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FUM13 Encodes a Short Chain Dehydrogenase/Reductase Required for C-3 Carbonyl Reduction during Fumonisin Biosynthesis in *Gibberella moniliformis*

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Fumonisins are polyketide-derived mycotoxins produced by the filamentous fungus *Gibberella moniliformis* (anamorph *Fusarium verticillioides*). Wild-type strains of the fungus produce predominantly four B-series fumonisins, designated FB_1, FB_2, FB_3, and FB_4. Recently, a cluster of 15 putative fumonisin biosynthetic genes (*FUM*) was described in *G. moniliformis*. We have now conducted a functional analysis of *FUM13*, a gene in the cluster that is predicted by amino acid sequence similarity to encode a short chain dehydrogenase/reductase (SDR). Mass spectrometric analysis of metabolites from *FUM13* deletion mutants revealed that they produce approximately 10% of wild-type levels of B-series fumonisins as well as two previously uncharacterized compounds. NMR analysis revealed that the new compounds are similar in structure to FB_3 and FB_4 but that they have a carbonyl function rather than a hydroxyl function at carbon atom 3 (C-3). These results indicate that the *FUM13* protein catalyzes the reduction of the C-3 carbonyl to a hydroxyl group and are the first biochemical evidence directly linking a *FUM* gene to a specific reaction during fumonisin biosynthesis. The production of low levels of FB_1, FB_2, FB_3, and FB_4, which have a C-3 hydroxyl, by the *FUM13* mutants suggests that *G. moniliformis* has an additional C-3 carbonyl reductase activity but that this enzyme functions less efficiently than the *FUM13* protein.

KEYWORDS: Fumonisin; *Gibberella moniliformis*; *Fusarium verticillioides*; mycotoxin; gene cluster

INTRODUCTION

Fumonisins are polyketide-derived secondary metabolites produced by the filamentous fungus *Gibberella moniliformis* Wineland (anamorph *Fusarium verticillioides* (Sacc.) Nirenberg). These toxins, and others such as the AAL toxins of *Alternaria alternata* f. sp. *lycopersici*, have been described as sphingosine analogue mycotoxins because of the structural similarities they share with the sphingolipid intermediate sphingosine. In animal cells, fumonisins have been shown to inhibit sphingosine *N*-acyltransferase thereby blocking sphingolipids biosynthesis (*1*). *G. moniliformis* causes ear and stalk rot of maize but can also be present in maize tissues without causing disease symptoms (*2*). Fumonisin induces leukoencephalomalacia, pulmonary edema, and cancer when ingested by horses, swine, and laboratory rodents, respectively (*3*, *4*), and in some parts of the world where maize is a dietary staple, fumonisins have been implicated in human esophageal cancer (*4*).

Recently, a fumonisin biosynthetic gene cluster has been described on chromosome 1 in *G. moniliformis* (*5*). This cluster consists of 15 coregulated genes (*FUM1* and *FUM6* through *FUM19*). Amino acid sequence analysis indicated that 11 of the clustered genes are predicted to encode enzymes that catalyze biosynthetic reactions; two are predicted to encode transporters; and the two remaining genes are predicted to encode longevity assurance factors, which may have a self-protection function. To date, six *FUM* genes have been examined via disruption analysis. However, these analyses have not revealed the exact function of any of the genes. Disruptions of *FUM1* (formerly *FUM5*), *FUM6*, and *FUM8*, which are predicted to encode a polyketide synthase, cytochrome P450 monooxygenase, and amino transferase, respectively, blocked fumonisin production but did not lead to the accumulation of identifiable fumonisin intermediates (*6*, *7*). Disruption of *FUM17* and *FUM18*, which are predicted to encode longevity assurance factors, had no effect on fumonisin production (*5*). Disruption of *FUM19*, which is predicted to encode an ABC transporter, resulted in a subtle alteration in the ratios of FB_1, FB_2, and FB_3 produced (*5*).

FUM13 is predicted to encode a 369-AA polypeptide with sequence similarity/identity to short chain dehydrogenases/reductases (SDRs). SDRs include enzymes that catalyze the dehydrogenation or reduction of various substrates, such as alcohols, steroids, sugars, and aromatic compounds (*8*). Homology between any two SDRs is generally low. However, two regions are conserved. An amino-terminal nucleotide binding site (Thr-Gly-X_{2-3}-Gly-X_{1-2}-Gly) and the active site, which varies from a conserved triad of Ser, Tyr, and Lys residues to

