TF Fum21 was identified by Brown et al. (2007). Its activity is essential for fumonisin biosynthesis: deletion of *FUM21* resulted in low expression of *FUM1* and *FUM8* in the early stages of growth (Brown et al. 2007). Recently, Montis et al. (2013) identified a putative TF-binding motif (CGGMTA) in *F. verticillioides* which is relevant for transcriptional activation of *FUM1* and is present in several fumonisin producers, but also non-producers (Montis et al. 2013). However, it was not shown which regulator might bind to this motif (Flaherty and Woloshuk 2004; Brown et al. 2007). In *F. fujikuroi*, this motif is not present in the promoter of *FUM1*.

In the present work, we gained more insight into the regulation of fumonisin biosynthetic genes in *F. fujikuroi*. We show that production levels of fumonisins are very low in *F. fujikuroi* compared to *F. verticillioides* despite the high homology of the cluster genes. However, constitutive expression of the cluster internal TF Fum21 strongly activated the fumonisin biosynthetic genes and led to 1000-fold elevated fumonisin yields. Similarly to *F. verticillioides*, fumonisin biosynthetic genes are subject to nitrogen metabolite repression in an AreA-dependent manner. In addition, we provide evidence that the second nitrogen-responsive GATA TF AreB is also essential for fumonisin gene expression and biosynthesis. To study the hierarchy between the impact of the cluster internal TF Fum21 and the two global regulators AreA and AreB, we over-expressed *FUM21* in the background of the *AREA* and *AREB* deletion mutants. We show that the fumonisin biosynthesis was only partially restored in these double mutants, indicating that the activity of both GATA factors is essential for the action of Fum21.

Material and methods

Strains and cultivation conditions

The *F. fujikuroi* wild-type (WT) strain IMI58289 (Commonwealth Mycological Institute, Kew, UK), and the mutants Δ*areA* (Tudzynski et al. 1999) or Δ*areB* (Michielse et al. 2014), were used as parental strains for gene knock-out and over-expression experiments. For comparative analyses of SM production and SM gene expression, the WT strains of *F. fujikuroi* and *F. verticillioides* (strain M-3125, provided

by D. Brown, U. S. Department of Agriculture) as well as the *F. fujikuroi* Δ*areA* and Δ*areB* mutants were cultivated in different conditions. For general maintenance, all strains were grown on solid complete medium (CM; Pontecorvo et al. 1953). To grow strains for genomic DNA isolation, a cellophane layer was placed on the surface of solidified CM and the colonies were grown for 2 to 5 days. For fungal transformation, gene expression, and metabolite analyses, the indicated strains were grown in liquid cultures. Two agar plugs from CM plates were pre-cultivated for 3 days in 300-mL Erlenmeyer flasks containing 100 mL Darken medium (Darken et al. 1959) at 28 °C and 180 rpm on a rotary shaker in darkness. As main culture, 100 mL of defined ICI medium (Imperial Chemical Industries Ltd., UK; Geissman et al. 1966) were prepared with different nitrogen sources and concentrations replacing the original nitrogen source, 6/60 mM L-glutamine (Gln), maize flour (2 g/L; Frießinger Mühle GmbH, Bad Wimpfen, Germany) or ground, lyophilized maize seedlings (2 g/L). The cultures were inoculated with 500 μL of the pre-culture and shaken for 3 to 7 days under conditions described above. For the germination of maize (*Zea mays*) seedlings, the kernels were surface sterilized (Glenn et al. 2008), incubated for 3 days at 28 °C in the dark on water agar (1.5 %) and incubated for an additional 3 days in the presence of a 12 h light–12 h dark cycle at room temperature. Prior to preparation of protoplasts of *F. fujikuroi*, 500 μL of the pre-culture were transferred to 100 mL ICI medium containing 10 g/L fructose (instead of glucose), and 0.5 g/L $(NH_4)_2SO_4$ as nitrogen source. These cultures were incubated for no longer than 16 h.

The *Saccharomyces cerevisiae* strain FY834 (Winston et al. 1995) was used for recombinational cloning of vectors (Colot et al. 2006; Schumacher 2012), and the *Escherichia coli* strain TOP10F′ (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation.

Standard molecular techniques

Total DNA from *F. fujikuroi* was isolated following the protocol of Cenis (1992) from lyophilized and ground mycelium. Plasmid DNA from *S. cerevisiae* and *E. coli* was isolated using the NucleoSpin® Plasmid Kit (Machery-Nagel, Düren, Germany). Large amounts of plasmid for fungal transformation were isolated from *E. coli* using the Nucleobond® Xtra Midi Kit (Machery-Nagel, Düren, Germany). For standard DNA amplification by polymerase chain reaction (PCR), BioTherm™ DNA Polymerase (GeneCraft, Lüdinghausen, Germany) was used. Large fragments were amplified using TaKaRa LA Taq® DNA Polymerase (Takara Bio, Saint-Germain-en-Laye, France) and in case proof reading was essential, Phusion® High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) was applied. All polymerases were used according to the manufacturers' instructions. After isolation from yeast and propagation in *E.coli*, all vectors used for either over-expression or point mutation were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Prior to RNA isolation, strains were cultivated for 3 to 5 days in liquid ICI medium, the mycelium lyophilized and then ground under liquid nitrogen. The isolation was carried out using the TRI Reagent™ (Sigma-Aldrich, Deisenhofen, Germany). In order to analyze gene expression levels via Northern blot (Church and Gilbert 1984), 20 μg of isolated total RNA were separated in a 1 % (*w*/*v*) denaturating agarose gel (Sambrook et al. 1989) and subsequently transferred on to a nylon membrane (Nytran™ SPC, Whatman, Sanford, USA). The blotted membrane was hybridized with ^{32}P-labeled probes (Sambrook et al. 1989). Fragments of several genes involved in fumonisin biosynthesis were used as template. The following primer pairs were used for amplification: fum1_F/fum1_R for *FUM1*, fum6_F/fum6_R for *FUM6*, fum8_F/fum8_R for *FUM8*, and fum21_F/fum21_R2 for *FUM21*. Total genomic DNA of *F. fujikuroi* served as template for PCR reactions. All primers are listed in Table S1.

