

# Transcription of Genes in the Biosynthetic Pathway for Fumonisin Mycotoxins 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 fumonisin (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*.

Fumonisin is 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 tricarballic 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 organisms like filamentous fungi, such clustering occurs and is maintained probably because it facilitates horizontal transfer and coordinated transcriptional regulation of the genes therein (29).

The FB biosynthetic gene cluster consists of 17 transcriptionally coregulated genes designated *FUM1* through *FUM3* and *FUM6* through *FUM21* (with *FUM2* and *FUM3* later found to be the same as *FUM12* and *FUM9*, respectively) (1, 5, 18, 26). While the functions of some of these genes are only hypothesized, others have been characterized experimentally. In particular, *FUM1* plays a key role in biosynthesis because its product is a polyketide synthase involved at an early step in the assembly of the FB backbone (19). *FUM8* encodes an aminoacyl transferase that catalyzes the formation of the full-length C<sub>20</sub> molecule by adding a carbanion derived from the  $\alpha$ -carbon of alanine to the polyketide backbone generated by Fum1; the disruption of these two genes blocks FB production (1, 26).

Knowledge about the regulatory mechanism controlling FB

biosynthesis at the molecular level is also increasing. The FUM cluster contains—just upstream of *FUM1*—a gene coding for a protein with a regulatory function, which is required for FB synthesis. This is Fum21, a predicted Zn(II)<sub>2</sub>Cys<sub>6</sub> DNA-binding protein and narrow-domain transcription factor that positively regulates *FUM* gene expression (5). Several additional genes located outside the cluster appear to impact the regulation of FB biosynthesis, both positively and negatively; these encode broad-domain transcription factors and global regulators of transcription (reviewed in reference 17).

Besides *trans*-acting transcriptional regulators, epigenetic factors, including modification of chromatin and nucleosome structure, can also provide transcriptional control (30). Changes in chromatin structure, particularly the switch from hetero- to euchromatin during chromatin remodeling, are often associated with transcriptional activation of genes localized in the remodeled chromatin stretch (33). Recent studies have begun to identify molecular players in chromatin remodeling in fungi, which seems to impact SM synthesis more extensively than primary cell functions. Epigenetic control of fungal SM has been confirmed for sterigmatocystin, penicillin, and terrequinone A (22, 28). Those studies highlighted the importance, in fungal SM, of reversible posttrans-

Received 5 July 2011 Accepted 15 November 2011

Published ahead of print 23 November 2011

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doi:10.1128/EC.05159-11

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 Velvet* 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 which *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-CNR, Bari, Italy; <http://server.ispa.cnr.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 obtained and cultivated in liquid media in the dark and at 25°C for 7 days unless noted otherwise. Czapek medium does not induce FB production (per liter: 2 g NaNO<sub>3</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.4 g MgSO<sub>4</sub>, 0.5 g KCl, 0.016 g FeSO<sub>4</sub>, and 3 g sucrose), while GYAM (8 mM L-Asp, 1.7 mM NaCl, 4.4 mM K<sub>2</sub>HPO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 8.8 mM CaCl<sub>2</sub>, 0.05% yeast extract, 0.24 M glucose, and 5.0 mM malic acid) and fructose-containing medium (per liter: 0.5 g malt extract, 1 g yeast extract, 1 g peptone, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.3 g MgSO<sub>4</sub>, 0.3 g KCl, 0.05 g ZnSO<sub>4</sub>, 0.02 g CuSO<sub>4</sub>, and 20 g fructose) are inductive. To test *FUM1*, *FUM21*, and *FUM8* expression and FB production under histone-hyperacetylated conditions, we added the HDAC inhibitor trichostatin A (TSA; 1 μM) to Czapek medium. The cultures in 40 ml of medium were inoculated with 100 μl of a suspension of freshly harvested conidia (10<sup>6</sup> CFU/ml).

