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Molecular Characterization of the *afl-1* Locus in *Aspergillus flavus*†

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An unusual mutation at the *afl-1* locus, affecting aflatoxin biosynthesis in *Aspergillus flavus* 649, was investigated. The inability of strain 649 to produce aflatoxin was found to be the result of a large (greater than 60 kb) deletion that included a cluster of aflatoxin biosynthesis genes. Diploids formed by parasexual crosses between strain 649 and the aflatoxigenic strain 86 did not produce aflatoxin, indicating the dominant nature of the *afl-1* mutation in strain 649. In metabolite feeding experiments, the diploids did not convert three intermediates in the aflatoxin pathway to aflatoxin. Northern (RNA blot) analysis of the diploids grown in medium conducive for aflatoxin production indicated that the aflatoxin pathway genes *nor1*, *ver1*, and *omt1* were not expressed; however, there was low-level expression of the regulatory gene *aflR*. Pulsed-field electrophoresis gels indicated a larger (6 Mb) chromosome in strain 649 than the apparently homologous (4.9 Mb) chromosome in strain 86. The larger chromosome in strain 649 suggests that a rearrangement occurred in addition to the deletion. From these data, we propose that a trans-sensing mechanism in diploids is responsible for the dominant phenotype associated with the *afl-1* locus in strain 649. Such a mechanism is known in *Drosophila melanogaster* but has not been described for fungi.

Aflatoxins are decaketides produced by *Aspergillus flavus* and *Aspergillus parasiticus* (4). Members of this family of toxins are common contaminants on corn, tree nuts, peanuts, and cotton seed. While animal health is most affected by the toxic effects of aflatoxins, the potential carcinogenicity in humans has caused worldwide concern about aflatoxin contamination of food sources (14).

Biosynthesis of aflatoxin occurs by the polyketide pathway of secondary metabolism (4). Like that of other polyketides, aflatoxin biosynthesis starts with the condensation of acetate units by a reaction mechanism analogous to fatty acid biosynthesis. Recently, a gene from *A. parasiticus* that may code for the polyketide synthase responsible for these initial steps in aflatoxin biosynthesis was isolated (7). The synthase is similar to that involved in the biosynthesis of 6-methylsalicylic acid in *Penicillium patulum*, a large polypeptide with multiple enzymatic domains (1). The first stable intermediate of the aflatoxin biosynthetic pathway is norsolorinic acid, and this metabolite is converted to aflatoxin by an estimated 16 enzyme-catalyzed steps (4). Purification of the enzymes in the pathway has been difficult because they are produced in low quantities and are unstable, and convenient assays are lacking. Despite these difficulties, several of the enzymes have been purified (3, 11, 20, 22, 24).

Genetic studies on *A. flavus* were pioneered by K. E. Papa. *A. flavus* and *A. parasiticus* do not have a known teleomorph, but a parasexual cycle has been described for both species (26, 27). Among the many mutant strains of *A. flavus* generated and studied by Papa, 23 were aflatoxin-nonproducing mutants, and a number of spore color and auxotrophic mutants were derived from the common parental strain, PC-7 (2). Parasexual mating analysis was used to map more than 30 mutations to eight linkage groups (28–31). The mutations affecting aflatoxin biosynthesis are all recessive in diploids except for a mutation at the *afl-1* locus, which is dominant (2). Of the 11 aflatoxin loci mapped, 9 are in linkage group VII, 1 is in linkage group II, and 1 is in linkage group VIII. Papa was able to determine the order of several loci in linkage group VII as (*afl-1 nor1*), *leu7*, *afl-15*, *arg7*, *afl-17*, and the centromere. Crossing-over between *nor1* and *afl-1* was not detected; thus, it was not possible to establish which was closer to *leu7*. The specific locations of the other group VII loci have not been determined (2).