Plasmid construction

Yeast recombinational cloning was used to generate deletion, over-expression, and mutation vectors (Colot et al. 2006; Schumacher 2012). Directed gene deletion of *FUM21* was achieved by homologous recombination using a ca. 1 kb upstream (5′ flank) and downstream region (3′ flank) of the genes that were amplified using the primer pairs fum21_5F/5R and fum21_3F/3R, respectively. The hygromycin resistance cassette encoding the hygromycin B phosphotransferase gene *hph* under control of the strong *trpC* promoter from *Aspergillus nidulans* was amplified with the primer pair hph_F/hph_R from pCSN44 (Staben et al. 1989). *S. cerevisiae* strain FY834 was co-transformed with the three fragments and the *Eco*RI/*Xho*I digested pRS426 shuttle vector (Christianson et al. 1992) resulting in the knock-out vector pΔ*fum21* (Fig. S1). For constitutive expression of *FUM21*, the gene was amplified using the primer pair OEfum21_F/OEfum21_R and cloned downstream of the strong *oliC* promoter of the ATP synthase subunit 9 gene from *A. nidulans* (Ward and Turner 1986) using the *Nco*I/*Not*I digested vector pNDN-OGG (Schumacher 2012). As resistance marker, the nourseothricin resistance cassette, consisting of the *nat1* gene (nourseothricin acetyltransferase) under control of the strong *trpC* promoter from *A. nidulans*, was present in the resulting over-expression vector pOE::*FUM21* (Fig. S2). Constructs for directed point mutations in the *FUM1* promoter region were obtained by combination of the fragments amplified with the following primer pairs: MutPFum1_5'F/MutPFum1_5'R_hph (1), MutPFum1_PromF_hph/Mut2u3_R (2), Mut2u3F/MutPFum1_3'R (3), MutPFum1_PromF_hph/

Mut3_R (4), Mut3_F/MutPFum1_3'R (5), Mut_F1/MutPFum1_3'R (6), MutPFum1_PromF_hph/Mut_R1 (7), and MutPFum1_PromF_hph/MutPFum1_3'R (8) with the *Eco*RI/*Xho*I-digested pRS426 shuttle vector and the hygromycin resistance cassette. For pMut2/3, carrying point mutations in the second and third motif sequence, fragments (1), (2), and (3) were combined. The vector pMut3, carrying point mutations in the third motif sequence, combines the fragments (1), (4), and (5), while pMut_denovo, carrying point mutations in the de novo motif sequence, combines fragments (1), (6), and (7). For the negative control vector pMut0 without mutation, fragments (1) and (8) were combined (Fig. S3 and Fig. S6). Used primers are listed in Table S1.

Fungal transformation and confirmation of transformants

F. fujikuroi was transformed by protoplast transformation as published previously (Tudzynski et al. 1999). The *FUM21* deletion cassette was amplified from the isolated yeast DNA containing the vector p∆*fum21* using the primer pair fum21_5F/3R. The PCR product or, in the case of over-expression, 10 μg of circular vectors were used for transformation of ca. 10^7 protoplasts. For selection of transformants, 100 μg/mL hygromycin B (Calbiochem, Darmstadt, Germany) or 100 μg/mL nourseothricin (Werner-Bioagents, Jena, Germany) were applied. The putative transformants were confirmed by diagnostic PCR. ∆*fum21* knock-out mutants were tested for homologous integration of the deletion cassette and absence of the WT gene using the primer pairs fum21_5F_dia/pCSN44_trpC_T (5′ flank), fum21_3R_dia/pCSN44_trpC_P2 (3′ flank), and fum21_seq1/fum21_seq2 (WT genotype), respectively (Fig. S1). Possible additional ectopic integration of the resistance cassette was excluded in a Southern blot analysis using the 5′ flank (primer pair: fum21_5F/5R) as labeled probe on *Hin*dIII digested gDNA (Fig. S1). The presence of the over-expression construct of *FUM21* in the WT, ∆*areA*, and ∆*areB* backgrounds was tested with the primer pair PoliC_seqF2/fum21_seq1 (Fig. S2). The successful *FUM21* over-expression was verified by Northern blot analysis. For generation of point mutation mutants, 30 μg of the respective vector was digested with *Pvu*II and used for transformation of the *F. fujikuroi* WT and the OE::*FUM21* mutant. Point mutation of the putative DNA binding motifs in the *FUM1* promoter and absence of the WT sequence were verified by diagnostic PCR using the primer pairs: PromFum1_seq1/pCSN44_trpC_T (5′ flank), fum1_R/pCSN44_trpC_P2 (3′ flank) and PromFum1_seq1/fum21_F (WT genotype) and sequencing of the relevant region which was amplified using the primer pair PFum1_Mut_seqF1/PFum1_Mut_seqR1. All primers are listed in Table S1.

Prediction and identification of DNA binding sites

In order to identify over-represented promoter motifs in the gene cluster, we applied the de novo prediction by use of the algorithms MEME (Bailey et al. 2006), Weeder (Pavesi et al. 2004), and PhyloCon (Wang and Stormo 2003) and scanned for previously published binding motifs of the TRANSFAC database (Wingender et al. 2001). To specifically identify palindromic 5′-CGG[X_n]CCG-3′ binding sites, which are associated with Zn(II)2Cys6 transcription factors (MacPherson et al. 2006), we wrote a script that scans for CGG palindromes with a spacer of 0 to 30 bp between the CGG/CCG triplets. We selected the intergenic upstream sequence of genes with a maximum length of 1 kb as promoter sequence. Additionally, orthologous promoters of the closely related *F. verticillioides* were scanned for conserved motifs.

We determined the genome-wide occurrence of all candidate motifs and selected statistically over-represented motifs on the cluster promoters using Fisher's exact test (Fisher 1922). All resulting *p* values were corrected for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). Motifs with a corrected *p* value below 0.05 were selected as significantly over-represented promoter motifs.

