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Robert A. E. Butchko
USDA-ARS, butchkora@ncaur.usda.gov

Ronald D. Plattner
USDA-ARS

Robert H. Proctor
USDA-ARS

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Deletion Analysis of *FUM* Genes Involved in Tricarballylic Ester Formation during Fumonisin Biosynthesis

ROBERT A. E. BUTCHKO,* RONALD D. PLATTNER, AND ROBERT H. PROCTOR

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

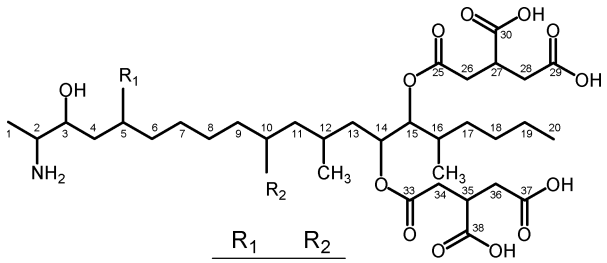
Fumonisins are carcinogenic mycotoxins produced by the maize ear rot pathogen *Gibberella moniliformis* (anamorph *Fusarium verticillioides*). These toxins consist of a linear polyketide-derived backbone substituted at various positions with an amine, one to four hydroxyl, two methyl, and two tricarballylic ester functions. In this study, we generated and characterized deletion mutants of *G. moniliformis* for five genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* in the fumonisin biosynthetic gene cluster. Functional analysis of mutants in four genes, predicted to encode unrelated proteins, affected formation of the tricarballylic esters. *FUM7* deletion mutants produced a previously undescribed homologue of fumonisin B_1 with an alkene function in both tricarballylic esters, *FUM10* and *FUM14* deletion mutants produced homologues of fumonisin B_3 and fumonisin B_4 that lack tricarballylic ester functions, and *FUM11* deletion mutants produced fumonisins that lack one of the tricarballylic ester functions. These phenotypes indicated specific roles for *FUM7*, *FUM10*, *FUM11*, and *FUM14* in fumonisin biosynthesis that are consistent with the predicted proteins encoded by each gene. Deletion of *FUM16* had no apparent effect on fumonisin production. The phenotypes of the deletion mutants provide further insight into the order of steps in fumonisin biosynthesis.

KEYWORDS: Fumonisin; *Gibberella moniliformis*; *Fusarium verticillioides*; *FUM* genes; mycotoxin; tricarballylic ester

INTRODUCTION

Fumonisins are polyketide-derived mycotoxins produced by the maize stalk and ear rot pathogen *Gibberella moniliformis* Wineland (anamorph *Fusarium verticillioides* (Sacc.) Nirenberg) (*1*). Fumonisins can disrupt sphingolipid biosynthesis by inhibiting the enzyme sphinganine *N*-acyltransferase (*2*−*5*). Because of the importance of sphingolipids in multiple cellular processes, the disruption of sphingolipid metabolism may be the mechanism by which fumonisins induce a variety of diseases in animals (*4*, *5*). Pulmonary edema in pigs, leukoencephalomalacia in horses, and cancer and neural tube defects in rodents (*2*, *4*, *6*, *7*) have been associated with the ingestion of fumonisin-contaminated maize. Fumonisins have also been implicated in human esophageal cancer (for review see ref *7*).

Most field isolates of *G. moniliformis* produce predominantly four B-series fumonisins (B_1, B_2, B_3, and B_4, **Figure 1**) (*8*, *9*). These mycotoxins are synthesized, at least in part, through the activity of enzymes encoded by the fumonisin biosynthetic (*FUM*) gene cluster. This cluster consists of 15 coregulated genes designated *FUM1*−*FUM3* (previously *FUM5*, *FUM12*, and *FUM9*, respectively (*10*−*12*)), *FUM6*−*FUM8*, *FUM10*, *FUM11*, *and FUM13*−*FUM19* that are located on chromosome I (*11*). The roles of some of the genes in the cluster have been confirmed by gene deletion analysis (*10*, *11*, *13*−*15*) and heterologous expression (*16*). Deletion of either *FUM1*, *FUM6*, or *FUM8* blocked accumulation of all fumonisins, indicating that these genes are required for fumonisin production (*14*, *15*). Their exact roles in biosynthesis have been inferred by the similarity of their sequences to genes of known function and through analysis of deletion mutants. For example, analysis of fumonisin production in co-cultures of *FUM6* and *FUM8* deletion mutants provided details of the early steps of fumonisin biosynthesis (*17*). In contrast, deletion of either *FUM2*, *FUM3*, or *FUM13* led to the accumulation of less oxygenated fumo-



	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. Major fumonisin homologues produced by wild-type strain M-3125.

* Author to whom correspondence should be addressed. Tel: 309-681-6073; fax: 309-681-6686; e-mail: butchkora@ncaur.usda.gov.

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nisins and provided insight into the exact function of these genes (*10*, *12*, *13*). For example, *FUM2* deletion strains produce only fumonisins B_2 and B_4, which lack the C-10 hydroxyl (the hydroxyl group at carbon atom 10) and indicate that the *FUM2*-encoded cytochrome P450 monooxygenase most likely catalyzes fumonisin C-10 hydroxylation (*12*). *FUM3* deletion mutants produce only fumonisins B_3 and B_4, which lack the C-5 hydroxyl and indicate that the *FUM3*-encoded dioxygenase most likely catalyzes fumonisin C-5 hydroxylation (*10*). *FUM13* deletion mutants produce 3-keto homologues of fumonisins B_3 and B_4 and indicate that the *FUM13*-encoded ketoreductase most likely catalyzes the reduction of the C-3 carbonyl to a C-3 hydroxyl (*13*). In contrast to *FUM2*, *FUM3*, and *FUM13*, deletion of *FUM17* and *FUM18* did not affect fumonisin production while deletion of *FUM19* subtly effected the ratio of fumonisin B_1 to B_3 produced (*11*). Deletion of *FUM15* had no observed effect on fumonisin production (*18*).