Recently, molecular biology techniques have been used to further study the *A. flavus* strains. Thus far, seven auxotrophic mutations have been complemented with cloned wild-type DNA: *thi1*, *arg2*, *lys4*, *pdx6*, *arg7*, *leu7*, and *his8* (15). Four genes (*nor1*, *ver1*, *omt1*, and *aflR*) involved in aflatoxin biosynthesis have been isolated from *A. flavus* and *A. parasiticus* (8, 9, 32, 35, 38, 40, 45). Three of these genes code for structural pathway enzymes: *nor1* is involved in the conversion of norsolorinic acid to averantin; *ver1* is involved in the conversion of versicolorin to sterigmatocystin; and *omt1* is involved in the conversion of sterigmatocystin to *O*-methylsterigmatocystin. The *aflR* gene appears to be a regulatory gene coding for a protein with a zinc cluster motif, suggesting a DNA-binding function (40). Nucleic acid sequence analysis of these genes indicates that the similarity between the *A. flavus* and *A. parasiticus* genes is greater than 95%. Also, it appears that the genes are present as a single cluster in the genome of these fungi. A map of the gene cluster in *A. flavus* and *A. parasiticus* was recently constructed, indicating the positions of the genes on a 60-kb fragment of genomic DNA (46).

The research presented here focuses on the repressor activity associated with the mutation at the *afl-1* locus in *A. flavus* 649. Research published by Papa (28, 29) showed that strain 649 does not produce aflatoxin. Furthermore, heterozygous diploid strains with both mutant (*afl-1*) and wild-type (*afl-1*$^+$) alleles also do not produce aflatoxin, indicating that the mutated allele (*afl-1*) is dominant in diploids. The biochemical and molecular mechanisms by which aflatoxin biosynthesis is disrupted in the *afl-1* mutant strains are not known. The pur-

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pose of this study was to characterize the effects of the *afl-1* locus on aflatoxin biosynthesis and gene expression in *A. flavus* 649.

MATERIALS AND METHODS

Fungal strains. Strains 649 (*tan leu7 afl-1*), 86 (*whi arg7*), and wild-type strain NRRL 3357 were obtained from the U.S. Department of Agriculture Northern Regional Research Center in Peoria, Ill. Fungi were maintained on potato dextrose agar (PDA).

Aflatoxin analysis. Coconut agar medium was used to detect aflatoxin-producing isolates (12). Aflatoxin can be visualized as a blue fluorescence in the medium around the fungal colony. Aflatoxin produced in liquid cultures was quantified by an enzyme-linked immunosorbent assay (33). The aflatoxin B_1 monoclonal antibodies and aflatoxin B_1-horseradish peroxidase conjugates needed for the assay were kindly supplied by J. Pestka (Michigan State University). The conversion of aflatoxin pathway intermediates by intact mycelia was analyzed by previously described methods (5, 32). In these experiments, aflatoxin was measured with a thin-layer chromatograph-fluorescence spectrophotometer after thin-layer chromatography.

Parasexual mating techniques. Heterokaryons were obtained by forcing stains 649 and 86 to grow together on minimal medium (Czapek-Dox agar medium) by previously described methods (26, 42). Stable diploids were recognized among a population of heterokaryons by their green conidial heads. Once green-conidia strains were isolated, proof of the diploid state was confirmed by forcing haploidization and recovery of both parental phenotypes (42).

Analysis of DNA and RNA. Fungal mycelium used for nucleic acid isolations was obtained from cultures grown by the petri dish method described previously (40). The methods for DNA and RNA isolation and analysis were also previously described (42). For pulsed-field gel electrophoresis, agarose plugs containing chromosomal DNA were prepared from protoplasts washed twice in buffer (25 mM Tris [pH 7.6], 1 M sorbitol, 25 mM EDTA). The plugs had a final protoplast concentration of 4×10^8 per ml. Plugs were allowed to solidify for 20 min at 4°C and then incubated in lysis buffer (0.5 M EDTA, 10 mM Tris [pH 8], 1% sodium *N*-lauroylsarcosinate containing 1 mg of proteinase K per ml) at 60°C for 24 h. The plugs were washed three times for 1.5 h total in 0.5 M EDTA and stored at 4°C.

