APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3567–3571 Vol. 62, No. 10
0099-2240/96/$04.00+0

Identification of Aflatoxin Biosynthesis Genes by Genetic Complementation in an *Aspergillus flavus* Mutant Lacking the Aflatoxin Gene Cluster†

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Received 6 May 1996/Accepted 15 July 1996

Aspergillus flavus **mutant strain 649, which has a genomic DNA deletion of at least 120 kb covering the aflatoxin biosynthesis cluster, was transformed with a series of overlapping cosmids that contained DNA harboring the cluster of genes. The mutant phenotype of strain 649 was rescued by transformation with a combination of cosmid clones 5E6, 8B9, and 13B9, indicating that the cluster of genes involved in aflatoxin biosynthesis resides in the 90 kb of *A. flavus* genomic DNA carried by these clones. Transformants 5E6 and 20B11 and transformants 5E6 and 8B9 accumulated intermediate metabolites of the aflatoxin pathway, which were identified as averufanin and/or averufin, respectively. These data suggest that *avfl*, which is involved in the conversion of averufin to versiconal hemiacetal acetate, was present in the cosmid 13B9. Deletion analysis of 13B9 located the gene on a 7-kb DNA fragment of the cosmid. Transformants containing cosmid 8B9 converted exogenously supplied *O*-methylsterigmatocystin to aflatoxin, indicating that the oxidoreductase gene (*ord1*), which mediates the conversion of *O*-methylsterigmatocystin to aflatoxin, is carried by this cosmid. The analysis of transformants containing deletions of 8B9 led to the localization of *ord1* on a 3.3-kb *A. flavus* genomic DNA fragment of the cosmid.**

Aflatoxins are polyketide-derived secondary metabolites that are highly toxic and carcinogenic in a variety of animal species and are suspected carcinogens in humans (10). These toxins are produced by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which infect important crops such as corn, cotton, peanuts, and tree nuts. Figure 1 illustrates the aflatoxin biosynthesis pathway, beginning with the first stable intermediate, norsolorinic acid. The conversion of norsolorinic acid to aflatoxin involves at least 16 enzymatic steps (2, 28). In recent years, aflatoxin research focused on the identification, cloning, and characterization of biosynthetic genes. A variety of approaches led to the isolation of several genes involved in aflatoxin biosynthesis, including the structural genes *pksA* (6), *fas1A* (14), *nor1* (22), *norA* (4), *ver1* (13), *ord2* and *avnA* (27, 28), and *omt1* (26) and the positive regulatory gene *aflR* (5, 17). Analysis of *A. flavus* and *A. parasiticus* genomic clones, which contained some of these genes, indicated that these genes are clustered and that their relative positions are similar in the clusters of the two fungal species (28).

In the present study, we propose the use of genetic complementation in an aflatoxin mutant strain of *A. flavus* as a means to identify and isolate genes involved in aflatoxin biosynthesis. Strain 649 was isolated by Papa (16), who used the parasexual cycle to map the dominant mutation (*afl1*) in this strain to a position near the *nor* locus in linkage group VII. Strain 649 has a deletion of at least 120 kb (25), and although the exact size of this deletion is not known, we determined that the entire cluster of aflatoxin biosynthesis genes is absent in this mutant (25). Southern analysis of chromosome blots indicated that the 4.9-Mb chromosome, where the cluster is located in aflatoxin-producing strains, has regions homologous to a 6-Mb chromosome in strain 649. These data suggest that the larger chromosome in strain 649 resulted from the acquisition of additional DNA as well as the loss of the cluster of aflatoxin biosynthesis genes (25).

The mechanism responsible for the dominant phenotype affecting aflatoxin biosynthesis in strain 649 is not known. One possibility is the aberrant expression in strain 649 of a repressor gene that could suppress aflatoxin production in diploids. This gene might be associated with the apparent chromosome rearrangement in linkage group VII, leading to a gene fusion at the break junction of the deletion that covers the aflatoxin gene cluster (25). A second hypothesis is the existence of a mechanism known as transsensing, by which wild-type alleles are inactivated in diploids because of chromosome pairing or allele proximity (25). This phenomenon has been reported for *Drosophila melanogaster* but not for filamentous fungi (25).