The functions for five other *FUM* genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16*, have been postulated on the basis of sequence similarity to genes of known function (*11*). The predicted *FUM10* and *FUM16* proteins have a high degree of similarity to acyl-CoA synthetases and either or both were predicted to catalyze the CoA activation of either the fumonisin polyketide or the tricarballylic ester precursor (*11*). The predicted *FUM11* protein exhibits a high degree of similarity to mitochondrial membrane-bound tricarboxylate transporters and was proposed to be involved in transporting tricarboxylate precursors of the tricarballylic esters to make them available for fumonisin production (*11*). The functions of the predicted proteins encoded by *FUM7* and *FUM14* in fumonisin biosynthesis was not obvious on the basis of their similarity to genes of known function; *FUM7* was predicted to encode a dehydrogenase and *FUM14* was predicted to encode a protein with similarity to the condensation domain of nonribosomal peptide synthetases (*11*). A detailed analysis of the function of the enzyme encoded by *FUM14* has been reported (*19*). The objective of this study was to determine the functions of *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* in fumonisin biosynthesis by analysis of individual gene deletion mutants.

MATERIALS AND METHODS

Strains and Media. *Gibberella moniliformis* strain M-3125 was used throughout this study (*20*). Other strains used in this study include M-5500 (*FUM1* mutant) (*21*) and the following deletion mutants: GfA3075 (*FUM6* deletion) (*15*), GfA3245 (*FUM8* deletion) (*15*), GMT9-206 (*FUM3* deletion) (*10*), and GfA2874 (*FUM2* deletion) (*12*). Strains were cultured on V8 juice agar medium for the production of conidia and in liquid GYEP (5% glucose, 0.1% yeast extract, 0.1% peptone) for production of mycelia for genomic DNA preparation and in cracked corn medium or liquid GYAM (*15*) for analysis of fumonisin production (*15*, *21*).

***FUM* Gene Deletion Constructs, Transformation, and Southern Analysis.** Deletion constructs for *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* were engineered utilizing approximately 1-kb flanking regions both upstream and downstream of the coding region as described previously for deletions of other *FUM* genes (*13*, *22*). Briefly, upstream and downstream regions were amplified by PCR and were subcloned into a single vector. **Table 1** lists the primers used to amplify each region of each gene. The hygromycin B resistance gene (*HygB*) (*23*) was then inserted between the flanking regions. The resulting vectors, pF7KOH, pF10KOH, pF11KOH, pF14KOH, and pF16KOH, were transformed separately into strain M-3125 as previously described (*14*). Primary transformants were first screened for hygromycin resistance and then by PCR to determine whether double homologous recombination, and therefore gene deletion, had occurred. Briefly, PCR primers were designed to amplify fragments corresponding to wild-type *FUM* genes (wild-type fragments) or fragments expected to result from replacement of the target *FUM* coding region with *HygB* (deletion fragments). Genomic DNA from transformants of each *FUM* gene was isolated and analyzed both for the presence of the deletion fragments and for the absence of the wild-type fragments. For each *FUM* gene analyzed in this study, two independently isolated deletion mutants, as determined by PCR, were selected for Southern blot analysis to confirm deletion of the targeted *FUM* gene and for LC-MS analysis to determine the effect of the *FUM* gene deletion on fumonisin production. **Figure 2** shows an example of a Southern blot for *FUM11* deletion mutants. Genomic DNA was isolated, digested with *BspHI*, electrophoresed, and blotted to nylon membrane. A portion of the *FUM11* coding region that was deleted was labeled with ^{32}P using the RediprimeII kit (Amersham Pharmacia Biotech) and was used to hybridize the Southern blot.

Table 1. Oligonucleotide Primers Used To Characterize *FUM* Gene Deletion Mutants

