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Fumonisin Production in the Maize Pathogen *Fusarium verticillioides*: Genetic Basis of Naturally Occurring Chemical Variation

ROBERT H. PROCTOR,* RONALD D. PLATTNER, ANNE E. DESJARDINS, MARK BUSMAN, AND ROBERT A. E. BUTCHKO

National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Fumonisins are polyketide-derived mycotoxins produced by the maize pathogen *Fusarium verticillioides*. Previous analyses identified naturally occurring variants of the fungus that are deficient in fumonisin C-10 hydroxylation or that do not produce any fumonisins. In the current study, gene deletion and genetic complementation analyses localized the C-10 hydroxylation deficiency to a cytochrome P450 monooxygenase gene in the fumonisin biosynthetic gene (*FUM*) cluster. Sequence analysis indicated that the hydroxylation deficiency resulted from a single nucleotide insertion that caused a frame shift in the coding region of the gene. Genetic complementation localized the fumonisin-nonproduction phenotype to the polyketide synthase gene in the *FUM* cluster, and sequence analysis indicated that the nonproduction phenotype resulted from a nucleotide substitution, which introduced a premature stop codon in the coding region. These results provide the first direct evidence that altered fumonisin production phenotypes of naturally occurring *F. verticillioides* variants can result from single point mutations in the *FUM* cluster.

KEYWORDS: *Fusarium verticillioides*; fumonisins; fumonisin biosynthesis; biosynthetic gene; polyketide synthase; cytochrome P450 monooxygenase

INTRODUCTION

Fumonisins are polyketide mycotoxins that can accumulate in maize and can cause animal health problems, including cancer and neural tube defects in laboratory rodents (*1*, *2*). Consumption of fumonisin-contaminated maize has also been epidemiologically correlated with human esophageal cancer and neural tube defects in some regions of the world (*3*, *4*). Fumonisins are produced by several relatively closely related species of the genus *Fusarium* (*5*, *6*). Of these fungal species, *F. verticillioides* (sexual state *Gibberella moniliformis*) has received the most attention because of its widespread occurrence on maize and its ability to cause maize ear and stalk rot (*7*). *F. verticillioides* typically produces fumonisins B_1, B_2, B_3, and B_4 (FB_1, FB_2, FB_3, and FB_4, respectively). These compounds share a linear 20-carbon backbone with an amine at carbon atom 2 (C-2) and tricarboxylic acid moieties esterified to C-14 and C-15 (*8*). The compounds differ from one another by the presence or absence of hydroxyl functions at C-5 and C-10 (**Figure 1**). FB_1 has both hydroxyls, FB_2 lacks the C-10 hydroxyl, FB_3 lacks the C-5 hydroxyl, and FB_4 lacks both the C-5 and C-10 hydroxyls. In general, FB_1 is the most abundant fumonisin (~70%) in naturally contaminated maize and in cultures of most field
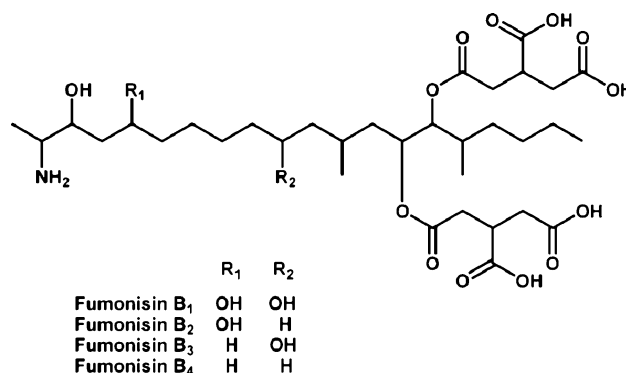
	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH
Fumonisin B_4	H	H

Figure 1. Chemical structures of fumonisins B_1, B_2, B_3, and B_4.

isolates of *F. verticillioides*, whereas FB_2, FB_3, and FB_4 are less abundant (*9*).

In surveys of *F. verticillioides* aimed at examining the diversity of fumonisin chemotypes and their potential in the biological control of fumonisin contamination, researchers have identified naturally occurring variants of the fungus with relatively rare chemotypes (*10*, *11*). One variant isolated from maize from Nepal produced no fumonisins; another variant isolated from maize from South Carolina was deficient in C-5 hydroxylation and, as a result, produced only FB_3 and FB_4; and a third variant, also isolated from maize from South Carolina,

* Corresponding author [telephone (309) 681-6380; fax (309) 681-6686; e-mail proctorh@ncaur.usda.gov].