We describe here both the functional complementation of strain 649 by transformation with overlapping cosmids harboring the aflatoxin gene cluster and the mapping of two new genes involved in aflatoxin biosynthesis in *A. flavus.*

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Strains 649 (*afl1 tan leu7*) and 86 (*whi arg7*) were provided by the USDA Northern Regional Research Center in Peoria, Ill. Strain 29-10D (*whi arg7 pyr*) was provided by G. A. Payne, North Carolina State University, Raleigh. Conidial suspensions were obtained from cultures grown in the dark on potato dextrose agar medium (Difco Laboratories, Detroit, Mich.). Clones 5E6, 20B11, 8B9, and 13B9 were isolated from an *A. flavus* (strain CRA02B) cosmid library constructed into vector pAF-1 that contains the *pyr4* gene of *Neurospora crassa* (17, 21). *A. flavus* DNA fragments contained in cosmid clones were subcloned into pBluescript KS(−) (Stratagene Laboratories). Subclones derived from cosmid 8B9 were WE3, with a 4-kb *Eco*RI fragment; WE4, with a 3.9-kb *Xba*I fragment that contains *aflR*; WE25, with a 5-kb *Kpn*I-*Eco*RI fragment; WE27, with an 18.7-kb *Cla*I fragment; WE32, with a 5.8-kb *Xba*I fragment; and WE33, with a 3.3-kb *Xba*I-*Sma*I fragment.

Parasexual crosses. The *pyr* mutation of aflatoxigenic strain 29-10D was moved to strain 649 by a parasexual cross (25). A haploid sector strain recovered

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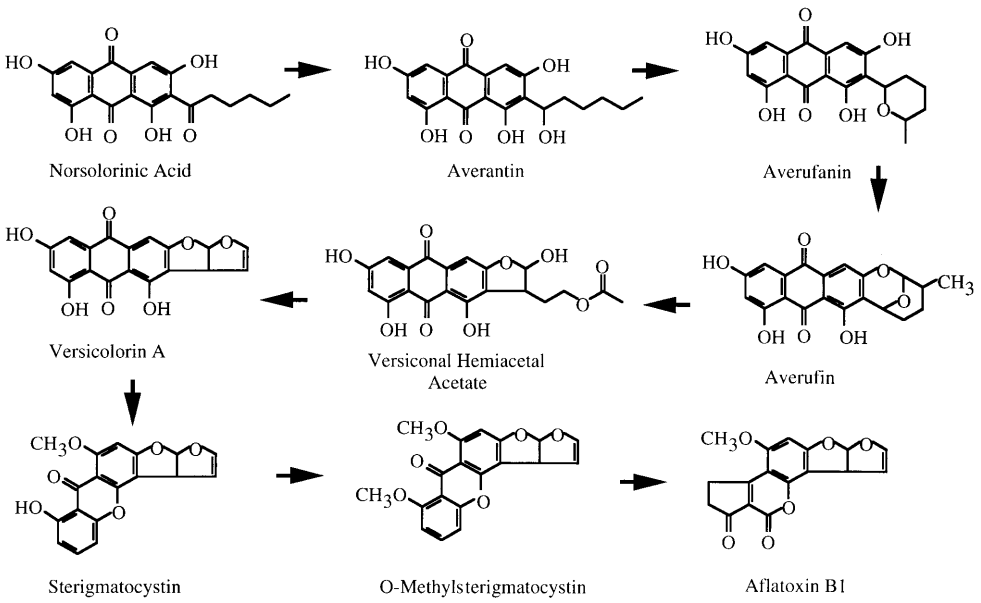

FIG. 1. Biosynthetic pathway of aflatoxin B_1, showing the major intermediates, beginning with the first stable intermediate (norsolorinic acid).

from this cross, designated 649 WAF2, had the same phenotype as strain 649 except that it required uracil for growth. Southern blot analysis of 649 WAF2 indicated that the aflatoxin gene cluster was deleted, as in strain 649. Also, diploids isolated from a cross between strain 649 WAF2 and strain 86 did not produce aflatoxin, indicating that the dominant phenotype associated with the *afl1* locus was maintained.

