The FlbA-regulated predicted transcription factor Fum21 of Aspergillus niger is involved in fumonisin production

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Abstract Aspergillus niger secretes proteins throughout the colony except for the zone that forms asexual spores called conidia. Inactivation of flbA that encodes a regulator of G-protein signaling results in colonies that are unable to reproduce asexually and that secrete proteins throughout the mycelium. In addition, the ΔflbA strain shows cell lysis and has thinner cell walls. Expression analysis showed that 38 predicted transcription factor genes are differentially expressed in strain ΔflbA. Here, the most down-regulated predicted transcription factor gene, called fum21, was inactivated. Growth, conidiation, and protein secretion were not affected in strain Δfum21. Whole genome expression analysis revealed that 63 and 11 genes were down- and up-regulated in Δfum21, respectively, when compared to the wild-type strain. Notably, 24 genes predicted to be involved in secondary metabolism were down-regulated in Δfum21, including 10 out of 12 genes of the fumonisin cluster. This was accompanied by absence of fumonisin production in the deletion strain and a 25% reduction in production of pyranonigrin A. Together, these results link FlbA-mediated sporulation-inhibited secretion with mycotoxin production.

Keywords Asexual development · Aspergillus · Fumonisin · Fungus · Mycotoxin · Secondary metabolism · Protein secretion

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Introduction

The genus *Aspergillus* consists of more than 300 species (Samson et al. 2014) that are among the most abundant fungi on the globe. Aspergilli degrade plant waste and as such play a role in carbon cycling in nature. Moreover, the genus includes opportunistic pathogens of plants, animals, and humans (Krijgsheld et al. 2013a). Enzymes secreted by *Aspergillus* play an important role in degradation of organic material and pathogenicity. The property to secrete high levels and a diversity of enzymes make aspergilli such as *Aspergillus niger* important cell factories for the production of proteins and metabolites (Meyer et al. 2011; Wösten et al. 2013).

Aspergilli form a mycelium consisting of a network of hyphae that grow at their apex and that branch subapically. The mycelium grows initially vegetative but at a certain moment asexual development is initiated (Krijgsheld et al. 2013a). The resulting conidia are the only spore type that are produced by *A. niger*. Conidiation starts with the formation of thick aerial hyphae called stalks. When a stalk has reached a certain height, its tip swells to form a vesicle. In many aspergilli, this structure buds resulting in a layer of metulae. The metulae in turn form a layer of phialides, from which chains of conidia develop. These spores are heterogeneous in composition, water dispersal efficiency, and germination rate (Teertstra et al. 2017) and give rise to new mycelia.

The zone forming the asexual conidia within an *A. niger* colony does not secrete proteins, while non-sporulating zones do release proteins into their environment (Krijgsheld et al. 2013b). Preventing conidiation of the colony by covering it with a polycarbonate membrane does not impact the spatial secretion pattern indicating that the capacity to sporulate but not the conidiation process itself inhibits secretion. FlbA was shown to impact spatial secretion in the colony (Krijgsheld et al. 2013b). This protein regulates the Gα subunit FadA by activating its GTPase activity. By doing so, it promotes asexual development and inhibits vegetative growth and autolysis (Wieser et al. 1994; Yu et al. 1996). Conidiation is abolished in the ΔflbA strain of *A. niger* and, as a consequence, protein secretion takes place throughout the colony (Krijgsheld et al. 2013b). Moreover, the cell wall of ΔflbA is thinner and intracellular proteins can be found in the culture medium, indicative of cell lysis. The ΔflbA strain therefore has a pleiotropic phenotype. Inactivation of flbA is accompanied by differential expression of 38 transcription factor genes, of which 18 are down-regulated and 20 are up-regulated (Krijgsheld and Wösten 2013). These downstream regulatory genes may impact one or more of the processes affected by FlbA. Here, the role of the most down-regulated predicted transcription factor gene, *fum21*, was studied. It is shown that *fum21* regulates production of the mycotoxins fumonisins. Thus, sporulation-inhibited protein secretion is linked to production of secondary metabolites via FlbA.