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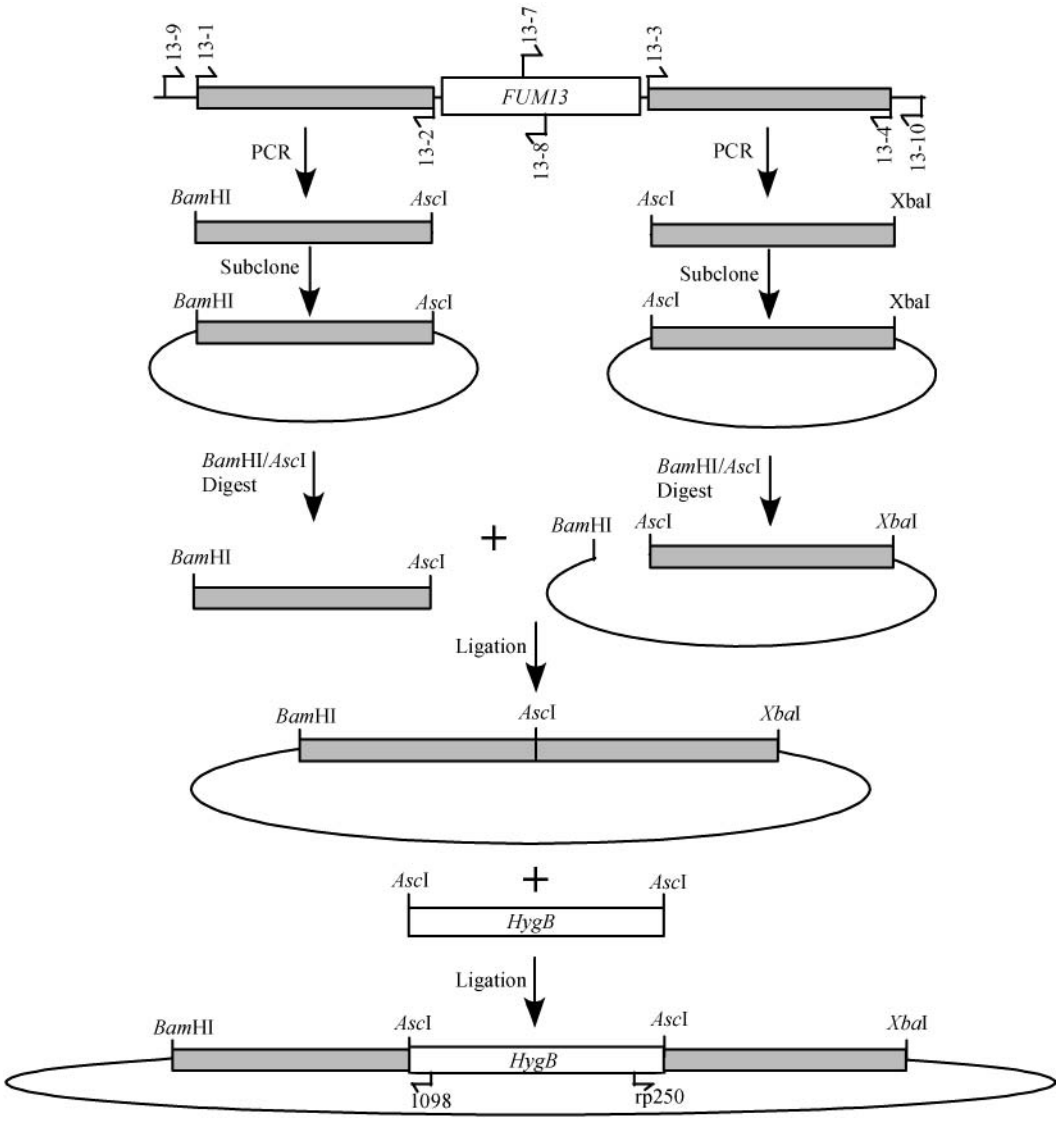


Figure 1. Engineering deletion vector pFUM13KOH. 13-1 through 13-4, 13-9 through 13-12, rp250, and 1098 indicate PCR primer sites. *FUM13* indicates the location of the *FUM13* coding region. *HygB* indicates the hygromycin resistance gene.

conservation of the Tyr residue only (*8*). In the current study, we characterized the function of the *G. moniliformis FUM13* by gene deletion analysis.

MATERIALS AND METHODS

Strains and Media. *G. moniliformis* wild-type strain M-3125 was used throughout this study (*9*). The fungus was cultured on solid V8 juice medium for the production of conidia, in liquid GYEP (5% glucose, 0.1% yeast extract, and 0.1% peptone) for genomic DNA preparation, and in cracked corn medium for analysis of fumonisin production (*7*, *10*).

Fumonisin Analysis. *G. moniliformis* strains were initially screened by culturing on cracked corn for 2 weeks. Fumonisins were extracted with acetonitrile:water (1:1) as previously described (*6*). Extracts were diluted 100-fold with acetonitrile:water, and 10 μL aliquots were analyzed by liquid chromatography/mass spectrometry (LC/MS) as previously described (*11*). For purification and isolation of fumonisins and related compounds, strains were cultured on cracked corn for 4 weeks and extracted as above. The extracts of six 50 g cultures were diluted with five volumes of water. This solution was fractionated using a preparative high-performance liquid chromatography (HPLC) column module (Ranin Instrument Co., Woburn, MA) at a flow rate of 5 mL/min using a linear solvent gradient from 20:80 (acetonitrile:water) to 50:50 over 120 min and a second wash gradient from 50:50 to 70:30 over 60 min followed by a final 60 min wash at 70:30. Fractions (50 mL) were collected during the first gradient wash. Fractions were analyzed by LC/MS, and those containing similar fumonisin homologues were combined from several batches. Combined fractions were extracted with methylene chloride to remove unwanted components and chromatographed again following the protocol above to obtain fractions containing purified fumonisins for structural characterization by NMR. NMR spectra were obtained with a Bruker (Billerica, MA) Avance 400 spectrometer equipped with a 5 mm inverse broadband Z-gradient probe (13C NMR, 100 MHz, 1H, 400 MHz).

