SHORT COMMUNICATION

Dominant Aflatoxin Mutant of *Aspergillus flavus*

By K. E. PAPA

Department of Plant Pathology and Plant Genetics, University of Georgia,
Athens, Georgia 30602, U.S.A.

(Received 4 December 1979)

A dominant mutant of *Aspergillus flavus* impaired in aflatoxin production, designated *afl-1* and linked to *leu* in linkage group VII, was recovered after treatment of conidia with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mutant *afl-1* failed to complement other non-allelic aflatoxin mutants in diploids and also restricted aflatoxin accumulation when paired with toxigenic strains in diploids. No detectable effect of *afl-1* was demonstrated in heterokaryons. This gene locus is apparently not involved in the extracellular degradation of aflatoxin.

INTRODUCTION

Induced mutants of *Aspergillus flavus* and *A. parasiticus* have been useful in the demonstration of the parasexual cycle (Papa, 1973, 1978), the establishment of linkage groups (Papa, 1976) and the study of aflatoxin biosynthesis (Lee *et al.*, 1976; Hsieh *et al.*, 1976; Singh & Hsieh, 1977). Bennett (1979) reported that mutants of *A. parasiticus* accumulating the aflatoxin precursors, versicolorin and norsolorinic acid, were recessive to wild-type. Similarly, a number of mutants of *A. flavus* impaired in aflatoxin biosynthesis were shown to be recessive (Papa, 1979). While determining the complementation pattern of these mutants, it was noted that one mutant (*afl-1*) failed to complement or only weakly complemented other mutants. Synthesis of diploids between *afl-1* and toxigenic strains led to the conclusion that *afl-1* was dominant.

The present study is an examination of *afl-1* in additional diploids and in heterokaryons. The possibility that *afl-1* may degrade aflatoxin is also considered.

METHODS

Strains. Mutants of *A. flavus* used in this study are listed in Table 1. The induction of auxotrophy and spore colour variation has been described previously (Leach & Papa, 1974; Papa, 1976). All auxotrophic spore-colour mutants produced aflatoxin B₁, low levels of B₂ and no detectable G toxins. Mutants *afl-1*, *afl-4* and *afl-b2* were recovered by exposing conidia of three auxotrophic spore-colour variants to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Papa, 1977, 1979). Mutants *afl-1* and *afl-4* do not accumulate aflatoxin and are non-allelic, mapping to linkage groups VII and II, respectively. The former is linked to *leu* and the latter to *w*. Mutant *afl-b2* accumulates more aflatoxin B₂ than B₁.

Media. The complete medium (CM) consisted of Czapek-Dox broth, 0.75% (w/v) malt extract, 0.25% (w/v) yeast extract and 1.5% (w/v) agar. The minimal medium (MM) was CM without the malt and yeast extracts. Appropriately supplemented MM was used for the identification of auxotrophs. The medium (YES) for production of aflatoxin consisted of 2% (w/v) yeast extract and 20% (w/v) sucrose.

Assay for aflatoxin. Aflatoxin extraction and assay procedures were similar to those described by Lillard *et al.* (1970) and Papa (1976). Aflatoxin levels were expressed as either µg aflatoxin or as µg aflatoxin (g mycelial dry wt)⁻¹.

Comparison of diploids and heterokaryons. Techniques involved in forcing heterokaryons and recovering diploids have been reported previously (Papa, 1976). From heterokaryons established on MM, conidia

Table 1. *Mutants of Aspergillus flavus*

Genotype	Spore colour	Auxotrophic requirement	Aflatoxin production
<i>afl-1 leu t</i>	Tan	Leucine	—
<i>afl-4 pdx t</i>	Tan	Pyridoxine	—
<i>afl-b2 pdx w</i>	White	Pyridoxine	+
<i>pdx w</i>	White	Pyridoxine	+
<i>pab w</i>	White	<i>p</i> -Aminobenzoic acid	+
<i>his w</i>	White	Histidine	+
<i>iv w</i>	White	Isoleucine/valine	+
<i>pro w</i>	White	Proline	+
<i>phe w</i>	White	Phenylalanine	+
<i>ad w</i>	White	Adenine	+
<i>met w</i>	White	Methionine	+
<i>thi w</i>	White	Thiamin	+

from heterokaryotic conidial heads were used to inoculate 250 ml Erlenmeyer flasks containing 50 ml YES medium. Five, four and two flasks were inoculated with each heterokaryon, diploid and haploid, respectively.