Materials and methods

Strains and culture conditions

*A. niger* strain MA234.1 (transient ku70::amdS) (Park et al. 2016) and its derivatives were grown at 30 °C using minimal medium (MM) (de Vries et al. 2004) containing 25 mM xylose as a carbon source and either (MM-XA) or not (MM-X) containing 1.5% agar. Alternatively, strains were grown on Czapek Yeast Auto-lysate (CYA) agar (Frisvad and Samson 2004), Yeast Extract Sucrose (YES) agar (Frisvad and Samson 2004), or transformation medium (TM; MM containing 0.5% yeast extract, 0.2% casaminoacids, and 25 mM glucose, pH 6) (Kusters-van Someren et al. 1991).

Conidia were harvested from 3-day-old MM-XA cultures. Liquid cultures inoculated with 5 × 10^8 spores were pre-grown for 16 h at 200 rpm in 300 ml TM in 500 ml Erlenmeyer flasks. After 16 h, 10 g wet weight mycelium was harvested, washed with 0.9% NaCl, and transferred to a 1 l Erlenmeyer flask containing 150 ml MM-X. The culture was shaken at 250 rpm and 30 °C for 4 h (RNA sequencing) or 24 h (SDS-PAGE).

For growth on agar media, strains were inoculated directly on the medium or grown in a layer of 1.25% agarose in between two perforated polycarbonate membranes (poles of 0.1 μm, diameter 76 mm; Profiltra, Almere, The Netherlands) (Wösten et al. 1991). These sandwiched cultures were inoculated in the center of the agarose layer by placing a 2 μl drop containing 10^4 conidia. The upper polycarbonate membrane was placed on top of the agarose layer 24 h after inoculation to prevent spreading of conidia.
Inactivation and complementation constructs of \textit{fum21}

For the construction of the \textit{fum21} deletion strain, 5' and 3' flanks of the gene were amplified from genomic DNA by PCR using primer pairs 1/2 and 3/4, respectively (Supplemental Table 1). The hygromycin resistance gene \textit{hph} was amplified from vector pAN7.1 (Punt et al. 1987) using primer pair 5/6 (Supplemental Table 1). Split marker fragments of this selection marker were created by fusion PCR (Arenthorst et al. 2015) using primer pairs 1/7 and 4/8 (Supplemental Table 1) for the 5' and 3' fragment, respectively.

For the construction of the \textit{fum21} complementation construct, the hygromycin resistance cassette contained in pAN7.1 was amplified using primers 9 and 10 (Supplemental Table 1) that contain XbaI sites at their 5' ends. The amplified 3 kb fragment that had a shorter promoter and terminator region as compared to pAN7.1 was cloned in the XbaI site of pUC20, resulting in vector pWR11. Gene \textit{fum21} with 1048 bp promoter and 444 bp terminator sequence was amplified from MA234.1 genomic DNA using Phusion polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and primer pair 11/12 (Supplemental Table 1). The amplified fragment was inserted in the SmaI site of pWR11 by using the InFusion\textsuperscript{HD} Cloning Kit (Clontech, Mountain View, CA, USA). This resulted in vector pDA1 containing the hygromycin resistance cassette and gene \textit{fum21}.

Transformation of \textit{A. niger}

Transformation of \textit{A. niger} was performed according to de Bekker et al. (2009). For inactivation of \textit{fum21}, the split marker fragments were transformed to strain MA234.1. Transformants were purified twice on MM-XA containing hygromycin. Deletion of \textit{fum21} was verified by Southern blot analysis using HindIII digested genomic DNA (Supplemental Fig. 1). For complementation, the disrupted \textit{kusA} gene in the \textit{Δfum21} strain was first restored by selecting AmdS loop-out strains on 5' fluoroacetamide (Carvalho et al. 2010) to facilitate ectopic integration. Since the \textit{fum21} deletion strain was already hygromycin resistant, the resulting \textit{Δfum21 kusA\textsuperscript{+}} strain was complemented by co-transforming vectors pDA1 and pMA357. The latter pJet1.2 (Thermo Fisher Scientific) based vector contains the \textit{amdS} gene and 3' regulatory sequences of \textit{Aspergillus nidulans} under control of the \textit{gpdA} promoter. The \textit{amdS} expression cassette (Meyer et al. 2007) was PCR amplified with primer pair 13/14 (Supplemental Table 1). Selection was done on MM containing 0.95 M sucrose, 15 mM CsCl, and 10 mM acetamide as sole nitrogen source. Integration of the wild-type copy of the gene was confirmed by Southern blot analysis (Supplemental Fig. 1).

