Transcription of Genes in the Biosynthetic Pathway for Fumonisins Is Epigenetically and Differentially Regulated in the Fungal Maize Pathogen *Fusarium verticillioides*

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When the fungal pathogen *Gibberella moniliformis* (anamorph, *Fusarium verticillioides*) colonizes maize and maize-based products, it produces class B fumonisins (FB) mycotoxins, which are a significant threat to human and animal health. FB biosynthetic enzymes and accessory proteins are encoded by a set of clustered and cotranscribed genes collectively named FUM, whose molecular regulation is beginning to be unraveled by researchers. FB accumulation correlates with the amount of transcripts from the key FUM genes, FUM1, FUM21, and FUM8. In fungi in general, gene expression is often partially controlled at the chromatin level in secondary metabolism; when this is the case, the deacetylation and acetylation (and other posttranslational modifications) of histones are usually crucial in the regulation of transcription. To assess whether epigenetic factors regulate the FB pathway, we monitored FB production and FUM1, FUM21, and FUM8 expression in the presence of a histone deacetylase inhibitor and verified by chromatin immunoprecipitation the relative degree of histone acetylation in the promoter regions of FUM1, FUM21, and FUM8 under FB-inducing and noninducing conditions. Moreover, we generated transgenic *F. verticillioides* strains expressing GFP under the control of the FUM1 promoter to determine whether its strength under FB-inducing and noninducing conditions was influenced by its location in the genome. Our results indicate a clear and differential role for chromatin remodeling in the regulation of FUM genes. This epigenetic regulation can be attained through the modulation of histone acetylation at the level of the promoter regions of the key biosynthetic genes FUM1 and FUM21, but less so for FUM8.

Fumonisins are a family of mycotoxins produced by the secondary metabolism (SM) of *Fusarium verticillioides* (teleomorph, *Gibberella moniliformis*) and *Fusarium proliferatum* that contaminate maize and maize-based products. Within the B series of these toxins (FB), FB1, FB2, and FB3 are the ones most frequently found under field conditions and have been linked to various animal and human mycotoxicoses (23). FB are polyketides consisting of a linear 19- or 20-carbon backbone with hydroxyl, methyl, and tri-carballylic acid moieties at various positions along the base chain (19). In filamentous Ascomycetes, genes involved in the biosynthesis of toxins (such as aflatoxins and trichothecenes) and of other secondary metabolites are frequently organized into clusters (6). Clustering is not observed for most biosynthetic genes in the SM of higher eukaryotes, with a few exceptions in plants (7, 21). In fungi in general, gene expression is often partially controlled at the chromatin level in secondary metabolism; when this is the case, the deacetylation and acetylation (and other posttranslational modifications) of histones are usually crucial in the regulation of transcription. To assess whether epigenetic factors regulate the FB pathway, we monitored FB production and FUM1, FUM21, and FUM8 expression in the presence of a histone deacetylase inhibitor and verified by chromatin immunoprecipitation the relative degree of histone acetylation in the promoter regions of FUM1, FUM21, and FUM8 under FB-inducing and noninducing conditions. Moreover, we generated transgenic *F. verticillioides* strains expressing GFP under the control of the FUM1 promoter to determine whether its strength under FB-inducing and noninducing conditions was influenced by its location in the genome. Our results indicate a clear and differential role for chromatin remodeling in the regulation of FUM genes. This epigenetic regulation can be attained through the modulation of histone acetylation at the level of the promoter regions of the key biosynthetic genes FUM1 and FUM21, but less so for FUM8.

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lational modifications of histones (the proteins around which DNA is wrapped to form nucleosomes) and of the enzymes that carry out such modifications (30).

Among the genes that encode enzymes responsible for reversible posttranslational modifications of histones, FvVE1 is the F. verticillioides homologue of the Aspergillus Vlvet gene (VeA). Its deletion compromises FB production, among other effects (14). In Aspergillus, the interaction between VeA and LaeA, a protein methyltransferase, regulates transcription of genes within clusters involved in SM synthesis. Researchers have hypothesized that LaeA is involved in chromatin remodeling through histone methylation (2, 4, 10). The diminished FB production by *F. verticillioides* strains lacking FvVE1 suggests that the expression of genes in the FUM cluster is regulated not only at the level of the individual inducible promoters, but also epigenetically.