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Table 1. *F. verticillioides* Strains Used in the Study

strain	fumonisin production[a]	genotype[a]	strain origin	parent strains		ref
M-3125	FB$_1$, FB$_2$, FB$_3$, FB$_4$	*Fum1-1, Fum2-1*	field isolate	N/A[b]		35
ISU-93-152	none	nd[b]	field isolate	N/A		31
109-R-14	FB$_2$, FB$_4$	*Fum1-1, Fum2-2*	meiotic progeny	M-3120[c] (♀)		13
				A-0822[c] (♂)		
982-R-50	FB$_2$, FB$_4$	*Fum1-1, Fum2-2*	meiotic progeny	GfA2364 (♀)	A.E.D., R.D.P., and R.H.P., unpublished results	
				648-R-183[c] (♂)		21
57-7-7	none	*Fum1-2, Fum2-1*	meiotic progeny	M-5500[c] (♀)		13
				M-3125 (♂)		
GfA2364[d]	none	*Fum2-1*	*FUM1* disruption mutant	N/A		20

a Fumonisin production phenotypes and genotypes were determined previously by LC-MS and meiotic analyses (*13, 21*). *Fum1-1* and *Fum2-1* are functional alleles. *Fum1-2* and *Fum2-2* are nonfunctional alleles that originated in strains M-5500 and A-0822, respectively (see footnote d for more details on these two strains) (*11, 13*).*b* nd indicates not determined, and N/A indicates not applicable. *c* Strain M-3120 was a field isolate from California, had the *Fum1-1/Fum2-1* genotype, and produced the wild-type complement of FB$_1$, FB$_2$, FB$_3$, and FB$_4$ (*35*). Strain A-0822 is a field isolate from South Carolina, had the *Fum1-1/Fum2-2* genotype, and produced FB$_2$ and FB$_4$ only (*11*). Strain 648-R-193 was a meiotic progeny derived from strain A-0822, had the *Fum1-1/Fum2-2* genotype, and produced FB$_2$ and FB$_4$ only (*13, 21*). Strain M-5500 was a field isolate from Nepal, had the *Fum1-2/Fum2-1* genotype, and produced no fumonisins. *d* Strain GfA2364 was generated from wild-type strain M-3125 by disruption of the *FUM1* gene (*20*). The *Fum1* genotype for strain GfA2364 is not included here because, on the basis of the results of the current study, it has neither the functional *Fum1-1* allele nor the nonfunctional *Fum1-2* allele.

was deficient in C-10 hydroxylation and, as a result, produced only FB$_2$ and FB$_4$. Meiotic analyses indicated that these altered production phenotypes were caused by nonfunctional alleles at three closely linked loci. The nonproduction phenotype resulted from a nonfunctional allele at the *Fum1* locus (*12*), the C-10 hydroxylation deficiency resulted from a nonfunctional allele at the *Fum2* locus, and the C-5 hydroxylation deficiency resulted from a nonfunctional allele at the *Fum3* locus (*13*).

Molecular genetic analysis of *F. verticillioides* has identified a fumonisin biosynthetic gene (*FUM*) cluster that consists of 15 coregulated genes, all of which exhibit a pattern of expression that is correlated with fumonisin production (*14*). The roles of some of the clustered *FUM* genes in fumonisin biosynthesis have been determined (*15−19*). Several studies have provided indirect evidence for relationships between natural variations in fumonisin production and the clustered *FUM* genes. Three lines of evidence suggest that the naturally occurring fumonisin-nonproduction phenotype may result from a mutation in or near the *FUM* cluster. First, fumonisin-nonproducing strains of *F. verticillioides* can be generated by disruption (inactivation) of three *FUM* genes, the polyketide synthase gene *FUM1* (previously *FUM5*), the cytochrome P450 monooxygenase gene *FUM6*, and the oxoamine synthase gene *FUM8* (*18, 20*). Second, meiotic analysis indicated that the naturally occurring mutation that caused the fumonisin-nonproduction phenotype was loosely linked to *FUM1* and therefore the *FUM* cluster (A.E.D., R.D.P., and R.H.P., unpublished results). Finally, introduction of cosmid clone 16-1, which had the entire *FUM1* gene, only part of *FUM6*, and no other known *FUM* genes, restored fumonisin production in strain 57-7-7, which has the naturally occurring nonproduction phenotype that originated in Nepal (*20*). In contrast, cosmid clone 2-2, which had only part of *FUM1* and no other known *FUM* genes, did not restore production to strain 57-7-7 (*20*). Although the latter results suggest that a mutation within *FUM1* is responsible for the naturally occurring nonproduction phenotype, they are inconclusive. Because cosmid clone 16-1 was only partially characterized, the results do not rule out the possibility that some other gene(s) in clone 16-1 restored fumonisin production. There is even less evidence for an association between the C-10 hydroxylation deficiency and genes in the *FUM* cluster. Neither cosmid clone 2-2 nor 16-1 restored production of the wild-type complement of FB$_1$, FB$_2$, FB$_3$, and FB$_4$ when introduced into a strain with the naturally occurring C-10 hydroxylation deficiency (*20*). Thus, the relationships of the fumonisin nonproduction

and C-10 hydroxylation-deficient phenotypes to the *FUM* gene cluster remain unresolved.

There is strong, albeit indirect, evidence for an association between the naturally occurring C-5 hydroxylation deficiency and the *FUM* cluster. The evidence was obtained from two laboratory-induced mutant strains of *F. verticillioides* that have the C-5 hydroxylation deficiency. The first mutant was generated by UV light mutagenesis (*21*), and the second was generated by specific deletion of a dioxygenase gene (*FUM3*) in the *FUM* cluster (*22*). Sequence analysis of the UV-light-induced mutant revealed a mutation in *FUM3* that was predicted to render the gene nonfunctional. Because both laboratory-generated mutations of *FUM3* resulted in the C-5 hydroxylation deficiency, it is likely that naturally occurring C-5 hydroxylation deficient strains also have a defect in *FUM3*.