RNA sequencing and analysis

Mycelium of biological duplicates of liquid cultures pre-grown in TM and transferred to MM-X was frozen in liquid nitrogen and ground for 1 min at 25 Hz with a Tissue Lyzer II (Qiagen, Venlo, The Netherlands). Samples were taken up in 1 ml TRIzol reagent (Invitrogen, Bleiswijk, The Netherlands) and incubated for 5 min at room temperature (RT). 0.2 ml chloroform was added and samples were centrifuged for 15 min at 4 °C and 12,000 × g after 2 min incubation at RT. Total RNA was precipitated from the resulting aqueous phase by addition of 0.5 ml isopropanol, incubation at RT for 10 min, and centrifugation for 10 min at 4 °C and 12,000 × g. RNA was washed with 1 ml 75% ethanol, left to dry, and resuspended in RNase-free water. RNA was purified using the NucleoSpin\textsuperscript{RNA} kit (Macherey-Nagel, Düren, Germany). Concentration and purity of RNA was checked using the Nanodrop ND-1000 (Thermo Fisher Scientific).

Library preparation, cluster generation, and sequencing of cDNA were performed by ServiceXS (Leiden, The Netherlands) using Illumina sequencing. The reads are deposited in NCBI GEO with accession number GSE93990. Tophat version 2.1.13 (Trapnell et al. 2009) was used to align sequence reads to the Aspni7 version of the \textit{A. niger} ATCC 1015 genome (Andersen et al. 2011), which was obtained from Mycocosm (Grigoriev et al. 2014). Functional annotation of the predicted genes was described previously (Teertstra et al. 2017). Cuffdiff (version 2.2.1), which is part of Cufflinks (Trapnell et al. 2010), was used to identify reads mapping to predicted genes and to identify differentially expressed genes. The bias correction method was used while running Cuffdiff (Roberts et al. 2011). The expression level of each predicted gene was normalized to fragments per kilobase of exon model per million fragments.
In addition to Cuffdiff’s requirements for differential expression the following requirements were applied: a $\geq$ 2-fold change and a minimal expression level of 4 FPKM in at least one of the samples. The quality of these results was analyzed using CummeRbund (Goff et al. 2013).

**Q-PCR**

Expression of *fum10* (proteinID 1117227), *fum8* (ProteinID 1117230), and *fum1* (ProteinID 1162446) in *A. niger* wild type, $\Delta$fum21, and 4 complemented strains was assessed by Q-PCR using beta-tubulin (ProteinID 208263) and 18S rRNA (GenBank sequence ID KC545869.1) as reference genes. Total RNA was isolated as described above from biological duplicates of CYA- and YES-grown sandwiched colonies, after which it was purified using NucleoSpin® RNA (Macherey-Nagel, Düren, Germany) and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). The cDNA (1 ng) was used for SYBR Green Q-PCR using 200 nM of the primer pairs 15/16 for *fum10*, (efficiency 98.7%), 17/18 for *fum8* (efficiency 104.4%), 19/20 for *fum1* (efficiency 109.7%), 21/22 for beta-tubulin (efficiency 102.1%), and 23/24 for 18S (efficiency 95.8%) (Supplemental Table 1). No-template controls (NTCs) were included as a negative control and total reaction volumes were 10 µl. Samples were run on a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Wilmington DE, USA) and analyzed using the ΔΔCt method.

**SDS-PAGE**

Proteins in culture medium were precipitated overnight at $-20^\circ C$ after adding 4 volumes of acetone. They were pelleted twice for 30 min at 4°C and 21,000 x g with an intermediate washing step using $-20^\circ C$ acetone. After drying the pellets, sample buffer (125 mM Tris pH 6.8, 4% sodium dodecyl sulfate (SDS), 17.4% glycerol, 5% β-mercaptoethanol, 200 µg/ml bromophenol blue) was added resulting in a 20-fold volume reduction when compared to the culture medium. Samples were incubated at 100 °C for 10 min and proteins were separated in 12.5% SDS-PAGE gels using Pierce™ Prestained Protein Molecular Weight Marker (Thermo Fisher Scientific) as reference. Gels were fixed with 50% methanol and 10% acetic acid for 10 min, stained overnight with 0.1% Coomassie Brilliant Blue R-250, and destained with 25% methanol, 10% acetic acid. Gels were imaged using the Universal Hood III with Image Lab software (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