Acetylation and deacetylation of histones are among the best understood posttranslational modifications involved in epigenetic control. The acetylation status of histones directs chromatin structure transitions and controls the affinity and accessibility of the transcriptional machinery to regulatory sequences in DNA. Therefore, histone acetyltransferases (HATs) and histone deacetylases (HDACs) play a key role in the transition between hetero- and euchromatin, hypoacetylation being associated with heterochromatin and gene silencing while hyperacetylation is associated with euchromatin formation and gene activation (32).

In this work, we investigated the role of histone acetylation and deacetylation in the expression of *FUM1, FUM21*, and *FUM8* and in the production of FB in *F. verticillioides*. Our results indicate that a hyperacetylated state of histones is associated with an increased expression of *FUM1* and *FUM21* but less so for *FUM8* and that acetylation levels of the histones around *FUM1* and *FUM21* promoter regions are wrapped increase under FB-inducing conditions.

**MATERIALS AND METHODS**

**Strains and media.** The *F. verticillioides* isolates used in this work were deposited in the collection of the Institute of Sciences of Food Production (ISPA-CNRI, Bari, Italy; http://server.ispa.cnri.it/ITEM/Collection). Strains VP2 (ITEM 10670), FR3 (ITEM 10679), and GE1 (ITEM 10681) are effective FB producers (34). Monoconidial cultures of all strains were inoculated with 100 conidia (10⁶ CFU/ml). Table I shows the basal medium (IM containing 5 mM instead of 10 mM glucose, and 100 µM acetoacetate (A. tumefaciens strain H9262). This medium was used as the backbone to generate a pCAM-*FUM1*-GFP expression vector. To do so, the *FUM1-GFP* cassette was excised by Clal and XbaI from pCAM-*FUM1*-GFP and subcloned into pBSK⁺. In this construct, a promoter swap was performed by Clal and NcoI excision of *FUM1* and subcloning of Clal/NcoI-digested *FUM1* in the same position. Finally, the whole pCAM-*FUM1*-GFP cassette was cut out by Clal and XbaI and inserted back into the pCAM-*FUM1*-GFP vector in place of the *FUM1*-GFP-fragment to generate the pCAM-*FUM1*-GFP construct (see Fig. S1 in the supplemental material).

**Agrobacterium-mediated Fusarium transformation.** pCAM-*FUM1*-GFP was transformed into *F. verticillioides* strain VP2 by using Agrobacterium tumefaciens strain EHA105 as described by Takken et al. (31) with minor modifications. One colony of *A. tumefaciens* harboring the construct was cultured overnight in minimal medium (MM; 10 mM KH₂PO₄, 10 mM KH₂PO₄, 2.5 mM NaCl, 2 mM MgSO₄, 0.7 M CaCl₂, 2H₂O, 9 µM FeSO₄, 4 mM (NH₄)₂SO₄, and 10 mM glucose) supplemented with kanamycin (50 µg/ml). Cells were then diluted to an optical density of 600 nm of 0.30 to 0.45 in induction medium [IM; MM plus 40 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.3, and 0.5% glycerol (vol/vol)] and incubated for 6 h at 28°C in the presence of 100 µM acetoxy-syringone (AS). At the same time, fungal conidia were harvested from 1-week-old shake cultures in Czapek liquid medium by filtration over Miracloth and diluted to 10⁶ CFU/ml in IM. Then, 100 µl volumes of *A. tumefaciens* cells were mixed with 100-µl volumes of the *F. verticillioides* spore suspension, and the mixtures were plated individually on ME-25 filters (0.45-µm pore size; Whatman) placed on agar-containing cocultivation medium (IM containing 5 mM instead of 10 mM glucose, and 100 µM AS). After 2 days at 25°C, filters were transferred onto agar-containing Czapek medium supplemented with hygromycin (100 µg/ml) and ceftaxime (200 µM). The presence of the correct expression cassette in the transformed colonies (named Fv-*P_FUM1* here) was detected by PCR analysis with promFUM1-F-Cla and promFUM1-R-Nco in combination with primers designed on the GFP sequence (GFP-F, GAACTGACCTTCACCAAGCAG GACCATGT, and GFP-R, GTGACCAACCTCTCACATCGG) followed by sequencing of the amplicons.