gene	primer name	sequence
		A. Deletion Construction
FUM7	rp499	GACACGCGTAGGTTCATCTGGCCCGAATC
	rp500	GACGGCGCGCCCACCAACTCAATTCCCTTCCC
	rp501	GACGGCGCGCCATGAGAACCATTGTTGCC
	rp502	GACGGCGCGCCGGTGGTTCTATGGGACCT
FUM10	rp511	GACACGCGTCAAGGAAATTGGCGCACATAG
	rp512	GACGGCGCGCCCGTGTTGATTGGGACGATTG
	rp513	GACGGCGCGCCTGAAGAAGCATATGCGCCATC
	rp514	GACCTCGAGTCGAGGACCGCAGATTAGAGA
FUM11	rp515	AGCAGAATTCTGCAACCCATTTCCGG
	rp516	GACGGCGCGCCAAGCAACGGAAACTAGCCGC
	rp517	GACGGCGCGCCTCGGGCATCGAGAGATACAG
	rp518	GACAAGCTTTACAACTTGACCGCGTCGAA
FUM14	rp532	GGACTCGAGGTAGCGGTAACATGACTGCATG
	rp533	CATGGCGCGCCAACATGGTTCTTGGGAACTCG
	rp534	CATGGCGCGCCGATCCCAGTCGTATCTCAGTCA
	rp535	GTAGCATGCGGACAAGACTTTGATCTGTACA
FUM16	rp519	GACGGATCCTGGCTTCCATTACGACGAAAC
	rp520	GACGGCGCGCCACCAGCGCTATACAACGGCT
	rp521	GACGGCGCGCCAGCTATCGGTTATCGGACCTG
	rp522	GACGGCGCGCCGATATGCCAATGTGCGTGAA
		B. Deletion Detection
FUM7	rp603	GTTGCTCTGATCGAACGACT
	rp304	GAGTTCCGGGAGTTTGCTTGG
	rp604	CCTGTCCAAAACGTATCCTG
	rp307	GAGGATGTCTGCACACAAG
FUM10	rp547	TGAATGGATGAGCCTCCT
	rp506	GACCTCGAGGGCAACAAACTCCCTG
	rp548	TTGCTGATGACGATGGGA
	rp515	AGCAGAATTCTGCAACCCATTTCCGG
FUM11	rp431	CATGGCGCGCCAAGAAGGGAGGGACTCGAGTCT
	rp430	CGTGGATCCTGCCAGAAGAATGCCGAACCT
	rp605	CATTACCTGGTATCACGCCG
	rp514	GACCTCGAGTCGAGGACCGCAGATTAGAGA
FUM14	rp529	CAAGTCGACTGGCCTATTGGAC
	rp609	GGAACAATCTCAACCATCTC
	rp606	GTATGATTGGTACCATACTG
	rp510	GACTCTAGACGATGAACAACTTTCCCG
FUM16	rp553	CACCAAGCGTATCGTATG
	rp554	TGCCGTGAGGTATAGTCTAC
	rp555	GTTCTTGGTCGCTAACGA
	rp556	CGGTGGCTAAGGTAATTAAG
HygB	rp250	CTGCTGCATTCCCATTCCCATCGT
HygB	1098	ACCAAGCCTATGCCTACAGCATCC

Fumonisin Analysis. To assess fumonisin production in deletion mutants, strains were cultured on cracked corn medium for 3 weeks and were extracted with acetonitrile:water (1:1) as previously described (*14*). The fumonisin content of culture extracts was determined by liquid chromatography−mass spectroscopy (LC-MS) as previously described (*24*). Liquid GYAM medium was also used to assay putative intermediates for their incorporation into the fumonisin biosynthetic pathway. Fumonisins and putative fumonisin biosynthetic intermediates produced by gene deletion mutants were dissolved in water and were added to

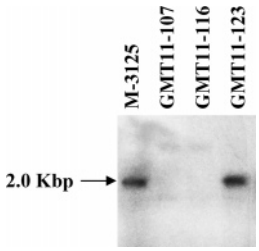

Figure 2. Southern analysis of *G. moniliformis* transformants with a deletion in *FUM11*. Genomic DNA was prepared from three transformants and from wild-type strain M-3125. Using a probe corresponding to the deleted portion of the gene, the absence of the *FUM11* coding region is illustrated in strains GMT11-107 and GMT11-116 compared to the wild-type strain M-3125 and a hygromycin resistant transformant in which the plasmid construct did not recombine at the *FUM11* locus.

20-mL liquid GYAM cultures of 5-day-old wild-type or mutant strains of *G. moniliformis* at a final concentration of 5 ng/μL. The cultures were incubated for a further 5 days, after which they were filtered through a 0.2-μm Nalgene filter and culture filtrate analyzed by LC-MS. Purification of a fumonisin-like metabolite from *FUM7* deletion mutants was done by preparative high-performance liquid chromatography (HPLC) on C18 columns (Ranin Instrument Co, Woburn, MA) for analysis by ^{13}C NMR and hydrolysis. NMR spectra were obtained with a Bruker (Billerica, MA) Avance 400 spectrometer equipped with a 5-mm inverse broad-band Z-gradient probe (^{13}C NMR, 100 MHz, 1H, 400 MHz).

RESULTS AND DISCUSSION

Phenotypes of *FUM* Deletion Mutants. When cultured on cracked corn, the two *FUM7* deletion mutants (GMT7-301 and GMT7-426) accumulate no fumonisin B_1, B_2, B_3, or B_4 but instead accumulate several metabolites with *m*/*z* 718 or *m*/*z* 702. These metabolites were not observed in culture of the wild-type parent strain M-3125. Fumonisin B_1 (*m*/*z* 722) less four hydrogen atoms would have an *m*/*z* of 718 while fumonisin B_2 and B_3 (*m*/*z* 706) less four hydrogen atoms would have an *m*/*z* of 702. One possible explanation for the loss of four hydrogen atoms could be the presence of two carbon−carbon double bonds instead of two carbon−carbon single bonds in each fumonisin-like metabolite. To determine whether the mass loss occurred within the tricarballylic moiety or within the fumonisin backbone, one of the metabolites with an *m*/*z* 718 was purified by HPLC and was subjected to potassium hydroxide-mediated hydrolysis, which removes the tricarballylic ester chains from the backbone. LC-MS analysis of the hydrolyzed product indicates that it was hydrolyzed fumonisin B_1, which has hydroxyl functions at C-14 and C-15 instead of tricarballylic esters. The generation of hydrolyzed fumonisin B_1 by hydrolysis suggests that the tricarballylic moiety of the *m*/*z* 718 metabolite contains the structural difference that accounts for the mass difference observed between the new metabolite and fumonisin B_1.

