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Robert H. Proctor  
_USDA-ARS, proctorh@ncaur.usda.gov_

Ronald D. Plattner  
_USDA-ARS_

Anne E. Desjardins  
_USDA-ARS_

Mark Busman  
_USDA-ARS_

Robert A. E. Butchko  
_USDA-ARS_

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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. DESIARDINS, 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 B₁, B₂, B₃, and B₄ (FB₁, FB₂, FB₃, and FB₄, 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). FB₁ has both hydroxyls, FB₂ lacks the C-10 hydroxyl, FB₃ lacks the C-5 hydroxyl, and FB₄ lacks both the C-5 and C-10 hydroxyls. In general, FB₁ is the most abundant fumonisin (~70%) in naturally contaminated maize and in cultures of most field isolates of *F. verticillioides*, whereas FB₂, FB₃, and FB₄ 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 FB₁ and FB₄; and a third variant, also isolated from maize from South Carolina,
was deficient in C-10 hydroxylation and, as a result, produced only FB2 and FB4. 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 o xoamine 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 ofcosmid 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 FB1, FB2, FB3, and FB4 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).

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Table 1. F. verticillioides Strains Used in the Study

<table>
<thead>
<tr>
<th>strain</th>
<th>fumonisin production</th>
<th>genotype</th>
<th>strain origin</th>
<th>parent strains</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-3125</td>
<td>FB2, FB5, FB4</td>
<td>Fum1-1, Fum2-1</td>
<td>field isolate</td>
<td>N/A</td>
<td>35</td>
</tr>
<tr>
<td>ISU-93-152</td>
<td>none</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>field isolate</td>
<td>N/A</td>
<td>31</td>
</tr>
<tr>
<td>109-R-14</td>
<td>FB2, FB4</td>
<td>Fum1-1, Fum2-2</td>
<td>meiotic progeny</td>
<td>M-3120&lt;sup&gt;c&lt;/sup&gt; (2)</td>
<td>13</td>
</tr>
<tr>
<td>982-R-50</td>
<td>FB2, FB4</td>
<td>Fum1-1, Fum2-2</td>
<td>meiotic progeny</td>
<td>A-0822&lt;sup&gt;c&lt;/sup&gt; (d)</td>
<td>13</td>
</tr>
<tr>
<td>57-7-7</td>
<td>none</td>
<td>Fum1-2, Fum2-1</td>
<td>meiotic progeny</td>
<td>GIA2364&lt;sup&gt;c&lt;/sup&gt; (c)</td>
<td>21</td>
</tr>
<tr>
<td>GIA2364&lt;sup&gt;d&lt;/sup&gt;</td>
<td>none</td>
<td>Fum2-1</td>
<td>FUM1 disruption mutant</td>
<td>N/A</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 for more details on these two strains) (11, 13). nd indicates not determined, and N/A indicates not applicable. *Strain M-3120 was a field isolate from California, had the Fum1-1/Fum2-1 genotype, and produced the wild-type complement of FB1, FB2, FB3, and FB4 (36). Strain A-0822 was a field isolate from South Carolina, had the Fum1-1/Fum2-2 genotype, and produced FB2 and FB4 only (11). Strain 648-R-193 was a meiotic progeny derived from strain A-0822, had the Fum1-1/Fum2-2 genotype, and produced FB2 and FB4 only (13, 21). Strain M-5500 was a field isolate from Nepal, had the Fum1-2/Fum2-1 genotype, and produced no fumonisins. *Strain GIA2364 was generated from wild-type strain M-3125 by disruption of the FUM1 gene (20). The Fum1 genotype for strain GIA2364 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.