**Nucleic acid protocols.** Genomic DNA was extracted with the following cetyltrimethylammonium bromide (CTAB) method. Whole deep-frozen mycelium mats from 7-day cultures in 40 ml of Czapek medium were ground with a mortar and pestle and transferred to 2 ml Eppendorf tubes. One ml of CTAB buffer (2% [wt/vol] CTAB, 2% [wt/vol] PVP K30 [sigma 81420], 100 mM Tris-HCl [pH 8.0], 25 mM EDTA, 2.0 M NaCl, and 2% [vol/vol] β-mercaptoethanol) and 1 µl of proteinase K were added to each tube, and the tubes were then incubated for 10 min at 42°C and subsequently for 10 min at 65°C, with mixing every 3 min. An 800-µl volume of chloroform–isoamyl alcohol was added, and the tubes were incubated on ice for 10 min. Tubes were then centrifuged for 10 min at 5,700 × g. A 600-µl volume of each upper phase was transferred to new 1.5-ml Eppendorf tubes to which 200 µl of 30% polyethylene glycol (PEG)
and 100 μl of 5 M NaCl were added. Samples were then centrifuged for 15 min at 17,500 × g, the pellets were washed twice with 600 μl of 75% ethanol, and samples were centrifuged for 5 min at 17,500 × g. Pellets were then dried under slight vacuum, and DNA was finally dissolved in 20 μl of double-distilled H2O. Tubes were kept at 4°C overnight and then incubated at 40°C for 2 h. Total genomic DNA of wild-type F. verticillioides strain VP2 and two independent Fv-P PROM transformants were prepared for Southern analysis as follows. Genomic DNA (5–μg samples) was digested by HindIII, SacI, or XbaI, electrophoresed on a 1.2% agarose gel, and transferred onto a nitrocellulose membrane (Whatman/3MM) with a vacuum blotting pump (VacuGene XL, vacuum blotting system; Amersham). A 404-bp, digoxigenin (DIG)-labeled DNA probe (digoxigenin-11-UTP; Roche) was produced with primers SB-F (GAACCGGAAAT TCGATGCTG) and SB-R (GGACTTGAAGAAGTCGCTG) according to the manufacturer’s instructions and was used to hybridize the fungal DNA according to the DIG application manual from Roche.