Gene Deletion and Transformation. The *FUM13* gene deletion vector, pFUM13KOH, was constructed by amplifying approximately 1 kbp regions flanking both ends of the *FUM13* coding region from cosmid cloned DNA following a polymerase chain reaction (PCR)-based approach developed by Brown et. al (*12*). Primers 13-1 and 13-2 (**Figure 1** and **Table 1**) were used to amplify the region upstream of the *FUM13* start site. Primers 13-3 and 13-4 were used to amplify the region downstream of the *FUM13* stop codon. Each primer included a restriction enzyme site (**Table 1**) that was used in subsequent subcloning steps. Primer 13-1 incorporated a *Bam*HI site, primers 13-2 and 13-3 incorporated *Asc*I sites, and primer 13-4 incorporated an *Xba*I site. **Figure 1** shows the successive subcloning steps. The PCR products were subcloned into plasmid vector pT7-Blue (Novagen). The *Bam*HI/*Asc*I fragment containing the upstream flanking region was subcloned into the vector containing the downstream flanking region. This created

Table 1. PCR Primer Sequences Used in This Study[a]

name	sequence
13-1	GAC*GGATCC*ATGCGCAGCGTACTACTCCG
13-2	GAC*GGCGCGCC*ATAAGTAAGATGACCAACGTGAGGACT
13-3	GAC*GGCGCGCC*AGTACCTTGGCTCGTCTGC
13-4	GAC*TCTAGA*CGATGAACAACTTTCCCG
13-7	CAACGACCTTACCACTACA
13-8	CGTCGAAATGTAGTGGTAAG
13-9	AAGGGTTACGCTGCTATCCC
13-10	CGACGGAATATGCTAGTCTA
rp250	CTGCTGCATTCCCATTCCCATCGT
1098	ACCAAGCCTATGCCTACAGCATCC

[a] Italics indicate restriction endonuclease sites engineered for subcloning procedures. GGATCC, recognition sequence for *Bam*HI; GGCGCGCC, recognition sequence for *Asc*I; and TCTAGA, recognition sequence for *Xba*I.

a vector containing the upstream and downstream flanking regions in the appropriate orientation separated by an *Asc*I site. Last, a 2.5 kbp *Asc*I fragment containing the hygromycin B resistance gene (*HygB*) (*13*) was subcloned into the *Asc*I site of the *FUM13* deletion vector. All PCR products were generated using *Pfu* polymerase (Stratagene), and their nucleotide sequences were determined to verify the absence of any inadvertent mutations. Vector pFUM13KOH was designed such that homologous recombination of the vector with regions upstream and downstream of the *FUM13* coding region would result in replacement of the entire coding region with the *HygB* resistance gene.

Transformation of *G. moniliformis* strain M-3125 was carried out using the protoplast method previously described with circular plasmid DNA and the modifications noted below (*6*). Protoplasts were regenerated on a high osmotic medium (0.1% yeast extract, 0.1% casein-enzyme hydrolysate, 0.8 M sucrose, and 1.6% agar), and hygromycin resistant transformants were selected by overlaying with 1% water agar containing 300 μg/mL hygromycin B. Putative transformants were subsequently transferred to YEPD (0.3% yeast extract, 1% peptone, and 2% glucose) amended with 150 μg/mL hygromycin B. To ensure that each transformant represented only a single strain, all transformants were regrown from a single conidium prior to nucleic acid and fumonisin analysis.

Nucleic Acid Analysis of Transformants. Genomic DNA, for use in PCR, was prepared from mycelia grown on V8 juice agar. The mycelium was scraped from the agar surface and placed in 250 μL of extraction buffer (200mM Tris, pH 8.0, 250 mM NaCl, 25mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)) in a 1.7 mL Eppendorf tube, ground with a micropestle, and incubated for 10 min at 75 °C. After the mixture was heated, 250 μL of a 1:1 mixture of TRIS-saturated phenol:chloroform:isoamyl alcohol (25:24:1) was added and the mixture was vortexed for 30 s. The aqueous phase was separated by centrifugation and removed to a fresh tube. Genomic DNA was purified from this solution using UltraBind following the manufacture's directions (Mo Bio, Solana Beach, CA).

PCR primers were designed to allow the amplification of bands specific to both 5′ and 3′ recombination events. Primer pairs 13-9/13-8 and 13-10/13-7 were used to detect the presence of an intact wild-type *FUM13* at the 5′ and 3′ ends, respectively, of the *FUM13* coding region. Primer pair 13-9/1098 was used to detect homologous recombination between the 1 kb region immediately upstream of the *FUM13* coding region and the deletion vector. Likewise, primer pair 13-10/rp250 was used to detect homologous recombination between the 1 kb region immediately downstream of the *FUM13* coding region and the deletion vector. The position of each primer is shown in **Figure 1**, and the primer sequences are shown in **Table 1**.