Chromosomes were separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis (10) with an LKB Pulsaphor 2015 electrophoresis apparatus. Electrophoresis was carried out in 1% electrophoresis-grade agarose (Bethesda Research Laboratories) prepared and run in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 0.1 mM EDTA) at 40 V and 12°C, with switching intervals of 4,500 s for 168 h and 3,600 s for an additional 168 h. Gels were stained with ethidium bromide and Southern blotted by standard techniques (25). All autoradiographs were digitally scanned with a Sharp JX450 color scanner with the Macintosh version of Adobe Photoshop software. Images were adjusted to optimize contrast by using the program's auto function.

RESULTS

Genetic analysis. A total of 19 independent diploids were isolated from a parasexual mating of strain 649 with the aflatoxigenic strain 86. None of these diploids produced visible fluorescence indicative of aflatoxin when grown on coconut agar medium. Two diploids (A and B) were selected for more detailed analyses. The aflatoxin induction profile of these two diploids, along with those of strains 649, 86, and NRRL 3357, was determined by a medium replacement technique. The *A. flavus* strains were initially grown in a peptone mineral salts medium (PMS) that supports growth of the fungus but not aflatoxin production (6). The medium was then replaced with sucrose low salts (SLS) medium, which does support aflatoxin production (34). Under these conditions, aflatoxin was detected in cultures of strains 86 and NRRL 3357 18 h after replacement with the SLS medium (Fig. 1). The pattern of induction and accumulation was similar to that in previously reported experiments with aflatoxin-producing strains (32). No accumulation of aflatoxin was observed in cultures of strain 649, diploid A, or diploid B. These data support previous studies showing that the mutation in strain 649 affecting aflatoxin production is genetically dominant in diploids (29).

Papa (31) mapped the *afl-1* locus to linkage group (LG) VII in *A. flavus*. Also mapped to LG VII were the *arg7* and *leu7* loci. The phenotypes of 50 haploid sectors derived from the haploidization of diploids A and B indicated that the mutated *afl-1* locus segregated with the *arg7* and *leu7* loci. Forty percent of the sectors had parental phenotypes (10 sectors each), and the remaining sectors arose by apparent recombinations of the white and tan conidia color loci, which map to linkage groups II and IV, respectively. Despite the limited number of sectors analyzed, these data support the contention of Papa that the *afl-1*, *leu7*, and *arg7* loci are in the same linkage group.

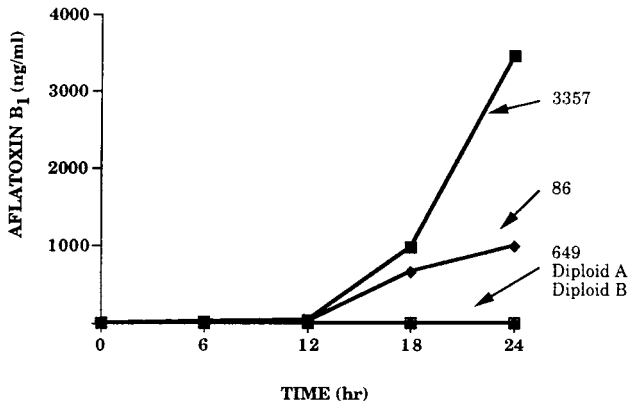

FIG. 1. Aflatoxin B_1 accumulation in cultures of *A. flavus* NRRL 3357, 86, and 649 and diploids A and B. The fungi were grown in PMS medium in petri plates for 3 days at 28°C, then resuspended in SLS medium, and grown at 28°C. Culture filtrates were sampled at 6-h intervals and assayed for aflatoxin B1.

Biochemical analysis. The biochemical phenotype of the *afl-1* mutant has not been studied. A classic method for determining if a mutation affects enzymes in a biochemical pathway is by metabolite feeding experiments. The pathway intermediates norsolorinic acid, versicolorin A, and sterigmatocytin were fed to mycelia of strains 649, 86, NRRL 3357, diploid A, and diploid B. These metabolites are intermediates from the early, middle, and late steps in the pathway. Strain 649, diploid A, and diploid B did not convert any of the intermediates to aflatoxin (Table 1), indicating that the enzymes for aflatoxin biosynthesis were not functioning in these strains. In contrast, strains 86 and NRRL 3357 produced aflatoxin under these conditions.