**Localization of protein secretion**

Protein secretion was monitored as described (Krijsgheld et al. 2013b) by labelling sandwiched colonies that had been grown on MM-XA for 6 days and transferred for 24 h to fresh MM-XA containing $^{14}$C-amino acids (NEC-850E amino acid mixture, L-$^{14}$C(U)-, Perkin Elmer, Waltham MA, USA).

**Protease activity**

*A. niger* was grown as a sandwiched colony (see above) on MM containing 33% skimmed milk and 1.5% agar. After 5 days of growth, the sandwiched culture was removed and presence of halos monitored.

**Secondary metabolite profiling**

CYA and YES plates were inoculated in duplicate with $10^4$ conidia. After 7 days of growth at 25 °C, 56-mm wide mycelial plugs were taken along the diameter of the colony and freeze-dried. Secondary metabolites were extracted in duplicate from the plugs with ethylacetate/dichloromethane/methanol (3:2:1, vol/vol/vol) with 1% formic acid using ultrasonic treatment for 50 min (Smedsgaard 1997). The extracts were transferred to a 2 ml dram vial and taken up in 300 µl methanol after removing the organic solvents by evaporation. Pyranonigrin A, pyranonigrin X (i.e. pyranonigrin B, C, D, E, or S), kotanin, BMS 192548, aurasperone B, tensidol B, and funalenone were analyzed using ultra-high performance liquid chromatography (UHPLC) (Houbraken et al. 2012). These compounds were identified using diode array detection (UV spectra from 190-600 nm) with purified compounds as standard. The relative quantity of the metabolites was estimated based on absorption at 210 nm (milli absorption units, mAU). Fumonisin B$_2$, B$_4$, and B$_6$ were quantified by UHPLC-High Resolution Mass Spectrometry (UHPLC-HRMS) using an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a
diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 μm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B, increased to 100% B in 15 min, and held for 2 min at this composition, returned to 10% B in 0.1 min and held for 3 min at this composition (0.35 ml min⁻¹, 60 °C). An injection volume of 1 μl was used. MS detection was performed on an Agilent 6540 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 l min⁻¹, sheath gas temperature of 300 °C and flow of 12 l min⁻¹. Capillary and nozzle voltage were set at 4000 and 500 V, respectively. Mass spectra were recorded at 10, 20, and 40 eV as centroid data for m/z 85–1700 in MS mode and m/z 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30 methanol:water was infused in the second sprayer using an extra LC pump at a flow of 15 μl min⁻¹ using a 1:100 splitter. The solution contained 1 μM tributylamine (Sigma-Aldrich) and 10 μM Hexakis (2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The [M + H] + ions (m/z 186.2216 and 922.0098, respectively) of both compounds was used (Kildgaard et al. 2014; Klitgaard et al. 2014).

Results

Functional characterization of fum21

Thirty eight predicted transcription factor genes are differentially expressed in xylose-grown colonies of ΔflbA when compared to wild type (Krijgsheld and Wösten 2013). Of these genes, An01g06900 (Cerqueira et al. 2014) is the most down-regulated predicted transcription factor gene with a 22, 42, and 31 fold-change in the central, middle, and the outermost concentric zone of the colony, respectively (Krijgsheld and Wösten 2013). This gene showed a bidirectional hit with the fumonisin regulator fum21 of Fusarium (Proctor et al. 2013) showing 29% identity at amino acid level and sharing the GAL4 DNA binding domain and the Middle Homology Region (MHR) domain (Supplemental Fig. 2). The fact that An01g06900 (i.e. fum21) is located in the predicted fumonisin gene cluster of A. niger (Supplemental Fig. 2) supports a role of this gene in fumonisin production.

