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Linkage among Genes Responsible for Fumonisin Biosynthesis in *Gibberella fujikuroi* Mating Population A

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Most naturally occurring strains of the fungus *Gibberella fujikuroi* mating population A produce high levels of the mycotoxin fumonisin B_1 (FB_1), which is oxygenated at both carbons C-5 and C-10. Some strains, however, produce only FB_2 or FB_3, suggesting that they lack the ability to hydroxylate position C-10 or C-5, respectively. Genetic analysis indicates that these different phenotypes are due to single gene defects at closely linked loci designated *fum2* and *fum3*. Further allelism tests indicate that both *fum2* and *fum3* are closely linked to *fum1*, a previously identified gene that regulates fumonisin production. The recovery frequency of FB_1-producing progeny from cross 510 between *fum1* and *fum2* mutations suggests a map distance of approximately 6.2 cM between these two loci. Amplified fragment length polymorphism analysis of parents and progeny of cross 510 was employed to confirm that the FB_1-producing strains are recombinant progeny. We conclude that *fum1*, *fum2*, and *fum3* constitute a fumonisin biosynthetic gene cluster on chromosome 1 of the restriction fragment length-map of *G. fujikuroi*.

Fumonisins are a family of amino-polyalcohols that contaminate maize-based foods and feeds worldwide. Fumonisins are produced by several closely related species of *Fusarium* that can grow within maize tissues without causing visible symptoms of disease (10). Interest in fumonisins is due primarily to the discovery that they are potent inhibitors of sphingolipid biosynthesis (17) and that they can impair animal health (10). Fumonisins were discovered only in 1988 (1), and their biochemistry and toxicology are areas of active research. The biosynthesis of fumonisins is believed to occur through condensation of an amino acid (alanine) to an acetate-derived precursor (2, 3). Branched-chain methyl groups are added at C-12 and C-16 by an *S*-adenosylmethionine transferase (3). The number and order of the steps of the biosynthetic pathway that lead to oxygenation of the acetate-derived backbone and subsequent esterification are unknown. To date, no fumonisin biosynthetic enzymes have been purified and no genes have been cloned.

The most prevalent fumonisin homolog in naturally contaminated maize is fumonisin B_1 (FB_1), which has free or esterified hydroxyl groups at carbon atoms C-3, C-5, C-10, C-14, and C-15. Less-oxygenated fumonisin homologs also occur naturally, but they are usually much less abundant than FB_1. These homologs include FB_2, which lacks the C-10 hydroxyl group; FB_3, which lacks the C-5 hydroxyl group; and FB_4, which lacks both the C-5 and C-10 hydroxyl groups (Fig. 1) (14). If fumonisin biosynthesis is similar to the biosynthesis of other fungal secondary metabolites such as aflatoxins and trichothecenes (4, 5, 18), then less-oxygenated homologs, such as FB_4, FB_3, and FB_2, are likely biosynthetic precursors of the most highly oxygenated homolog, FB_1.

We have undertaken studies of the genetics and molecular biology of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) in an effort to better understand fumonisin biosynthesis (6, 7, 13). *G. fujikuroi* mating population A is haploid, and its heterothallic sexual cycle facilitates classical genetic analysis. In addition, transformation systems and a restriction fragment length polymorphism map of this species (18) facilitate molecular genetic analysis. Classical genetic analysis is based on the use of mutant strains that differ in the phenotype under study. Although most (>95%) naturally occurring strains of *G. fujikuroi* mating population A produce predominately FB_1, extensive strain surveys have identified variants with unusual fumonisin production phenotypes. Previously, maize collected in Nepal yielded strains that consistently produce no detectable FB_1, FB_2, FB_3, or FB_4 (11). Genetic analysis of two of these strains showed that changes at a single locus (or group of closely linked loci), designated *fum1*, could block fumonisin production (7). Marker-based mapping has localized the *fum1* locus to a region of the largest chromosome (chromosome 1) of *G. fujikuroi* mating population A (18). Recently, maize collected in South Carolina yielded two new classes of fumonisin production variants: strain A-0822, which produces FB_2 and FB_4 but no FB_1 or FB_3; and strain A-0819, which produces FB_3 and FB_4 but no FB_1 or FB_2 (13). The objective of this study was to discern the genetic basis of these two new unusual phenotypes, so that these strains could be used to further elucidate the fumonisin biosynthetic pathway. A preliminary report of some of these results was presented previously (6).