**FUM1 promoter cloning procedure.** The putative promoter sequence for *FUM1* (*P<sub>FUM1</sub>*) was retrieved from the NCBI database (AF155773). The primers promFUM1-F (TTATCTGATATGTACGTTCTGGGG) and promFUM1-R (GTGCAACGTTGATGATATCAGTAG) were used to amplify about 1 kbp upstream of the *FUM1* start codon. The 5' ends of the primers corresponded to nucleotides -1040 and -1 with respect to ATG (21061 and 22100 in AF155773). PCR amplification was performed on genomic DNA of *F. verticillioides* strain VP2 immobilized on Whatman FTA cards in a 25-μl final volume, with promFUM1-F and

promFUM1-R (0.4 μM each), 0.625 U of GoTaq polymerase (5 U/μl; Promega), 1× PCR buffer (Promega), MgCl<sub>2</sub> (1.5 mM), and deoxy-nucleoside triphosphates (0.4 mM each). The amplification program was as follows: 1 cycle at 94°C (3 min); 30 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C; and 1 cycle at 72°C (10 min). After amplification, PCR products were visualized by agarose gel electrophoresis (1% wt/vol in 0.5× TBE [per liter: 5.4 g Tris base, 2.75 g boric acid, and 2 ml of 0.5 M EDTA; pH 8.0]) in the presence of ethidium bromide. The amplicon was recovered with a NucleoSpin Extract II kit (Macherey-Nagel) and sequenced on both strands by Genelab (ENE, Rome, Italy) after T/A cloning in pGEM-T (Promega). Once the identity of the amplicon was confirmed, *Clal* and *NcoI* restriction sites were added by PCR with modified primers (promFUM1-F-*Clal*, GGATCGATGTTGCTGCTTGTTA TACT; promFUM1-R-*NcoI*, CCATGGGTCGAACGTTGATGATATCAG TAG), and the amplified cDNA was sequenced again. The pCAM-*P<sub>TOXA</sub>*::*GFP* vector expresses green fluorescent protein (GFP) constitutively under the control of the *ToxA* promoter (*P<sub>TOXA</sub>*) in fungal cells and was used as the backbone to generate a pCAM-*P<sub>FUM1</sub>*::*GFP* expression vector. To do so, the *P<sub>TOXA</sub>*::*GFP* cassette was excised by *Clal* and *XbaI* from pCAM-*P<sub>TOXA</sub>*::*GFP* and subcloned into pBSK<sup>+</sup>. In this construct, a promoter swap was performed by *Clal* and *NcoI* excision of *P<sub>TOXA</sub>* and subcloning of *Clal/NcoI*-digested *P<sub>FUM1</sub>* in the same position. Finally, the whole *P<sub>FUM1</sub>*::*GFP* cassette was cut out by *Clal* and *XbaI* and inserted back into the pCAM-*P<sub>TOXA</sub>*::*GFP* vector in place of the *P<sub>TOXA</sub>*::*GFP*-fragment to generate the pCAM-*P<sub>FUM1</sub>*::*GFP* construct (see Fig. S1 in the supplemental material).