Gene fum21 was deleted in A. niger strain MA234.1, resulting in strain Δfum21 (Supplemental Fig. 1). Growth and conidiation were not affected in the deletion strain. Biomass of 7-day-old wild-type and Δfum21 sandwiched colonies was 17.20 ± 3.4 mg and 17.43 ± 2.0 mg (± SD, n = 7, p > 0.05), while colony diameter was 4.6 ± 0.63 cm and 4.9 ± 0.29 cm (± SD, n = 6, p > 0.05). Conidiation in the two strains took place in the sub-peripheral zone and the center of the colonies (Fig. 1). Growth and conidiation were not affected when colonies were grown on YES (data not shown). Degradation of skimmed milk (data not shown) and the number of spores that were produced also did not differ (Fig. 1e). These data show that conidiation and sporulation-inhibited secretion are still functional in Δfum21.

Strain Δfum21 and its progenitor were grown for 7 days on CYA. Fumonisins B₂, B₄, and B₆ could be extracted from mycelial plugs from different zones of wild-type colonies. In contrast, Δfum21 did not produce these metabolites (Fig. 2a). Similar results were obtained when colonies were grown on YES medium (data not shown). Production of pyranonigrin A was also reduced by 25% in the deletion strain but production of other secondary metabolites was not affected when Δfum21 and the wild type were grown on CYA (Fig. 2b) and YES (data not shown) medium.

Gene expression analysis

Gene expression in Δfum21 and the wild-type progenitor strain was assessed by RNA sequence analysis after growth in TM for 16 h followed by transfer to MM-X for 4 h. This analysis revealed that only 74 genes were differentially expressed in Δfum21 relative to the wild type. Of these genes, 63 were down-regulated and 11 were up-regulated in the deletion strain (Table 1; Supplemental Table 2). Of the up-regulated genes, 7 have a predicted signal sequence for secretion including genes encoding lipases, a protease, a cellobiohydrolase and an anti-fungal protein. Of the down-regulated genes, 12 genes encode a protein without annotation and 24 genes are predicted to be involved in secondary metabolism. Of the latter genes,
20 are located in the secondary metabolism clusters 1 (3 out of 21 genes down-regulated), 12 (4 out of 13 genes down-regulated), 70 (3 out of 6 genes down-regulated), and 15 (10 out of 12 genes down-regulated). The secondary metabolites produced by the proteins encoded by clusters 1 and 12 are unknown but clusters 70 and 15 are predicted to be involved in TAN-1612 and fumonisin production, respectively (Li et al. 2011; Khaldi and Wolfe 2011). The down-regulated genes in the fumonisin cluster included fum10, fum8, and fum1 (Table 2), which are predicted to be involved in the early catalytic steps of fumonisin synthesis in Fusarium (Proctor et al. 2003). Q-PCR analysis showed that their expression was absent in Δfum21 of A. niger and restored in two complemented strains (data not shown).

Expression of the 74 differentially expressed genes in Δfum21 was assessed in the central, intermediate, and outer zones of wild-type and ΔflbA colonies using data of Krijgsheld and Wösten (2013). Six out of 11 up-regulated genes in Δfum21 were down-regulated in ΔflbA (Supplemental Table 3). Conversely, 10 out of the 63 down-regulated genes in Δfum21 were up-regulated in ΔflbA. In addition, 13 of the down-regulated genes in Δfum21 were also down-regulated in ΔflbA among which all genes of the fumonisin cluster (Supplemental Table 4). The genes in the fumonisin cluster were highly expressed at the periphery of xylose-grown colonies of A. niger, 6 of which were statistically significantly more highly expressed (i.e. exhibited $>2$-fold higher expression levels) in this zone when compared to the more inner zones. Gene fum21 was not differentially expressed in zones of the wild-type colonies.