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This page contains information about the genetic basis of fumonisin variation in F. verticillioides, including a table of fumonisin-producing strains used in the study, and a discussion of the molecular genetic analysis and the results of the study. The work was aimed at elucidating the molecular genetic basis of the fumonisin-nonproduction phenotype and other variations in fumonisin production in different strains of F. verticillioides. The study also contributed to understanding fumonisin biosynthesis and the biological significance of different fumonisin chemotypes in the ecology of F. verticillioides and in maize agriculture.
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, pAFUM12-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′-CGTGGATCCCTGCCAGAAGAATGCGCCAACCT-3′ (underlined sequence is a BamHI site) and 5′-CATGGCGCCGCAAGAGGGAGGATCGCTGTCC-3′ (underlined sequence is an AscI site), and the primers used to amplify the downstream fragment were 5′-CATGGCGCCGCAAGACAGGT-GAATGGGATTC-3′ (underlined sequence is an AscI site) and 5′-GTCGAGCTACAGCAGATACGACATGTC-3′ (underlined sequence is a SacI site). The two amplicons were cloned into the same vector (pT7Blue) with the BamHI, SacI, and AscI 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 introduced via the PCR primers and/or present in the pT7Blue multiple cloning sequence. This cloning step restored the orientation and position introduced via the PCR primers and/or present in the pT7Blue multiple cloning sequence. The cloning sequence. This cloning step restored the orientation and position introduced via the PCR primers and/or present in the pT7Blue multiple cloning sequence. The cloning sequence. This cloning step restored the orientation and position introduced via the PCR primers and/or present in the pT7Blue multiple cloning sequence.

**Construction of the FUM12 Deletion Vector.** The second fragment was obtained by digestion with *SacI* and spanned from 1473 bp upstream to 1580 bp downstream of the *FUM1* start codon. The *FUM1* gene is 3.7 kb. The *FUM1* deletion vector, pFCUM1-Hyg, was constructed 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 (Invitrogen Life Technologies). The resulting vector with the *NotI* site to yield pFUM12-Hyg. The 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 *NotI* fragment carrying the hygromycin resistance gene *HygB* was introduced into the resulting vector with the *NotI* site in the polyclinor region of pCR XL TOPO. *HygB* was modified from pUCH2-8 (26, 27) by introduction of *NotI* sites at both ends of the gene by PCR amplification with primers 5′-CGATGCGCCGCCACCGCAGCAGTGCA-3′ and 5′-GCATGCGCCGCCAGTGAAGTTCGTA-3′ (underlined sites are unmodified).

The FUM1 complementation vector was constructed from two overlapping DNA fragments obtained by restriction enzyme digestion of cowid clone 1-1 (J4, 20). The first fragment was obtained by *DraI* digestion of clone 1-1 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 *SpeI* digestion of clone 1-1 and spanned from 1473 bp upstream to 1580 bp downstream of the FUM1 start codon. The *DraI* fragment was cloned into pBlueScript II (Stratagene), and the resulting construct was cut with *SpeI* and then ligated to the *SpeI* 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 (26). 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 (23).