**Agrobacterium-mediated Fusarium transformation.** pCAM-*P<sub>FUM1</sub>*::*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 K<sub>2</sub>HPO<sub>4</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM NaCl, 2 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.7 mM CaCl<sub>2</sub> · 2H<sub>2</sub>O, 9 μM FeSO<sub>4</sub>, 4 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 10 mM glucose] supplemented with kanamycin (50 mg/liter). Cells were then diluted to an optical density at 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 acetosyringone (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 10<sup>5</sup> 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 cefotaxime (200 μM). The presence of the correct expression cassette in the transformed colonies (named Fv-*P<sub>FUM1</sub>* here) was detected by PCR analysis with promFUM1-F-*Clal* and promFUM1-R-*NcoI* in combination with primers designed on the *GFP* sequence (GFP-F, GAACCCAGCAG GACCATGT, and GFP-R, GTGACCACCTTCACCTACGG) 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  $\mu\text{l}$  of 5 M NaCl were added. Samples were then centrifuged for 15 min at  $17,500 \times g$ , the pellets were washed twice with 600  $\mu\text{l}$  of 75% ethanol, and samples were centrifuged for 5 min at  $17,500 \times g$ . Pellets were then dried under slight vacuum, and DNA was finally dissolved in 20  $\mu\text{l}$  of double-distilled  $\text{H}_2\text{O}$ . Tubes were kept at  $4^\circ\text{C}$  overnight and then incubated at  $40^\circ\text{C}$  for 2 h. Total genomic DNA of wild-type *F. verticillioides* strain VP2 and two independent Fv-P<sub>FUM1</sub> transformants were prepared for Southern analysis as follows. Genomic DNA (5- $\mu\text{g}$  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 (CAACCGGAAT TTCGATGCTG) and SB-R (GGACTTGAAGAAGTCGTGCT) 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* (ACACCAAAGCCTCTACAGGTGA and AGGTATC GGGCACCGCT), *TUB2* (TGCTCATTTCCAAGATCCGCG and GTAG TTGAGGTCACCGTAGGAGG), and *FUM8* (AGCACAGACGGCGGAG AAGTT and TGAGTTGTCGCTCGCTTGTG) 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 (AAAATGTCGGTTCGAGGTGAC) for *FUM21* and primers GFPb-F (ATGGTGAGCAAGGGCGA) and GFPb-R (GTGCTGCTTC ATGTGGTCGG) 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 software, version 2.1 (Applied Biosystems). Each reaction mixture consisted of the following: 5  $\mu\text{l}$  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  $\mu\text{l}$ . PCR cycling conditions consisted of 10 min at  $95^\circ\text{C}$  (1 cycle) and 15 s at  $95^\circ\text{C}$  followed by 1 min at  $60^\circ\text{C}$  (40 cycles). Transcript abundance values for *FUM1*, *FUM21*, *FUM8*, 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 pGEM-T vector (Promega); the quantity of plasmid in the first point of the standard curve for each target gene was 25 pg, corresponding to 1.02 pg and  $14.4 \times 10^6$  copies of *FUM1*, 1.13 pg and  $14.4 \times 10^6$  copies of *FUM21*, 0.89 pg and  $14.6 \times 10^6$  copies of *FUM8*, 1.89 pg and  $14.0 \times 10^6$  copies of *GFP*, and 1.80 pg and  $14.0 \times 10^6$  copies of *TUB2*.

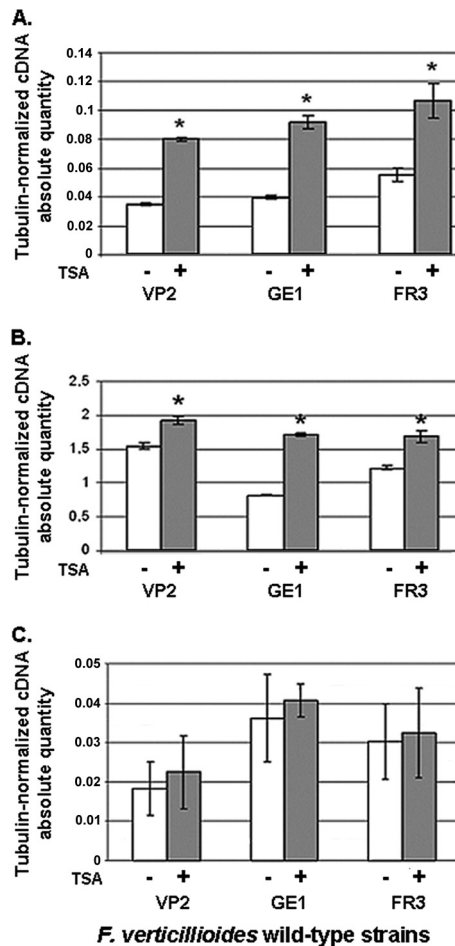
**Chromatin immunoprecipitation (ChIP).** The ChIP procedure was performed essentially as described by the manufacturer of the EZ ChIP chromatin immunoprecipitation kit (Upstate, Charlottesville, VA). A 40-ml volume of Czapek, Czapek-TSA, or fructose-enriched medium was inoculated with 100  $\mu\text{l}$  of a suspension of freshly harvested conidia of *F. verticillioides* VP2 ( $10^6$  CFU/ml) and incubated for 7 days at  $25^\circ\text{C}$  in the dark. Protein cross-linking was performed at harvest by incubating the cultures for 10 min in 1% formaldehyde (final concentration in the growth medium). The cross-linking reaction was stopped by addition of 0.125 M Gly (final concentration). The mycelium was rinsed in PBS (20 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  and 150 mM NaCl, pH 7.5) and then frozen in liquid nitrogen. Frozen mycelium (0.5 g) was ground in liquid nitrogen with a mortar and pestle, and the powder was resuspended in 2 ml of ice-cold DNA extraction buffer (Upstate) according to the manufacturer's instructions. Chromatin was sheared by sonication in an ultrasonic bath (ELMA T460/H) with 5 pulses of 60 s each at full power. Cross-linked DNA and proteins were immunoprecipitated with antibodies against hyperacetylated histone H4 (Penta; Upstate) on the cross-linked DNA. Chromatin was then washed and eluted, and cross-links were reversed