The objective of this study was to elucidate the molecular genetic basis of the fumonisin-nonproduction and C-10 hydroxylation-deficient phenotypes that occur in natural variants of *F. verticillioides*. The results of this study will contribute to understanding fumonisin biosynthesis and the significance of different fumonisin chemotypes in the ecology of *F. verticillioides* and in maize agriculture. Some of the results of the current study have been previously reported in an abstract (*23*).

MATERIALS AND METHODS

Fungal Strains and Media. Previously identified strains of *F. verticillioides* used in this study and their fumonisin-production phenotypes are listed in **Table 1**. Strain ISU-93-152 was provided by Professor Donald White (University of Illinois). For production of conidia, strains were grown on V-8 juice agar medium (*24*) for 7−10 days, and for DNA preparation, strains were grown in liquid GYEP medium (0.3% glucose, 0.1% yeast extract, 0.1% peptone) for 2−3 days.

Nucleic Acid Manipulations. *F. verticillioides* genomic DNA was prepared from dried mycelia with the DNeasy Plant Mini Kit as described by the manufacturer (Qiagen, Valencia, CA). All DNA plasmids used as transformation vectors in deletion or complementation experiments were purified from *Escherichia coli* cells with the Midi Prep Kit (Qiagen). All *Fusarium*-derived DNA fragments that were amplified by Polymerase Chain Reaction (PCR) and used in the construction of vectors were amplified with *PfuUltra* (Stratagene, La Jolla, CA) or *Pfx* (Invitrogen Life Technologies, Carlsbad, CA) DNA polymerase. The nucleotide sequences of such fragments were determined to confirm that no errors were introduced during PCR. Sequencing reactions were done with the BigDye Terminator Cycle Sequencing (version 3.1, Applied Biosystems, Foster City, CA)

protocol. Sequencing reactions were passed through a Sephadex G-50 column (Amersham Biosciences AB, Piscataway, NJ), dried in a vacuum centrifuge, and resuspended in HiDi (Applied Biosystems) before electrophoretic analysis on a 3100 Genetic Analyzer (Applied Biosystems). Transformants of *F. verticillioides* were examined by both PCR and Southern blotting according to standard methods (*25*). Labeling of hybridization probes for Southern blots was done with the Prime-a-Gene system (Promega, Madison, WI) or Ready-to-Go (Amersham Biosciences AB) protocol.

Vector Construction, Transformation, and Meiotic Crosses. The *FUM12* deletion vector, pΔFUM12-Hyg, was constructed using the same approach previously described to construct the *FUM3* and *FUM13* deletion vectors (*16*, *22*). The 1.4-kb regions immediately up- and downstream of the *FUM12* coding region were amplified and cloned into pT7Blue (Invitrogen Life Technologies). The primers used to amplify the upstream fragment were 5′-CGTGGATCCTGCCAGAA-GAATGCCGAACCT-3′ (underlined sequence is a *Bam*HI site) and 5′-CATGGCGCGCCAAGAAGGGAGGGACTCGAGTCT-3′ (underlined sequence is an *Asc*I site), and the primers used to amplify the downstream fragment were 5′-CATGGCGCGCCAAGCACAGGT-TGAGTGGGATTG-3′ (underlined sequence is an *Asc*I site) and 5′-GTCGAGCTCAGCACAGCATAGCCCACATGTC-3′ (underlined sequence is a *Sac*I site). The two amplicons were cloned into the same vector (pT7Blue) with the *Bam*HI, *Sac*I, and *Asc*I sites that were introduced via the PCR primers and/or present in the pT7Blue multiple cloning sequence. This cloning step restored the orientation and position of the two fragments found in the *F. verticillioides* genome except that the two fragments were separated by an *Asc*I site instead of the *FUM12* coding region. The *HygB* gene was then inserted between the upstream and downstream fragments via the *Asc*I site to yield pΔFUM12-Hyg. Construction of the *FUM3* (= *FUM9*) deletion vector, pFUM9KOH, was described previously (*22*).

The *FUM12* complementation vector, pCFUM12-Hyg, was prepared by PCR amplification of a fragment spanning from 1052 bp upstream of the *FUM12* start codon to 680 bp downstream of the *FUM12* stop codon. The 3460-bp amplicon was cloned into the pCR XL TOPO vector (Invitrogen Life Technologies). A 2489-bp *Not*I fragment carrying the hygromycin resistance gene *HygB* was introduced into the resulting vector with the *Not*I site in the polylinker region of pCR XL TOPO. *HygB* was modified from pUCH2-8 (*26*, *27*) by introduction of *Not*I sites at both ends of the gene by PCR amplification with primers 5′-CGATGCGGCCGCACCGGCTGCACATGTCAAGG-3′ and 5′-GCATGCGGCCGCACAGTTAAATTGCTAACGCAGTC-3′ (*Not*I sites are underlined).