Synthesis of *N*-acetylated fumonisin FB1

For synthesis of FA_1, 10 mg (1.38 mmol) of FB_1 (isolated according to Hübner et al. (2012)) were incubated with acetic anhydride (2 mmol) using trimethylamine (1.42 mmol) as chemical catalyst in 10 mL 1:1 methanol/chloroform (*v*/*v*). The reaction mixture was incubated under a protective argon atmosphere for 48 h at room temperature under constant stirring. The reaction product was evaporated, resolved in water, and analyzed by HPLC-MS/MS.

HPLC-MS/MS analysis of fumonisins

To prepare samples for HPLC-MS/MS measurements, the supernatants of fungal cultures were filtered through 0.45-μm membrane filters (BGB Analytik, Schloßböckelheim, Germany). For quantification of fumonisins, isotopically labeled FB_1 (d_6-FB_1) was used as internal standard (Lukacs et al. 1996). Measured FB_x and FA_x levels were adjusted to the dry weight of the fungal mycelium. An API 4000 QTrap mass spectrometer (Sciex, Darmstadt, Germany) coupled to an Agilent 1100 series HPLC and a QTRAP 5500 mass spectrometer (Sciex, Darmstadt, Germany) coupled to a Nexera™ system (Shimadzu, Duisburg, Germany) were used for the detection of different fumonisins in multiple reaction monitoring (MRM) mode. Data acquisition was performed with Analyst 1.6.2 software (Sciex). Chromatographic separation was done on a 150 × 2 mm, 3-μm, Hyperclone C_8 3 μm BDS 130 Å column with a 4 × 3 mm i.d. guard column

(Phenomenex, Aschaffenburg, Germany) using a binary gradient of 1 % (*v*/*v*) formic acid in acetonitrile (solvent A) and 1 % (*v*/*v*) formic acid in water (solvent B), similar to the method published by Bergmann et al. (2013). The injection volume was 10 μL, and the autosampler was cooled to 4 °C. The flow rate was set to 250 μL/min with the following gradient: 35 % solvent A (0 min), 95 % A (8 min), 95 % A (10 min), subsequent return to the start conditions of 35 % A (10.5 min), and equilibration (15 min). For electrospray ionization, the ion voltage was set to +5500 V in the positive mode, and nitrogen was used as curtain gas (20 psi) and collision gas (5×10^{-5} Torr for API 4000 or medium for QTRAP 5500). Zero-grade air was used as a nebulizer gas (35 psi) and as a drying gas (45 psi) heated to 350 °C. Two MRM transitions were measured for identification and quantitation (first transition mentioned) of each analyte of interest with a duration of 50 ms: FB_1 $[M + H]^+$ 722 → 334 and 722 → 352, FB_2 and FB_3 $[M + H]^+$ 706 → 336 and 706 → 318, FB_4 $[M + H]^+$ 690 → 320 and 690 → 338, FA_1 $[M + H]^+$ 764 → 394 and 764 → 334, FA_2 and FA_3 $[M + H]^+$ 748 → 378 and 748 → 336, as well as d_6-FB_1 $[M + H]^+$ 728 → 358 and 728 → 340. According parameters for declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and collision cell exit potential (CXP) were the following in Volt: DP 121, EP 10, CEP 35, CE 55, CXP 8.

Results

F. fujikuroi and *F. verticillioides* differ in quantity and quality of produced fumonisins

Previously, we have shown that *F. fujikuroi* produces only small amounts of fumonisin under laboratory conditions compared to *F. verticillioides* (Wiemann et al. 2013). The reasons for these differences are not known as the *FUM* cluster organization is almost the same (Fig. 1b), and the level of protein sequence identity is very high between both species (Fum1 86 %, Fum8: 83 %, Fum6 85 %, Fum21: 69 %).

In order to study the functionality of the *FUM* gene cluster in *F. fujikuroi* in more detail, we first compared the fumonisin production of both *Fusaria* in liquid culture. For *F. verticillioides*, it has been shown that *FUM* gene expression is subject to nitrogen metabolite repression (Kim and Woloshuk 2008). To confirm the nitrogen-dependent fumonisin production also for *F. fujikuroi*, the two WT strains were grown in standard synthetic ICI medium with high (60 mM) and low (6 mM) concentrations of nitrogen in the form of glutamine (Gln). Total B-type fumonisin levels (FB_x) were measured by HPLC-MS/MS after 7 days of cultivation as a sum of the two main fumonisins, FB_1 and FB_2 (Fig. 2). Only traces of FB_3 and FB_4 were detected in most samples (data not shown). As expected, highest levels of FB_x were produced under nitrogen-limiting conditions in both strains, although *F. fujikuroi* produces only about 1–10 % of the FB_x level of *F. verticillioides* (Fig. 2). However, while *F. verticillioides* consistently produces FB_1 as the main product, *F. fujikuroi* synthesizes FB_1 and FB_2 in a more balanced ratio mostly tending to FB_2 as main compound (Fig. 2).

HPLC-MS/MS measurements revealed also signals of *N*-acetylated forms of fumonisins (FA_1 and FA_2) in the supernatants as, e.g., described for *F. verticillioides* (Lazzaro et al. 2013). Mass spectrometric transitions were verified in comparison to FA_1 which was synthesized from FB_1. Peak ratios representing $\sum FA_x$ ($FA_1 + FA_2$) reflect the ratio of $\sum FB_x$ comparing different strains and were therefore not included in further descriptions (Fig. 2; Fig. S4). Measurements in the WT compared to the mutant strains are in general presented as the sum of the two major fumonisins FB_1 and FB_2 ($\sum FB_x$).

Maize supplements induce fumonisin production in *F. verticillioides*, but not in *F. fujikuroi*

Recently, it has been shown that different plant extracts induce fumonisin production in *F. proliferatum* (Stępień et al. 2015). To establish whether the different host plants (maize versus rice) make the difference in FB_x production yields between *F. verticillioides* and *F. fujikuroi*, we studied the effect of maize-derived supplements on FB_x production in the low-producing rice pathogen *F. fujikuroi* and the high-producing maize pathogen *F. verticillioides*. Equal amounts of maize flour and germinated maize seedlings, respectively, were added as sole nitrogen source to synthetic ICI liquid medium. The FB_x contents of the culture filtrates were measured by HPLC-MS/MS after 7 days of cultivation and compared to those produced in the presence of 6 mM Gln (inducing conditions). Interestingly, the addition of germinated maize seedlings led to about a fivefold increase in FB_x levels in *F. verticillioides*, whereas the addition of maize flour had no effect (Fig. 3). It was notable that the addition of maize seedlings resulted also in production of detectable amounts of FB_3 in *F. verticillioides* of about 4–7 % compared to FB_1 (data not shown). In *F. fujikuroi*, none of the supplements had any effect on produced FB_x concentrations (Fig. 3). Although the strains showed varying growth in the different media, no direct correlation of growth rate to produced FB_x levels was found (data not shown).