according to the manufacturer's instructions. Quantification was performed by qPCR under conditions that mirrored those for RT-qPCR but with immunoprecipitated DNA as a template (samples normalized to fresh weight). The primers used here were targeted to either the *FUM1*, *FUM21*, or *FUM8* promoter region; their sequences were CTCTATGCC AGCCCTGACTC (promFUM1b-F), GGGACAGTAACGTCCGAAAA (promFUM1b-R), CAGCCATTATCCATCCAGGT (promFUM21-F), GTTCGGATAACACGGCAGTT (promFUM21-R), CTTCTCTCTCT GTGCCAAG (promFUM8-F), and CTGGGGGTATTTCGGAAGTT (promFUM8-R). We quantified the abundance of target DNA by the relative standard curve method (Applied Biosystems) with *TUB2* as the endogenous reference for normalization. Two biological and three analytical repeats were performed for each condition, and standard errors were calculated.

**HPLC analysis of fumonisins.** To detect FB1 production under control and histone-hyperacetylated conditions, we inoculated  $10^8$  conidia of the wild-type strains of *F. verticillioides* VP2, FR3, and GE1 into 40 ml of Czapek medium with the addition of TSA (1  $\mu\text{M}$ ). The quantity of FB1 was determined after 7- or 20-day-old cultures were filtered through a 0.8- $\mu\text{m}$ -pore-size filter. Samples were prepared for analysis by adding 15 ml of methanol to 5 ml of culture supernatant and shaking overnight. After centrifugation at 16,100 rpm for 10 min, 1 ml of the supernatant was evaporated in a speed vacuum concentrator at  $40^\circ\text{C}$ . The residue was redissolved in 500  $\mu\text{l}$  of methanol-water (1:1, vol/vol) and defatted with 250  $\mu\text{l}$  of cyclohexane. Chromatographic separation was performed by high-pressure liquid chromatography (HPLC) on a Kinetex C<sub>18</sub> column (50.0 by 2.1 mm; particle size, 2.6  $\mu\text{m}$ ; Phenomenex, Aschaffenburg, Germany) at  $40^\circ\text{C}$  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  $\mu\text{l}/\text{min}$ . FB1 was quantified using tandem mass spectrometry with positive electrospray ionization (ESI) and a 500MS ion trap (Varian, Darmstadt, Germany). The protonated molecular ion 722.5  $m/z$   $[\text{M}+\text{H}]^+$  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  $\mu\text{g}/\text{liter}$  FB1 in the medium. The concentration of FB1 in samples was determined by using standard calibration curves prepared with 10 concentrations from 0.1  $\mu\text{g}/\text{liter}$  to 1  $\text{mg}/\text{liter}$  (Romer Labs, Tulln, Austria). Samples with FB1 concentrations exceeding the highest standards were diluted to less than 500  $\mu\text{g}/\text{ml}$ .