The *FUM1* complementation vector was constructed from two overlapping DNA fragments obtained by restriction enzyme digestion of cosmid clone 16-1 (*14*, *20*). The first fragment was obtained by *Dra*I digestion of clone 16-1 and spanned from 29 bp upstream of the *FUM1* start codon to 753 bp downstream of the *FUM1* stop codon. The second fragment was obtained by *Spe*I digestion of clone 16-1 and spanned from 1473 bp upstream to 1580 bp downstream of the *FUM1* start codon. The *Dra*I fragment was cloned into pBluescript II (Stratagene), and the resulting construct was cut with *Spe*I and then ligated to the *Spe*I fragment described above. The resulting construct, pCFUM1, consisted of the entire *FUM1* coding region plus 1473 bp of 5′-flanking and 753 bp of 3′-flanking DNA cloned in pBlueScript II. This plasmid was introduced into *F. verticillioides* by cotransformation with plasmid pUCH2-8 as previously described (*28*). pUCH2-8 carries the hygromycin phosphotransferase gene *HygB*, which facilitates selection of transformants by their ability to grow on the antibiotic hygromycin (*26*, *27*).

Transformation of *F. verticillioides* was done with the protoplast method described previously (*20*). Transformants were selected by their ability to grow on hygromycin B at 150 μg/mL (*20*). Sexual crosses of *F. verticillioides* were done with the carrot agar method described previously (*13*, *29*).

Fumonisin Analysis. Strains were grown in 10-g cracked maize kernel cultures and or in 20-mL GYAM medium cultures as previously described (*18*). After a 3-week incubation, cracked maize kernel cultures were extracted with 5 mL of acetonitrile/water (1:1, v/v) per gram of culture. After a 2-week incubation, GYAM cultures were filtered

through 0.2-μm Nalgene filters. Extracts and filtrates were analyzed by HPLC−mass spectrometry (MS) for the presence of fumonisins (*30*). The HPLC system employed an Intersil ODS3 column (10 cm, 5 μm), a flow rate of 0.3 mL min^{-1}, and a gradient solvent system that began with water/methanol/acetic acid (65:35:0.35) and changed to water/methanol/acetic acid (5:95:0.35) over 10 min. The solvent was maintained at the latter ratio for 15 min and then returned to the former ratio over a period of 1 min. The HPLC column was coupled to an API Source of a Finnigan LCQ Deca MS System (ThermoQuest, San Jose, CA) operated in the electrospray (ESI) mode. The MS interface capillary temperature was 255 °C, and the spray voltage was 4.5 kV. The MS scanned for ions from 250 to 950 mass units. FB$_1$, FB$_2$, FB$_3$, and FB$_4$ were identified by retention time in comparison to standards and by their ESI spectra.

RESULTS

Identification of the Fumonisin C-10 Hydroxylase Gene. The *FUM12* gene is located in the *FUM* gene cluster and encodes a cytochrome P450 monooxygenase (*14*). To determine whether this gene is required for fumonisin biosynthesis, we deleted the *FUM12* coding region in wild-type *F. verticillioides* strain M-3125 via transformation with deletion vector pΔFUM12-Hyg. The vector was designed so that homologous recombination between *FUM12* sequences in pΔFUM12-Hyg and in the *F. verticillioides* genome would replace the *FUM12* coding region with the *HygB* gene and thereby delete the coding region. One hundred and twenty-two hygromycin-resistant putative transformants were analyzed by PCR, and only two (strains GfA2874 and GmT201) yielded the amplification products expected for deletion of the *FUM12* coding region and its replacement with *HygB*. The deletion was confirmed by Southern blot analysis in which genomic DNA was digested with restriction endonuclease *Sna*BI and, after electrophoresis and blotting, hybridized to a probe that spanned from 1032 bp upstream to 933 bp downstream of the *FUM12* start codon. The probe hybridized to a 2.9-kb DNA fragment in wild-type strain M-3125 but to a 3.7-kb fragment in the *FUM12* deletion mutants GfA2874 and GmT201 (**Figure 2A**). On the basis of known sequence data, the expected size of the *Sna*BI fragment with *FUM12* in wild-type *F. verticillioides* is 2.9 kb, and the expected size of the *Sna*BI fragment in which the *FUM12* coding region is replaced by the *HygB* gene is 3.7 kb.

LC-MS analysis of cracked maize culture extracts revealed that the two *FUM12* deletion mutants produced only FB$_2$ and FB$_4$, both of which lack the C-10 hydroxyl. In contrast, the progenitor strain M-3125 and transformants in which *FUM12* was not deleted produced the wild-type complement of fumonisins including the C-10-hydroxylated FB$_1$ and FB$_3$. In this experiment, the levels of fumonisins produced in two cultures of M-3125 were 5300 and 12200 μg of FB$_1$, 1900 and 3700 μg of FB$_2$, and 900 and 1300 μg of FB$_3$ per gram of cracked maize culture. The level of FB$_2$ produced by the two *FUM12* disruption mutants was 2400 (GfA2874) to 9200 (GmT201) μg per gram of cracked maize culture. The presence of FB$_4$ was noted but not quantified in this experiment. Given that cytochrome P450 monooxygenases often catalyze hydroxylation reactions, these results indicate that the *FUM12*-encoded monooxygenase catalyzes fumonisin C-10 hydroxylation.

