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Genetic Transformation System for the Aflatoxin-Producing Fungus *Aspergillus flavus*†

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A heterologous transformation system was developed for *Aspergillus flavus* with efficiencies greater than 20 stable transformants per μg of DNA. Protoplasts of uracil-requiring strains of the fungus were transformed with plasmid and cosmid vectors containing the *pyr-4* gene of *Neurospora crassa*. Transformants were selected for their ability to grow and sporulate on medium lacking uracil. Vector DNA appeared to integrate randomly into the genome of *A. flavus* with a tendency for multiple, tandem insertion. Transformants with single or multiple insertions were stable after five consecutive transfers on medium containing uracil. Uracil-requiring recipient strains were obtained either by UV-irradiating conidia and selecting colonies resistant to 5-fluoro-orotic acid or by transferring the mutated *pyr* locus to strains by parasexual recombination. This is the first report of a transformation system for an aflatoxin-producing fungus. The transformation system and the availability of aflatoxin-negative mutants provide a new approach to studying the biosynthesis and regulation of aflatoxin.

Aflatoxins are toxic and carcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus* on a number of food and feed sources. Contamination with aflatoxin can be a serious problem before and after harvest. There are no effective control procedures for preventing preharvest contamination with aflatoxin, and decontamination is either ineffective or not economical. New control methods may evolve once more is known about the biochemistry and the genetics of aflatoxin biosynthesis. Present experimental approaches for studying the enzymology and genetics of aflatoxin biosynthesis are arduous, and progress has been slow. The difficulties stem from the lack of sensitive enzyme assays and the lack of a sexual reproduction cycle in the fungus. Recent developments in the molecular biology of filamentous fungi may provide a more effective approach for studying aflatoxin biosynthesis. Genetic transformation systems developed for such filamentous fungi as *Neurospora crassa*, *Ustilago maydis*, and *Aspergillus nidulans* have resulted in the isolation of a growing number of genes by complementation of specific mutations with DNA libraries (11, 15, 25, 29). Such a transformation system for *A. flavus* could facilate the isolation of genes by a similar strategy.

Successful transformation systems rely on cloning vectors with genetic markers suitable for positive selection of transformants. Two classes of selection systems are being used with filamentous fungi. One class utilizes resistance to metabolic inhibitors such as hygromycin (10, 14, 23, 28) and benomyl (27) for selection. The other class utilizes the conversion of auxotrophic mutations such as *trpC* (29), *argB* (3, 8, 9, 21) and *pyr* (1, 4, 26) to prototrophy. The first class of markers is the most desirable because any fungal strain sensitive to the inhibitors can be used as a potential recipient in transformation. In contrast, the second class of markers requires recipient strains to have an auxotrophic mutation, which in many cases is difficult to obtain. Unfortunately, it has not been possible to use markers from the first class in *A. flavus*. The fungus is insensitive to most available metabolic inhibitors, and the benomyl resistance genes from *N. crassa* and *A. nidulans* are not sufficiently expressed in *A. flavus* for selection of transformants (C. P. Woloshuk and G. A. Payne, unpublished data). Therefore, a study was undertaken to develop a transformation system for *A. flavus* by complementation of an auxotrophic mutation. This report describes the isolation of strains of *A. flavus* containing a *pyr* mutation and the transformation of these strains with vectors containing the *pyr-4* gene of *N. crassa* as a selectable marker. A strategy is also discussed for isolating genes effecting aflatoxin biosynthesis in *A. flavus* by this transformation system.

MATERIALS AND METHODS

Fungal strains and media. Strains 650 (*tan leu afl-2*), 774 (*white pdx afl-21*), and 827 (*white lys nor*) of *A. flavus* were obtained from the collection of K. E. Papa (16–20), which has been deposited at the U.S. Department of Agriculture Northern Regional Research Center in Peoria, Ill. The designation *afl* denotes genes for aflatoxin biosynthesis. Potato dextrose agar (PDA; Difco Laboratories, Detroit, Mich.) was used for maintenance of the strains and for obtaining conidia. Other media used were Czapek solution agar (MM) and coconut medium (5). When required for growth, lysine (10 mM), leucine (10 mM), pyridoxine (10 mM) and uracil (10 mM) were added to media. Cultures were incubated at 36°C.

Isolation of mutants containing *pyr* mutation. Conidia of *A. flavus* 650, an aflatoxin nonproducer, were irradiated with UV light (500 μW/cm^2) and plated on PDA containing uracil (10 mM) and 5-fluoro-orotic acid (FOA) (1 mg/ml). Resistance to FOA was used as a positive screen for isolating mutants containing *pyr* mutations (2). Colonies resistant to FOA were subsequently screened for uracil auxotrophy.