**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 (PR34N43 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  $60^\circ\text{C}$  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 ( $10^6$  CFU/ml), and incubating them overnight at  $27^\circ\text{C}$  before sowing. Assays were performed in plant growth chambers at  $30^\circ\text{C}$  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 $\times$  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.

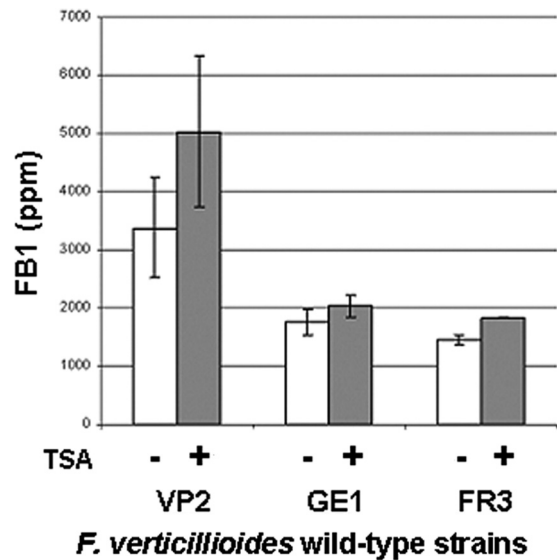
**Statistical analysis.** qPCR data were subjected to analysis of variance, and the means were compared with the least significant difference test with SGWIN software.



**FIG 1** Effects of the HDAC inhibitor TSA on FUM gene expression in wild-type VP2, FR3, and GE1 strains of *F. verticillioides*. The strains were cultivated in the non-FB-inducing Czapek medium in the absence or in the presence of 1  $\mu$ M TSA. After 7 days of growth, the amounts of transcripts of *FUM1* (A), *FUM21* (B), and *FUM8* (C) were estimated by the absolute quantification method in RT-qPCR. For each gene, expression was normalized to *TUB2* transcript abundance. Mean and standard error were calculated with data from three biological and three analytical replicates. Asterisks indicate a *P* value of <0.05.