MATERIALS AND METHODS

Fungal cultures. Strain numbers, original hosts, and geographic origins of the field strains used in this study are given in Table 1. Strains with the prefix M were obtained from the Fusarium Research Center, The Pennsylvania State University, University Park. Strains with the prefix A were obtained from J. F. Leslie, Kansas State University, Manhattan. All strains originated from single microconidia. Crosses for genetic analysis were made on carrot agar, and random ascospores were dissected freehand at ×150 with a dissecting microscope. To avoid contamination with conidia during ascospore isolation, individual perithecia were removed from culture plates and transferred to a Buchner funnel lined with sterile cloth. The perithecia were rinsed well with several aliquots of sterile water, and then each perithecium was transferred to a drop of water on a plate of 5% agar. The perithecium was crushed to allow large rosettes of asci to exude into the water. Individual ascospores were then separated with a mounted eyelash. Each ascospore was isolated by the cutting of a box in the surrounding agar and was transferred to a culture tube.

AFLP. Amplified fragment length polymorphism (AFLP) was carried out as

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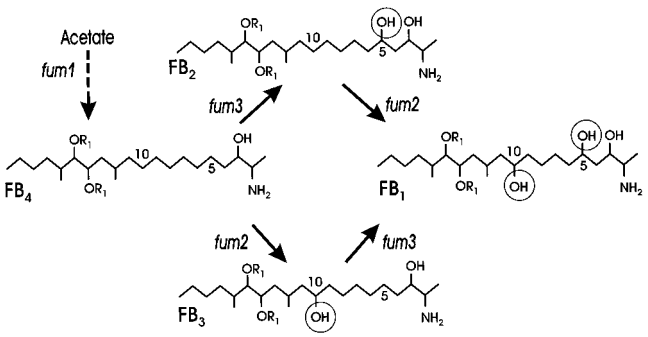

FIG. 1. Proposed model for fumonisin biosynthesis based on the genetic data in this study. R_1 designates tricarballylic acid esters.

described previously (16) with the modifications noted below. Fungal genomic DNA was isolated as described previously (8), except that after the chloroform extraction, the aqueous phase was incubated with RNase for 30 min at room temperature, combined with an equal volume of 5 M LiCl, incubated for 15 min on ice, and centrifuged at 16,000 × *g* for 10 min. DNA was precipitated from the supernatant with 2.5 volumes of 95% ethanol and resuspended in Tris-EDTA (TE) buffer (15). One-half microgram of this DNA was digested with the restriction enzymes *Mse*I and *Eco*RI and ligated to the *Mse*I and *Eco*RI adapters as described previously (16), except that the *Eco*RI adapter was biotinylated. Biotinylated ligation products were separated from nonbiotinylated products by incubation of the ligation mix with streptavidin beads (Promega) suspended in 2× STEX (200 mM NaCl, 20 mM Tris-HCl, 2 mM EDTA, 0.2% Triton X-100 [pH 8.0]) at room temperature for 30 min (20). The beads were washed three times with STEX and resuspended in 200 ml of 10 mM Tris · HCl–0.1 mM EDTA (pH 8.0) (15). Five microliters of the resulting solution was used as a template in 20-μl PCR mixtures containing the following: 1 ng of ^{32}P end-labeled *Eco*RI primer, 30 ng of *Mse*I primer, 50 mM deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, and 10 μg of gelatin per ml. The first PCR cycle consisted of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min. In cycles 2 to 10, the conditions were the same, except that the annealing temperature was lowered 1°C per cycle (to 56°C in cycle 10). In cycles 11 to 33, the annealing temperature was held at 56°C, and all other conditions were as indicated above. The *Mse*I primers had the nucleotide sequence 5′-GATGA GTCCTGAGTAAX$_1$X$_2$-3′, where X_1 and X_2 are the nucleotide pairs AG, GG, and TG in primers MSE4, MSE14, and MSE15, respectively. *Eco*RI primers had the sequence 5′-CTGCGTTACCAATTCX$_1$X$_2$-3′, where X_1 and X_2 are the nucleotide pairs AG and AC in primers ECOR4 and ECOR6, respectively. Samples were analyzed by polyacrylamide gel electrophoresis and autoradiography as described previously (16).