Cluster-specific transcription factor *FUM21* crucially regulates fumonisin biosynthesis

In *F. verticillioides*, the narrow-domain Zn(II)2Cys6 TF Fum21 plays an essential role for activating the expression of the *FUM* cluster genes. Deletion of the *FUM21* gene led to almost complete inhibition of fumonisin production

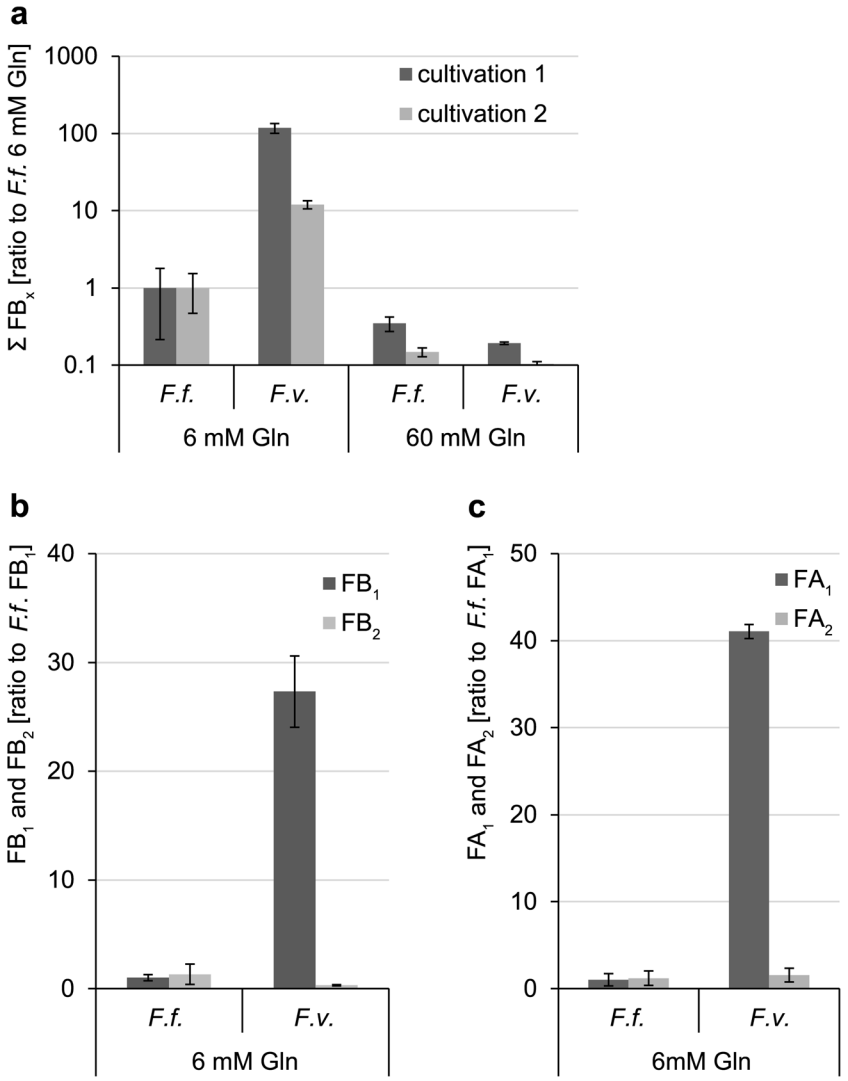


Fig. 2 Comparison of fumonisin production of *F. fujikuroi* and *F. verticillioides*. The two wild-type strains were cultivated for 7 days in liquid ICI with 6 and 60 mM Gln, respectively, as sole nitrogen source. Fumonisin levels ($FB_1 + FB_2 = \Sigma FB_x$), as well as FB_1, FB_2, and FA_1 and FA_2 separately, were analyzed in triplicate via HPLC-MS/MS from the culture supernatant using d_6-FB_1 as internal standard. Both strains show repression of FB_x under high nitrogen, and under inducing conditions (6 mM Gln) *F. fujikuroi* only produces 1–10 % compared to *F. verticillioides* (**a**). *F. verticillioides* clearly produces FB_1 as main compound whereas *F. fujikuroi* shows a more balanced ratio mostly tending to FB_2 as main compound (**b**). In all samples measured in 6 mM Gln, the ratio of FA_1 to FA_2 was about the same as FB_1 to FB_2 (**c**). *F.f. F. fujikuroi*, *F.v. F. verticillioides*

(Brown et al. 2007). One possibility for differing fumonisin production levels between the two species might be a low activity of Fum21 in *F. fujikuroi*. However, sequence comparison revealed no significant differences. The predicted intron/exon structure of the *FUM21* homolog in *F. fujikuroi* (FFUJ_09240) was confirmed experimentally by cDNA sequencing revealing a 2067-bp cDNA encoding 688 amino acids (aa) compared to 722 aa in *F. verticillioides*. Both proteins contain a Zn(II)2Cys6 domain (Zn_clus, PF00172) and a domain typical for fungal TFs (Fungal_trans, PF04082) identified using the Pfam algorithm (Finn et al. 2014).