Discussion

Most wild-type A. niger strains produce fumonisin in liquid and solid cultures (Frisvad et al. 2007, 2011). Fumonisins are potent mycotoxins that exhibit neurotoxicity, hepatotoxicity, and nephrotoxicity in various animal models. They have also been linked to tumor formation in humans (esophageal cancer) and animals (Stockmann-Juvala and Savolainen 2008). The fumonisin biosynthesis cluster of A. niger has been proposed to originate from a horizontal gene transfer event (Khaldi and Wolfe 2011). The Fusarium cluster consists of up to 17 genes depending on the species (Proctor et al. 2003, 2008; Brown et al. 2007; Wiemann et al. 2013). Of these genes, expression of at least fum1 and fum8 is controlled by the
transcriptional regulator Fum21 (Brown et al. 2007). A bidirectional BLAST showed that gene An01g06900 of A. niger is the orthologue of fum21 of Fusarium. Inactivation of fum21 in A. niger did not impact vegetative growth, mycelium morphology, conidiation, and (spatial) secretion of proteins. In contrast, Δfum21 of A. niger did not produce fumonisin, while expression of 10 out of 12 genes of the fumonisin gene cluster was reduced. These data show that Fum21 controls fumonisin production as was previously shown in Fusarium (Brown et al. 2007).

The function of Fum21 of A. niger and Fusarium verticillioides is remarkably similar despite the evolutionary distance between these species that belong to the Sordariomycetes and the Eurotiomycetes, respectively. Both proteins activate fumonisin production, while not affecting other processes such as growth and sporulation (Brown et al. 2007). In addition, both fum21 homologs are regulated by genes involved in asexual development; i.e. the VeA homolog FvVE1 in F. verticillioides (Myung et al. 2009) and flbA in A. niger. It should be noted that deletion of fum21 in A. niger completely abolished fumonisin production, while some fumonisin can still be detected in the fum21 deletion strain of F. verticillioides. In the latter case the transcription factor genes pac1 and zfr1 also impact biosynthesis of this secondary metabolite (Shim and Woloshuk 2001; Flaherty et al. 2003; Flaherty and Woloshuk 2004; Bluhm and Woloshuk 2006). A. niger has orthologues of both genes. Future studies should confirm a role of these genes in fumonisin production. They might for instance regulate fum15 and fum19 that were shown not to be regulated by fum21. Results also showed that Fum21 of A. niger affects production of the secondary metabolite pyranonigrin A. Indeed, absence of Fum21 of A. niger affected expression of genes of three other secondary metabolite clusters. Whether Fum21 of F. verticillioides also affects expression of other secondary metabolite clusters is not known.

As mentioned above, fum21 is down-regulated in ΔflbA (Krijgsheld and Wösten 2013). This implies that FlbA is not only involved in conidiation, spatial secretion of proteins, composition of the secretome, cell wall architecture, and lysis of hyphae (Krijgsheld et al. 2013b) but also in controlling secondary metabolism. Indeed, all genes of the fumonisin cluster are down-regulated in ΔflbA (Krijgsheld and Wösten 2013). The fact that Fum21 does not control expression of 2 out of 12 genes of the cluster implies that FlbA impacts expression of another transcription factor involved in fumonisin production. Of interest, the pac1 homologue of A. niger (known as pacC) is up-regulated in ΔflbA (Krijgsheld and Wösten 2013). Possibly, this transcription factor is a repressor of genes in this mycotoxin cluster.

LaeA was initially identified as a regulator of secondary metabolism in A. nidulans (Bok and Keller

![Fig. 2 Amount of fumonisin B2, B4, and B6 (FB2, FB4, and FB6, respectively) (a) and other secondary metabolites (b) in arbitrary units (a.u.), produced by the wild-type strain MA234.1 (open bars) and Δfum21 (gray shaded bars) in CYA medium. Asterisk indicates significant differences (p < 0.05) between the two strains indicated by the horizontal line below the asterisk.](image)
**Table 1** Differentially expressed genes in liquid shaken cultures of Δfum21 and the wild-type strain (for full dataset, see Supplemental Table 2)