Preliminary ^{13}C nuclear magnetic resonance (NMR) analysis of the purified *m*/*z* 718 metabolite provided additional support to this hypothesis, however, the exact nature of the compounds is not clear. Broad shouldered peaks at the frequencies expected for the carbon atoms in the tricarballylic of the molecules indicate a complex mixture of more than one isomer present in the solution. Plattner et al. describe the carbon shift for propane-1,2,3-tricarboxylic acid and indicate that there is a pH-dependent equilibrium between the open-chain structure and a cyclic structure (*25*). Broad peaks in the NMR spectra indicate that the double bonds can be present between more than one pair of adjacent carbon atoms as well as the result of cyclization of the molecule (*25*). A more detailed NMR analysis of this fraction is required to fully characterize this compound, and experiments are underway. The chemical shifts for all other carbons in the NMR spectra were identical to fumonisin B_1. Taken together, these results support the structure in **Figure 3**, referred to as tetradehydro-fumonisin B_1, which includes a carbon−carbon double bond in the esters attached to C-14 and C-15.

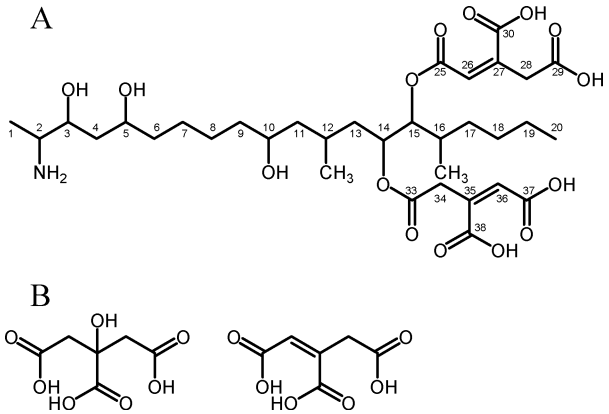

Figure 3. Panel **A** shows a predicted structure of the fumonisin-like compound, tetradehydro-fumonisin B1, accumulated by *FUM7* deletion mutants cultured on cracked corn. One possible confirmation of the tricarballylic side chains is shown; however, the positions of each of the putative C–C double bonds have not been determined. If such double bonds are present in these side chains, it is equally likely that the bonds are between carbons 26 and 27 as it is possible they are between carbons 27 and 28 on each tricarballylic molecule, likewise for the double bonds on the tricarballylic ester on carbon 14, i.e., it is equally likely that the bonds are between carbons 35 and 36 as it is possible they are between carbons 34 and 35. Panel **B** shows the structures of two putative precursors of the tricarballylic esters of fumonisins, citrate on the right and *cis*-aconitate on the left.

The *FUM10* (GMT10-206 and GMT10-213) and *FUM14* (GMT14-106 and GMT14-109) deletion mutants did not accumulate the wild-type complement of fumonisins B_1, B_2, B_3, and B_4 but instead accumulated two metabolites with retention times, masses, and mass spectra identical to hydrolyzed fumonisin B_3 and hydrolyzed fumonisin B_4 when cultured on cracked corn. Analysis of similar *FUM14* deletion mutants has been reported previously (*19*). Hydrolyzed fumonisins B_3 and B_4 are identical in structure to fumonisins B_3 and B_4 except that they have hydroxyl functions at C-14 and C-15 instead of tricarballylic ester functions.

The *FUM11* deletion mutants (GMT11-107 and GMT11-116) synthesized the wild-type complement of fumonisins, as well as a significant quantity of metabolites with retention times, masses, and mass spectra consistent with half-hydrolyzed and keto half-hydrolyzed homologues of fumonisins B_1, B_2, B_3, and B_4 when cultured on cracked corn. The half-hydrolyzed fumonisin homologues are identical to B-series fumonisins except that they lack one of the tricarballylic side chains and are detectable only at very low levels in culture filtrates of the wild-type strain. In half-hydrolyzed homologues the hydroxyl function is at either C-14 or C-15 instead of a tricarballylic ester, while keto half-hydrolyzed homologues have a carbonyl function at either C-14 or C-15 instead of a tricarballylic ester. We measured the amounts of fumonisin B_1, half-hydrolyzed fumonisin B_1, and keto half-hydrolyzed fumonisin B_1 in the wild-type strain and the two *FUM11* deletion mutants. In the wild-

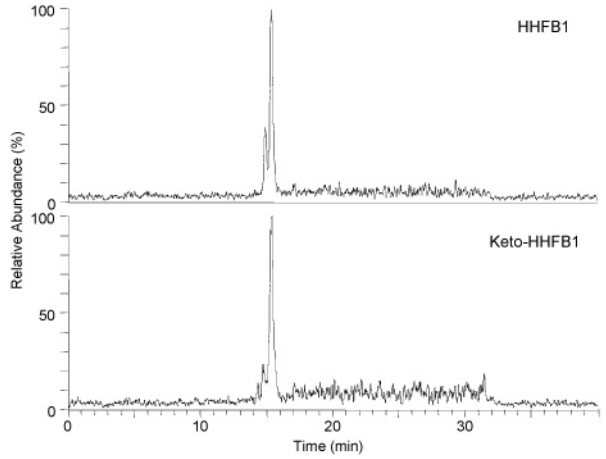

Figure 4. LC-MS analysis of extracts from *FUM11* deletion mutant GMT11-116. Top panel shows the peak at 15.35 min with an *m*/*z* of 564 corresponding to half-hydrolyzed fumonisin B_1. The bottom panel shows the peak at 15.41 min with an *m*/*z* of 562 corresponding to keto half-hydrolyzed fumonisin B_1.