Complementation of the Naturally Occurring C-10 Hydroxylation Deficiency. The inability of *FUM12* deletion mutants to hydroxylate the fumonisin backbone at C-10 suggests that strains with the naturally occurring C-10 hydroxylation deficiency may have a mutation within *FUM12*. To address this possibility, we used transformation to introduce a wild-type copy of *FUM12* into two strains, 109-R-14 and 982-R-50, with the
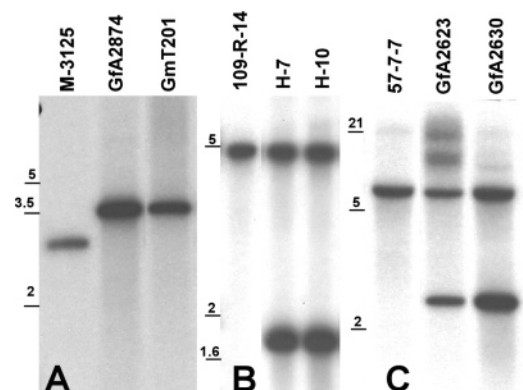
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Figure 2. Southern blots of complementation and deletion strains. (**A**) Deletion of *FUM12* coding region. Lanes: M-3125, wild-type strain; GfA2874 and GmT201, *FUM12* deletion mutants. Under the conditions of this analysis, the wild-type strain was expected to yield a 2.9-kb band and strains in which the *FUM12* coding region was replaced by the *HygB* gene were expected to yield a 3.7-kb band. (**B**) Complementation of the C-10 hydroxylation-deficient phenotype by transformation with the wild-type *FUM12* gene in pCFUM12-Hyg. Lanes: 109-R-14, C-10 hydroxylation-deficient progenitor strain; H7 and H10, strains generated by transformation of strain 109-R-14 with pCFUM12-Hyg. Under the conditions of this analysis, the endogenous *FUM12* gene was expected to be present as a 4.6-kb band and the *FUM12* in vector pFUM12-Hyg was expected to be present on a 1.7-kb band. (**C**) Complementation of the fumonisin-nonproducing phenotype by transformation with the wild-type *FUM1* gene in vector pCFUM1. Lanes: 57-7-7, strain with naturally occurring nonproduction phenotype; GfA2623 and GfA2630, strains generated by transformation of 57-7-7 with pCFUM1. Under the conditions of this analysis, the endogenous *FUM1* from strain 57-7-7 was expected yield a 6.9-kb band and the *FUM1* from vector pCFUM1 was expected to yield a 2.7-kb band. Within panels **A**–**C**, each lane is from the same blot, but lanes between those shown have been removed. Numbers to the left of each panel indicate the positions of selected molecular weight markers (kb) generated by digestion of phage λ DNA with *Eco*RI and *Hin*dIII.

naturally occurring C-10 hydroxylation deficiency. These two strains are meiotic progeny derived from the original C-10 hydroxylation-deficient field isolate A-0822, which was isolated from maize grown in South Carolina (**Table 1**). The meiotic progeny were used in this experiment instead of the original field isolate because they were more amenable to genetic analysis. The wild-type copy of *FUM12* was present in complementation vector pCFUM12-Hyg, and transformants were selected by their ability to grow on hygromycin B-amended media.

LC-MS analysis of GYAM culture filtrates revealed that 9 of 11 109-R-14 transformants and 4 of 7 982-R-50 transformants produced the wild-type complement of FB_1, FB_2, FB_3, and FB_4. In contrast, strains 109-R-14 and 982-R-50 and the remaining transformants produced only FB_2 and FB_4. In this experiment, the 15 complemented transformants of fumonisin production ranged from 7 to 312 μg of FB_1, from 1 to 43 μg of FB_2, from 1 to 49 μg of FB_3, and from <1 to 6 μg of FB_4 per milliliter of GYAM medium. Fumonisin production by the two progenitor strains 109-R-14 and 982-R-50 ranged from 73 to 84 μg of FB_2 and from 2 to 6 μg of FB_4 per milliliter of GYAM medium.

The presence of the complementation vector in transformants was confirmed by Southern blot analysis (**Figure 2B**). In this analysis, genomic DNA was digested with *Eco*RI, electrophoresed, blotted, and hybridized to a ^{32}P-labeled probe corresponding to nucleotides 816–1593 of the *FUM12* coding region. On the basis of known sequence data, the region of the

endogenous *FUM12* complementary to the hybridization was predicted to be present as a 4.6-kb *Eco*RI fragment, whereas the same region in the complementation vector pCFUM12-Hyg was predicted to be present as a 1.7-kb *Eco*RI fragment. In the Southern analysis, strains 109-R-14 and 982-R-50 had the 4.6-kb fragment only, but all transformants with wild-type fumonisin production had both the 4.6- and 1.7-kb fragments (**Figure 2B**). Thus, introduction of a wild-type *FUM12* gene into strains with the naturally occurring C-10 hydroxylation deficiency could restore wild-type fumonisin production.