| Protein id | Δfum21 | Wild type | Functional annotation |
|------------|--------|-----------|-----------------------|
| Down-regulated genes in Δfum21 |
| 1166045 | 0 | 13.21 | AAA+ -type ATPase |
| 1182124 | 0 | 4.89 | Major facilitator superfamily transporter |
| 225717 | 0 | 29.92 | fum21 |
| 1117227 | 0.30 | 519.60 | Peroxisomal acyl-CoA synthetase; fum10 orthologue |
| 1117230 | 0.25 | 368.97 | α-Oxoglutarate synthase; serine palmitoyltransferase; fum8 orthologue |
| 1182116 | 0.39 | 573.20 | Fe-containing alcohol dehydrogenase type IV; fum7 orthologue |
| 1142053 | 1.09 | 1433.24 | No annotation |
| 1162446 | 0.25 | 167.04 | Polyketide synthase; fum1 orthologue |
| 1162442 | 2.19 | 913.32 | NAD-dependent epimerase/dehydratase; fum13 orthologue |
| 1101614 | 0.98 | 236.55 | Cytochrome p450; fum6 orthologue |
| 1142051 | 8.34 | 1607.79 | No annotation |
| 1166044 | 2.75 | 347.93 | No annotation |
| 1172265 | 1.62 | 191.62 | Oxidoreductase |
| 1162443 | 6.66 | 564.86 | CoA-dependent acyltransferase; fum14 orthologue |
| 1186369 | 0.57 | 19.06 | Ca^{2+}-modulated nonselective cation channel polycystin |
| 51907 | 3.73 | 110.86 | Predicted 3-ketosphinganine reductase |
| 1181633 | 0.49 | 12.69 | SWI-SNF chromatin-remodeling complex protein |
| 1082505 | 1.48 | 32.99 | Major facilitator superfamily transporter |
| 1142861 | 1.59 | 33.92 | Chloroperoxidase |
| 1159889 | 0.93 | 17.88 | O-methyltransferase |
| 1087288 | 2.37 | 39.12 | Taurine catabolism dioxygenase TauD |
| 45784 | 1.70 | 25.17 | Ca^{2+}-modulated nonselective cation channel polycystin |
| 1169210 | 5.86 | 72.09 | Glutathione S-transferase-like protein |
| 1116476 | 2.31 | 28.18 | CDR ABC transporter |
| 1089440 | 2.52 | 29.10 | Major facilitator superfamily transporter |
| 1112167 | 0.60 | 6.38 | Polyketide synthase AdaA |
| 1181632 | 2.79 | 26.80 | No annotation |
| 1115620 | 1.27 | 12.02 | C-type lectin |
| 189113 | 8.60 | 80.47 | NmrA-like family protein |
| 1005100 | 2.33 | 21.65 | No annotation |
| 1186279 | 20.64 | 191.85 | No annotation |
| 1103854 | 1.34 | 11.77 | Glycosyl transferase |
| 1139199 | 4.02 | 34.97 | Mono-oxygenase, FAD-binding/aromatic ring hydroxylase |
| 1186352 | 2.74 | 23.65 | Molecular chaperone |
| 1109756 | 1.59 | 13.54 | Kinesin-related protein |
| 1187549 | 1.99 | 16.75 | Integral membrane protein |
| 1125454 | 0.71 | 5.80 | Dihydroxy-acid dehydratase |
| 1184525 | 3.92 | 31.30 | Non-ribosomal peptide synthetase |
| 1124090 | 2.40 | 18.02 | Tryptophan synthase |
| 1015414 | 1.95 | 14.50 | Short chain dehydrogenase |
| 1139200 | 33.67 | 239.67 | AdaD |
| 1187587 | 2.19 | 15.55 | AAA+ -type ATPase |
| 1152279 | 4.10 | 28.71 | Major facilitator superfamily transporter |
2004). It controls expression of several gene clusters, including clusters involved in production of sterigmatocystin, penicillin, and lovastatin. LaeA is also involved in secondary metabolism in *A. niger*. It does not impact fumonisin production but it represses production of the compounds BMS-192548 and aspernigrin A, while activating production of asperrubrol, atromentin, and JBIR86 (Niu et al. 2015). Experimental data showed that production of aurasperone B, funalenone, kotanin, and tensidol are neither regulated by Fum21 (this work) nor by LaeA (Niu et al. 2015). This implies that other regulatory genes are involved in the production of these secondary metabolites. The fact that expression of *laeA* is not affected by FlbA in *A. niger* implies that its regulation is different from that of *fum21*. Together,