To study the role of Fum21 in *F. fujikuroi*, *FUM21* deletion and over-expression strains were generated and analyzed for FB_x production and *FUM* gene expression. FB_x production was completely abolished in the Δ*fum21* mutant. In contrast, the constitutive high expression of *FUM21* strongly induced gene expression of *FUM* cluster genes (shown for *FUM1*, *FUM6*, and *FUM8*) and resulted in up to 1000-fold elevated FB_x levels under low nitrogen (6 mM Gln) and even under repressing conditions (60 mM Gln) (Fig. 4, Fig. S5). Interestingly, *FUM21* is also expressed in the WT though slightly slower than in OE::*FUM21*. However, this WT expression level seems not to be sufficient for proper expression of the other *FUM* cluster genes. To study this phenomenon in more detail, a time course experiment was performed showing that the constitutive *oliC* promotor causes high expression of *FUM21* from day 1 until day 7, whereas in the WT *FUM21* expression starts only at day 2, peaks at day 4, and then

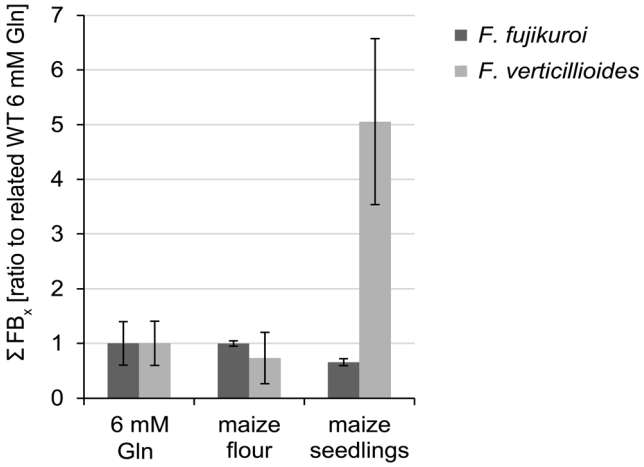


Fig. 3 Comparison of *F. fujikuroi* and *F. verticillioides* fumonisin production in media containing different maize supplements. The *F. fujikuroi* and *F. verticillioides* wild-type strains were cultivated for 7 days in liquid ICI with 6 mM Gln and 2 g/L maize flour or maize seedlings as sole nitrogen source, respectively. Fumonisin levels ($FB_1 + FB_2 = \Sigma FB_x$) of the three strains were analyzed in triplicate via HPLC-MS/MS from the culture supernatant using d_6-FB_1 as internal standard

decreases again (Fig. S5). Accordingly, *FUM6* and *FUM8* were also highly expressed from day 1 in OE::*FUM21* compared to the weak expression in the WT (Fig. S5).

Despite the high FB_x levels, growth of the OE::*FUM21* was not significantly inhibited compared to the WT, neither in liquid culture nor on solid ICI medium, indicating that fumonisins have no toxic effect on the producing fungus (data not shown).

To gain more insight into the mode of action of the Zn(II)2Cys6 TF Fum21 in *F. fujikuroi*, we searched for potential Fum21 DNA binding sites. Recently, a conserved motif has been reported in the promoters of the *FUM* cluster genes in *F. verticillioides* (Montis et al. 2013). Although an identical motif (CGGATA|TATCCG) is not present in the promoter region of *FUM1* in *F. fujikuroi*, we identified a very similar significantly over-represented (p value <0.05, Fisher's exact test with Benjamini-Hochberg correction for multiple testing) motif with a consensus of 5′-ATCCGA-3′. The motif was predicted independently by the two algorithms MEME (Bailey et al. 2006) and Weeder (Pavesi et al. 2004) within the promoter regions of all 15 *FUM* genes in *F. fujikuroi* (Fig S6). Comparing the locations of these similar consensus sequences in the 5′-upstream regions of the *FUM* genes in both fungi revealed that only 7 of the 28 sites of the newly identified motif match with the sites found by Montis et al. (2013) limiting the promotor region to the intergenic 5′-upstream regions with a maximum of 1 kb.

Furthermore, many studies show that Zn(II)2Cys6 proteins recognize highly related elements containing trinucleotide sequences such as CGG triplets in single or repeat forms, in either a symmetrical or an asymmetrical format (MacPherson et al. 2006). Searching for those typical 5′-CGG[X_n]CCG-3′ motifs with repeated CGG triplets, we found the motif 5′-CGG[X_{17}]CCG-3′ upstream of 7 out of the 15 *FUM* genes. We found this motif significantly enriched compared to the genome-wide distribution (p value <0.05, Fisher's exact test with Benjamini-Hochberg correction for multiple testing) and even present twice in the promoter region of *FUM1* (Fig. S6). In *F. verticillioides*, the same consensus was also identified in most of the *FUM* gene promoters (Fig S6).

To prove their functionality, the postulated Fum21 binding motifs were mutated by directed single nucleotide exchange in the promoter of *FUM1*. 5′-ATCCGA-3′ was changed to 5′-ATC**A**G**C**-3′. The two 5′-CGG[X_{17}]CCG-3′ motifs were mutated separately and also both simultaneously (−572: CGG➔**A**G**T** and/or −543: CCG➔**A**C**T**), and the mutated fragments were homologously integrated into the *locus* of the *FUM1* promoter in the WT as well as the OE::*FUM21* mutant strains. To be sure that potential changes were not due to homologous integration of the transformed fragment carrying the hygromycin resistance cassette, the same construct without any mutation was also inserted as a control (Fig. S3).

FB_x levels of two independent mutant strains per mutation were measured in triplicates and compared to the recipient WT and OE::*FUM21* strains. Surprisingly, both the integration of the non-mutated control constructs and the mutated promoter fragments led to about 40–50 % reduced production of FB_x (Fig. S6). Therefore, the different mutations seem to have no specific influence on fumonisin biosynthesis in *F. fujikuroi* (Fig. S6). The general reduction of FB_x levels in all transformed strains seems to result from the insertion of the resistance cassette upstream of the *FUM1* promoter what clearly hinders efficient activation of the *FUM* cluster and metabolite biosynthesis.

Both AreA and AreB are critical for the action of Fum21 and efficient fumonisin production in *F. fujikuroi*

As FB_x production in *F. fujikuroi* is strictly regulated by nitrogen availability, we studied the potential role of the major GATA-type nitrogen regulator AreA and the recently characterized second nitrogen-responsive GATA TF AreB (Michielse et al. 2014) in fumonisin regulation. Both, *FUM* gene expression (shown for *FUM1*, *FUM6* and *FUM8*) as well as FB_x production were completely inhibited in both the Δ*areA* and Δ*areB* deletion mutants, indicating that the two global GATA-type TFs act as positive regulators of fumonisin biosynthesis (Fig. 5). Indeed, several double GATA sequence elements are present in the regions upstream of the *FUM* genes, which might represent binding motifs for AreA and AreB. In the promoter region of the cluster-specific TF *FUM21* five double GATA motifs are located, and two are present in the promoter region of *FUM1* (Fig. S6).