Fungal transformation. Protoplasts of *A. flavus* were transformed by the polyethylene glycol-calcium chloride method described by Woloshuk et al. (24). Transformants were selected on medium lacking uracil.

Analysis of aflatoxin and pathway intermediates. Transformed strains were evaluated for aflatoxin production by two methods. Aflatoxin was detected under UV irradiation as a blue fluorescence around fungal colonies grown on coconut agar medium (8). Aflatoxin and intermediate metabolites of the pathway were also extracted from culture media, separated by thin-layer chromatography, and detected under UV irradiation (14).

Metabolite conversion experiments were conducted as previously described (1, 3) with some modifications. One gram of mycelia was incubated at 28°C for 20 h with constant shaking in AM medium (1) supplemented with 5% glucose and 10 μg of either sterigmatocystin (ST) or *O*-methylsterigmatocystin (OMST). Aflatoxin pathway intermediates were extracted from mycelia with acetone and chloroform (7) and separated by thin-layer chromatography with an ether-methanol-water (96:3:1, vol/vol/vol) solvent system. Aflatoxin, ST, and OMST had R_f values of 0.37, 0.94, and 0.44, respectively.

Unknown metabolites that accumulated in some transformants were purified by thin-layer chromatography and analyzed on a Finnigan 4000 (Finnigan MAT, San Jose, Calif.) mass spectrometer by both 70-eV electron impact and isobutane chemical ionization. Samples were volatilized off a direct insertion probe.

Nucleic acid isolation and analysis. Fungal genomic DNA was obtained from cultures grown in petri dishes containing potato dextrose broth (Difco Laboratories) (25). Total RNA was purified 18 h after cultures grown on peptone mineral salts (PMS) medium were shifted to a medium conducive to aflatoxin production, consisting of PMS plus 60 g of glucose per liter (PMSG) (25). Methods for nucleic acid isolation, Northern (RNA) analysis, and Southern analysis were as described previously (24, 25). Radiolabelled probes were a 0.5-kb *Pst*I fragment of *nor1*, a 1.1-kb *Eco*RI fragment from *aflR*, a 0.9-kb *Eco*RV fragment from *omt1*, and WE13, a 0.9-kb *Xba*I-*Eco*RI fragment from cosmid 13B9. All autoradiographs were digitally scanned with a Sharp JX450 color scanner by using the Macintosh version of Adobe Photoshop software. Images were adjusted to optimize contrast.

RESULTS

Transformation of strain 649. Strain 649 WAF2 was transformed with overlapping cosmids harboring the aflatoxin gene cluster from *A. flavus* to determine if the *afl1* mutation could be complemented. The cosmids were defined by the known aflatoxin biosynthesis genes that each contains (Fig. 2). Cosmid 5E6 contains the genes for the putative polyketide synthase (*pks*) and *nor1*; cosmids 20B11 and 8B9 contain *aflR*, *ver1*, and *omt1*; and cosmid 13B9 contains *ver1* and *omt1*. Total genomic DNA was analyzed by Southern blots, each with either *nor1*, *omt1*, *aflR*, or WE13 as a radiolabelled probe (Fig. 2 and 3).