Analysis of fumonisin production. The production of FB_1, FB_2, and FB_3 by field strains and ascospore progeny was assessed after 4 weeks' growth on autoclaved maize kernels by high-performance fluorescence liquid chromatography as previously described (10). Detection limits for fumonisins were 5 μg/g of culture material. FB_4 was not quantitated in this study.

RESULTS

Segregation of fumonisin production phenotypes. Crosses were made between strains of *G. fujikuroi* mating population A that differ in their ability to produce FB_1, FB_2, and FB_3 on autoclaved maize kernels. The characteristics of the field strains of *G. fujikuroi* that were used in this study are presented in Table 1. An outline of the principal crosses in this study is presented in Fig. 2, and a summary of the segregation of fumonisin production among progeny of these crosses is presented in Table 2. Because FB_4 was found to be produced by all tested strains that produced the more oxygenated fumonisins, FB_4 was not a useful marker for genetic analysis of fumonisin biosynthesis and was not quantitated in this study.

In cross 109, strain A-0822, which produces FB_2 but no FB_1 or FB_3, was crossed to strain M-3120, which produces predominately FB_1 with lower levels of FB_2 and FB_3. Cross 109 was of low fertility, with 43% viability of random ascospore progeny. The parents and 40 progeny of cross 109 were scored for their ability to produce FB_1 and FB_2. Parent A-0822 produced only FB_2 at 1,030 μg/g of culture material; 20 progeny produced FB_2 at 7,100 ± 4,090 μg/g (mean ± standard deviation) but produced no FB_1 and little or no FB_3. Parent M-3120 produced FB_1, FB_2, and FB_3 at a total of 9,130 μg/g; 19 progeny produced FB_1, FB_2, and FB_3 at a total of 6,190 ± 370 μg/g. Qualitative assessment of FB_4 revealed it to be produced by all progeny that produce FB_1. One progeny produced no detectable fumonisins. This 20:19 (1:1, $\chi^2 = 0.5$) segregation from cross 109 defines a single locus, or group of closely linked loci, designated *fum2* that determines whether FB_1 and FB_3, fumonisins hydroxylated at C-10, are produced.

In cross 397, strain A-0819, which produces FB_3 but no FB_1 or FB_2, was crossed as a male to strain M-3125, which produces predominately FB_1, with lower levels of FB_2 and FB_3. Cross 397 was of low fertility, with 36% viability of random ascospore progeny. The parents and 40 progeny from cross 397 were scored for their ability to produce FB_1, FB_2, and FB_3. Parent A-0819 produced only FB_3 at 3,670 and 13,990 μg/g in two replicate tests; 16 progeny produced FB_3 at 11,080 ± 8,780 μg/g but no FB_1 and little or no FB_2. Parent M-3125 produced FB_1, FB_2, and FB_3 at a total of 12,260 and 10,400 μg/g in two replicate tests; 23 progeny produced FB_1, FB_2, and FB_3 at a total of 6,920 ± 4,360 μg/g. One progeny produced no detectable fumonisins. This 23:16 (1:1, $\chi^2 = 2.8$) segregation defines a single locus, or group of closely linked loci, designated *fum3* that determines whether FB_1 and FB_2, fumonisins hydroxylated at C-5, are produced.