Southern blots were performed using standard protocols (*14*) to confirm the deletion of the *FUM13* coding region in transformants. High molecular weight genomic DNA was prepared with the DNeasy plant kit (Qiagen) from GYEP-grown mycelia. DNA of selected transformants was doubly digested with *Xho*I/*HindIII*. Digested DNA was electrophoresed and blotted to nylon membrane. The hybridization probe consisted of a 1381 bp *Xho*I/*Hind*III fragment spanning 779 bp of the 5′ end of *FUM13* plus 602 bp upstream of the start codon. DNA was labeled with ^{32}P using the RediprimeII kit (Amersham Pharmacia Biotech).

RESULTS

***FUM13* Encodes a SDR.** Preliminary RPS-BLAST (*15*) analysis of *FUM13* and subsequent protein alignment compiled with DNAMAN software (Lynnon BioSoft) revealed that the predicted *FUM13* protein shares significant similarity to SDRs. The amino acid identity between any two SDRs is typically low, between 10 and 30% (*8*). This is true for the predicted *FUM13* protein, which shares from 14 to 25% identity with the four proteins to which it is most similar (**Figure 2**). The *FUM13* protein has a putative N-terminal nucleotide binding region at AA 12-18 and a putative active site Tyr residue at AA 119 (**Figure 2**). Alignment with the proteins shown in **Figure 2** illustrates the diversity of the enzymatic reactions catalyzed by SDR proteins. A tblastn search using the *FUM13* nucleotide sequence to search the translated database at NCBI reveals many new homologies to predicted protein sequences arising from the ever-growing database of ESTs. These proteins share significantly more sequence similarity/identity at the amino acid level than the proteins presented in **Figure 2**; however, there are no experimental data to support their function as dehydrogenases/reductases.

Deletion of *FUM13*. We generated deletion mutants of *G. moniliformis FUM13* by transformation of wild-type strain M-3125 with vector pFUM13KOH. This vector was constructed so that its recombination with homologous sequences on both sides of the *FUM13* coding region would result in replacement of the coding region with the hygromycin resistance gene, *HygB*. A PCR strategy was designed to distinguish between transformants with an intact wild-type *FUM13* and those in which the *FUM13* coding region had been deleted. Transformants were first assayed for loss of a PCR product indicative of the wild-type *FUM13* and then for gain of a PCR product indicative of recombination between the vector and the *FUM13* flanking sequences. PCR analysis of 35 hygromycin B resistant isolates recovered following transformation revealed that in two (GMT-13-208 and GMT-13-224) the *FUM13* coding region had been replaced by *HygB*. The remainder had integrated only at one side of *FUM13* or the vector integrations elsewhere in the genome. In subsequent Southern blot analyses, we included one representative transformant (GMT-13-212) that contained a single homologous integration event at the 5′ end of *FUM13* and another representative transformant (GMT-13-201) that contained an ectopic integration of the vector elsewhere in the genome. Southern analysis confirmed the deletion of the *FUM13* coding region in transformants GMT-13-208 and GMT-13-224 and the presence of the wild-type *FUM13* band in transformants GMT-13-201 and GMT-13-212 (**Figure 3**). Replacement of the *FUM13* coding region with *HygB* results in the loss of a 1381 bp band and the gain of a 3305 bp band (containing *HygB*) when hybridized with the 1381 bp *Xho*I/*Hind*III fragment described in the Materials and Methods.

Fumonisin Analysis. The two *FUM13* deletion mutants, the 5′ integration transformant, the ectopic integration transformant, and their wild-type progenitor strain, M-3125, were cultured on cracked corn to assess their ability to produce fumonisins. Initial LC/MS analysis of cracked maize culture extracts indicated that production of FB_1, FB_2, FB_3, and FB_4 was reduced by at least 90% in the *FUM13* deletion mutants as compared to the wild-type strain and the other transformants. Fumonisin levels in uninoculated cracked corn were below detectable levels.