Production of aflatoxin and pathway intermediates. Aflatoxin was produced only by transformants containing the three-cosmid combination 5E6, 8B9, and 13B9 (Table 1). Aflatoxin production by these transformants was similar to that of the toxigenic strain 86 (data not shown). Transformants having cosmids 5E6 and 20B11 or cosmids 5E6 and 8B9 did not produce aflatoxin, but they accumulated yellow metabolites in culture medium (Table 1). These pigments were extracted and analyzed by thin-layer chromatography. Two metabolites with R_f values of 0.7 and 0.4 were present in the media of transformants containing cosmids 5E6 and 20B11. In contrast, transformants containing cosmids 5E6 and 8B9 produced only a metabolite with an R_f value of 0.7. These yellow pigments were purified and analyzed by mass spectrometry. The resulting spectra (Fig. 4) indicated that these metabolites were averufin (9) and averufanin (15), with molecular mass ions of 368 and 370, respectively. Transformants containing cosmids 5E6 and 8B9 accumulated only averufin, whereas those containing 5E6 and 20B11 accumulated both metabolites.

Northern analysis. Total RNA was purified from mycelia 18 h after cultures were shifted from a growth medium that

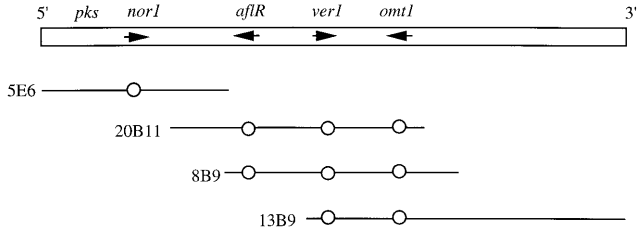

FIG. 2. Schematic diagram of the *A. flavus* aflatoxin biosynthesis cluster, indicating the relative positions of the genomic DNA carried by cosmid clones 5E6, 20B11, 8B9, and 13B9 and the locations of the genes within the cosmid DNA.

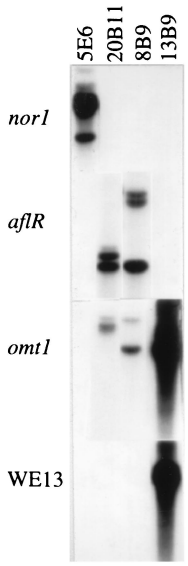

FIG. 3. Southern analysis of 649 WAF2 transformant strains containing different aflatoxin biosynthesis genes. DNA (3 μg) from transformant strains 5E6, 20B11, 8B9, and 13B9 was digested with *Bam*HI, fractionated on agarose gels, and analyzed by Southern hybridization with radiolabelled DNA fragments of either *nor1*, *aflR*, *omt1*, or WE13 (specific for cosmid 13B9) as probes.

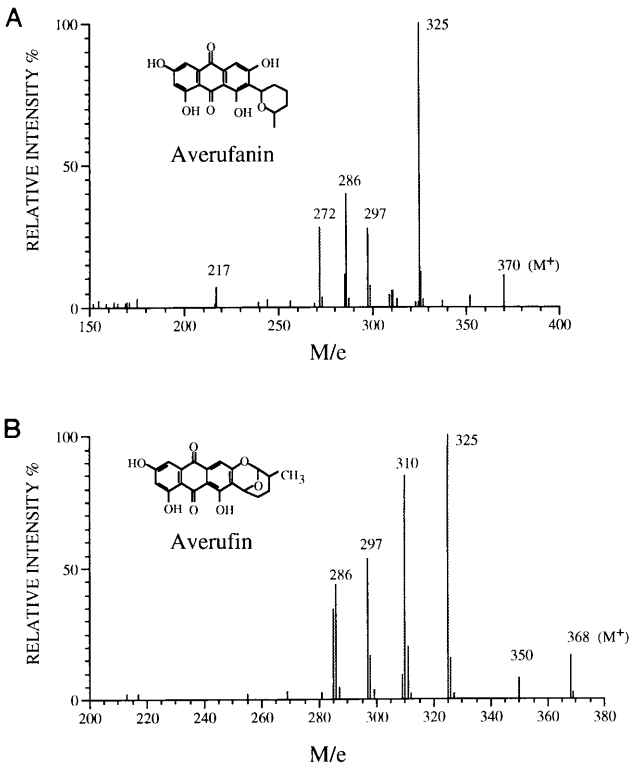

FIG. 4. Mass spectra of two yellow pigments isolated from culture media of transformants containing 5E6 and 20B11 (A) and 5E6 and 8B9 (B).