To gain insight into the hierarchy between the cluster-specific TF and the two global GATA regulators, all three

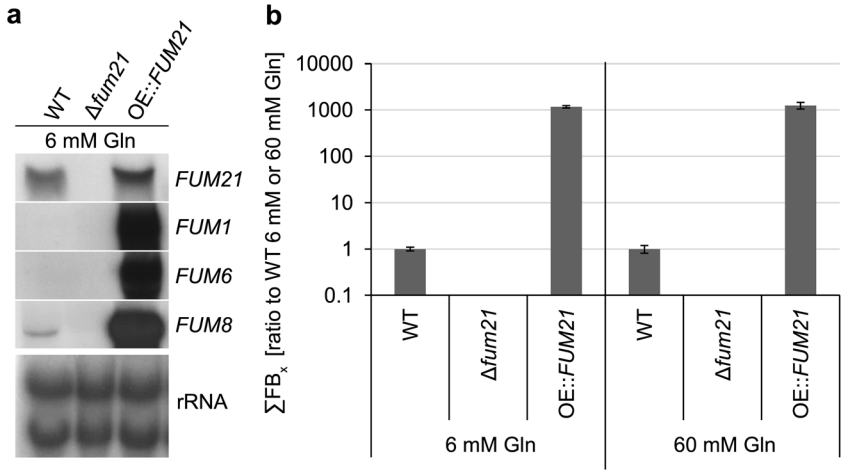


Fig. 4 Analysis of the cluster-specific Zn(II)2Cys6 transcription factor Fum21. The *F. fujikuroi* wild type (WT), Δ*fum21*, and OE::*FUM21* were cultivated for 3 or 7 days, respectively, in liquid ICI medium (6 mM Gln). The expression of *FUM21* itself and the essential cluster genes *FUM1* (PKS), *FUM6* (P450 monooxygenase), and *FUM8* (aminotransferase) is not detectable in Δ*fum21* and strongly induced in OE::*FUM21* (**a**). Fumonisin levels ($FB_1 + FB_2 = \Sigma FB_x$) of the three strains were analyzed in triplicate via HPLC-MS/MS from the culture supernatant using d_6-FB_1 as internal standard (**b**)

having a strong influence on fumonisin regulation, *FUM21* was constitutively over-expressed in the backgrounds of Δ*areA* and Δ*areB*, respectively. The expression of *FUM* genes (shown for *FUM1*, *FUM6*, and *FUM8*) was only partially restored in the OE::*FUM21*/Δ*areA* and OE::*FUM21*/Δ*areB* double mutants compared to the single Δ*areA* and Δ*areB* mutants. Both the expression of *FUM* genes and FB_x levels were significantly less than in the OE::*FUM21* mutant (Fig. 5).

Discussion

According to bioinformatic analyses, the *F. fujikuroi* genome contains 47 key genes for biosynthesis of SM, demonstrating the great potential of the fungus to produce a large variety of these bioactive compounds (Wiemann et al. 2013). One of them is the *FUM* gene cluster which exhibits a high level of conservation to that of the main fumonisin producer *F. verticillioides*. Fumonisins, especially those belonging to the

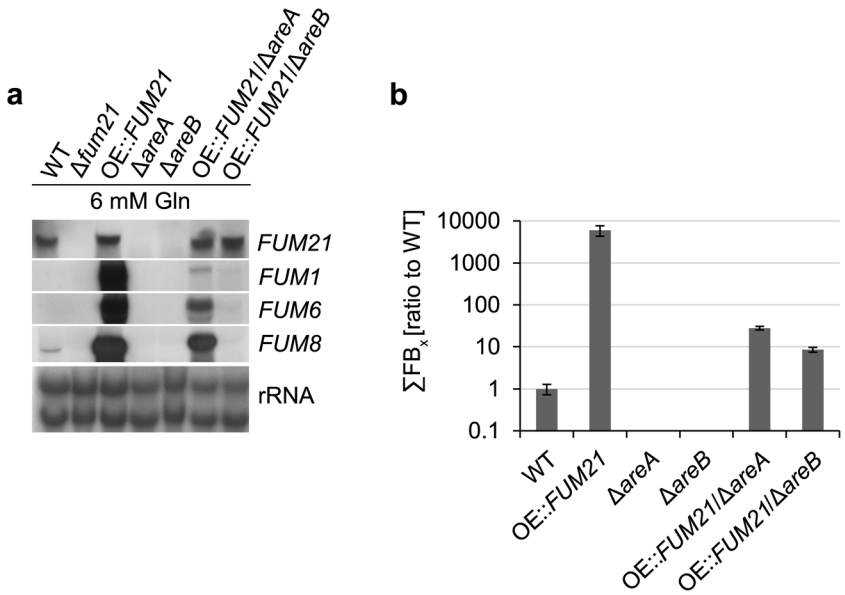


Fig. 5 Influence of the global GATA transcription factors AreA and AreB on regulation of FB_x biosynthesis. The *F. fujikuroi* wild type (WT), OE::*FUM21*, Δ*areA*, Δ*areB* as well as OE::*FUM21*/Δ*areA* and OE::*FUM21*/Δ*areB* were cultivated for 3 or 7 days, respectively, in liquid ICI medium (6 mM Gln). The expression of *FUM21* (transcription factor) and the *FUM* cluster genes *FUM1* (PKS), *FUM6* (P450 monooxygenase) and *FUM8* (aminotransferase) and was analyzed (**a**). Additionally, fumonisin levels ($FB_1 + FB_2 = \Sigma FB_x$) of the strains were measured in triplicate via HPLC-MS/MS from the culture supernatant using d_6-FB_1 as internal standard (**b**)

B series fumonisins, are very potent mycotoxins responsible for food and feed contamination of maize products. They have been associated with severe diseases in humans, including esophageal cancer and neural tube defects (Desai et al. 2002; Marasas et al. 2004). To get a deeper insight into factors affecting FB_x production in the low-producing fungus *F. fujikuroi*, we studied the complex regulation of fumonisin biosynthesis, and in particular, the role of pathway-specific and broad regulators and the interplay between them.