Degradation study. A total of 68 250 ml Erlenmeyer flasks containing 50 ml YES medium were used. Aflatoxin (1844 µg B₁ and 165 µg B₂) was added to each of 60 flasks; 8 flasks received no aflatoxin. The toxin was dissolved in benzene/acetonitrile (98:2, v/v) and added as 1 ml samples. The solvent was removed during the sterilization of the culture medium by autoclaving. Half of 48 flasks (40 with toxin and 8 without toxin) were inoculated with *afl-1* and half with *afl-4*. The remaining 20 flasks containing aflatoxin were not inoculated and served as controls. The cultures and controls were coded, randomized, incubated for 7 d at 27 °C in the dark, and then stored frozen.

RESULTS AND DISCUSSION

Diploids and heterokaryons were formed between 10 toxigenic strains and the aflatoxin mutant *afl-1* (Table 2). The average reduction in total aflatoxin in diploids and heterokaryons was 87% and 10.9%, respectively, when compared with each *afl*⁺ haploid. Among the diploids, the smallest reduction (38.1%) was realized in the diploid involving the *his w* haploid, a low producer of aflatoxin. Differences between the total aflatoxin of each diploid and its *afl*⁺ haploid component were statistically highly significant. The heterokaryons, however, did not differ significantly from their *afl*⁺ haploids. Diploids heterozygous for *afl-b2* yielded more aflatoxin B₁ than B₂, in agreement with earlier studies showing that *afl-b2* is recessive (Papa, 1977). The *afl-b2* allele was expressed in heterokaryons, however, resulting in the production of more aflatoxin B₂ than B₁.

The determination of the influence of heterokaryosis on aflatoxin production was obviously imprecise in that heterokaryons tend to break down on YES medium. Furthermore, the method of inoculation, which involved sampling heterokaryotic conidial heads (recognized by their mixture of white and tan conidial chains), provided for only a low frequency of heterokaryotic conidia to be present in the inoculum. Earlier studies revealed that 15 to 28% of the conidia from heterokaryons were heterokaryotic (Papa, 1973, 1978). Nevertheless, some heterokaryotic conidial heads were seen on most cultures growing on YES medium. Whether or not the degree of heterokaryosis was sufficient to test the effect of the dominant *afl-1* allele in heterokaryons is not certain. However, the lack of expression of *afl-1* was not surprising since non-allelic aflatoxin mutants that complemented well in diploids all failed to complement in heterokaryons (unpublished results). If *afl-1* produces an enzyme that intracellularly degrades aflatoxin, then a high degree of heterokaryosis would probably be required before its effect could be detected. In contrast, if *afl-1* codes for an extracellular enzyme which degrades aflatoxin, heterokaryosis would not be as necessary as would a sufficient growth of the *afl-1* mutant in mixtures with *afl*⁺ haploids.

To determine if *afl-1* could degrade aflatoxin, it was inoculated into flasks containing

Table 2. *Aflatoxins in diploids and heterokaryons formed between mutant afl-1 and 10 haploid afl⁺ strains*

The amounts of aflatoxins B₁ and B₂, expressed as μg (g mycelial dry wt)⁻¹, are the means of 2, 4 and 5 samples for the *afl⁺* haploids, diploids and heterokaryons, respectively. The percentage changes in the total concentration of aflatoxin (B₁ + B₂) for diploids and heterokaryons compared with the corresponding haploids are also shown.

Haploid <i>afl⁺</i> strains			Diploids			Heterokaryons		
Genotype	B ₁	B ₂	B ₁	B ₂	% change	B ₁	B ₂	% change
<i>afl-b2 pdx w</i>	620	3438	235	18	-93.8	731	2661	-16.4
<i>pdx w</i>	1744	77	160	12	-90.6	1272	77	-25.9
<i>pab w</i>	4451	90	123	8	-97.1	4402	179	+ 1.0
<i>his w</i>	261	28	169	10	-38.1	237	10	-14.5
<i>iv w</i>	3508	94	128	8	-96.2	3017	58	-14.6
<i>pro w</i>	2931	44	222	7	-92.3	2845	114	- 0.5
<i>phe w</i>	3375	74	195	4	-94.2	2856	51	-15.7
<i>ad w</i>	2396	36	331	7	-86.1	2070	46	-13.0
<i>met w</i>	2765	40	244	10	-90.9	2111	95	-21.4
<i>thi w</i>	1986	35	185	6	-90.6	2207	63	+12.3
	Mean				-87.0			-10.9

Table 3. *Degradation of aflatoxin by two aflatoxin mutants*

Initially, 1844 μg aflatoxin B₁ and 165 μg B₂ were added where indicated. Results show the mean $\pm$ standard error for 20 samples, except those for *afl-1* and *afl-4* which were based on 4 samples each.