does not support aflatoxin production (PMS) to an aflatoxin-inducing medium (PMSG). Aflatoxin was detected in the media of strain 86 and 649 WAF2 transformants containing cosmids 5E6, 8B9, and 13B9 (data not shown). Transformants with cosmids containing the *aflR* gene (20B11 or 8B9) exhibited expression of *omt1* (Fig. 5). In transformants with 5E6 or 13B9, the expression of *nor1* or *omt1* was not detected, respectively. However, in transformant strains containing both 5E6 and 8B9, significant levels of expression of both aflatoxin biosynthesis genes were detected (Fig. 5).

Metabolite feeding studies. Mycelia of different transformant strains were incubated in aflatoxin-inducing medium with exogenously supplied ST or OMST. Transformants containing cosmid 8B9 converted both ST and OMST to aflatoxin (Table 1). In contrast, transformants containing cosmid 20B11 converted ST to OMST but not to aflatoxin (Table 1). These data indicated that the gene encoding oxidoreductase (*ord1*), which is involved in the last step of the pathway, is located at the 3′ region of cosmid 8B9 (Fig. 2). It was noteworthy that transformants containing cosmid 13B9, which carries the *ord1* region but not *aflR*, could not convert OMST to aflatoxin. The efficiency of conversion for both ST and OMST was estimated to be about 30%.

TABLE 1. Phenotypes of 649 WAF2 transformants containing overlapping cosmids harboring the aflatoxin gene cluster

Cosmid(s)	Pigment production	Aflatoxin production	Aflatoxin production[a] by feeding with:	
			ST	OMST
5E6	No	No	—	—
8B9	No	No	Yes	Yes
20B11	No	No	No[b]	No
13B9	No	No	No	No
5E6 and 20B11	Yes (yellow)	No	—	—
5E6 and 8B9	Yes (yellow)	No	—	—
5E6, 8B9, and 13B9	No	Yes	—	—

[a] —, transformants not fed.
[b] Transformant converted ST to OMST.

Mapping of new genes involved in aflatoxin biosynthesis. To determine the location of the oxidoreductase gene responsible for conversion of OMST to aflatoxin (Fig. 1), various DNA fragments from 8B9 were cotransformed with the *aflR* gene (present in plasmid WE4) into strain 649 WAF2. The resulting transformants were tested for their ability to produce aflatoxin when exogenously supplied with OMST. A 3.3-kb DNA fragment (WE33) located at the 3′ region of cosmid 8B9 (Fig. 6) contained the gene that encodes the ORD1 oxidoreductase protein.

Transformants containing cosmids 5E6 and 8B9 accumulated averufin (Fig. 4). In contrast, aflatoxin was produced by transformants containing the cosmid combination 5E6, 8B9, and 13B9. These data suggest that a gene we have designated *avf1*, which is responsible for the conversion of averufin to versiconal hemiacetal acetate (Fig. 1), is located on cosmid 13B9. To determine the location of *avf1*, several DNA fragments from cosmid 13B9 were cotransformed with cosmids 5E6 and 8B9 into 649 WAF2. The resulting transformants were screened for aflatoxin production. The gene was mapped to a 7-kb fragment of a 13-kb DNA region in cosmid 13B9 (data not shown).