Allelism tests of *fum1*, *fum2*, and *fum3*. The *fum1* locus was previously identified by segregation ratios of fumonisin production in crosses between strains M-3120 and M-3125, which produce high levels of FB_1, FB_2, and FB_3, and fumonisin-nonproducing strains from Nepal (7). To establish linkage relationships among *fum1*, *fum2*, and *fum3*, the three classes of mutants were crossed to each other. Because of the poor female fertility of the field strains M-5500, A-0819, and A-0822, female fertile progeny of a suitable mating type and fumonisin phenotype were selected as parents for allelism tests.

First, in cross 459, strain A-0819 (*fum1*$^+$ *fum2*$^+$ *fum3*$^-$) was crossed to strain 109-R-14, a *fum1*$^+$ *fum2*$^-$ *fum3*$^+$ progeny of strain A-0822. Cross 459 was of high fertility, with 85% viability of random ascospore progeny. Forty-nine progeny were tested for fumonisin production. Twenty-nine progeny produced only FB_2 at 7,670 ± 3,500 μg/g; 20 progeny produced only FB_3 at

TABLE 1. Characteristics of field strains of *G. fujikuroi* in this study

Strain	Geographic origin	Host	Mating type	Fumonisin phenotype	Putative fumonisin genotype
M-3120	Calif.	Sorghum	A$^+$	FB_1 FB_2 FB_3	*fum1*$^+$*fum2*$^+$*fum3*$^+$
M-3125	Calif.	Maize	A$^-$	FB_1 FB_2 FB_3	*fum1*$^+$*fum2*$^+$*fum3*$^+$
M-5500	Nepal	Maize	A$^+$	None	*fum1*$^-$*fum2*$^+$*fum3*$^+$
A-0819	S.C.	Maize	A$^+$	FB_3	*fum1*$^+$*fum2*$^+$*fum3*$^-$
A-0822	S.C.	Maize	A$^-$	FB_2	*fum1*$^+$*fum2*$^-$*fum3*$^+$

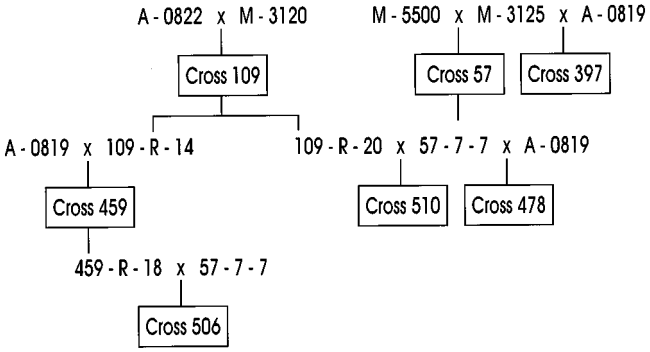

FIG. 2. Principal crosses used in this study.

6,080 ± 3,040 μg/g. No recombinant, FB_1-producing progeny were recovered. This result indicated that the *fum2* and *fum3* mutations are closely linked or allelic.

Next, in cross 510, strain 109-R-20 (*fum1*$^+$ *fum2*$^-$ *fum3*$^+$) was crossed to strain 57-7-7, a *fum1*$^-$ *fum2*$^+$ *fum3*$^+$ progeny of cross 57 (M-5500 × M-3125). Cross 510 was of moderate fertility with 65% viability of random ascospore progeny. Sixty-five progeny were tested for fumonisin production. Thirty progeny produced FB_2 at 7,800 ± 2,970 μg/g but no FB_1 or FB_3; 33 progeny produced no fumonisins. Two recombinant progeny, designated 510-R-11 and 510-R-53, that produced wild-type levels (10,780 and 4,150 μg/g) and ratios of FB_1, FB_2, and FB_3 were recovered from cross 510, indicating that the *fum1* and *fum2* mutations are closely linked but not allelic. The percentage of recombination between *fum1* and *fum2* (4 of 65) is estimated to be 6.2%. Approximately two progeny are assumed to be *fum1*$^-$ *fum2*$^-$ double mutants which cannot be distinguished because *fum1* is epistatic to *fum2*.