type strain, half-hydrolyzed fumonisin B_1 and keto half-hydrolyzed fumonisin B_1 constitute only 1.2% and 2.1%, respectively, of fumonisin B_1 produced. However, in the *FUM11* deletion mutant GMT11-107, half-hydrolyzed fumonisin B_1 and keto half-hydrolyzed fumonisin B_1 constitute 15% and 6% of fumonisin B_1, respectively, while strain GMT11-116 produced 26% and 10% of fumonisin B_1, respectively. **Figure 4** shows the LC-MS analysis of extracts from a *FUM11* deletion mutant.

Like *FUM10*, *FUM16* is predicted to encode an acyl-CoA synthetase and therefore could be involved in activation of the tricarboxylic acid molecules with CoA (*11*). However, in contrast to the *FUM10* deletion mutants as well as the *FUM7*, *FUM11*, and *FUM14* deletion mutants, the *FUM16* (GMT16-215 and GMT16-230) deletion mutants did not exhibit altered fumonisin production relative to the wild-type progenitor strain M-3125 when cultured on cracked corn. While *FUM10* is required for tricarballylic ester formation, *FUM16* is not.

Intermediate Study. Metabolites produced by the *FUM* deletion mutants generated in this study may be fumonisin biosynthetic intermediates or end points of the truncated fumonisin biosynthetic pathway. To test the hypothesis that tetradehydro-fumonisin B_1 is an intermediate in the fumonisin biosynthetic pathway, the purified tetradehydro-fumonisin B_1 was added to liquid cultures of *FUM3*, *FUM6*, and *FUM8* deletion mutants and a naturally occurring fumonisin nonproducer with a point mutation in *FUM1* (*12*, *21*). We predicted that if tetradehydro-fumonisin B_1 is a fumonisin biosynthetic intermediate it would be metabolized to fumonisin B_1 or other fumonisins. After 5 days of incubation, LC-MS analysis of culture filtrates indicated that none of the tetradehydro-fumonisin B_1 was converted to fumonisin B_1 (data not shown) and remained in the culture filtrate. In contrast, cultures of the mutants were able to convert fumonisin B_3 to fumonisin B_1 indicating that *FUM3* was still functional in these strains. These results indicate that tetradehydro-fumonisin B_1 is not an intermediate in the pathway but instead is a shunt or end product produced only after the deletion of *FUM7*.

Here, we have described the phenotypes of deletion mutants for five *FUM* genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16*. Our data indicate that the activities of enzymes encoded by four *FUM* genes are involved in the formation of the tricarballylic ester functions at C-14 and C-15 of the fumonisin backbone.

FUM10 and *FUM14* deletion mutants had the same fumonisin production phenotype; they produce hydrolyzed fumonisins B_3 and B_4 that both lack tricarballylic ester functions. The predicted functions of the proteins encoded by *FUM10* and *FUM14*, on the basis of their similarity to genes of known function, together with the mutant phenotype suggest roles for both genes in fumonisin biosynthesis. *FUM10* is predicted to encode an acyl-CoA synthetase (*11*), and we propose that the *FUM10* protein catalyzes the CoA activation of tricarballylic acid or a tricarballylic acid precursor. The protein encoded by *FUM14* has homology to nonribosomal peptide synthetase condensation domains (*11*), which catalyze peptide bond formation (*19*). The function of *FUM14* has been demonstrated by expression and isolation of the *FUM14* protein in *Escherichia coli* and in vitro esterification of hydrolyzed fumonisin B_1 (*19*). On the basis of these observations, we propose that the protein encoded by *FUM14* catalyzes the esterification of the CoA-activated tricarballylic acids to the fumonisin backbone. The accumulation of only hydrolyzed forms of fumonisins lacking the C-5 hydroxyl group (hydrolyzed fumonisins B_3 and B_4) in the *FUM10* and *FUM14* deletion mutants rather than hydrolyzed B_1 suggests that esterification of the tricarballylic acid must occur before the hydroxylation of C-5. This is consistent with previous work that indicated that C-5 hydroxylation is the last step in the fumonisin biosynthetic pathway (*13*, *26*).

FUM7 deletion mutants did not produce typical B-series fumonisins but instead produced multiple metabolites that are four mass units less than fumonisin B_1 or B_2/B_3. Analysis of one of the metabolites with *m*/*z* 718 indicated that it was identical to fumonisin B_1 except that each tricarballylic ester contained an alkene function. On the basis of these results, we propose that they all have an alkene function in both tricarballylic esters and refer to them as tetradehydro-fumonisins (**Figure 3**). These results also suggest that the protein encoded by *FUM7* is involved in reducing the alkene function and, further, that prior to their esterification to the fumonisin backbone the tricarballylic acid precursors have an alkene function. On the basis of its predicted sequence, the *FUM7* dehydrogenase belongs to the iron-containing alcohol dehydrogenases protein family. Another member of this class of enzymes is maleylacetate reductase, which catalyzes the reversible reduction of an alkene to an alkane (*27*). Thus, there is precedence for this class of iron-containing alcohol dehydrogenases catalyzing alkene reductions. Different positions of the alkene function within the tricarballylic ester or different isomeric arrangements of the alkene could explain why *FUM7* mutants produce multiple metabolites with *m*/*z* 718 or 702 rather than a single homologue for fumonisin B_1, B_2, and B_3. Further analyses of each metabolite are required to determine whether this is the case. Similarly, detailed analysis of the enzyme encoded by *FUM7* will be useful for clarifying the steps in the pathway for which it is responsible.