Parasexual transfer of *pyr* locus. Heterokaryons were formed by coinoculation of strain 650-33 (*tan leu afl-2 pyr*) with 774 or 827 onto MM. Heterokaryons were identified as prototrophs producing mixed-color conidial heads (white

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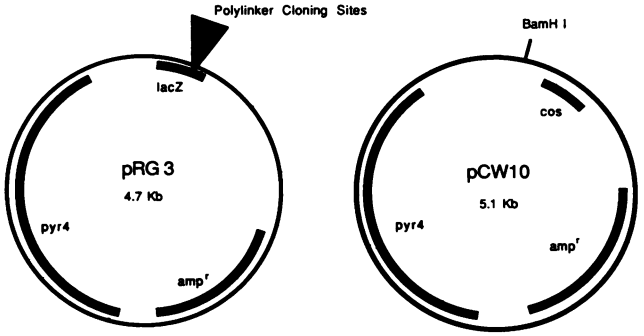

FIG. 1. Maps of plasmid vector pRG3 (gift of G. S. May) and the cosmid vector pCW10. *pyr-4* is a 2.0-kb fragment from *N. crassa* inserted at the *Nde*I site of pUC19. pCW10 was constructed by the insertion of *COS* (400 base pairs) into the *Pst*I site of pRG3.

and tan). Diploids appeared in the population of heterokaryons at a low frequency, but were distinguished from heterokaryons by green conidial heads. The diploids were isolated and subsequently induced to segregate into fast-growing haploid sectors by growing them on PDA containing uracil and fluorophenylalanine (0.5 mM) or benomyl (0.5 μg/ml). Conidia from the haploid sectors were isolated, and their phenotypes were determined by comparing their growth on MM containing various nutritional supplements. Those strains having similar phenotypes as 774 and 827 but requiring uracil for growth were saved for further studies.

Analysis of aflatoxin. Conidia (5×10^5) were inoculated into 25 ml of coconut medium without agar and incubated as static cultures at 28°C for 5 days. The cultures were extracted with acetone (60 ml) overnight. The mycelial mat was removed and used for determination of dry weight. The filtrate was extracted with chloroform, the chloroform extract was dried, and the residue was dissolved in chloroform-hexane (1:1) and applied to a Sep-Pak cartridge (Waters Associates, Inc., Milford, Mass.). After the cartridge was washed with chloroform-hexane (3:1), aflatoxin was eluted with hexane-acetone (1:1). The hexane-acetone extract was dried, and the residue was dissolved in acetonitrile-water (1:1) and then filtered through a 0.45-μm-pore-size Acrodisc CR (Gelman Sciences, Inc., Ann Arbor, Mich.). Aflatoxins were separated by high-performance liquid chromatography on a C_{18} column with a mobile phase consisting of water-tetrahydrofuran-acetic acid (78:20:2). Aflatoxins were identified and quantified by comparison with pure standards (Sigma Chemical Co., St. Louis, Mo.).

Plasmid and cosmid DNA. *A. flavus* was transformed with both a plasmid and a cosmid vector. The plasmid pRG3 was obtained from G. May, Baylor University. Cosmid pCW10 was constructed by the insertion of the 400-base-pair lambda *cos* fragment of pMUA10 (13) into the *Pst*I site of pRG3 (Fig. 1).

Transformation experiments. Transformations of *Escherichia coli* were performed by the $RbCl_2$-$CaCl_2$ method described by Maniatis et al. (12). Procedures used in transformations of *A. flavus* were a modification of methods developed for *A. nidulans* (15). Conidia (10^7) from strains of *A. flavus* with *pyr* mutations were inoculated into 500-ml flasks containing 250 ml of potato dextrose broth and uracil and incubated for 12 to 18 h on a rotary shaker at 36°C. A 1-g (wet weight) sample of mycelium from these cultures was digested in 20 ml of an enzyme solution containing Novozyme (5 mg/ml; Novo Industries), β-glucuronidase (1,200 U/ml; Sigma), 7% NaCl, 20 mM $CaCl_2$, and 10 mM $NaPO_4$ (pH 5.8). After inoculation at 30°C for 3 h, protoplasts were filtered through cheesecloth and a 30-μm screen, washed two times with buffer containing 0.4 M ammonium sulfate, 1% sucrose, and 50 mM potassium citrate (pH 6.0), and suspended in 0.6 M KCl–50 mM $CaCl_2$–10 mM potassium morpholine ethanesulfonic acid (MES) (pH 6.0) to a final concentration of 10^7 protoplasts per ml. Plasmid or cosmid DNA dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA (TE) was added to 100 μl of protoplasts followed by 50 μl of 25% polyethylene glycol solution (50 mM $CaCl_2$, 0.6 M KCl, 10 mM Tris [pH 7.5], 25% polyethylene glycol 8000). After incubation of the mixture on ice for 15 min, 1 ml of 50% PEG solution (same as 25% polyethylene glycol solution except contained 50% polyethylene glycol) was added, and the mixture was incubated for an additional 15 min at room temperature. The transformation mixture was then suspended in molten MM (50°C) containing 0.4 M ammonium sulfate and the appropriate nutritional supplements and incubated in culture plates at 36°C.