Finally, in crosses 478 and 506, respectively, strain A-0819 and a progeny, 459-R-18 (both of which are *fum1*$^+$ *fum2*$^+$ *fum3*$^-$), were crossed to strain 57-7-7 (*fum1*$^-$ *fum2*$^+$ *fum3*$^+$). Crosses 478 and 506 gave 61 and 50% viability, respectively, of random ascospore progeny. Including both crosses, 11 progeny produced FB_3 at 9,420 ± 3,960 μg/g but no FB_1 or FB_2; 27 progeny produced no fumonisins. The segregation ratios of crosses 478 and 506 are slightly skewed ($\chi^2 = 9.0$, $\chi^2 = 27.0$) from 1:1, with fumonisin-nonproducing progeny in excess. Ascospore viability was low in both crosses, and differences in ascospore viability in these crosses may be skewing the ratios observed. The excess nonproducers are unlikely to be recombinant, double mutants, because no corresponding FB_1-producing recombinants were recovered in either cross. Thus, the *fum1* and *fum3* mutations appear to be closely linked or allelic.

Confirmation of recombinant genotypes. The strains of *G. fujikuroi* employed in this study are, or are derived from, unmarked field isolates. As a result, the two recombinant FB_1-producing strains recovered from cross 510 did not have genetic markers to help confirm that they were indeed progeny of strains 109-R-20 (*fum1*$^+$ *fum2*$^-$ *fum3*$^+$) and 57-7-7 (*fum1*$^-$ *fum2*$^+$ *fum3*$^+$). Such markers would have been particularly useful, since the preponderance of *G. fujikuroi* field isolates produce FB_1. To obtain confirmatory evidence that the FB_1-producing strains, 510-R-11 and 510-R-53, recovered from cross 510 were progeny of strains 109-R-20 and 57-7-7, we identified molecular genetic markers generated by AFLP.

In AFLP, (i) genomic DNA is digested with two restriction enzymes, (ii) oligonucleotide linkers are ligated to each end of the resulting restriction fragments, and (iii) the linker-restriction fragment-linker chimeras are used as templates in PCR to amplify the fragments (16). The amplification products constitute AFLP fragments, and individual fragments can serve as molecular markers when they are present in some strains but absent in others. Differences in AFLP markers among two or more strains result from differences in the positions of restriction enzyme recognition sequences. Oligonucleotides used as PCR primers in AFLP are complementary to the linker and restriction enzyme recognition sequences and also have two selective nucleotides at their 3′ ends (16). Selective nucleotides

TABLE 2. Summary of the segregation of different fumonisin production phenotypes[a]

Cross no.	Parental		No. of random ascospore progeny in each fumonisin phenotype class				
	Female and male pair	Fumonisin phenotype	FB_1 FB_2 FB_3	FB_2	FB_3	No fumonisins	Total progeny
57	M-3125 (female) M-5500 (male)	FB_1 FB_2 FB_3 No fumonisins	31	0	0	25	56[b]
109	M-3120 (female) A-0822 (male)	FB_1 FB_2 FB_3 FB_2	19	20	0	1	40
397	M-3125 (female) A-0819 (male)	FB_1 FB_2 FB_3 FB_3	23	0	16	1	40
459	109-R-14 (female) A-0819 (male)	FB_2 FB_3	0	29	20	0	49
478	57-7-7 (female) A-0819 (male)	No fumonisins FB_3	0	0	6	11	17
506	57-7-7 (female) 459-R-18 (male)	No fumonisins FB_3	0	0	5	16	21
510	57-7-7 (female) 109-R-20 (male)	No fumonisins FB_2	2	30	0	33	65

[a] Maize cultures of all strains were incubated for 4 weeks and analyzed for fumonisins by high-performance liquid chromatography (6, 10).
[b] Data were derived from references 6 and 7.