F. fujikuroi and *F. verticillioides* differ in quantity and quality of produced fumonisins

In *F. fujikuroi*, all 15 *FUM* cluster genes are highly conserved compared to the *F. verticillioides* cluster genes except for the missing *FUM17* and *FUM20* genes. *FUM17* is predicted to encode a longevity assurance factor, but was shown to be not essential for fumonisin biosynthesis in *F. verticillioides* (Proctor et al. 2003), and *FUM20* has no predicted function or even an open reading frame (Brown et al. 2005; Wiemann et al. 2013). Therefore, strong similarities in FB_x biosynthesis and its regulation have been expected. However, *F. fujikuroi* produces only 1–10 % of the FB_x levels of *F. verticillioides* under inducing low-nitrogen conditions.

Nevertheless, nitrogen repression of FB_x biosynthesis, as reported for *F. proliferatum* and *F. verticillioides* (Shim and Woloshuk 1999; Kohut et al. 2009), was also true for *F. fujikuroi* because it produced significantly less FB_x in the presence of high (60 mM Gln) nitrogen compared to low nitrogen (6 mM Gln).

Most fumonisin-producing *Fusarium* field isolates produce FB_1 and FB_2 as main products (Summerell and Leslie 2011). In contrast to *F. verticillioides* M-3125, which produces mainly FB_1, the ratio in *F. fujikuroi* was more balanced, tending to a higher level of FB_2. This fumonisin was found to be comparably toxic to FB_1 in jimsonweed (*Datura stramonium*), and even more toxic in rat hepatoma cells and turkey lymphocytes (Abbas et al. 1993; Gutleb et al. 2002). High levels of FB_2 were also reported for some *Aspergillus* and several *Fusarium* isolates, including some of *F. verticillioides* (Frisvad et al. 2007; Matić et al. 2013). In *Aspergillus niger*, the absence of FB_1 can result from the lack of the monooxygenase encoding gene *FUM2* (Susca et al. 2010).

Although *F. fujikuroi* and *F. verticillioides* are similar plant pathogens, they have evolved different host specificities: whereas *F. fujikuroi* infects rice (Leslie and Summerell 2006; Wiemann et al. 2013), *F. verticillioides* is mainly a maize pathogen (Nelson 1992). As might be expected, FB_x production was only stimulated in the maize pathogen *F. verticillioides* by addition of maize supplements. One component contained in maize kernels, amylopectin, was shown to activate FB_1 biosynthesis in *F. verticillioides* (Bluhm and Woloshuk 2005). However, in our experiments, only ground maize seedlings showed a stimulating effect, suggesting a specific signal probably emerging during germination.

Fum21 strongly regulates fumonisin biosynthesis in *F. fujikuroi*

The cluster-specific narrow-domain Zn(II)2Cys6 TFs Fum21 in *F. fujikuroi* and *F. verticillioides* are 69 % identical at the protein level. Both show an identical domain structure consisting of the typical Zn(II)2Cys6 domain (PF00172) and a second conserved domain (PF04082), which is typical for fungal TFs. Deletion of *FUM21* in *F. fujikuroi* resulted in the expected loss of FB_x production, as it was first described for Δ*fum21* mutants in *F. verticillioides* (Brown et al. 2007). On the contrary, over-expression of cluster-specific TFs, e.g., the apicidin F TF Apf2, is a common approach to activate silent or only faintly expressed gene clusters (Niehaus et al. 2014a). *APF2* over-expressing strains produce up to six times more product than the WT under inducing conditions and show even slightly enhanced product levels under repressing conditions (Niehaus et al. 2014a). For FB_x biosynthesis, such an effect was not reported so far. In fact, constantly high expression of *FUM21* in *F. fujikuroi* led to a strong activation of cluster gene expression (shown for *FUM1*, *FUM6*, and *FUM8*) and a 1000-fold elevation of FB_x levels compared to the WT under inducing (6 mM Gln) as well as repressing (60 mM Gln) conditions. It is surprising that *FUM21* transcripts are clearly detectable in the WT while the Fum21-regulated cluster genes are not or only weakly expressed. Accordingly, only low FB_x levels are detectable in the WT. Apparently, the constitutive strong expression of *FUM21* in OE::*FUM21* starting already from day 1 is the reason for the significantly elevated FB_x level. In addition, a different transcription start introduced by the constitutive *oliC* promotor or changed transcript stability due to the used *Botrytis cinerea* glucanase (*gluc*) terminator (Schumacher 2012) could have an impact on production yield.

Hence, even the low-level producer *F. fujikuroi* can be a potent producer of the mycotoxins fumonisins, leading to potentially high contamination of rice products. Fum21 activators or activating conditions are crucial for high fumonisin contamination, making Fum21 and its regulation a suitable target for further studies dealing with fumonisin control.

In order to better understand the regulatory mechanism underlying the *FUM* cluster gene activation by Fum21 in *F. fujikuroi*, putative binding motifs were analyzed. Though the exact motif previously reported by Montis et al. (2013) is not present in the promoter of *FUM1* in *F. fujikuroi*, a very similar motif (5′-ATCCGA-3′) was found in the 5′-upstream regions of all cluster genes. The positions of both motifs overlap only in one fourth of the predicted sites. Additionally, a typical Zn(II)2Cys6 binding motif, 5′-CGG[X_{17}]CCG-3′, was identified in seven *FUM* genes, including the *FUM1* promoter.

However, the mutation of each of these motifs in either OE::*FUM21* or WT background did not have any inhibitory effect on produced FB_x levels. Already the introduction of the negative control construct without mutation led to a reduction of the FB_x content of about 40–50 % which also occurred in all tested mutants. Recently, Visentin et al. (2012) showed by GFP fusion that the genomic localization and thereby probably the chromatin status of the promoter regions of the key genes *FUM1* and *FUM21* in *F. verticillioides* is important for activation of the gene expression. We assume that the interaction of further regulators involved in the regulation of fumonisin biosynthesis with the *FUM1* promoter region is hindered by the introduction of the resistance cassette leading to the overall FB_x reduction in the transformants. However, additional experiments will have to test this hypothesis, though from other fumonisin-producing strains several eligible regulators are known (reviewed in Woloshuk and Shim 2013).