Molecular analysis. To determine the effect of *afl-1* on transcription of pathway genes, total RNA was isolated from the cultures at various times after replacement of the medium (Fig. 1). Northern blots were probed with the aflatoxin biosynthesis genes *aflR*, *nor1*, *ver1*, and *omt1* and the *adh1* gene (41) from *A. flavus*. At 18 h, when aflatoxin was being produced by strains 86 and NRRL 3357, no detectable accumulation of the *nor1*, *ver1*, and *omt1* transcripts was observed in RNA from strain 649, diploid A, or diploid B (Fig. 2). In contrast, there was a marked accumulation of these transcripts in strains 86 and

TABLE 1. Conversion of metabolites to aflatoxin B_1 by mycelia of five strains of *A. flavus*

Metabolite added	Aflatoxin (ng/g [wet wt] of mycelia) produced by strain[a]:				
	649	Diploid A	Diploid B	86	3357
None (control)	0	0	0	5,535	5,706
NOR (25 μg)	0	0	0	4,542	6,082
VER A (15 μg)	0	0	0	3,740	7,060
ST (10 μg)	0	0	0	6,846	7,639

[a] Values are for three experiments with two replications of each.

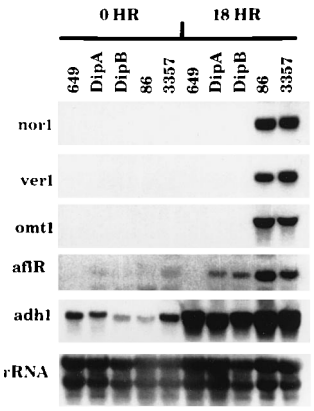

FIG. 2. Accumulation of RNA transcripts during aflatoxin biosynthesis. Strains NRRL 3357, 86, and 649 and two diploids (DipA and DipB) were grown in PMS medium in petri plates for 3 days at 28°C, then resuspended in SLS medium, and grown at 28°C (see the legend to Fig. 1). Total RNA isolated from mycelia collected at time zero and 18 h was analyzed with the genes *nor1*, *ver1*, *omt1*, *aflR*, *adh1*, and *rRNA* as radiolabeled probes.

NRRL 3357. Transcripts of the regulatory gene *aflR* were detectable in diploid A and diploid B after 18 h of incubation; however, the amount of transcript accumulation in the diploid strain was less than in the two aflatoxin-producing strains. No *aflR* transcript was detected in the RNA from strain 649. Finally, nearly equal amounts of *adh1* gene transcripts accumulated in all strains, including strain 649.

To determine if the lack of transcription of pathway genes in strain 649 was due to a deletion of one or more genes, genomic DNA from strain 649 was hybridized with probes made from the *nor1* and *ver1* genes. No hybridization was detected with these probes (Fig. 3). In contrast, these probes hybridized to DNA from the aflatoxin-producing strains 86 and NRRL 3357 and the two diploids (Fig. 3). These results suggest that a deletion in the genome of strain 649 occurred near the aflatoxin gene cluster, so that the *ver1* and *nor1* genes are no longer present in strain 649. The genomic DNA from the aflatoxin gene cluster has been cloned on three overlapping cosmids (Fig. 4) in a genomic DNA library from strain NRRL 3357. In order to delimit the size of the deletion, DNA fragments (WE9 and WE11), representing the ends of the gene cluster, were isolated from cosmids 20B11 and 5E6 and used as radiolabeled probes. Neither of these probes hybridized to genomic DNA from strain 649 (data not shown). The *leu7* gene is known to be distal but linked to the aflatoxin gene cluster. A cosmid containing the *leu7* gene was used as a probe in Southern analysis of genomic DNA from strain 649. The resulting Southern blots