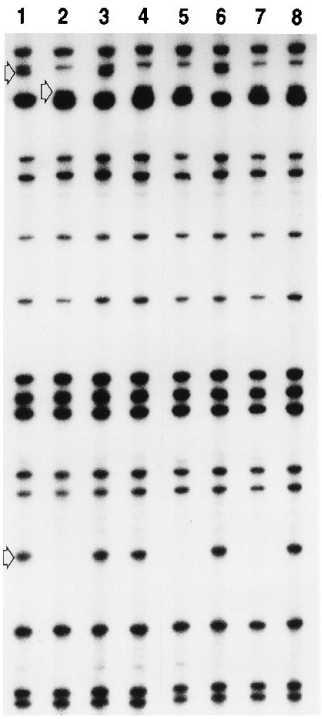

FIG. 3. Portion of an autoradiogram showing AFLP fragments amplified with primers ECOR6 and MSE15. The templates used in the PCR were derived from genomic DNA of the parents and six selected progeny from cross 510. Lanes: 1, FB_2-producing parental strain 109-R-20; 2, fumonisin-nonproducing parental strain 57-7-7; 3 and 4, recombinant FB_1-producing progeny 510-R-11 and 510-R-53, respectively; 5 and 6, FB_2-producing parent-type progeny 510-R-6 and 510-R-12, respectively; 7 and 8, fumonisin-nonproducing parent-type progeny 510-R-5 and 510-R-9, respectively. Arrows indicate AFLP markers present in only one parent but inherited by one or both recombinant progeny and by one or more of the parent-type progeny.

limit the number of AFLP fragments that are amplified by PCR, making it easier to see individual fragments. Thus, the only templates amplified with primers with selective nucleotides are those that have at each end a linker followed by the restriction enzyme recognition sequence next to two nucleotides complementary to the selective nucleotides. Furthermore, AFLP fragments amplified by PCR can be varied by changing the selective nucleotides on the PCR primers.

Two hundred seventy-two distinct AFLP fragments were amplified from the two parental strains from cross 510 with four primer pairs. With few exceptions, fragments that were amplified from both parents were amplified from all six progeny that were examined, including the two recombinant FB_1-producing strains, 510-R-11 and 510-R-53. All fragments that were amplified from the recombinant progeny were also amplified from one or both of the parental strains. In addition, fragments that were amplified from only one parent were amplified from at least some of the progeny, including strains 510-R-11 and 510-R-53. Figure 3 shows a portion of an AFLP autoradiogram in which AFLP markers that are present in only one parental strain were also present in one or both of the recombinant progeny. These markers appeared to be inherited by the recombinant, FB_1-producing strains in the same manner that they were by the parent-type progeny. Therefore, the AFLP data indicate that the FB_1-producing strains, 510-R-11 and 510-R-53, are indeed progeny of strains 109-R-20 and 57-7-7.

To obtain a more quantitative assessment of the AFLP data, we determined the number of differences in AFLP fragments that were amplified from the eight strains examined. We expected that more distantly related strains (i.e., two unrelated parents) would exhibit a greater number of differences than more closely related strains (e.g., a parent and offspring). Among the 272 distinct AFLP fragments amplified in this study, 194 were present in both parents, while 78 were present in only one parent or the other. Thus, there were 78 differences in AFLP fragments between the two parents (Table 3). As expected, fewer differences in AFLP fragments occurred in all other pairwise comparisons of more closely related individuals (i.e., parent versus progeny and progeny versus progeny). When an FB_1-producing progeny (510-R-11 or 510-R-53) and a parent-type progeny were compared, the number of AFLP differences were within the same range as the number of differences (23 to 53) that occurred when two parent-type progeny were compared. Similarly, when a recombinant progeny and a parent were compared, the numbers of AFLP fragment differences were similar to the numbers of differences observed when a parent and a parent-type progeny were compared. In the comparison of 109-R-20 (parent) and 510-R-11 (recombinant progeny), however, there was a slightly greater number of differences observed than in any other parent-progeny comparison (Table 3). Nevertheless, this examination of the AFLP data is also consistent with the proposed parent-offspring relationship between the parents (109-R-20 and 57-7-7) and FB_1-producing strains (510-R-11 and 510-R-53) from cross 510.