***FUM12* Sequence in a C-10 Hydroxylation-Deficient Strain.** Complementation of the naturally occurring C-10 hydroxylation deficiency via transformation with a wild-type *FUM12* indicates that there is a mutation within *FUM12* in the deficient strains. To address this possibility, the entire *FUM12* coding region was amplified by PCR from strain 109-R-14 and then sequenced. The sequence analysis revealed six nucleotide differences in the amplified *FUM12* coding region compared to the previously described wild-type *FUM12* sequence (Gen-Bank accession AF155773). All differences were confirmed in two independently amplified PCR products. Five of the differences were nucleotide substitutions; two of these altered and three did not alter the amino acid specificity of the codons in which they occurred. The sixth difference was a T insertion between nucleotides 72 and 73 of the *FUM12* coding region. The insertion caused a frame shift that introduced multiple stop codons into the *FUM12* coding region. Such a frame shift would cause incorrect translation as well as truncation of the *FUM12*-encoded cytochrome P450 monooxygenase. Thus, the frame shift mutation would almost certainly render *FUM12* nonfunctional.

Generation of C-5 and C-10 Hydroxylation-Deficient Strain. If the *FUM12*-encoded P450 monooxygenase catalyzes fumonisin C-10 hydroxylation and, as previously demonstrated, the *FUM3*-encoded dioxygenase catalyzes C-5 hydroxylation (*17*, *22*), a double mutant in which both *FUM12* and *FUM3* are nonfunctional should be deficient in both C-5 and C-10 hydroxylation. To test this hypothesis, the *FUM3* gene was deleted in the C-10 hydroxylation-deficient strain 982-R-50. Construction of the *FUM3* deletion vector and analysis of transformants were done as described previously for *FUM3* deletion in wild-type strain M-3125 (*22*). PCR analysis indicated that *FUM3* was deleted in only 1 of 52 transformants examined. LC-MS analysis of cracked maize extracts of this *FUM3* deletion mutant revealed that it produced only FB_4, which lacks both the C-5 and C-10 hydroxyls (**Figure 3**). As far as we are aware, this fumonisin production phenotype has not been described previously. In this experiment, the progenitor strain 982-R-50 produced 38 μg of FB_2 and 11 μg of FB_4 per milliliter of GYAM and the *FUM3* deletion mutant (in the genetic background of strain 982-R-50) produced 21 μg of FB_4 per milliliter of GYAM medium.

Characterization of a Naturally Occurring Fumonisin-Nonproducing Variant. LC-MS analysis of cracked maize cultures of *F. verticillioides* field isolate ISU-93-152 (**Table 1**) revealed that it did not produce detectable levels of fumonisins. In the same experiments, wild-type strain M-3125 produced approximately 8000 μg of FB_1, FB_2, and FB_3 combined per gram of cracked maize culture. To examine the inheritance of the fumonisin-nonproducing phenotype, strain ISU-93-152 was sexually crossed to wild-type strain M-3125. Of 20 single-ascospore progeny examined, 6 produced the wild-type complement of FB_1, FB_2, and FB_3 and 14 did not produce fumonisins. Strain ISU-93-152 was also crossed with strain GfA2364, a
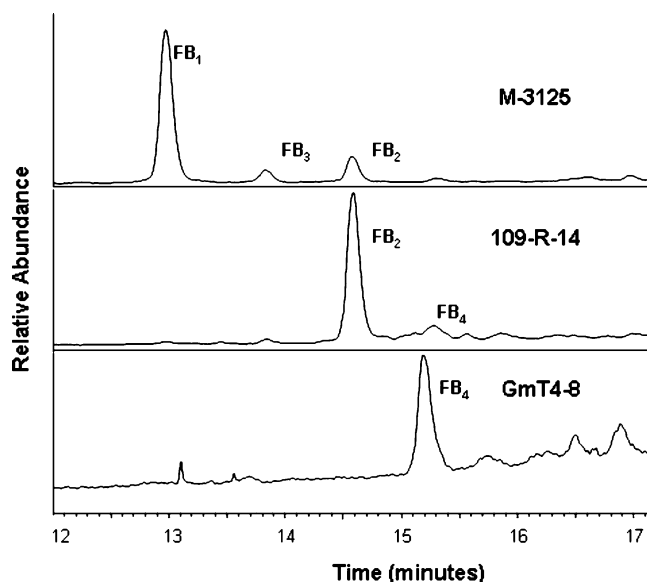
Figure 3. Total ion chromatograms from LC-MS analysis of GYAM culture filtrates of wild-type strain M-3125, C-10 hydroxylation deficient strain 982-R-50, and strain GmT4-8, which was generated by deletion of *FUM3* in strain 982-R-50.

fumonisin-nonproducing mutant generated by disruption of the polyketide synthase gene, *FUM1*, in the fumonisin biosynthetic gene cluster (*14*). All 19 progeny examined from this second cross exhibited the same fumonisin-nonproduction phenotype of the two parental strains. Therefore, no recombination between the mutation in ISU-93-152 and the disrupted *FUM1* gene was evident. Although these genetic analyses included only limited numbers of progeny, they are consistent with the fumonisin-nonproduction phenotype of strain ISU-93-152 being a heritable trait that results from a mutation(s) that is (are) at least loosely linked to the *FUM1* gene and therefore the *FUM* cluster.