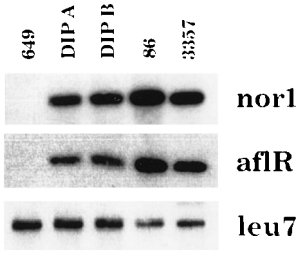

FIG. 3. Analysis of genomic DNA from *A. flavus* NRRL 3357, 86, and 649 and diploids A and B. Fungal DNA was digested with *Bam*HI, electrophoresed, blotted, and hybridized with radiolabeled *nor1* and *aflR* genes and a 4-kb DNA fragment from a cosmid containing the *leu7* gene.

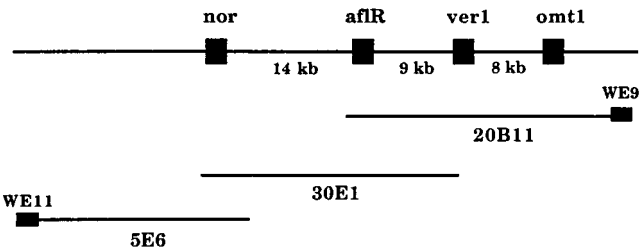

FIG. 4. Diagram showing the aflatoxin biosynthesis gene cluster in *A. flavus* and three overlapping cosmid clones. Regions WE9 and WE11 are subcloned DNA fragments from the ends of the respective cosmids.

showed a hybridization band pattern that was identical for all the strains (Fig. 3), indicating that strain 649 contains DNA linked to the aflatoxin gene cluster.

Separation of the chromosomes of strains 86 and 649 by pulsed-field electrophoresis revealed a single difference between the karyotypes (Fig. 5). Strain 649 contained a unique band of 6 Mb, whereas strain 86 contained a unique 4.9-Mb band. Seven other chromosome bands were identical in both strains. Southern analysis with the *nor1* gene as a radiolabeled probe resulted in a 4.9-Mb band of hybridization in strain 86 and no hybridization with strain 649 chromosomes (Fig. 5). In contrast, a probe containing the *leu7* gene hybridized to both the 4.9-Mb chromosome in strain 86 and the 6-Mb chromosome in strain 649.

DISCUSSION

The objective of this study was to characterize an unusual mutant strain of *A. flavus* isolated 16 years ago by K. E. Papa (28, 29). Papa generated the aflatoxin-nonproducing strain 649 by nitrosoguanidine treatment of a leucine auxotroph derived from PC-7 by UV mutagenesis (26). This mutation behaved differently from all other aflatoxin mutations in that it was

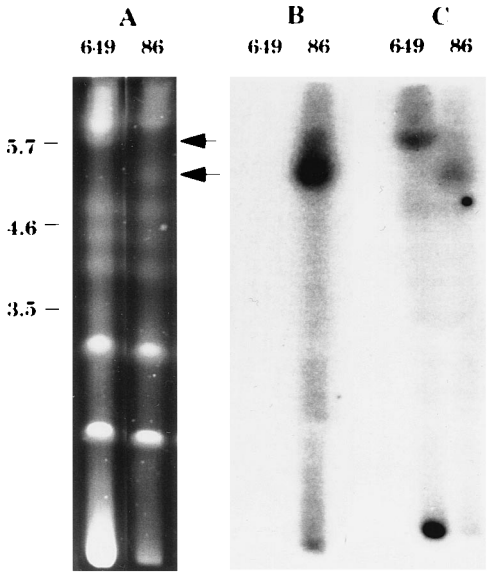

FIG. 5. CHEF electrophoretic separation and Southern blot analysis of chromosomal DNA from strains 649 and 86. The gel was stained with ethidium bromide (A), blotted, and hybridized with radiolabeled *nor1* (B) or a 4-kb DNA fragment from a cosmid containing the *leu7* gene (C). Chromosomal DNA from *Schizosaccharomyces pombe* was used as size standards (in kilobases). Arrows indicate chromosomes showing hybridization to the probes.