RNA was isolated and cDNA was prepared as described previously (9). Primers for FUM1 (CAAGCGGTCACATCCATG), TUB2 (TGTCTCATTTCTAAGAGCCG and GTAG TTGAGTGTCGGTAGAGG), and FM8 (GACACAGACGGCAGGAG AAGTT and TGGAGTGTCGGTCGCTGTTTGTG) were taken from the literature (8, 37); they amplify fragments of 128, 233, and 107 bp, respectively. We designed primers FUM21-F (GCCATCATTGCAACACATTC) and FUM21-R (AAAAATGTCGGTCGAGGTGAC) for FUM21 and primers GFP-R-F (ATGGTGAGCAAGGGCGA) and GFP-R-R (GTGCTGCTTCC ATGGTGTCGG) for GFP, which amplify PCR bands of 143 and 246 bp, respectively. Reverse transcription-quantitative PCRs (RT-qPCRs) were performed in a StepOne real-time PCR system (96-well format), and data were collected with StepOne version 2.1 (Applied Biosystems). Each reaction mixture consisted of the following: 5 μl of Power SYBR green PCR master mix (Applied Biosystems), forward and reverse primers for a single gene (500 nM each), cDNA template (corresponding to about 100 ng of mRNA), and nuclease-free water added to a final volume of 10 μl. PCR cycling conditions consisted of 10 min at 95°C (1 cycle) and 15 s at 95°C, followed by 1 min at 60°C (40 cycles). Transcript abundance values for FUM1, FUM21, FM8, and TUB2 were the means from three biological replicates and three analytical repetitions for each strain and experimental condition. We calculated transcript amounts by the absolute quantification method in qPCR (Applied Biosystems) with TUB2 as the endogenous reference for normalization. To design the standard curve, we used 1:5 serial dilutions of the different target genes cloned into the endogenous reference for normalization. To design the standard curve method (Applied Biosystems) with biological replicates and three analytical repetitions for each strain and 40-ml volume of Czapek, Czapek-TSA, or fructose-enriched medium was incubated at 17,500 rpm at 27°C overnight and then incubated at 27°C overnight. The pellets were washed twice with 600 μl of 70% ethanol, and samples were centrifuged for 5 min at 17,500 × g to wash methanol. The residue was redissolved in 500 μl of methanol-water (1:1, vol/vol) and defatted with 250 μl of cyclohexane. Chromatographic separation was performed by high-pressure liquid chromatography (HPLC) on a Kinetex C18 column (50.0 by 2.1 mm; particle size, 2.6 μm; Phenomenex, Aschaffenburg, Germany) at 40°C using a linear gradient of 40% to 98% methanol in water (with 7 mM acetic acid in both solvents) over 3 min, followed by 3 min of washing with 98% phase B and 5 min of equilibrating at the starting conditions. The flow rate was set to 200 μl/min. FB1 was quantified using tandem mass spectrum detection with positive electrospray ionization (ESI) and a 500MS ion trap (Varian, Darmstadt, Germany). The protonated molecular ion 722.5 m/z [M+H]+ was used as precursor ion and m/z 686.4, m/z 528.4, and m/z 352.5 were selected as product ions. The limit of quantification, defined as the toxin concentration exceeding the signal of the background noise by 10 times, was 2 μg/liter FB1 in the medium. The concentration of FB1 in samples was determined by using standard calibration curves prepared with 10 concentrations from 0.1 to 1 μg/liter (Romer Labs, Tulln, Austria). Samples with FB1 concentrations exceeding the highest standards were diluted to less than 500 μg/ml.

Inoculation experiments and microscopy. All experiments were performed with three plants per triplicate pot per treatment and were repeated at least twice. For seed inoculation, maize kernels (PR34N34 hybrid; Pioneer HiBred), collected from healthy ears and determined to be Fusarium free, were surface disinfested for 5 min in 5% NaClO (vol/vol), rinsed with sterile distilled water, and subsequently heat shocked by placing them in a 60°C water bath for 5 min for internal sterilization. Inoculations were performed by placing sterilized seeds in a petri dish (10-cm diameter), flooding them with 10 ml of the conidial suspension (10⁶ CFU/ml), and incubating them overnight at 27°C before sowing. Assays were performed in plant growth chambers at 30°C in continuous dark, conditions under which FB accumulation in the roots becomes easily detectable (35). Microscopic observations were performed every other day, starting at 7 days after sowing, until plants were 4 weeks old. Hand-made longitudinal and transversal sections of roots were first observed with an epifluorescence microscope (Olympus BX40; Leica) equipped with a U-MSWB filter set (excitation, 450 to 480 nm; emission, >515 nm). Samples were then scanned with a Leica TCS SP2 confocal microscope equipped with a long-distance 40× water immersion objective (HCX Apo 0.80). The Ar laser band of 488 nm was used to excite GFP, whose signal was detected at the specific emission window of 500 to 530 nm.

Statistical analysis. qPCR data were subjected to analysis of variance, and the means were compared with the least significant difference test with SGWIN software.
RESULTS

Transcripts of FUM1 and FUM21 are significantly more abundant under HDAC-inhibiting conditions. To determine the role played by HDACs in the regulation of FB production in F. verticillioides, we followed a pharmacological approach at first; i.e., we analyzed the expression levels of three key genes in the FB biosynthetic pathway in the presence of the standard inhibitor of HDACs, TSA. The transcript abundance of FUM1, FUM21, and FUM8 was quantified by RT-qPCR on RNA extracted after 1 week of growth on Czapek medium (non-FB-inducing) or Czapek medium amended with 1 μM TSA. The results for all three field isolates of F. verticillioides analyzed (VP2, FR3, and GE1) indicated significantly larger amounts of FUM1 and FUM21 mRNAs in the presence of TSA than in its absence (Fig. 1A and B), while the induction of FUM8 transcript accumulation was less convincing (Fig. 1C). When FB production was checked in samples grown 7 or 20 days in Czapek medium in the absence or presence of TSA (Fig. 2; also, see Fig. S2 in the supplemental material), a trend mirroring FUM1 and FUM21 expression was observed, but differences were never significant and were not always obvious for all three strains and in all independent experiments performed (for example, see isolate GE1 in Fig. 2 and also in Fig. S2 in the supplemental material).