The production of tetradehydro-fumonisins by *FUM7* mutants raises the question, what is the substrate of the *FUM7* dehydrogenase? One possible substrate is *cis*-aconitate because of the similarity of its structure to the tricarballylic ester of the putative alkene-containing tetradehydro-fumonisins (**Figure 3**). *cis*-Aconitate is an intermediate in the mitochondrial citric acid cycle and thus a primary metabolite that could be utilized in fumonisin biosynthesis. However, it is not clear how *cis*-aconitate could give rise to multiple isomers of tetradehydro-fumonisins. Thus, it is possible that some tricarboxylic acids other than *cis*-aconitate is the tricarballylic ester precursor.

FUM11 deletion mutants produced the wild-type complement of fumonisins, however, they also produce elevated levels of

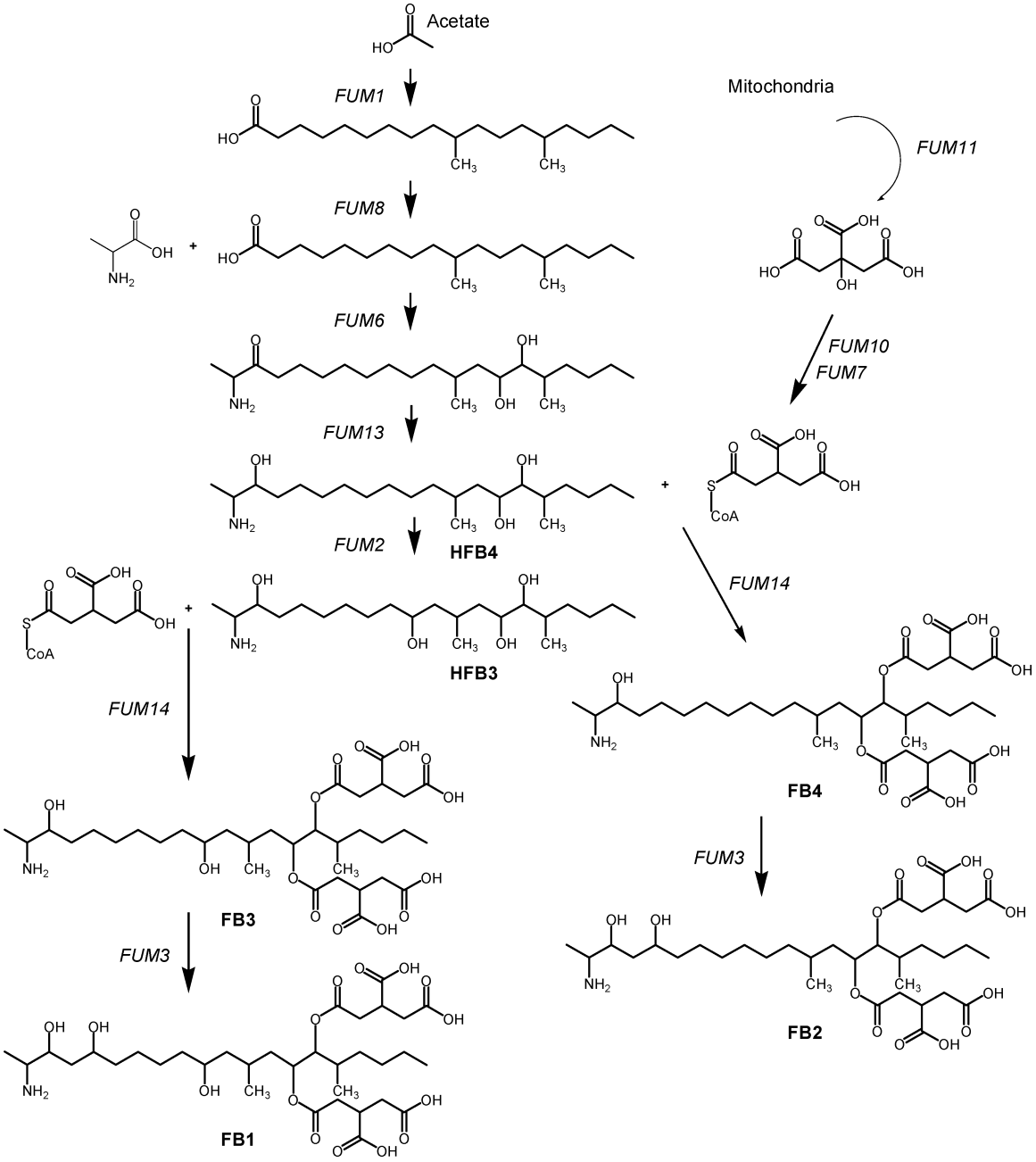

Figure 5. Proposed fumonisin biosynthetic pathway.

half-hydrolyzed and keto half-hydrolyzed fumonisins relative to the wild-type progenitor strain. The predicted *FUM11* protein shares similarity to tricarboxylate transporters suggesting that it might be involved in the transport of tricarboxylic acid precursors of the tricarballylic esters from the inner mitochondria lumen, where they would be produced as a result of the citric acid cycle, to the cytoplasm where they would presumably be accessible to the fumonisin biosynthetic machinery (*11*). The production of the wild-type complement of fumonisins by *FUM11* deletion mutants indicates that *FUM11* is not essential for the procurement of the tricarballylic acid precursor molecules. However, elevated production of half-hydrolyzed and keto half-hydrolyzed fumonisins suggests that the availability of tricarballylic acid precursors could be limited and therefore that the protein encoded by *FUM11* may aid in making the tricarballylic acid precursors available.