dominant; diploids formed between strains carrying it and aflatoxin-producing strains did not produce aflatoxin. The genetic data presented in this study confirmed Papa's conclusion that the *afl-1* locus from strain 649 is dominant in diploids, resulting in a non-aflatoxin-producing phenotype. We further characterized this locus and showed that strain 649 contains a deletion. Southern analysis of genomic DNA from strain 649 indicated a deletion at the *nor1* locus. Papa originally mapped the *afl-1* locus to a position near the *nor1* locus on LG VII (2). The exact size of the deletion is not known, but we have determined that the entire aflatoxin biosynthesis gene cluster, nearly 60 kb, is deleted in strain 649. Also, Southern analysis of chromosome blots indicated that the 4.9-Mb chromosome harboring the *nor1* gene (9) in aflatoxin-producing strain 86 (also derived from strain PC-7) is not present in the *afl-1* mutant strain 649. In addition to this deletion, strain 649 contains a larger chromosome (6 Mb) that is not present in strain 86. A *leu7* probe, which also maps to LG VII (15), hybridized to both the 4.9-Mb and the 6-Mb chromosomes, suggesting that these chromosomes contain homologous regions. A previous study showed that LG VII also corresponded to the 4.9-Mb chromosome in *A. flavus* 656-2 (also derived from strain PC-7) (15). These data suggest that the larger chromosome in strain 649 resulted from the acquisition of additional DNA as well as loss of the cluster of aflatoxin biosynthesis genes. From Papa's genetic data on LG VII, which showed that the *afl-1* locus is distal to *leu7*, *arg7*, and the centromere, we speculate that the chromosome is intact from the *leu7* locus to the centromere and that the additional DNA is located distal to *leu7*. Because there are no genetic markers distal to the *nor1* locus, it is impossible to determine the size of the deletion.

The lack of genes required for aflatoxin biosynthesis in strain 649 readily explains the inability of this strain to produce aflatoxin. The apparent repression of aflatoxin production in the diploids with this strain is not as easily explained. Evidence presented here, indicating a deletion of the aflatoxin gene cluster in strain 649 and an apparent rearrangement event in the same chromosome, leads us to hypothesize transvection or trans-sensing as a possible mechanism. Transvection is a genetic phenomenon first described for *D. melanogaster* by E. B. Lewis in 1954 to explain the aberrant phenotypes that arose from rearrangement at the bithorax gene complex (BX-C) locus. Transvection is the inactivation of alleles due to chromosome pairing or allele proximity. The term trans-sensing has recently been suggested to describe a similar phenomenon involving somatic pairing of chromosomes at several loci in *D. melanogaster* (36). The most noted example of trans-sensing involves the *white* (*w*) gene and *zeste* (*z*) gene loci (43, 44). The *white* gene product is required for normal red eye color, and the *zeste* gene product appears to be a DNA-binding protein affecting the transcription of the *white* gene. A mutation in *zeste* (z^1) results in flies with yellow eyes. This phenotype occurs only when there is w^+/w^+ pairing. If one of the w^+ alleles is displaced to another position in the genome, pairing at w^+ is not possible and the phenotype is red eyes. Another example of trans-sensing is the trans-inactivation associated with a rearrangement mutation affecting the *brown* (*bw*) gene in *D. melanogaster* (13, 19). The phenotype of homozygous mutants (*bw*/*bw*) is the same as that of a heterozygous mutant (bw^+/*bw*), indicating a dominant phenotype for the mutation. An ectopic insertion of a wild-type copy of the *brown* gene (bw^+) into the genome of a heterozygous mutant results in restoration of the wild-type phenotype. Finally, Huntington's disease, a human neurodegenerative disease, has been proposed to result from a trans-sensing phenomenon (23).