Histones localized at the FUM1 and FUM21 promoters are significantly hyperacetylated under FB-inducing conditions. To assess whether the effects of TSA on gene transcription mirror what happens under FB-inducing conditions (and therefore whether hyperacetylated histone proteins are directly localized at the promoter of FUM1 and FUM21 genes when their transcription is active), we performed a ChIP assay with a commercial antibody targeted to the hyperacetylated form of histone H4. We focused our analysis on the promoter regions of FUM1, FUM21, and FUM8 (here named P_FUM1, P_FUM21, and P_FUM8, respectively) of F. verticillioides isolate VP2 grown in the absence (negative control) or presence (positive control) of TSA and in fructose-containing, FB-inducing medium. The amount of immunoprecipitated DNA fragments containing the target sequences was quantified by qPCR with the comparative threshold cycle (Ct) method after normalization on the quantity of DNA fragments containing the TUB2 gene. The quantities of immunoprecipitated DNA fragments containing P_FUM1 and P_FUM21 were significantly greater in the presence of TSA (positive control) and in FB-inducing medium than under noninducing control conditions (Fig. 3A and B). Data for P_FUM8 followed the same trend, but differences were minor and not significant (Fig. 3C).

Transcription driven by P_FUM1 is increased under FB-inducing conditions but is more intense when P_FUM1 is ectopically located in the genome. We reasoned that if some key FUM genes are epigenetically repressed by the locally nonpermissive
chromatin state under non-FB-inducing conditions (i.e., if their position in the *F. verticillioides* genome is important for their expression pattern), the activity of their cognate promoter regions should be influenced by their genomic location. To test this hypothesis, we used P$_{FUM1}$ from the toxigenic *F. verticillioides* strain VP2 to drive expression of the GFP cDNA after random reinsertion into the same genomic background by *A. tumefaciens*-mediated transformation of conidia. Hygromycin-resistant fungal colonies (Fv-P$_{FUM1}$) were selected and subjected to transgene copy number assessment by Southern blot analysis. Two independent transformants carrying a single insertion were retained (Fv-P$_{FUM1}$-1 and -3) (see Fig. S2 in the supplemental material). We tested the functionality of the P$_{FUM1}$:GFP expression cassette by checking the fluorescence of the transgenic isolate Fv-P$_{FUM1}$-1 during growth on maize kernels, a test that mimics natural conditions of FB accumulation. The fungus was grown on cracked kernels for 2 weeks at high humidity, and aerial hyphae were observed under UV light with an optical epifluorescence microscope. Fv-P$_{FUM1}$-1 mycelium was heavily and homogeneously fluorescent under these experimental conditions (images not shown), in agreement with the high FB1 and FB2 levels recorded in the substrate and comparable to the amounts produced by the parental wild-type strain (see Table S1 in the supplemental material). Homogeneously fluorescent mycelium was also visible in fructose-containing medium (images not shown), whereas fluorescence in GYAM was mostly restricted to cells involved in asexual reproduction (Fig. 4A). Also, fluorescent mycelium could be observed in the cortex and vascular system of roots emerging from Fv-P$_{FUM1}$-1-infected seeds. Intense fluorescence was visible only in etiolated plantlets, starting from 3 weeks after sowing (Fig. 4B); under these conditions, FB1 can be detected in infected roots (35). Therefore, the qualitative pattern of fluorescence indicated that the P$_{FUM1}$ was active when expected.