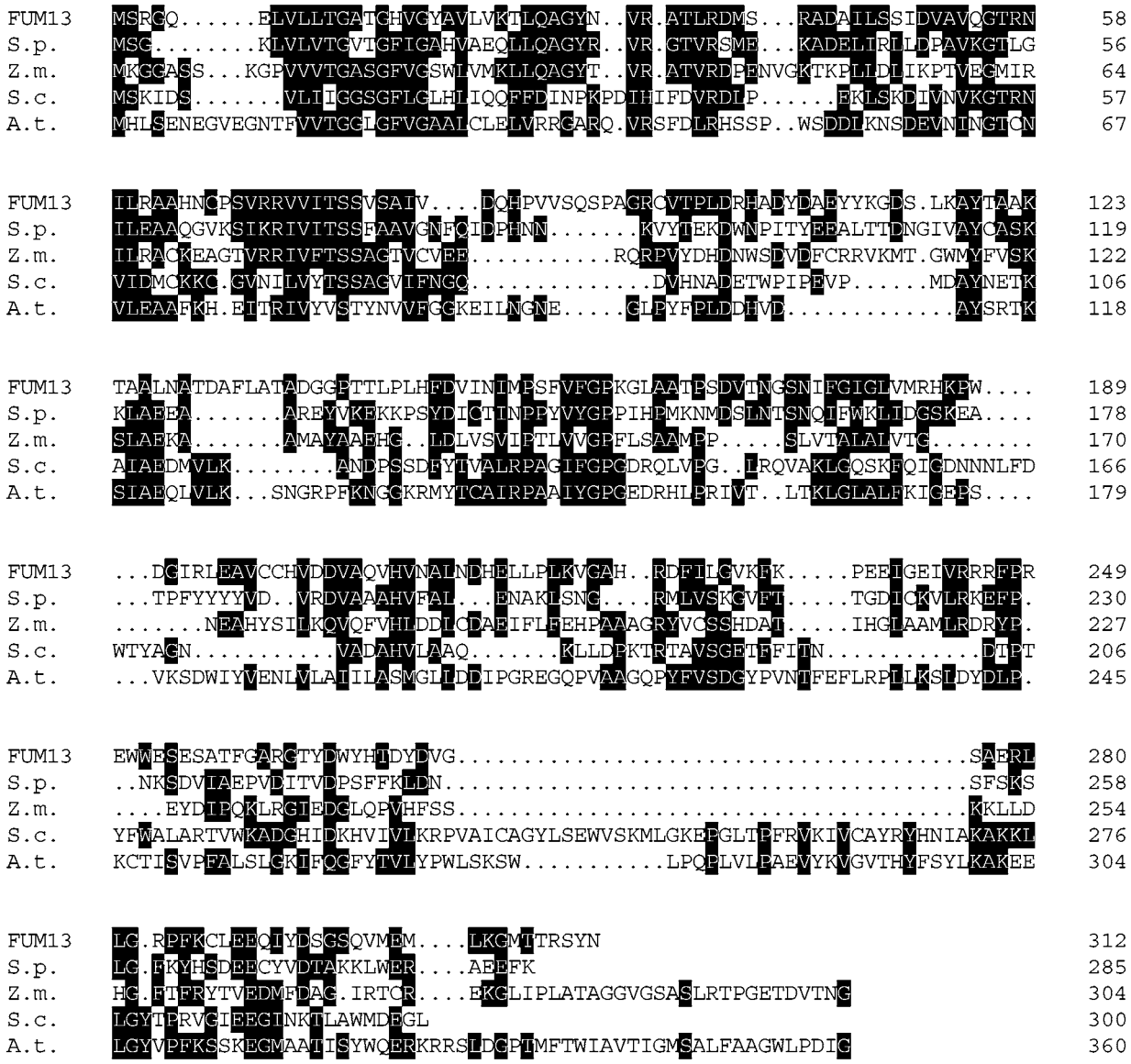


Figure 2. Amino acid comparison between *FUM13* and four closely related SDR-like proteins. S.p., *Schizosaccharomyces pombe* putative cinnamoyl-CoA reductase (GI:19114893); Z.m., *Zea mays* dihydrokaempferol 4-reductase (GI:7427698); S.c., *S. cerevisiae* putative 3β-hydroxysteroid dehydrogenase (GI:1723793); A.t., *Arabidopsis thaliana* putative steroid dehydrogenase (GI:2459443).

The LC/MS analysis of *FUM13* deletion mutants also revealed that they produce several metabolites not seen in the wild-type strain (**Figure 4**). Relative amounts of these metabolites were approximately 60−80% of the total amount of fumonisins B_1, B_2, and B_3 from the wild-type. The two major metabolites observed in the deletion strains had protonated molecular ions at *m*/*z* 704 and 688. These molecular ions correspond to a loss of two mass units from FB_2/FB_3 and FB_4, respectively, and are consistent with the presence of a carbonyl group at C-3 of the fumonisin backbone. These two new metabolites were proposed to be 3-keto homologues of FB_3 and FB_4 based on their HPLC retention times. After the *m*/*z* 704 metabolite was purified, it was hydrolyzed with 2 N KOH in methanol. LC/MS analysis of the resulting mixture revealed a component that eluted slightly earlier than the expected retention time of hydrolyzed FB_3 with a signal at *m*/*z* 388, which is consistent with the molecular weight of 387 expected for hydrolyzed 3-keto FB_3. Similarly, the LC/MS of hydrolyzed products of the purified *m*/*z* 688 metabolite revealed a component with a signal at *m*/*z* 374, which is consistent with the expected molecular weight of hydrolyzed 3-keto FB_4.