The loose linkage to the *FUM* gene cluster raises the possibility that the fumonisin-nonproduction phenotype of strain ISU-93-152 results from a mutation within the cluster. To address this possibility, ISU-93-152 was transformed independently with two overlapping cosmid clones (6B and 4-5), each of which carried an incomplete set of cluster genes. Cosmid clone 6B carried a complete copy of *FUM1*, *FUM6*, and *FUM7* but no other cluster genes, and cosmid clone 4-5 carried all of the cluster genes (*FUM3*, *FUM7*, *FUM8*, and *FUM10−FUM19*) except for *FUM1* and *FUM6* (*14*). Five hygromycin-resistant isolates recovered following transformation with each cosmid clone were analyzed for fumonisin production. Four isolates recovered following transformation with clone 6B were restored to fumonisin production, but none of the isolates recovered following transformation with clone 4-5 were restored to fumonisin production. These results indicate that clone 6B has a wild-type copy of the gene that is mutated in strain ISU-93-152 and that clone 4-5 does not. The fact that disruption of *FUM1* and *FUM6* blocks fumonisin production (*18*) combined with the presence of these two genes in clone 6B suggests that the nonproduction phenotype of strain ISU-93-152 results from a mutation in one of them.

Complementation of Fumonisin-Nonproduction Phenotype with *FUM1*. If the naturally occurring fumonisin-nonproduction phenotype results from a mutation within *FUM1* or *FUM6*, introduction of a wild-type copy of these two genes into a nonproducing strain should restore fumonisin production. To determine whether nonproduction can be restored by *FUM1*, we used transformation to introduce a wild-type copy of *FUM1*

into two strains (ISU-93-152 and 57-7-7) with the naturally occurring nonproduction phenotype. Strain ISU-93-152 is a field isolate obtained from maize grown in Iowa (*31*), and strain 57-7-7 is a progeny of the fumonisin-nonproducing field isolate, M-5500, from Nepal (**Table 1**). We used strain 57-7-7 rather than M-5500 because it was more amenable to genetic analysis and had been used successfully in previous transformation studies (*20*).

The wild-type *FUM1* used in this study was present in complementation vector pCFUM1, which was cotransformed (*28*) into strains ISU-93-152 and 57-7-7. Transformants were selected by their ability to grow on hygromycin B-amended media. LC-MS analysis of cracked maize culture extracts of 19 hygromycin-resistant transformants recovered following transformation of strain 57-7-7 with pCFUM1 revealed that 15 produced the wild-type complement of FB$_1$, FB$_2$, and FB$_3$ (the presence of FB$_4$ was not determined). The remaining four transformants had the fumonisin-nonproduction phenotype of progenitor strain 57-7-7. Likewise, LC-MS analysis of culture extracts of 23 isolates recovered following transformation of strain ISU-93-152 revealed that two produced the wild-type complement of fumonisins. The levels of fumonisins produced by the 17 complemented transformants of 57-7-7 and ISU-93-152 ranged from 240 to 4500 μg of FB$_1$, from 21 to 1463 μg of FB$_2$, and from 62 to 3067 μg of FB$_3$ per gram of cracked maize culture (FB$_4$ was detected but not quantified in this experiment). Southern blot analysis of a subset of transformants derived from both nonproducing progenitor strains confirmed the presence of the complementation vector in fumonisin-producing transformants (**Figure 2C**). For this analysis, genomic DNA was digested with *Xba*I and the hybridization probe spanned from 503 bp upstream to 1051 bp down stream of the *FUM1* translation start site. On the basis of known sequence data, the region of the endogenous *FUM1* complementary to the hybridization probe should have been present as a 6.9-kb *Xba*I fragment, whereas the same region in vector pCFUM1 should have been present as a 2.7-kb *Xba*I fragment. The Southern blot analysis revealed that all fumonisin-producing transformants had both the 6.9- and 2.7-kb fragments. The progenitor strains and selected nonproducing transformants had the 6.9-kb fragment only. These results indicate that the naturally occurring fumonisin-nonproduction phenotype in strains 57-7-7 and ISU-93-152 could be complemented by introduction of a wild-type copy of *FUM1*.

***FUM1* Sequence in a Fumonisin-Nonproducing Strain.** Complementation of two strains with the naturally occurring fumonisin-nonproduction phenotype via introduction of a wild-type copy of *FUM1* suggests that these strains have a mutation within their *FUM1* gene that renders the gene nonfunctional. To address this possibility, we amplified and sequenced the entire 8.16-kb *FUM1* coding region from fumonisin-nonproducing strain 57-7-7. The sequence analysis revealed 20 nucleotide differences in the *FUM1* coding region of strain 57-7-7 compared to the previously reported wild-type *FUM1* sequence (GenBank accession AF155773). All differences consisted of single nucleotide substitutions, 12 of which affected and 7 of which did not affect the amino acid specificity of the codons in which they occurred. Only one of the nucleotide substitutions, a G-to-A transition at nucleotide 5415 of the protein coding region, disrupted *FUM1* by introducing a premature stop codon that would stop translation prior to synthesis of the enoyl reductase, keto-reductase, and acyl carrier domains of the polyketide synthase encoded by *FUM1* (*20*). Thus, the presence of the G-to-A transition at nucleotide 5415

would almost certainly render *FUM1* nonfunctional. The presence of the G-to-A transition was confirmed by sequence analysis of a 700-bp amplicon that spanned nucleotide 5415.