## 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  $\mu$ M 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

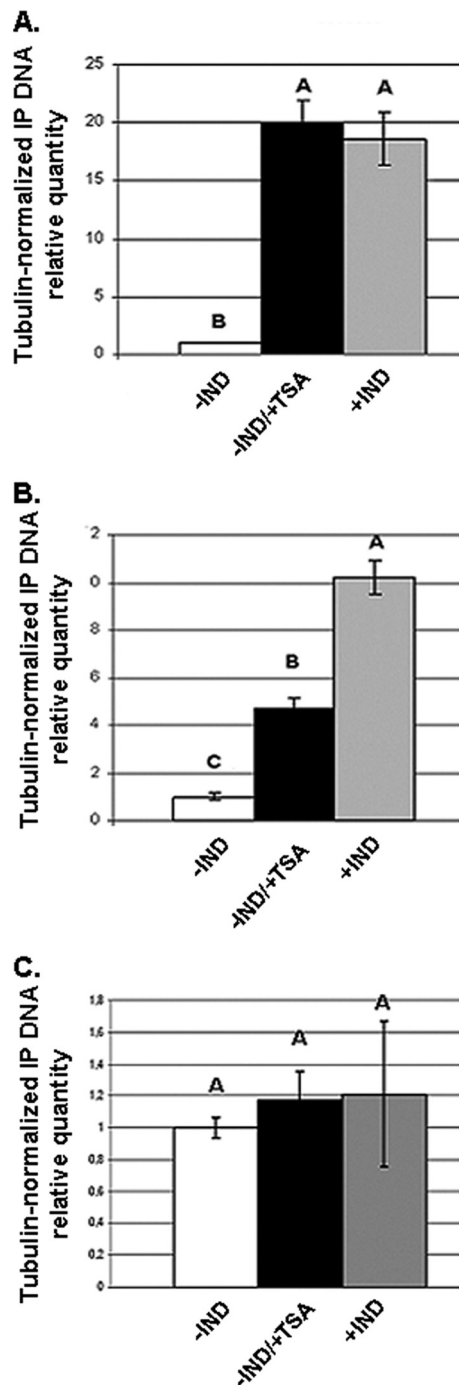


**FIG 2** HPLC quantification of FB1 production in wild-type VP2, FR3, and GE1 strains of *F. verticillioides*. Values are the means of three independent culture batches for each strain, grown for 7 days in liquid Czapek medium (40 ml, inoculated with  $10^5$  spores). The role of HDACs in FB1 production was tested by adding the HDAC inhibitor TSA to the medium to a final concentration of 1  $\mu$ M. Means and standard deviations were calculated with data from three biological replicates.

(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 ( $C_T$ ) 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



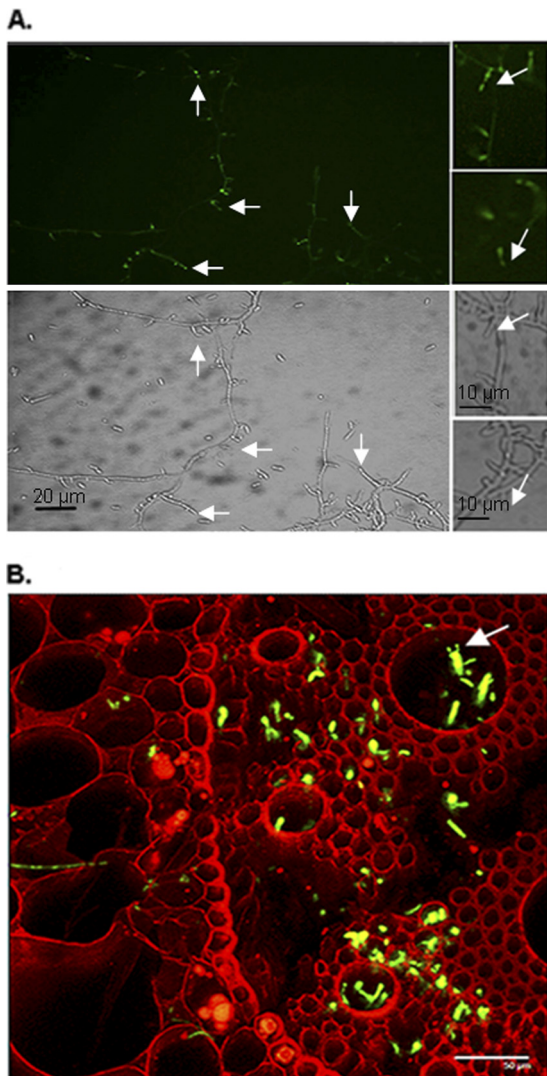
**FIG 3** Relative acetylation levels of the *FUM1* (A), *FUM21* (B), and *FUM8* (C) gene promoters under different conditions. Strain VP2 was cultured for 7 days in Czapek medium, in Czapek medium containing 1  $\mu$ M TSA, an HDAC inhibitor, or in fructose-enriched, FB-inducing medium. Acetylation levels were analyzed by ChIP with antibodies specific to hyperacetylated histone H4 (Penta). The abundance of target DNA was quantified by the comparative  $C_T$  method (Applied Biosystems) with *TUB2* as the endogenous reference for normalization. Error bars indicate the standard errors of two biological and three technical repetitions. Different letters indicate significant differences ( $P < 0.01$ ). -IND, non-FB-inducing medium; +IND, FB-inducing medium.