NMR analysis revealed that the proton spectrum of the putative 3-keto-FB_3 homologue was identical to the FB_3 spectrum except for three signals that were consistent with the presence of a C-3 carbonyl rather than a C-3 hydroxyl on the 3-keto homologue. First, the FB_3 spectrum included a signal at 3.45 ppm corresponding to the C-3 hydrogen. The spectrum of the putative 3-keto homologue does not include this signal and, therefore, is consistent with the absence of a C-3 hydrogen. Second, the FB_3 spectrum had a signal at 1.3 ppm corresponding to the hydrogen atoms at C-1. In the spectrum of the 3-keto homologue, this second signal was shifted to 1.49 ppm, a shift that was consistent with the C-1 hydrogen atoms being β to a carbonyl group rather than to a hydroxyl group. Finally, the FB_3 spectrum included a signal at 3.1 ppm corresponding to the hydrogen at C-2. In the spectrum of the 3-keto homologues, this third signal was shifted to 4.14 ppm, a shift that was consistent with the C-2 hydrogen being adjacent to a carbon rather than to a hydroxyl carbon.

The carbon NMR spectra of FB_3 (*16*) and the 3-keto-FB_3 homologue were also identical except that the signal in the FB_3 spectrum at 73.10 ppm, which corresponds to C-3, was shifted

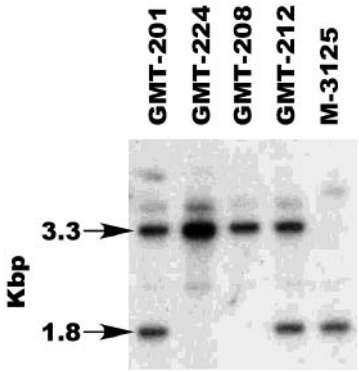
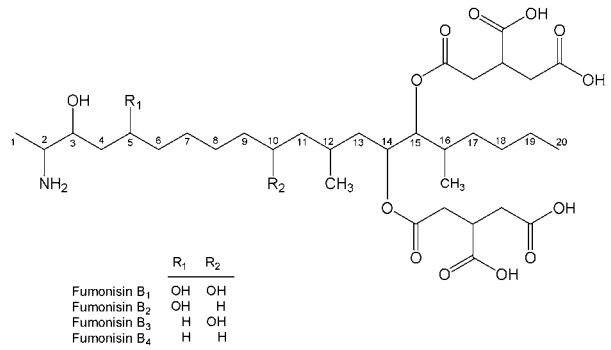
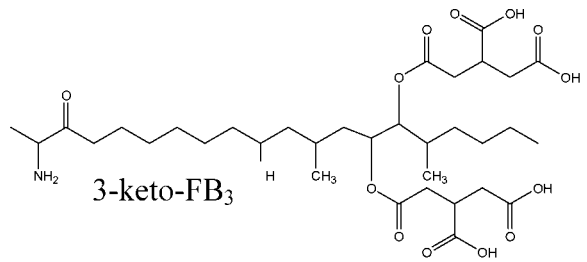
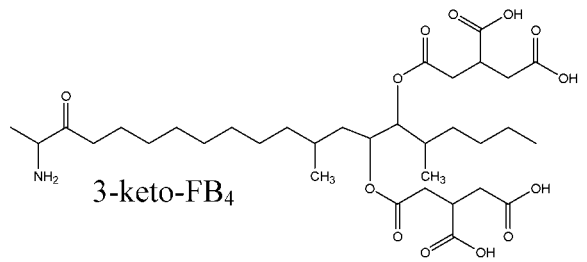


Figure 3. Southern analysis of *G. moniliformis* transformants. Genomic DNA was prepared from four transformants plus the wild-type progenitor strain as described in the Materials and Methods. Using a probe to the 5′ end of the gene, strain M-3125 shows the hybridization of the 1381-bp wild-type *FUM13* band. Strains GMT-224 and GMT-208 contain deletions of *FUM13* as indicated by the shift of the hybridizing band to 3305-bp and the absence of the wild-type hybridization band. Strains GMT-201 and GMT-212 harbor ectopic copies of the deletion construct and thus have both the wild-type *FUM13* hybridization band and the 3305-bp band.



A.





B.

Figure 4. **(A)** Major fumonisin homologues produced by wild-type strain M-3125. **(B)** Major fumonisin homologues produced by *G. moniliformis FUM13* deletion mutants.

to 207.64 ppm in the spectrum of the keto homologue. **Table 2** lists the carbon shifts for fumonisin B_3 (*16*, *17*) and the 3-keto-FB_3 homologue that accumulates in the *FUM13* deletion mutant. This shift at carbon atom 3 is consistent with the presence of a carbonyl carbon rather than a hydroxyl carbon.

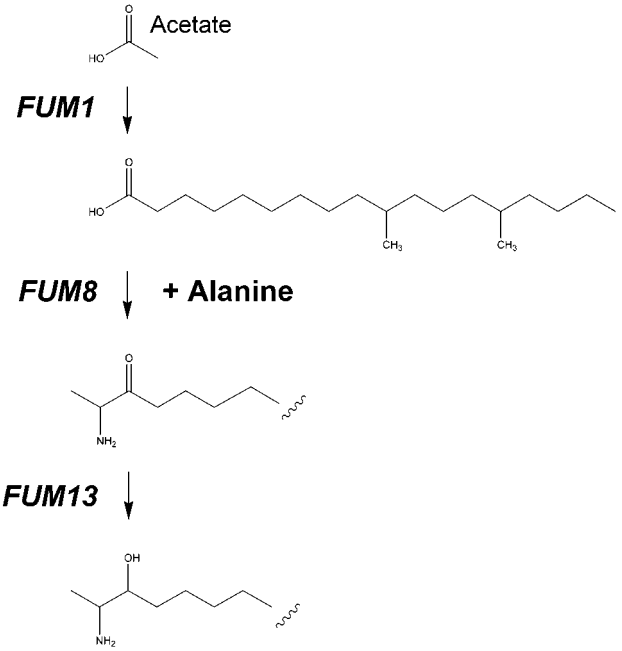


Figure 5. Proposed role of the *FUM13* protein in the modification of the carbonyl end of the putative fumonisin polyketide during fumonisin biosynthesis.