Sequence analysis of the same 700-bp region of *FUM1* in strain ISU-93-152 revealed that this strain did not differ from the wild type at nucleotide 5415. In addition, overlapping $1.5-2$ kb DNA fragments amplified by PCR from along the entire length of the *FUM1* coding region in strain ISU-93-152 and wild-type strain did not exhibit a noticeable difference in size when viewed on an ethidium bromide-stained agarose gel (data not shown). These data indicate that the mutation in the ISU-93-152 *FUM1* gene responsible for the nonproduction phenotype is not a large (>50 bp) insertion or deletion in the coding region.

DISCUSSION

Natural variation in mycotoxin production within individual fungal species and between closely related species has received considerable attention because of the potential impact of such variation on human and animal health, on international trade, on biological control of mycotoxin contamination, and on understanding mycotoxin biosynthesis. In the cases of aflatoxins and trichothecenes, the molecular genetic basis of some of the variation has been demonstrated. For example, some field isolates of *Aspergillus flavus* do not produce aflatoxins because of a single nucleotide substitution that introduces a premature stop codon in the coding region of the polyketide synthase gene in the aflatoxin biosynthetic gene cluster (*32*). In field isolates of *F. graminearum*, production of the trichothecenes nivalenol and 4-acetylnivalenol versus deoxynivalenol can result from multiple deletions and insertions in a monooxygenase gene (*Tri13*) and an acyltransferase gene (*Tri7*) in the trichothecene biosynthetic gene cluster (*33*, *34*). Prior to the current study, characterization of the molecular genetic basis of natural variation in fumonisin production in *F. verticillioides* has been incomplete. The results reported here, however, provide direct evidence that this variation can result from point mutations in genes in the fumonisin biosynthetic gene cluster.

Three lines of evidence from the current study indicate that the C-10 hydroxylation-deficient phenotype resulted from a mutation within the *FUM12* gene. First, laboratory-induced *FUM12* deletion mutants exhibited the C-10 hydroxylation deficiency; second, the naturally occurring deficiency was complemented by transformation with a wild-type copy of *FUM12*; and third, the *FUM12* coding sequence in a strain with the naturally occurring deficiency had a frame shift mutation predicted to render the *FUM12*-encoded monooxygenase nonfunctional. Previous meiotic analyses determined that the naturally occurring C-10 hydroxylation deficiency was caused by a nonfunctional allele at the *Fum2* locus (*13*). Thus, the data reported in the current study also indicate that the meiotically defined *Fum2* locus and the molecularly defined *FUM12* are the same gene. Because *Fum2* was described prior to *FUM12*, we propose that hereafter the gene be designated *FUM2*. Furthermore, because the results of this study revealed that the monooxygenase encoded by *FUM2* is responsible for fumonisin C-10 hydroxylation, they further define the functions of the *FUM* cluster in *F. verticillioides*.

Complementation experiments in the current study also localized the naturally occurring fumonisin nonproduction phenotype in two strains with distant geographic origins to the polyketide synthase gene, *FUM1*, in the *FUM* cluster. Furthermore, sequence analysis indicated that the nonproduction phenotype in strain 57-7-7 most likely resulted from a nucleotide substitution that introduced a premature stop codon in the *FUM1*

coding region. Restoration of wild-type fumonisin production to strain ISU-93-152 by transformation with *FUM1* indicated that the mutation responsible for the fumonisin-nonproduction phenotype in ISU-93-152 was located in *FUM1*. Limited sequence analysis demonstrated that the mutation was not located in the same position as in strain 57-7-7 and did not resolve whether the mutation in ISU-93-152 was in the coding, promoter, or termination region of *FUM1*. Previously reported meiotic analyses demonstrated that the fumonisin-nonproduction phenotype in 57-7-7 resulted from a nonfunctional allele at the *Fum1* locus (*13*, *21*). Thus, the results from the current study combined with the previous meiotic analysis indicate that the molecularly defined *FUM1* gene and the meiotically defined *Fum1* locus are the same gene. In keeping with the current designation of *FUM* and other *F. verticillioides* genes, we propose that hereafter the gene be referred to as *FUM1*.

When compared to the published sequences for wild-type *F. verticillioides*, *FUM1* in strain 57-7-7 and *FUM2* (= *FUM12*) in strain 109-R-14 included nucleotide substitutions in addition to those that introduced the premature stop codon in *FUM1* and frame shift in *FUM2*. Some of these other changes affected the amino acid specificity of the codons in which they occurred. However, it is not clear whether the resulting amino acid substitutions would affect the activity of the *FUM1* or *FUM2* enzymes. Regardless, the premature stop codon in *FUM1* of 57-7-7 and the frame shift in *FUM2* of 109-R-14 would cause such profound changes in the corresponding proteins that the proteins would be nonfunctional.

Most field isolates of *F. verticillioides* produce the full complement of FB_1, FB_2, FB_3, and FB_4 (*11*). The scarcity of the nonproduction and C-5 and C-10 hydroxylation-deficient phenotypes in natural populations of the fungus may be an indication that FB_1 production contributes to the competitiveness of the fungus. Characterization of fumonisin biosynthetic genes and elucidation of the genetic basis of naturally occurring altered fumonisin production phenotypes should contribute to understanding the role, if any, of the toxins in the ecology of *F. verticillioides*.

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