The types of fumonisins and fumonisin-like compounds that accumulate in *FUM* gene deletion mutants reported here shed further light on the order of steps in the biosynthetic pathway. The inability of *F. verticillioides* to metabolize the purified tetradehydro-fumonisin B_1 suggests that this metabolite is not a fumonisin biosynthetic intermediate. This in turn suggests that the *FUM7* protein-catalyzed reduction of the carbon−carbon double bond in the tricarballylic acid precursor occurs before esterification to the fumonisin backbone. *FUM7*, however, is not required for esterification to the fumonisin backbone.

Figure 5 shows a proposed fumonisin biosynthetic pathway. Some of the steps in the pathway are based on previously published data (*12*−*17*). The results of the current study provide evidence for the following additional steps in the proposed fumonisin biosynthetic pathway. The predicted *FUM11* tricarboxylate transporter makes a tricarboxylic acid precursor available for fumonisin biosynthesis. The exact nature of the precursor is not completely clear from this work, but candidates include citrate or *cis*-aconitate (**Figure 3**) (*19*). If the precursor is citrate, the *FUM7* dehydrogenase could remove the C-3

hydroxyl of citrate to form tricarballylic acid either before or after the CoA activation by the *FUM10* acyl CoA synthetase and *FUM14* catalyzed esterification of CoA-activated tricarballylic acid to the C-14 and C-15 hydroxyls of the fumonisin backbone. Without *FUM7*, chemical dehydration of the hydroxyl group of citric acid could yield a mixture of products available for CoA activation and esterification resulting in the complex mixture of esters that are observed in the *FUM7* deletion mutant culture extract. Alternatively, if the precursor is *cis*-aconitate, *FUM7* may function to reduce the double bond. In this alternate proposal, feeding studies with tetradehydro-fumonisin B_1 suggests that *FUM7* cannot function on the tricarballylic ester and must therefore act before the *FUM14*-mediated esterification. The fumonisin phenotype of the *FUM7* deletion mutants leaves opportunity for future experiments to describe the fine details of this portion of the fumonisin biosynthetic pathway. Additional studies involving isolation of the gene products and cell-free reactions or producing additional transgenic strains with multiple gene deletions will be needed to determine the exact precursor and order of the steps in the formation of the tricarballylic esters.

Mycotoxin contamination of food and feed products remains a problem. Understanding the biochemistry and genetics of secondary metabolism provides an avenue for determining how these toxins are produced and may lead to a better understanding of why they are produced. We have exploited the tools of molecular biology to dissect the fumonisin biosynthetic pathway in *G. moniliformis*. Analysis of *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* deletion mutants in this study has led to a better understanding of the biochemical reactions required for formation of fumonisins. Further clarification of the fumonisin biosynthetic pathway requires analysis of the activities of enzymes encoded by *FUM* genes (*16*, *19*, *28*).

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LITERATURE CITED

(1) Marasas, W. F. O. Fumonisins: History, world-wide occurrence and impact. In *Fumonisins in Food*; Jackson, L. S., DeVaries, W., Bullerman, L. B., Eds.; Plenum Publishing Co.: New York, 1996.

(2) Marasas, W. F. O.; Riley, R. T.; Hendricks, K.; Stevens, V. L.; Sadler, T. W.; Gelineau-van Waes, J.; Missmer, S. A.; Cabrera, J.; Torres, O.; Gelderblom, W. C. A.; Allegood, J.; Martínez, C.; Maddox, J.; Miller, J. D.; Starr, L.; Sullards, M. C.; Roman, A. V.; Voss, K. A.; Wang, E.; Merrill, A. H. Fumonisins disrupt shingolipid metabolism, folate transport and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *J. Nutr.* **2004**, *134*, 711−716.

(3) Merrill, A. F.; Sullards, M. C.; Wang, E.; Voss, K. A.; Riley, R. T. Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Envrion. Health Perspect.* **2001**, *109 S2*, 283−289.

(4) Riley, R. T.; Wang, E.; Schroeder, J. J.; Smith, E. R.; Plattner, R. D.; Abbas, H.; Yoo, H. -S.; Merrill, A. F. Evidence for disruption of sphingolipid metabolism as a contributing factor in the toxicity and carcinogenicity of fumonisins. *Nat. Toxins* **1996**, *4*, 3−15.

(5) Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, A. H. Inhibition of sphingolipid biosynthesis by fumonisins: implications for diseases associated with *Fusarium moniliforme*. *J. Biol. Chem.* **1991**, *266*, 14486−14490.

(6) Howard, P. C.; Eppley, R. M.; Stack, M. E.; Warbritton, A.; Voss, K. A.; Lorentzen, R. J.; Kovach, R. M.; Bucci, T. J. Fumonisin B1carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. *Environ. Health Perspect.* **2001**, *109 S2*, 277−282.