Table 2. ^{13}Carbon NMR Shift for 3-Keto Fumonisin B_3

carbon	FB_3	3-keto-FB_3	carbon	FB_3	3-keto-FB_3
1	15.9q	20.7q	18	30.7t	30.0t
2	53.5d	44.6d	19	23.8t	24.0t
3	73.1d	207.6d	20	14.4q	15.8q
4	34.6t	39.1t	21	16.0q	16.1q
5	26.2t	24.2t	22	20.6q	20.8q
6	26.1t	29.7t	25	173.1s	173.3s
7	26.8t	30.6t	26	36.6t	39.2t
8	29.6t	26.2t	27	38.6d	39.4d
9	39.3t	39.2t	28	36.1t	36.8t
10	69.9d	70.0d	29	177.0s	176.7s
11	44.5t	44.7t	30	175.2s	178.2s
12	26.9d	30.0d	33	173.0s	173.2s
13	36.4t	37.1t	34	36.6t	36.8t
14	73.1d	73.0d	35	38.6d	39.1d
15	78.8d	78.8d	36	36.1t	37.1t
16	34.9d	33.1d	37	176.6s	176.2s
17	33.1t	33.1t	38	175.0s	177.7s

DISCUSSION

On the basis of the chemical structures of fumonisins and precursor feeding studies, fumonisin biosynthesis is predicted to include at least five groups of biochemical reactions: (i) synthesis of a linear polyketide with a single terminal carbonyl function and methyl groups at C-10 and C-14; (ii) condensation of the polyketide with alanine; (iii) reduction of the polyketide carbonyl to a hydroxyl; (iv) hydroxylation of 2−4 polyketide carbons; and (v) esterification of six-carbon tricarboxylic acids to two of the hydroxyls. In the current study, the accumulation of 3-keto homologues of FB_3 and FB_4 in *FUM13* deletion mutants indicates that the SDR encoded by *FUM13* catalyzes the reduction of the polyketide carbonyl to a hydroxyl during fumonisin biosynthesis (**Figure 5**). To our knowledge, this is the first evidence directly linking a gene in the fumonisin gene cluster with a specific biochemical reaction in fumonisin biosynthesis. The functions of most of the genes in the fumonisin gene cluster were predicted based on the results from BLAST sequence comparison (*15*). However, previous attempts to

confirm the predicted functions of five of the genes (*FUM6*, *FUM8*, *FUM17*, *FUM8*, and *FUM19*) by gene disruption were not successful. For example, in the cases of *FUM6* and *FUM8*, disruption blocked fumonisin production but did not result in accumulation of unusual fumonisin homologues that would provide evidence for the activities of the enzymes encoded by these genes (*6*, *7*). By contrast, in this study, the accumulation of 3-keto FB_3 and 3-keto FB_4 homologues provides evidence that *FUM13* encodes a C-3 carbonyl reductase.

The 3-keto fumonisin homologues produced by *FUM13* deletion mutants provide further evidence that fumonisin biosynthesis uses a biosynthetic pathway analogous to that of the sphingolipid intermediate, sphinganine (*18*). Sphinganine synthesis begins with the condensation of palmitoyl-CoA and Ser to yield 3-ketosphinganine. The carbonyl of 3-ketosphinganine is then reduced to a hydroxyl to yield sphinganine. In *Saccharomyces cerevisiae*, a 3-ketosphinganine reductase encoded by *TSC10* catalyzes this latter reaction and is predicted to be an SDR based on sequence analysis (*19*). Even though the *TSC10* and *FUM13* proteins are both SDRs and they catalyze analogous reactions, the two proteins are only about 10% identical over their entire length (data not shown).