DISCUSSION

In the present study, aflatoxin biosynthesis was restored in strain 649 WAF2 by functional expression of genes present on three overlapping DNA clones, each carried by one of the cosmids 5E6, 8B9, and 13B9. These three cosmids cover about 90 kb of genomic DNA harboring a cluster of genes involved in

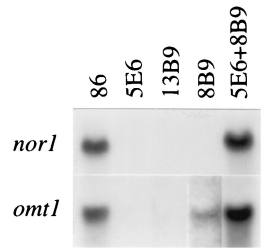

FIG. 5. Expression of aflatoxin biosynthesis genes in 649 WAF2 transformants. Total RNA (20 μg) from strain 86 and from transformants containing 5E6, 8B9, 13B9, and both 5E6 and 8B9 was fractionated on agarose gels and analyzed by Northern hybridization using labelled fragments of either *nor1* or *omt1* as probes.

aflatoxin biosynthesis (28). Strain 649 WAF2 has a DNA deletion that includes this 90-kb genomic region (25). Complementation of the mutant phenotype in strain 649 WAF2 indicated that this gene cluster resides in this 90-kb DNA region, and therefore, the genomic DNA on either side of this region is not involved in aflatoxin biosynthesis.

When cosmids were individually transformed into 649 WAF2, none of the resulting transformants produced aflatoxin. However, those transformants with cosmids containing the *aflR* gene (8B9 or 20B11) exhibited gene expression and enzyme activities associated with other aflatoxin biosynthesis genes carried by the cosmids. The expression of aflatoxin genes was not detected in transformants lacking the *aflR* gene (5E6 or 13B9). These data support the premise that *aflR* is a positive transcriptional regulator of aflatoxin genes (23). Further evidence for this role was the functional expression of aflatoxin biosynthesis genes on cosmids 5E6 and 13B9 when these cosmids were cotransformed with cosmid 8B9.

A cDNA encoding a cytochrome P-450-type enzyme was recently isolated from *A. parasiticus* and named *ord1* because it encodes the oxidoreductase involved in the last step of aflatoxin biosynthesis (28). However, disruption of the gene in an aflatoxigenic strain showed that *ord1* mediated the conversion of averantin to averufanin (27). This *A. parasiticus* gene was subsequently renamed *avnA* (27). In the present study, functional expression of genes present on cosmid 8B9 facilitated the identification and mapping of the oxidoreductase gene *ord1*. When mycelia of 8B9 transformants were incubated with ST or OMST, these metabolites were converted to aflatoxin. Analysis of transformants containing different 8B9 deletion clones located the oxidoreductase gene on a 3.3-kb DNA fragment. Furthermore, transformants containing this DNA fragment required a copy of *aflR* to convert OMST to aflatoxin, indicating that AFLR regulates ORD1 activity. Preliminary analysis showed that a single transcript is derived from the 3.3-kb DNA region. We also obtained a partial nucleotide sequence of the gene, and a search of various databases for similarity indicated that this gene likely encodes a P-450-type monooxygenase (18).

The 649 WAF2 transformants with cosmids 5E6 and 8B9 or cosmids 5E6 and 20B11 accumulated averufanin and/or averufin, respectively. In contrast, the transformant with 5E6, 8B9, and 13B9 produced aflatoxin, suggesting that *avf1*, which is responsible for converting averufin to versiconal hemiacetal acetate, was present in cosmid 13B9. The presence of averufanin in the culture of transformants containing 5E6 and 20B11 could be the result of a block at averufin. Transformants containing 5E6 and 8B9 accumulated less averufin (18), perhaps resulting in low or nondetectable levels of averufanin. By cotransformation of 649 WAF2 with cosmids 5E6 and 8B9 and DNA fragments from 13B9, the gene was located in a 7-kb DNA region.

Yu et al. (28) showed that the order of genes contained in the aflatoxin gene cluster generally parallels the reaction steps in the biosynthetic pathway. Accordingly, genes involved in earlier steps (*pksA*, *fas1A*, and *nor1*) are located at the 5′ region of the cluster (28), followed by genes involved in intermediate steps (*ver1* and *avnA*) and genes involved in the last steps of the pathway (*omt1* and *ord1*), which map at the 3′ region of the cluster. However, *avf1* represents an exception to this rule, because it was located not in the middle of the cluster, as one might predict according to the enzyme activity of its protein product, but at the 3′ end of the cluster, beyond the genes involved in the last step of the pathway.