To obtain quantitative insight into P$_{FUM1}$ activity in transformants Fv-P$_{FUM1}$-1 and -3, we used absolute RT-qPCR to quantify the abundance of the mRNAs coding for Fum1 (and therefore controlled by the endogenous promoter within the FUM cluster) and transgenic GFP (controlled by the P$_{FUM1}$ promoter at an ectopic location). For both independent transformants, a statistically significant difference between the amount of transcripts coding for Fum1 and GFP was obvious in Czapek medium (Fig. 5A); the same was seen in fructose-containing medium (Fig. 5B). In the latter case, a hyperinduction of GFP transcription under FB-inducing versus noninducing conditions was recorded. However, the amount of GFP transcripts accumulated by the action of the constitutive promoter P$_{TOXA}$ remained about 170- and 1,500-fold higher than by P$_{FUM1}$ under FB-inducing and noninducing conditions, respectively (Fig. 5A, rightmost column).

**DISCUSSION**

In filamentous fungi, genes needed for the production of secondary metabolites are often grouped in clusters of coordinately regulated, functionally related genes. The evolutionary reasons for clustering have been often debated; the explanation that is best supported by experimental data is that clustering facilitates transcriptional coregulation (11). This hypothesis is based on the assumption that the contiguous arrangement of genes cooperating in a metabolic pathway facilitates their coordinated expression. Chromatin modifiers may regulate such large genomic regions by establishing or removing chromatin-based gene-silencing signa-
Recent studies on the highly characterized cluster for aflatoxin biosynthesis in Aspergillus parasiticus have confirmed crucial epigenetic events in the regulation of the cluster (25). The role played by the HDAC HdaA (28) and by the protein methyltransferase LaeA, which activate transcription (3), are examples of the newly acquired knowledge about epigenetic regulation of SM in Aspergillus nidulans. In particular, because of its similarity to histone methyltransferases, LaeA was suggested to be directly involved in chromatin remodeling (3). Williams and colleagues (36) proposed a similar conserved mechanism by analyzing the SM regulation in unrelated fungi (Alternaria and Penicillium).

In the genus Fusarium, knowledge about the regulation of clustered genes for SM is also increasing. Many positive and negative regulatory factors have been identified for the FB biosynthetic genes in F. verticillioides, but to our knowledge, their regulation by chromatin remodeling has not been previously investigated. The only hint in this direction comes from the fact that FvVE1 mutants show a deregulation of FB production. FvVe1 is likely the functional orthologue of VeA in Aspergillus and should exert its effects through interaction with the already mentioned putative histone methyltransferase LaeA (14). A preliminary characterization of the phenotype of F. verticillioides mutants lacking the putative homologue of LaeA apparently confirms its influence on the regulation of SM (R. A. E. Butchko, S. P. McCormick, M. Busman, B. Tudzynski, and P. Wiemann, presented at the 26th Fungal Genetics Conference, Pacific Grove, CA, March 15 to 20, 2011), but sound data have not been published yet. A possible indication of the cooperation between epigenetic factors and trans-acting proteins in the regulation of FUM genes comes also from the detailed characterization of fum21 mutants of F. verticillioides (5). Rather unexpectedly, small amounts of transcripts of FUM1 and (in one of three independent transformants) of FUM8 were detected after

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**FIG 4** (A) Fluorescence micrographs of the F. verticillioides transformed strain Fv-P$_{FUM1}$-1 grown in FB-inducing GYAM (pH 3) for 1 week. Conidia, conidiophores, and phialides are the most intensely labeled (white arrows). The lower panels are the corresponding bright-field images. (B) Roots of etiolated maize seedlings colonized by GFP-expressing hyphae; confocal images were taken under UV light 3 weeks after sowing of Fv-P$_{FUM1}$-1-infected seeds. Blastospores budding from a growing hyphal tip in a xylem vessel are indicated by a white arrow. Plant cell walls fluoresce in red, mycelium in green (total depth, 235 μm; resolution, 0.36 × 0.36 × 1.5 μm).