Similarities between the structures and the biosynthesis of fumonisins and sphinganine suggest that the fumonisin C-3 oxygen is derived from acetate via a polyketide (*5*). This hypothesis is supported by mass spectrometric data on FB_1 produced by *G. moniliformis* cultures in $^{18}O_2$ or $H_2{}^{18}O$ feeding experiments carried out previously by Caldas et al. (*20*). When $H_2{}^{18}O$ was fed to the cultures, up to seven ^{18}O atoms were incorporated into FB_1. Upon hydrolysis, which cleaves the tricarballylic esters from the fumonisin backbone, the FB_1 backbone lost all but one of the ^{18}O atoms. These results indicate that only one ^{18}O atom was attached directly to the fumonisin backbone and that the others were incorporated into the tricarballylic esters. In contrast, when $^{18}O_2$ was fed to the cultures, up to four ^{18}O atoms were incorporated into FB_1. Upon hydrolysis, the FB_1 backbone retained all four ^{18}O atoms. Although the Caldas et al. paper did not state it, the data presented (*20*) are most consistent with four of the five oxygen atoms attached directly to the FB_1 backbone being derived from $^{18}O_2$ and the fifth being derived from $H_2{}^{18}O$. We propose that the C-3 oxygen is derived from $H_2{}^{18}O$. C-3 of the fumonisin backbone corresponds to C-1 of the polyketide precursor. In nascent polyketides, the C-1 position typically has a carbonyl function that corresponds to the carbonyl function of the final acetate incorporated into a polyketide. If in the feeding studies described by Caldas et al. (*20*) the ^{18}O atom from $H_2{}^{18}O$ was incorporated into acetate (e.g., via exchange of oxygen between water and carboxylic acid), it could be incorporated into the polyketide precursor of fumonisins via the activity of the polyketide synthase encoded by *FUM1* (*6*). In contrast, the oxygen atoms at C-5, C-10, C-14, and C-15 of the fumonisin backbone could be derived from O_2 by the activities of the monooxygenases (*FUM6*, *FUM12*, and *FUM15*) and dioxygenase (*FUM9*) encoded by the fumonisin gene cluster (*5*). These oxygenases utilize O_2 to catalyze the formation of hydroxyl functions. This seems likely for the C-10 and C-14 oxygen atoms because they do not correspond to acetate carbonyls and therefore should be added to the fumonisin backbone after it is formed. Likewise, the C-5 oxygen is absent in FB_3 and FB_4 and therefore is also most likely added to the backbone after it is formed.

Although the *FUM13* protein is most likely to catalyze the reduction of C-3 carbonyl of fumonisins in *G. moniliformis*, it is not essential for fumonisin production. This was evident by the production of low levels of FB_1, FB_2, FB_3, and FB_4 in the *FUM13* deletion mutants. It is possible that these C-3-hydroxylated fumonisins are produced in the deletion mutants because of a partial redundancy of *FUM13*. That is, *G. moniliformis* may produce another enzyme(s) with the same activity as *FUM13*. Fumonisin production by *FUM17*, *FUM18*, and *FUM19* disruption mutants indicates that these genes may also have redundant functions (*5*). Nevertheless, the low levels of C-3-hydroxylated fumonisins produced by the *FUM13* mutants indicate that if a second enzyme with C-3 carbonyl reductase activity exists, its activity is less than that of the *FUM13* protein. A possible candidate for a second enzyme with this activity is 3-ketosphinganine reductase (*19*). As noted above, the carbonyl reduction of 3-ketosphinganine is analogous to the C-3 carbonyl reduction during fumonisin biosynthesis. Although a pathway for sphingolipid biosynthesis has not been described in *G. moniliformis*, it is likely that the fungus produces these lipids and has a *TSC10* homologue because other *Fusarium* species produce sphingolipids (*21*).

The accumulation of 3-keto homologues of FB_3 and FB_4 but not the equivalent FB_1 and FB_2 homologues in cultures of *FUM13* deletion mutants suggests that during fumonisin biosynthesis the C-3 carbonyl reduction must occur before the C-5 hydroxylation. The C-5 hydroxyl is absent in FB_3, FB_4, and their 3-keto homologues but present in FB_1 and FB_2. If the C-5 hydroxylation could occur before the C-3 carbonyl reduction, *FUM13* mutants would be expected to accumulate 3-keto homologues of FB_1 and FB_2 along with those of FB_3 and FB_4. In contrast, the C-10 position of the fumonisin backbone can be hydroxylated independently of C-3 carbonyl reduction. This is evident because the only structural difference between the 3-ketoFB_3 and the 3-ketoFB_4 is the C-10 hydroxyl; it is present in 3-ketoFB_3 and absent in 3-ketoFB_4. These findings are consistent with precursor feeding experiments that indicated that the C-10 hydroxylation occurs earlier in fumonisin biosynthesis and that the C-5 hydroxylation occurred late in the biosynthesis (*22*).

Over the past decade, it has become evident that genes involved in the biosynthesis of a given fungal secondary metabolite tend to be clustered into groups of coregulated genes. Gene clusters have been described for mycotoxin biosynthetic pathways as well as for antibiotic biosynthetic and metabolite utilization pathways in fungi (*23*, *24*). The description and characterization of these gene clusters have facilitated elucidation of the biochemical pathways for the corresponding metabolites and provided insight into the regulatory mechanisms of metabolite production or utilization (*12*, *23*, *25*). The recent description of a fumonisin biosynthetic gene cluster in *G. moniliformis* (*5*) has allowed us to further characterize the biochemical pathway leading to the formation of fumonisins. *FUM13*, predicted to encode a SDR, is part of this biosynthetic gene cluster in *G. moniliformis* and through deletion analysis has been shown to be involved in fumonisin biosynthesis. Currently, we are disrupting the remaining genes in the *FUM* gene cluster to determine their functions in fumonisin biosynthesis. These studies should also provide further insight into the sequence of biochemical reactions that lead to the formation of fumonisins.

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