| Protein id | Δfum21 | Wild type | Functional annotation |
|------------|--------|-----------|----------------------|
| 1162650    | 21.21  | 146.94    | Aegerolysin          |
| 1200239    | 5.00   | 34.01     | NmrA-like family protein |
| 1223842    | 1.36   | 9.10      | hypothetical FAD/FMN-containing dehydrogenase |
| 1186845    | 0.79   | 5.13      | No annotation        |
| 1187028    | 192.14 | 1228.87   | No annotation        |
| 1181350    | 3.78   | 21.89     | *Aspergillus kawachii* d-alanine-d-alanine ligase orthologue |
| 1157348    | 5.074  | 28.54     | UDP-glucose 4-epimerase |
| 1186355    | 2.26   | 12.65     | FAD-linked oxidase    |
| 1155959    | 6.55   | 34.31     | MNNG and nitrosoguanidine resistance protein |
| 1141103    | 32.82  | 162.69    | trkA-N domain dehydrogenase |
| 1107461    | 1.26   | 6.04      | Chitinase             |
| 1187643    | 9.17   | 43.25     | No annotation        |
| 1099871    | 4.00   | 18.48     | Flavin-containing monoxygenase |
| 1124492    | 3.68   | 16.34     | Phosphoglycerate mutase |
| 1135815    | 9.66   | 41.71     | Serine/threonine kinase |
| 52063      | 18.67  | 80.01     | No annotation        |
| 1150465    | 11.56  | 48.79     | No annotation        |
| 1161325    | 56.49  | 217.67    | Integral membrane protein |
| 1141963    | 25.56  | 92.48     | Glutathione S-transferase |
| 1145979    | 10.39  | 35.46     | GMC oxidoreductase    |
| 1184413    | 92.14  | 22.85     | Serine/threonine kinase |
| 1184369    | 139.39 | 31.11     | Lipase               |
| 1156756    | 32.65  | 6.04      | No annotation        |
| 1185088    | 26.99  | 4.98      | No annotation        |
| 1180662    | 357.01 | 65.37     | Lipase               |
| 1181154    | 89.30  | 13.98     | No annotation        |
| 1183897    | 535.00 | 81.16     | Antifungal protein   |
| 1117716    | 35.428 | 5.03      | Glycoside hydrolase family 7 protein CbhB |
| 1187764    | 19.37  | 2.5476    | No annotation        |
| 1146836    | 6.24   | 0.69      | Hypothetical FAD/FMN-containing dehydrogenase |
| 1164071    | 108.91 | 11.66     | Peptidase G1, eqolisin |

Both strains had been grown in TM for 16 h followed by 4 h in MM-X. Gene expression is expressed as Fragments Per Kilobase Of Exon Per Million Fragments Mapped (FPKM).
these results indicate that \textit{fum21}, \textit{laeA}, and other transcriptional regulators are involved in secondary metabolite production in \textit{A. niger}. These genes are potential targets to improve \textit{A. niger} as a cell factory by minimizing production of mycotoxins.

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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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**Table 2** Expression of the genes of the fumonisin cluster in liquid shaken cultures of \textit{Δfum21} and the wild-type strain

| Protein ID | Functional annotation | Expression | \(Δfum21\) | Wild type |
|------------|-----------------------|------------|-------------|-----------|
| 1101614    | \textit{fum6}         | 0.98       | 236.55      |           |
| 51907      | \textit{sdr1}         | 3.73       | 110.86      |           |
| 1117227    | \textit{fum10}        | 0.30       | 519.60      |           |
| 1182116    | \textit{fum7}         | 0.39       | 573.20      |           |
| 1182117    | \textit{fum3*}        | 0.28       | 894.82      |           |
| 1117230    | \textit{fum8}         | 0.25       | 368.97      |           |
| 1162442    | \textit{fum13}        | 2.19       | 913.32      |           |
| 1162443    | \textit{fum14}        | 6.66       | 564.86      |           |
| 225717     | \textit{fum21}        | 0          | 29.92       |           |
| 1117235    | \textit{fum15*}       | 65.98      | 40.50       |           |
| 1182122    | \textit{fum19*}       | 218.64     | 146.37      |           |
| 1162446    | \textit{fum1}         | 0.25       | 167.04      |           |

Both strains had been grown in TM for 16 h followed by 4 h in MM-X. Gene expression is expressed as Fragments Per Kilobase Of Exon Per Million Fragments Mapped (FPKM). Gray shading indicates genes that are down-regulated in \textit{Δfum21} when compared to the wild-type strain. Asterisk indicates genes that are not significantly differentially expressed.
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