**FIG 5** FUM1 and/or GFP transcript abundance in the Fv-P$_{FUM1}$-1 and -3 transformants and in the Fv-P$_{TOXA}$::GFP strain grown for 1 week in Czapek medium (non-FB-inducing) (A) and grown for 20 days in fructose-enriched, FB-inducing medium (B). For each gene, transcript amounts were estimated by the absolute quantification method in RT-qPCR after normalization to TUB2 transcripts. Means and standard errors were calculated with data from three biological and three analytical replicates. Asterisks indicate a P value of <0.01.
2 days of growth in FB-inducing medium. This suggests that factors and/or mechanisms other than Fum21 would underlie the leaky FUM1::FUM8 transcription. A similar behavior was found in mutants of *Fusarium sporotrichioides* lacking Trt6 (a Cys2His2 Zn finger protein), in which a low-level transcription of the trichoecene biosynthetic genes *TRI4* and *TRI5* was still detectable (20).

Our research demonstrated that the acetylation level of the histones H4 around which the promoter regions of *FUM*1 and *FUM21* are wrapped plays a role in the regulation of their transcription, while this is far less obvious for *FUM8*. Several reports demonstrated that HATs and HDACs can be recruited to specific sites in the genome by sequence-specific transcription factors (15, 24). Thus, HATs were shown to be associated with the H3K4 methylation protein complex (*Compass*), which in turn is involved in the initiation of transcription through RNA polymerase II in *Saccharomyces cerevisiae* (13). On the other hand, histone hypoacetylation is often related to transcriptional silencing and chromatin compaction (16). In the current study, the quantity of *FUM1* and *FUM21* transcripts was greater when WT strains were grown in the presence of TSA, an inhibitor of HDACs, than under the control conditions (in non-FB-inducing medium alone), whereas *FUM8* induction was less clear under these conditions. FB1 production by WT strains grown in the presence of TSA tended to show a similar trend (though not for all strains in all experiments), but the lack of statistically significant differences suggests that other mechanisms downstream or independent of *FUM1* and *FUM21* gene activation may prevent unwanted FB synthesis and/or secretion, when environmental conditions (besides the state of chromatin at the *FUM1* and *FUM21* loci) signal the fungus that this branch of its SM should be inactive. Several options are available to the cell for this purpose, and these options include other epigenetic mechanisms acting in parallel with histone acetylation and decacylation; mRNA splicing and modification of mRNA stability and translatability; modification of posttranslational protein stability, solubility, and activity; and metabolite extrusion into the growth medium. Among these, only alternative splicing (of *FUM8*, *FUM11*, *FUM12*, *FUM14*, *FUM15*, *FUM16*, *FUM18*, and *FUM21* transcripts) has been proven experimentally (5, 18). However, our data specifically suggest that at least *FUM8* and possibly other crucial *FUM* genes are less responsive to chromatin remodeling by HDACs and/or HATs than *FUM1* and *FUM21*. On the other hand, our results concern the role of histone acetylation but do not allow us to rule out the possibility that other epigenetic marks may keep *FUM8* repressed under non-FB-inducing conditions [as indirectly suggested by the data reported by Brown and colleagues (5)].

We also showed by ChIP that in *F. verticillioides*, both in the presence of TSA and under FB-inducing conditions, decacylation of histone proteins linked to the promoter region of the key FUM genes *FUM1* and *FUM21* is suppressed when FB synthesis is induced. The effect of TSA treatment indicates that the increase of histone acetylation under FB-inducing conditions, especially on *P*<sub>FUM1</sub>, is caused by the downregulation of HDACs rather than by the stimulation of HATs. This suggests that under noninducing conditions the acetylation status of histones in this region is kept relatively low by constitutive, reciprocal activities of HATs and HDACs and that FB-inducing conditions cause a shift in this equilibrium. That histone acetylation is involved in the regulation of the aflatoxin cluster in *A. parasiticus* and of the sterigmatocystin cluster in *A. nidulans* was also demonstrated by ChIP assays (22, 25). The HDAC-encoding gene *HDF1* of *Fusarium graminearum* was also very recently shown to influence deoxynivalenol production, along with virulence, sexual and asexual reproduction, and development (12).