DISCUSSION

To facilitate the study of fumonisin biosynthesis, we have tried to identify variants of *G. fujikuroi* mating population A

TABLE 3. Differences in AFLP fragments amplified from the parents (57-7-7 and 109-R-20) and selected progeny from cross 510

Strain	Fumonisin phenotype	No. of AFLP fragments that differ between strain pair[a]							
		57-7-7	109-R-20	510-R-11	510-R-53	510-R-6	510-R-12	510-R-5	510-R-9
57-7-7	None	0							
109-R-20	FB_2	78	0						
510-R-11	FB_1 FB_2 FB_3	20	64	0					
510-R-53	FB_1 FB_2 FB_3	40	40	30	0				
510-R-6	None	32	48	38	46	0			
510-R-12	None	39	47	41	51	45	0		
510-R-5	FB_2	50	34	46	38	48	49	0	
510-R-9	FB_2	57	27	53	37	53	36	27	0

[a] Values are the number of AFLP fragments that differ between a given pair of strains. A value of 0 indicates no difference (as when a strain is compared with itself). Pairwise comparisons were made between the strains listed horizontally (above) and the same set of strains listed vertically (to the left). Primer pairs ECOR4 and MSE4, ECOR4 and MSE14, ECOR6 and MSE14, and ECOR6 and MSE15 were used to amplify the 272 AFLP fragments used in this analysis.

that are blocked in the production of FB_1. Because fumonisins are nonessential for fungal growth in vitro, there is no obvious direct selection method for blocked mutants; thus, blind selection and screening of strains are unavoidable. Furthermore, although fumonisins can be produced to high levels in various liquid culture systems, the production by many strains in liquid culture is at a lower level and is more variable than that in maize kernel culture (13). Therefore, long-term (4 weeks) growth on maize kernels, although cumbersome, is advisable for confirmation of the product profiles of fumonisin homologs, such as FB_1, FB_2, FB_3, and FB_4. In the past, screening for mycotoxin biosynthetic mutants has been facilitated by the availability of rapid and specific immunoassays. However, although immunoassays for FB_1 are available, the antibodies developed to date have not been specific for FB_1 (12). Cross-reactivity of FB_1 antibodies with homologs such as FB_2 and FB_3 has limited their usefulness for mutant screening and has forced reliance on more difficult and time-consuming chemical detection methods. All together, these technical difficulties have made the identification and characterization of fumonisin biosynthetic mutants a laborious and slow process.