**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. FB₁, FB₂, FB₃, and FB₄ 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 monoxygenase (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 pAFUM12-Hyg. The vector was designed so that homologous recombination between FUM12 sequences in pAFUM12-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-hygroycin-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 *SnaBI* 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 *SnaBI* fragment with FUM12 in wild-type *F. verticillioides* is 2.9 kb, and the expected size of the *SnaBI* 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 FB₂ and FB₄, 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 FB₁ and FB₃. In this experiment, the levels of fumonisins produced in two cultures of M-3125 were 5300 and 12200 μg of FB₁, 1900 and 3700 μg of FB₂, and 900 and 1300 μg of FB₃ per gram of cracked maize culture. The level of FB₄ produced by the two FUM12 disruption mutants was 2400 (GFA2874) to 9200 (GmT201) μg per gram of cracked maize culture. The presence of FB₄ was noted but not quantified in this experiment. Given that cytochrome P450 monoxygenases often catalyze hydroxylation reactions, these results indicate that the FUM12-encoded monoxygenase 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
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 FB1, FB2, FB3, and FB4. In contrast, strains 109-R-14 and 982-R-50 and the remaining transformants produced only FB2 and FB3. In this experiment, the 15 complemented transformants of fumonisins production ranged from 7 to 312 μg of FB1, from 1 to 43 μg of FB2, from 1 to 49 μg of FB3, and from <1 to 6 μg of FB4 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 FB2 and from 2 to 6 μg of FB4 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 EcoRI, electrophoresed, blotted, and hybridized to a 32P-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 EcoRI fragment, whereas the same region in the complementation vector pCFUM12-Hyg was predicted to be present as a 1.7-kb EcoRI 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 (GenBank 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 point mutation 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 FB4, 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 FB2 and 11 μg of FB3 per milliliter of GYAM and the FUM3 deletion mutant (in the genetic background of strain 982-R-50) produced 21 μg of FB4 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 FB1, FB2, and FB3 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 FB1, FB2, and FB3 and 14 did not produce fumonisins. Strain ISU-93-152 was also crossed with strain GfA2364, a
FUM1 production phenotype results from a mutation within FUM1. The loose linkage to the FUM gene cluster raises the possibility that the fumonisin-nonproduction phenotype of strain ISU-93-152 resulted 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 FB1, FB2, and FB3 (the presence of FB4 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 fumonisin. 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 FB1, from 21 to 1463 μg of FB2, and from 62 to 3067 μg of FB3 per gram of cracked maize culture (FB4 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 XbaI 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 XbaI fragment, whereas the same region in vector pCFUM1 should have been present as a 2.7-kb XbaI 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 \textit{FUM1} nonfunctional. The presence of the G-to-A transition was confirmed by sequence analysis of a 700-bp ampiclon that spanned nucleotide 5415. Sequence analysis of the same 700-bp region of \textit{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 \textit{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 \textit{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 \textit{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 \textit{F. graminearum}, production of the trichothecenes nivalenol and 4-acetyldeoxynivalenol versus deoxynivalenol can result from multiple deletions and insertions in a monooxygenase gene (\textit{Tri13}) and an acyltransferase gene (\textit{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 \textit{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 \textit{FUM12} gene. First, laboratory-induced \textit{FUM12} deletion mutants exhibited the C-10 hydroxylation deficiency; second, the naturally occurring deficiency was complemented by transformation with a wild-type copy of \textit{FUM12}; and third, the \textit{FUM12} coding sequence in a strain with the naturally occurring deficiency had a frame shift mutation predicted to render the \textit{FUM12}-encoded monooxygenase nonfunctional. Previous meiotic analyses determined that the naturally occurring C-10 hydroxylation deficiency was caused by a nonfunctional allele at the \textit{Fum2} locus (13). Thus, the data reported in the current study also indicate that the meiotically defined \textit{Fum2} locus and the molecularly defined \textit{FUM12} are the same gene. Because \textit{Fum2} was described prior to \textit{FUM12}, we propose that hereafter the gene be designated \textit{FUM2}. Furthermore, because the results of this study revealed that the monooxygenase encoded by \textit{FUM2} is responsible for fumonisin C-10 hydroxylation, they further define the functions of the \textit{FUM} cluster in \textit{F. verticillioides}.

Complementation experiments in the current study also localized the naturally occurring fumonisin nonproduction phenotype in two strains with distinct geographic origins to the polyketide synthase gene, \textit{FUM1}, in the \textit{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 \textit{FUM1} coding region. Restoration of wild-type fumonisin production to strain ISU-93-152 by transformation with \textit{FUM1} indicated that the mutation responsible for the fumonisin-nonproduction phenotype in ISU-93-152 was located in \textit{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 \textit{FUM1}. Previously reported meiotic analyses demonstrated that the fumonisin-nonproduction phenotype in 57-7-7 resulted from a nonfunctional allele at the \textit{Fum1} locus (13, 21). Thus, the results from the current study combined with the previous meiotic analysis indicate that the molecularly defined \textit{FUM1} gene and the meiotically defined \textit{Fum1} locus are the same gene. In keeping with the current designation of \textit{FUM} and other \textit{F. verticillioides} genes, we propose that hereafter the gene be referred to as \textit{FUM1}.

When compared to the published sequences for wild-type \textit{F. verticillioides}, \textit{FUM1} in strain 57-7-7 and \textit{FUM2 (=FUM12)} in strain 109-R-14 included nucleotide substitutions in addition to those that introduced the premature stop codon in \textit{FUM1} and frame shift in \textit{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 \textit{FUM1} or \textit{FUM2} enzymes. Regardless, the premature stop codon in \textit{FUM1} of 57-7-7 and the frame shift in \textit{FUM2} of 109-R-14 would cause such profound changes in the corresponding proteins that the proteins would be nonfunctional.

Most field isolates of \textit{F. verticillioides} produce the full complement of FB1, FB2, FB3, and FB4 (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 FB1 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 \textit{F. verticillioides}.

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**LITERATURE CITED**