To investigate how the location of FUM genes affects their regulation, we generated transformants of *F. verticillioides* harboring a GFP gene under the control of the *FUM1* promoter *P*<sub>FUM1</sub> and inserted at random loci by agroinfection. In these constructs, ectopic *P*<sub>FUM1</sub> would not be affected by the chromatin remodeling mechanisms that acted on the native locus of the FUM cluster. The amounts of transcripts of GFP and of the endogenous *FUM1*, which are under the control of the same promoter but in different genomic locations, were significantly different under both noninductive and inductive conditions. In other words, the number of mRNA molecules transcribed under the control of *P*<sub>FUM1</sub> seems to be severalfold higher if *P*<sub>FUM1</sub> is located outside rather than within the FUM cluster. As an alternative or complementary explanation to epigenetic control, the higher GFP mRNA levels could be due in principle to a higher stability of the GFP *versus* FUM1 transcripts. However, if the GFP signal is higher than the FUM1 signal under noninducing conditions merely because the GFP transcript is more stable than the *FUM1* transcript, then induction in fructose-amended medium should lead to proportionate increases of both signals. This was not the case: the FUM1 signal increased about 122- to 300-fold, while the GFP signal increased only about 9- to 16-fold. This strongly indicates that GFP transcript is partly derepressed under noninducing conditions and that epigenetic control specifically suppresses expression at the *FUM1* locus, under noninducing conditions. Furthermore, both the pharmacological approach and ChIP experiments agree in indicating a role for local chromatin structure in regulating *FUM1* transcription. It follows that epigenetic mechanisms may not be the only but are the most likely explanation for our data.

Examination of the fluorescence of *P*<sub>FUM1::GFP</sub> fusions on cracked maize kernels and in low-pH GYAM confirmed that the promoter was induced during growth in/on these media even when located ectopically. The Fv-*P*<sub>FUM1</sub>-1 transformant grown on maize kernels fluoresced homogeneously in all hyphal compartments examined, while conidia, conidiophores, and phialides showed preferential GFP accumulation at pH 3 (GYAM). These last results are in agreement with the hypothesis that FB and conidiation may be linked under certain conditions (27). When Fv-*P*<sub>FUM1</sub>-1 was observed microscopically under UV light during root infection, no fluorescence could be detected in the early phases of the interaction. Instead, intensely fluorescent mycelium could be seen colonizing root tissues of etiolated plants at later times, when insufficient light had substantially debilitated the plants. Because the *P*<sub>FUM1</sub> transcriptional strength is about 170-fold weaker than that of a constitutive promoter such as *P*<sub>ToxA</sub> (Fig. 5A) even under FB-inducing conditions, visual assessment of fluorescence is inevitably a lower-resolution test for *P*<sub>FUM1</sub> activation than RT-qPCR. However, when fluorescence becomes detectable, then transcription of the endogenous *FUM1* gene should be proceeding at a high rate as well. Our microscopic observations of GFP fluorescence in planta confirm that *P*<sub>FUM1</sub> activity was highest in tissues devoid of active defenses, such as dying roots of etiolated plants and autoclaved kernels *in vitro*.

In summary, our study indicates that the regulation of FB biosynthesis involves chromatin remodeling at the level of the FUM gene cluster. In particular, we propose that HDACs (so far un-
characterized in *F. verticillioides*) directly change the degree of histone acetylation in the promoter region of *FUM1* and *FUM2*, thus influencing the chromatin state in this genomic region. Because the levels of induction obtained with TSA treatment are significantly higher than the levels observed by growing the fungus under noninducing conditions, but still much lower than those attained under standard FB-inducing conditions, histone acetylation does not appear to be the only mechanism responsible for *FUM1* and *FUM2* regulation. Whether other posttranscriptional histone modifications are involved remains to be determined, but epigenetic controls add to the classical regulatory mechanisms provided by transcription factors and their cognate, cis-acting binding domains on promoter regions, which remain indiscernible regardless of their location in the genome, as shown here for *P_FUM1*.

These two regulatory levels likely cooperate to ensure that FUM genes are transcribed in a coordinated and timely manner and that FB production is achieved at the right time, in the right quantity, and at a minimal metabolic cost for the cell.

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I.V. and F.C. designed the experiments. I.V., V.M. (Fig. 2 and 3B), and K.D. (Fig. 2 and Fig. 52 in the supplemental material) performed the experiments and/or analyzed the samples. I.V. and F.C. analyzed the data. C.A., P.K., G.T., and F.C. funded the work and/or provided logistical help. I.V. and F.C. wrote the paper.

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