Surveys of more than 350 genetically fertile strains of *G. fujikuroi* mating population A isolated from various substrates in Central America, India, Mexico, Nepal, Taiwan, China, and the United States have found that more than 95% of these naturally occurring strains produce predominately FB_1 (7, 9, 11, 13). These surveys, however, have identified a small number of FB_1-deficient variants whose phenotypes segregate as single genetic loci in crosses with strains that produce high levels of FB_1. To date, four different classes of putative fumonisin biosynthetic pathway genes have been identified by this classical genetic approach. The first class, *fum1*, is represented by a group of strains from Nepal that produce no detectable FB_1, FB_2, FB_3, or FB_4. The second class, *fum4*, is represented by one strain from Kansas that acts to generally reduce, but not always completely block, fumonisin production. *fum4* is not allelic with *fum1*, but it is closely linked, giving a 4.3% frequency of recombination (13). In this paper, we genetically characterize two new classes of mutations, *fum2* and *fum3*, that affect hydroxylation at C-10 and C-5, respectively, but that have no effect on the overall levels of fumonisins produced. We also report here that *fum2* and *fum3* are closely linked to *fum1* and thus are located on chromosome 1 of *G. fujikuroi* mating population A. Xu and Leslie (18) estimated the average kilobase/centi-Morgan (cM) ratio for chromosome 1 to be 58 kb/cM for their mapping cross (which utilized parents derived from M-3120 and M-5538). Thus, *fum4* could be at a distance of approximately 250 kb from *fum1* and *fum2* could be at a distance of 360 kb from *fum1*. These map distances must be considered tentative and do not indicate gene order. However, both *fum2* and *fum4* appear to map closer to *fum1* than any of the restriction fragment length polymorphism and random amplified polymorphic DNA markers identified to date, the four closest of which map from 7.8 to 25 cM away from *fum1* (6). Identification of more closely linked DNA band markers is in progress and will be necessary before *fum* genes in this region are accessible to map-based cloning strategies.

The results of AFLP provide confirming evidence that the FB_1-producing strains 510-R-11 and 510-R-53 are indeed recombinant progeny of parents 57-7-7 and 109-R-20, because a number of AFLP fragments that were unique to each parent were inherited by one or both of the recombinant progeny. Furthermore, the number of DNA polymorphisms between these recombinant progeny and the parents was similar to the number of polymorphisms between the parents and other progeny from the cross. If 510-R-11 and 510-R-53 were contaminants, one would expect them to contain AFLP fragments that are not present in the parental strains and to differ from one another and the parental strains as much as the parental strains differ from one another.

The genetic analysis presented in this paper provides support for two hypothesis. First, close linkage of *fum1*, *fum2*, *fum3*, and *fum4* indicates that these genes may constitute a gene cluster on chromosome 1 of *G. fujikuroi* mating population A. It would not be unexpected if this were so, because genes that control biosynthesis of sterigmatocystin and aflatoxins by *Aspergillus* species and of trichothecenes by *Fusarium* species are also organized in complex clusters (4, 5, 19). The functional significance of gene clustering for these mycotoxin biosynthetic pathways is not clear. Gene clustering may facilitate the formation of chromosome structures involved in the regulation of gene expression or may reflect unique evolutionary origins of fungal toxin biosynthetic pathways.

Second, the genetic analysis supports the fumonisin biosynthetic scheme proposed in Fig. 1, although genetics alone cannot prove that this scheme is correct. It should also be noted that *fum* loci could encode regulatory genes rather than enzymes required directly for fumonisin biosynthesis. The simplest biosynthetic scheme consistent with the genetic data is that FB_4 can be converted to FB_1 via FB_2 or FB_3. *fum2* would thus control hydroxylation of C-10 and conversion of FB_4 to FB_3 and of FB_2 to FB_1. Therefore, in a *fum2*$^-$ mutant, neither FB_1 nor FB_3 could form. Complementarily, in a *fum3*$^-$ mutant, neither FB_1 nor FB_2 could form, because *fum3* controls hydroxylation of C-5 and conversion of FB_4 to FB_2 and of FB_3 to FB_1. If FB_1 biosynthesis is analogous to sphingosine biosynthesis, then alanine would be condensed with the coenzyme A ester of an 18-carbon fatty acid. However, it is not yet clear whether oxygenation and methylation occur before or after condensation with alanine, and preliminary efforts to distinguish these routes by feeding FB_2 and FB_3 to cultures of *G. fujikuroi* mating population A have been unsuccessful. The added compounds were neither degraded nor converted to FB_1 by the fungal cultures (12a). These data may indicate that hydroxylation occurs prior to condensation with alanine or may simply reflect poor uptake of fumonisins by fungal cells.

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