Beside the two motifs described above, a conserved motif with a significantly different consensus sequence (5′-AAATTGTCA-3′) was found in the promotor region of all cluster genes except for *FUM14* and *FUM15* and would be promising for future mutation experiments (motif3, Fig. S6a).

Fumonisin biosynthesis is regulated by a complex regulatory network in *F. fujikuroi*

In general, fumonisin biosynthesis has been shown to involve several levels of control, and *FUM* genes are the target of several regulators (reviewed in Woloshuk and Shim 2013). Among other factors, histone H4 acetylation of promoter regions of *FUM* cluster genes is important for their transcription in *F. verticillioides* (Visentin et al. 2012), and a role of the HOG MAP kinase pathway in regulation of fumonisin biosynthesis was described in *F. proliferatum* (Kohut et al. 2009). In *F. verticillioides* and *F. fujikuroi*, mutations in the *VEL1* gene, a homolog of the velvet gene *VEA* in *A. nidulans*, abolished fumonisin production on cracked maize (Myung et al. 2009; Wiemann et al. 2010). In addition, the pH regulator Pac1 was shown to repress the expression of *FUM* genes in *F. verticillioides* (Flaherty et al. 2003). Furthermore, deletion of *ZFR1*, encoding a Zn(II)2Cys6 TF of unknown function, resulted in a mutant strain producing only trace amounts of FB_1 (Flaherty and Woloshuk 2004). Studies in *F. verticillioides* and *F. fujikuroi* revealed the essential role of the cluster-specific TF Fum21 (Brown et al. 2007; this work). There are no reports describing the putative *cis*-element in the promoters of the fumonisin pathway genes that is specifically recognized by Fum21 so far. Bioinformatic analysis revealed two potential binding motifs for Fum21 in the promoters of the *F. fujikuroi FUM* genes. However, targeted replacement of the *FUM1* WT promoter for the mutated promoter fragments did not result in significant reduction of fumonisin production, neither in the WT nor in the OE::*FUM21* backgrounds. Systematic mutations of more putative binding motifs will be necessary to identify the correct Fum21 binding motifs.

Besides being regulated by the cluster-specific TF Fum21, fumonisin biosynthesis in *F. fujikuroi* was shown to be subject of nitrogen metabolite repression. Therefore, the two prominent GATA-type TFs involved in nitrogen-dependent regulation, AreA and AreB, were analyzed to establish their role in fumonisin regulation. In *F. verticillioides*, Δ*areA* mutants produced little to no fumonisin on cracked maize, while constitutive expression of *AREA* led to production of FB_1 even under nitrogen-sufficient conditions (Kim and Woloshuk 2008). Here, we have shown for the first time that not only AreA, but also the second nitrogen-responsive GATA TF, AreB, is essential for activation of the fumonisin gene cluster in *F. fujikuroi*. Deletion of either *AREA* or *AREB* resulted in total loss of fumonisin production. According to this observation, several double GATA sequence elements are present in the regulatory regions of *FUM* cluster genes. Fumonisins are only the second example, besides gibberellins, for a secondary metabolite gene cluster being a common target for both TFs under nitrogen-limiting conditions (Michielse et al. 2014). There are other gene clusters which are regulated only by one of the two GATA factors. Thus, AreB but not AreA is essential for apicidin F and fusaric acid biosynthesis under inducing nitrogen-sufficient conditions in *F. fujikuroi* (Niehaus et al. 2014a, b).

In addition to the global regulators AreA and AreB, we showed the strong activating effect of the pathway-specific TF Fum21. To gain insight into the role of AreA and AreB in the hierarchical gene regulatory network of global and pathways-specific TFs, double mutants were generated. In these double mutants, the *FUM21* over-expression was combined with the deletion of either *AREA* or *AREB*. Interestingly, loss of either AreA or AreB had a much stronger impact on fumonisin production than over-expression of *FUM21*. Compared to the 1000-fold elevation of FB_x in the OE::*FUM21* mutant, both double mutants produced only 1 % of the FB_x level measured with the sole over-expression of *FUM21*.

Studies on the nitrate reductase cluster in *A. nidulans* revealed a function of AreA in chromatin remodeling and showed its epistatic effect over the specific TF NirA at the promoters of the nitrate utilization gene cluster (Berger et al. 2008). A similar interconnection is conceivable for the mode of action of AreA and AreB at the fumonisin cluster. However, a putative link has to be further investigated.

Recently, a global regulator of secondary metabolism, Sge1, has been identified in *F. verticillioides* and *F. fujikuroi* which affects biosynthesis of multiple secondary metabolites including fumonisins (Brown et al. 2014; Michielse et al. 2015). In *F. fujikuroi*, deletion of *SGE1* resulted in significantly reduced expression levels of *FUM*, gibberellin, fusaric acid, fusarin, apicidin F, and bikaverin biosynthetic genes.

Conversely, over-expression of Sge1 led to a 98-fold increase in FB_x production. Interestingly, no FB_x production was recorded in the Δ*areA*/OE::*SGE1* double mutant underlining the dominating role of AreA in the complex fumonisin regulatory network (Michielse et al. 2015).

In summary, we showed that the *FUM* gene cluster and FB_x biosynthesis are strongly inducible by the cluster-specific TF Fum21. Using this approach, the naturally low fumonisin-producing rice pathogen *F. fujikuroi* was able to produce 1000-fold more FB_x than the WT. However, the binding motif for Fum21 still remains to be elucidated. Furthermore, we showed that nitrogen metabolite repression of fumonisin biosynthesis is mediated not only by the major nitrogen regulator AreA, but also a second GATA-type TF AreB. Both are essential for full activation of the fumonisin gene cluster and metabolite biosynthesis, and the loss of one of them obstructs the pathway-specific TF Fum21 from fully activating expression of *FUM* cluster genes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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