Mutant and treatment	Aflatoxin (μg) recovered from culture		
	B ₁	B ₂	Total
<i>afl-1</i>	0	0	0
<i>afl-4</i>	0	0	0
Toxin only	1072 $\pm$ 57.6	74 $\pm$ 4.8	1146 $\pm$ 60.6
<i>afl-1</i> + Toxin	605 $\pm$ 30.4	108 $\pm$ 25.0	713 $\pm$ 46.7
<i>afl-4</i> + Toxin	750 $\pm$ 79.6	83 $\pm$ 7.8	833 $\pm$ 58.3

aflatoxin (Table 3). A non-allelic recessive aflatoxin mutant (*afl-4*) was included for comparison. Neither mutant accumulated aflatoxin but a significant reduction in aflatoxin was observed in flasks that had been inoculated with *afl-1* or *afl-4*. Less aflatoxin was recovered in the control flasks than had been added. It is likely that the extraction and assay procedures did not lead to 100% recovery of toxin, and also that the recovery of toxin from flasks containing mycelium was even less efficient. The main point, however, is that the reduction of aflatoxin in flasks containing *afl-1* was not significantly different from that in flasks containing *afl-4*. In addition, mycelial mat weights for these two mutants when grown on YES medium did not differ significantly.

Doyle & Marth (1978*a, b*) showed that mycelium of *A. flavus* and *A. parasiticus* could degrade aflatoxin. Heating mycelium resulted in the loss of the degradative capacity, suggesting possible enzymic degradation of aflatoxin. In addition, they found that little or no aflatoxin was degraded by culture filtrates; however, the supernatant from homogenized mycelium could degrade aflatoxin. Consequently, the enzyme(s) involved in degradation is/are intracellular constituents of the mycelium with very little released into the culture medium.

These preliminary results indicated that *afl-1* was expressed only in diploids. Furthermore, degradation of aflatoxin by mycelium (Table 3) was not controlled by the dominant *afl-1* allele. Possibly, *afl-1* possesses an altered permeability which inhibits aflatoxin excretion from the cell or it may possess a regulatory role. The concentration of a regulatory gene product may be sufficiently high in diploids but too low in heterokaryons to effectively block synthesis.

Specific biochemical tests on *afl-I* will be pursued which should provide useful information concerning the control of aflatoxin biosynthesis.

I wish to thank Louise Miller for her capable technical assistance.

REFERENCES

- BENNETT, J. W. (1979). Aflatoxins and anthraquinones from diploids of *Aspergillus parasiticus*. *Journal of General Microbiology* **113**, 127–136.
- DOYLE, M. P. & MARTH, E. H. (1978*a*). Aflatoxin is degraded by mycelia from toxigenic and non-toxigenic strains of aspergilli grown on different substrates. *Mycopathologia* **63**, 145–153.
- DOYLE, M. P. & MARTH, E. H. (1978*b*). Aflatoxin is degraded by heated and unheated mycelia, filtrates of homogenized mycelia and filtrates of broth cultures of *Aspergillus parasiticus*. *Mycopathologia* **64**, 59–62.
- HSIEH, D. P. H., LIN, M. T., YAO, R. C. & SINGH, R. (1976). Biosynthesis of aflatoxin: conversion of norsolorinic acid and other hypothetical intermediates into aflatoxin B₁. *Journal of Agricultural and Food Chemistry* **24**, 1170–1174.
- LEAICH, L. L. & PAPA, K. E. (1974). Aflatoxins in mutants of *Aspergillus flavus*. *Mycopathologia et mycologia applicata* **52**, 223–229.
- LEE, L. S., BENNETT, J. W., CUCULLU, A. F. & ORY, R. L. (1976). Biosynthesis of aflatoxin B₁. Conversion of versicolorin A to aflatoxin B₁ by *Aspergillus parasiticus*. *Journal of Agricultural and Food Chemistry* **24**, 1167–1170.
- LILLARD, H. S., HANLIN, R. T. & LILLARD, D. A. (1970). Aflatoxigenic isolates of *Aspergillus flavus* from pecans. *Applied Microbiology* **19**, 128–130.
- PAPA, K. E. (1973). The parasexual cycle in *Aspergillus flavus*. *Mycologia* **65**, 1201–1205.
- PAPA, K. E. (1976). Linkage groups in *Aspergillus flavus*. *Mycologia* **68**, 159–165.
- PAPA, K. E. (1977). Genetics of aflatoxin production in *Aspergillus flavus*: linkage between a gene for a high B₂:B₁ ratio and the histidine locus on linkage group VIII. *Mycologia* **69**, 1185–1190.
- PAPA, K. E. (1978). The parasexual cycle in *Aspergillus parasiticus*. *Mycologia* **70**, 766–773.
- PAPA, K. E. (1979). Genetics of *Aspergillus flavus*: complementation and mapping of aflatoxin mutants. *Genetical Research* **34**, 1–9.
- SINGH, R. & HSIEH, D. P. H. (1977). Aflatoxin biosynthetic pathway: elucidation by using blocked mutants of *Aspergillus parasiticus*. *Archives of Biochemistry and Biophysics* **178**, 285–292.