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 *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

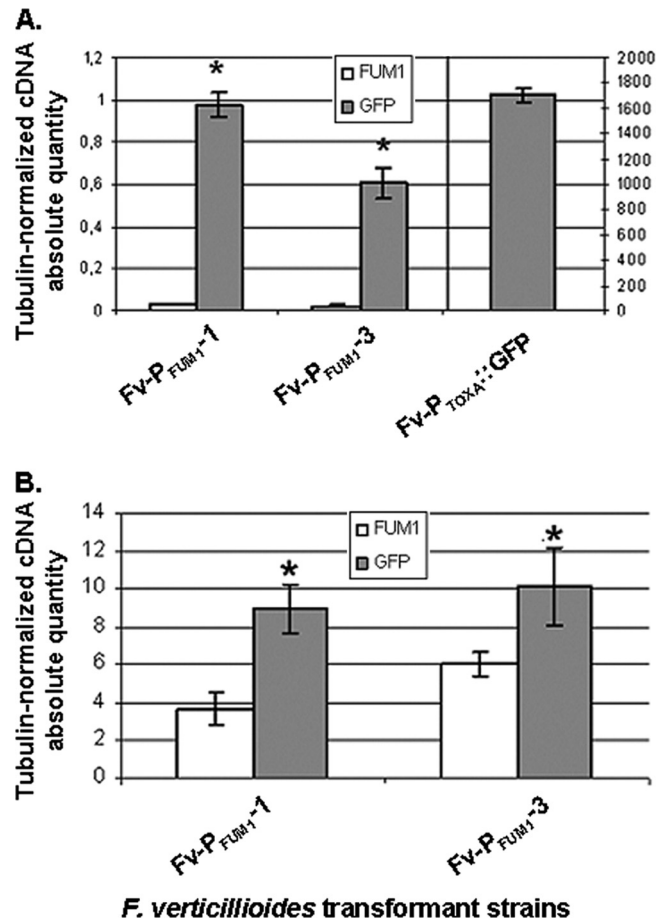
In filamentous fungi, genes needed for the production of secondary metabolites are often grouped in clusters of coordinately regulated, functionally related genes. The evolutionary reasons for clustering have been often debated; the explanation that is best supported by experimental data is that clustering 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-



**FIG 4** (A) Fluorescence micrographs of the *F. verticillioides* transformant strain Fv-P<sub>FUM1</sub>-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<sub>FUM1</sub>-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  $\mu$ m; resolution, 0.36  $\times$  0.36  $\times$  1.5  $\mu$ m).

tures. 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



**FIG 5** *FUM1* and/or *GFP* transcript abundance in the Fv-P<sub>FUM1</sub>-1 and -3 transformants and in the Fv-P<sub>TOXA::GFP</sub> 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.

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

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 Tri6 (a Cys2His2 Zn finger protein), in which a low-level transcription of the trichothecene 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 *FUM1* 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 deacetylation; 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. verticilliooides*, both in the presence of TSA and under FB-inducing conditions, deacetylation 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  $P_{FUM1}$ , 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. verticilliooides* harboring a *GFP* gene under the control of the *FUM1* promoter  $P_{FUM1}$  and inserted at random loci by agroinfection. In these constructs, ectopic  $P_{FUM1}$  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  $P_{FUM1}$  seems to be severalfold higher if  $P_{FUM1}$  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* transcription 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  $P_{FUM1}::GFP$  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- $P_{FUM1}$ -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- $P_{FUM1}$ -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  $P_{FUM1}$  transcriptional strength is about 170-fold weaker than that of a constitutive promoter such as  $P_{TOXA}$  (Fig. 5A) even under FB-inducing conditions, visual assessment of fluorescence is inevitably a lower-resolution test for  $P_{FUM1}$  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  $P_{FUM1}$  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 *FUM21*, 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 *FUM21* regulation. Whether other posttranscriptional histone modifications are involved remains to be determined, but epigenetic control adds to the classical regulatory mechanisms provided by transcription factors and their cognate, *cis*-acting binding domains on promoter regions, which remain inducible 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.

## ACKNOWLEDGMENTS

We are grateful to D. Valentino (DiVaPRA, Università di Torino) for technical help and to Martijn Rep (Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands) for the kind gift of pCAM-P<sub>TOXA</sub>::GFP.

This work was supported by Regione Piemonte with a contribution to I.V.'s research fellowship.

I.V. and F.C. designed the experiments. I.V., V.M. (Fig. 2 and 3B), and K.D. (Fig. 2 and Fig. S2 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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