A. flavus mutants originally isolated by Papa have significantly impacted our understanding of aflatoxin biosynthesis

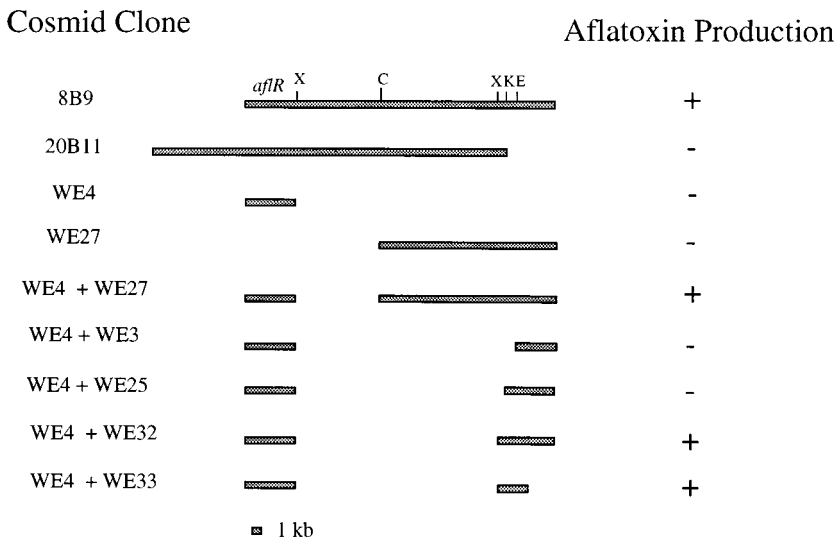

FIG. 6. Mapping of *ord1*. 649 WAF2 transformant strains containing *aflR* and each DNA fragment from cosmid 8B9 were exogenously fed OMST and evaluated for their ability to produce aflatoxin. X, *Xba*I; C, *Cla*I; K, *Kpn*I; E, *Eco*RI.

and secondary metabolism. These strains led to the isolation of a regulatory gene (*aflR*) involved in aflatoxin biosynthesis (17, 23) and the discovery of an aflatoxin biosynthesis gene cluster (28). We have located two new aflatoxin biosynthetic genes of *A. flavus* on the basis of their functional expression in strain 649 WAF2. A similar approach was used to isolate and characterize genes involved in the nitrate assimilation pathway in the unicellular green alga *Chlamydomonas reinhardtii* (12, 19, 20). *Chlamydomonas* mutant strains C2 and D2 have a DNA rearrangement and a deletion of DNA (20) harboring the nitrate reductase structural gene (*nit1*) (11), respectively. These strains facilitated the identification of a cluster of genes involved in nitrate assimilation (20). Further characterization of genes in this cluster, including functional expression of those genes in a strain with the C2 genetic background but containing the *nit1* gene, led to the identification and isolation of nitrate and nitrite transporters in *Chlamydomonas* spp. (12, 19). It is likely that strain 649 WAF2 can be used to answer questions about the function and regulation of aflatoxin biosynthesis genes and to study specific point mutations that affect the transcriptional regulation and active sites of enzymes. At this preliminary stage, the expression of genes in strain 649 WAF2 cannot conclusively answer the question concerning the dominant phenotype associated with the *afl1* locus. Data presented here support the hypothesis that a transsensing phenomenon is responsible for the dominant repression of aflatoxin biosynthesis in diploids of strain 649 WAF2. However, we cannot eliminate the possibility that the action of a repressor gene overexpressed in strain 649 WAF2 was inhibited by multiple copies of the positive regulatory gene *aflR* in the transformants.

ACKNOWLEDGMENTS

Mass spectrometric analyses were performed by Connie Bonham of the Purdue University Campus-wide Mass Spectrometry Center. We thank Robert A. Schwingendorf, Mark Weaver, and Irina Tikhonova for technical and intellectual input throughout the course of this work.

Support for this research was provided by the USDA NRI Competitive Grants Program, award 94-37201-1160.

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