(7) Marasas, W. F. O. Discovery and occurrence of the fumonisins:a historical perspective. *Environ. Health Perspect.* **2001**, *109 S2*, 239−243.

(8) Nelson, P. E.; Desjardins, A. E.; Plattner, R. D. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annu. Rev. Phytopathol.* **1993**, *31*, 233−252.

(9) Powell, R. G.; Plattner, R. D. Fumonisins. In *Alkaloids: Chemical and Biological Perspectives*; Pelletier, S. W., Ed.; Pergamon Press: Oxford, U.K., 1995.

(10) Butchko, R. A. E.; Plattner, R. D.; Proctor, R. H. *FUM9* is required for C-5 hydroxylation of fumonisins and complements the meiotically defined *Fum3* locus in *Gibberella moniliformis*. *Appl. Environ. Microbiol.* **2003**, *69*, 6935−6937.

(11) Proctor, R. H.; Brown, D. W.; Plattner, R. D.; Desjardins, A. E. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **2003**, *38*, 237−249.

(12) Proctor, R. H.; Plattner, R. D.; Desjardins, A. E.; Busman, M.; Butchko, R. A. E. Fumonisin production in the maize pathogen *Fusarium verticillioides*: genetic basis of naturally occurring chemical variation. *J. Agric. Food Chem.* **2006**, *54*, 2424−2430.

(13) Butchko, R. A. E.; Plattner, R. D.; Proctor, R. H. *FUM13* encodes a short chain dehydrogenase/reductase required for C-3 carbonyl reduction during fumonisin biosynthesis in *Gibberella moniliformis*. *J. Agric. Food Chem.* **2003**, *51*, 3000−3006.

(14) Proctor, R. H.; Desjardins, A. E.; Plattner, R. D.; Hohn, T. M. A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. *Fungal Genet. Biol.* **1999**, *27*, 100−112.

(15) Seo, J. A.; Proctor, R. H.; Plattner, R. D. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet. Biol.* **2001**, *34*, 155−165.

(16) Yi, H.; Bojja, R. S.; Fu, J.; Du, L. Direct evidence for the function of *FUM13* in 3-ketoreduction of mycotoxin fumonisins in *Fusarium verticillioides*. *J. Agric. Food Chem.* **2005**, *53*, 5456−5460.

(17) Bojja, R. S.; Cerny, R. L.; Proctor, R. H.; Du, L. Determining the biosynthetic sequence in the early steps of the fumonisin pathway by use of three gene-disruption mutants of *Fusarium verticillioides*. *J. Agric. Food Chem.* **2004**, *52*, 2855−2860.

(18) Proctor, R. H.; Butchko, R. A. E.; Plattner, R. D.; Brown, D. W.; Desjardins, A. E. Functional analysis of the fumonisin biosynthetic gene cluster in *Fusarium verticillioides*. *Mycopathologia* **2004**, *157*, 406.

(19) Zaleta-Rivera, K.; Xu, C.; Yu, F.; Butchko, R. A. E.; Proctor, R. H.; Hidalgo-Lara, M. E.; Raza, A.; Dussault, P. H.; Du, L. A bidomain nonribosomal peptide synthetase encoded by *FUM14* catalyzes the formation of tricarballylic esters in the biosynthesis of fumonisins. *Biochemistry* **2006**, *45*, 2561−2569.

(20) Leslie, J. F.; Plattner, R. D.; Desjardins, A. E.; Klittich, C. J. R. Fumonisin B_1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section Liseola). *Phytopathology* **1992**, *82*, 341−345.

(21) Desjardins, A. E.; Plattner, R. D.; Proctor, R. H. Linkage among genes responsible for fumonisin biosynthesis in *Gibberella fujikuroi* mating population A. *Appl. Environ. Microbiol.* **1996**, *62*, 2571−2576.

(22) Brown, D. W.; McCormick, S. P.; Alexander, N. J.; Proctor, R. H.; Desjardins, A. E. Inactivation of a cytochrome P-450 is a determinant of trichothecene diversity in *Fusarium species*. *Fungal Genet. Biol.* **2002**, *36*, 224−233.

(23) Turgeon, B. G.; Garber, R. C.; Yoder, O. C. Development of a fungal transformation system based on selection of sequences with promoter activity. *Mol. Cell. Biol.* **1987**, *7*, 3297−3305.

(24) Plattner, R. D.; Weisleder, D.; Poling, S. M. Analytical determination of fumonisins and other metabolites produced by *Fusarium moniliforme* and related species on corn. In *Fumonisins in Food*; Jackson, L. S., DeVaries, W., Bullerman, L. B., Eds.; Plenum Press: New York, 1996.

(25) Plattner, R. D.; Weisleder, D.; Shackelford, D. D.; Peterson, R.; Powell, R. G. A new fumonisin from solid cultures of *Fusarium moniliforme*. *Mycopathologia* **1992**, *117*, 23−28.

(26) Proctor, R. H.; Desjardins, A. E.; Plattner, R. D. Biosynthetic and genetic relationships of B-series fumonisins produced by *Gibberella fujikuroi* mating population A. *Nat. Toxins* **1999**, *7*, 251−258.

(27) Daubaras, D. L.; Saido, K.; Chakrabarty, A. M. Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Appl. Environ. Microbiol.* **1996**, *62*, 4267−4279.

(28) Ding, Y.; Bojja, R. S.; Du, L. Fum3p, a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins in *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **2004**, *70*, 1931−1934.

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