The mechanism by which transvection occurs is not known; however, the phenomenon suggests that regulation of gene expression can be influenced across two homologous chromosomes (18, 21, 36). Several hypotheses have been proposed to explain the mechanism of trans-inactivation at the *zeste* locus (16, 17, 36). One model proposes that the mutated zeste protein binds upstream of the *white* gene, forming self-aggregates and ultimately holding the two DNA molecules together and interfering with transcription (36, 44). The mechanism for this inactivation of the *brown* gene was hypothesized to result from the juxtaposing of heterochromatin DNA sequences to the wild-type *brown* gene. By an unknown mechanism, the heterochromatin interferes with the binding of a regulatory protein that normally activates transcription (13, 36). In strain 649 of *A. flavus*, a chromosomal rearrangement may have resulted in a positioning of teleomere DNA or heterochromatin-like DNA at or near the position in the chromosome that is normally occupied by the aflatoxin gene cluster. Such an event could cause a *cis* inactivation of a regulatory gene located near the chromosomal break junction in strain 649 and a *trans* inactivation of the wild-type aflatoxin genes in the homologous chromosome of diploids. No aflatoxin biosynthesis genes outside the aflatoxin gene cluster are known; however, Papa identified recessive mutations at seven different loci in LG VII (2). Preliminary studies of the strains having these mutated loci suggest that several affect the regulation of aflatoxin biosynthesis.

Although the repressive effects of teleomere DNA on gene expression have been described for *Saccharomyces cerevisiae* (37), transvection or trans-sensing has not been reported for filamentous fungi. The reason for this may be that most of the fungi used in genetic studies are haploid, and studies on gene expression in diploids have not been extensively pursued. Furthermore, mutational events that result in a detectable trans-sensing phenomenon may be as rare in fungi as they are in *D. melanogaster*. If the phenomenon observed at the *afl-1* locus is trans-sensing, then chromosome pairing must occur in fungal diploids, and as a result of this pairing, gene expression is influenced. Evidence to support chromosome pairing has come predominantly from studies on meiosis, in which homologous chromosome interactions are microscopically visible during prophase. However, a recent study using in situ fluorescence analysis in yeast cells indicated that homologous chromosomes are located near each other in the nucleus during interphase and may physically interact at all phases of the cell cycle (39). Such interactions could explain the basis for the mitotic recombination associated with the parasexual cycle and the dominant phenotype associated with the *afl-1* locus.

Other potential mechanisms also should be considered for the observed repression of aflatoxin biosynthesis in diploids. In this study, transcripts of the regulatory gene *aflR* were detected in the 649 diploids but in lower amounts than observed in the aflatoxin-producing strains. Expression of *aflR* in strains 650-33 and 656-2 was previously shown to be necessary for the expression of the other aflatoxin genes (32, 40). Assuming that a threshold expression of *aflR* is required, a mechanism involving the suppression of *aflR* could be responsible for the observed diploid phenotype. Although the suppression of *aflR* could occur by the trans-sensing mechanism described above, it could also result from the aberrant expression of a repressor gene in strain 649. This repressor gene might be associated with the apparent chromosome rearrangement in LG VII or associated with a bizarre gene fusion at the break junction of the deletion at the aflatoxin gene cluster. A possible candidate could be a gene coding for a transcriptional factor. If the repressor molecule were produced, one might expect no aflatoxin production in heterokaryons formed from strain 649 and an aflatoxin-producing strain. Previous studies by Papa indicated that het-

erokaryons produce aflatoxin. In our studies, the heterokaryon formed from strains 649 and 86 produced aflatoxin, but the amounts were lower than in strain 86. Furthermore, another heterokaryon formed from strains 649 and 176 (*whi phe*) produced aflatoxin at concentrations similar to those produced by the wild-type fungus. These observations suggest that a suppressor of aflatoxin produced by the nucleus of strain 649 does not effectively suppress the aflatoxin genes in the nuclei of the aflatoxin-producing strains used to produce the heterokaryons. Because it is not possible to ensure total stability of the heterokaryons, we cannot rule out that a suppressor of aflatoxin is being produced.

The work described in this paper has characterized the mutation in strain 649 by molecular techniques. Further research is needed to determine if the dominant phenotype associated with the mutation results from a trans-sensing phenomenon or by some other mechanism.

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