Hybridization analysis. Fungal DNA was isolated by a modified mini-preparation procedure (7). Frozen fungal tissue was ground to a powder in a mortar and suspended in a homogenizing buffer consisting of 100 mM LiCl, 10 mM EDTA, 10 mM Tris (pH 7.5), and 0.5% sodium dodecyl sulfate and then incubated for 10 min at 65°C. The suspension was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids were ethanol precipitated, pelleted by centrifugation, and dissolved in TE. After RNase treatment, the solution was again extracted with phenol-chloroform-isoamyl alcohol followed by extraction with chloroform-isoamyl alcohol. The DNA was then ethanol precipitated and redissolved in TE. Fungal DNA (5 μg) was digested to completion with *Eco*RI or *Bgl*II, electrophoresed through 0.7% agarose, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled DNA probes prepared by the method of Feinberg and Vogelstein (6). After hybridization, the filters were washed twice in 2× SSPE (0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], 1 mM EDTA)–0.1% sodium dodecyl sulfate at room temperature for 15 min and twice in 0.1× SSPE–0.1% sodium dodecyl sulfate at 65°C for 15 min. Hybridization was visualized by autoradiography of the filters.

Stability and radial growth of transformants. To determine the mitotic stability of the transformants, we consecutively mass transferred conidia for five generations on PDA containing uracil. Conidia from each generation were suspended in 0.05% Triton X-100 and plated on the same medium at a concentration to form 25 to 30 colonies per plate. Conidia from 100 of these colonies were transferred to medium lacking uracil and assayed for the ability to grow. Radial growth was determined by placing a 5-mm plug of the transformants in the center of a culture plate containing medium without uracil.

RESULTS

Isolation of mutants containing *pyr* mutations. Resistance to FOA was used as a positive screen for isolating mutants containing *pyr* mutations (2). Fifty FOA-resistant colonies of *A. flavus* 650 were isolated after treatment of conidia with UV light. Four isolates, designated 650-27, 650-29, 650-33, and 650-34, required uracil for conidial germination and growth. When inoculated to medium containing uracil, these isolates grew and sporulated normally. Populations of 650-27 and 650-33 were examined for reversion to uracil indepen-

TABLE 1. Genotype and aflatoxin production by haploid and diploid strains of *A. flavus*

Strain	Genotype[a]	Aflatoxin B_1 + B_2 (μg/g [dry wt])[b]
650-27	*t leu afl-2 pyr*	9.84
650-33	*t leu afl-2 pyr*	17.51
774	*w pdx afl-21*	1.10
774 × 650-33	$\frac{w}{+}\ \frac{+}{t}\ \frac{pdx}{+}\ \frac{+\ afl\text{-}21}{leu\ +}\ \frac{+}{afl\text{-}2}\ \frac{+}{pyr}$	317.52
774-1	*w pdx afl-21 pyr*	3.32
774-2	*w pdx afl-21 pyr*	2.86
774-2 × 650-33	$\frac{w}{+}\ \frac{+}{t}\ \frac{pdx}{+}\ \frac{+\ afl\text{-}21}{leu\ +}\ \frac{+}{afl\text{-}2}\ \frac{pyr}{pyr}$	92.10
827	*w lys nor*	15.75
827 × 650-33	$\frac{w}{+}\ \frac{lys}{+}\ \frac{+}{t}\ \frac{nor\ +}{+\ leu}\ \frac{+}{afl\text{-}2}\ \frac{+}{pyr}$	506.07
827-40	*w lys nor pyr*	19.79
3357	Wild type	366.48

[a] Locations of afl-2 and pyr are not known. All other genes are mapped.
[b] Each value represents the mean of four replications.

dence. No reversion was detectable in the greater than 10^9 conidia tested.

Transfer of *pyr* locus. The *pyr* locus of strain 650-33 was transferred into two other strains (774 and 827) by parasexual recombination. Diploids were selected from forced heterokaryons formed between 650-33 and these other strains. In the diploids, complementation occurred, resulting in wild-type phenotypes. The diploid strains were prototrophs with green conidial heads and produced aflatoxin at levels similar to those of a typical toxigenic strain (Table 1). Aflatoxin produced by diploid cultures ranged from 5 to 300 times the level produced by the parental haploid cultures. These diploids were subsequently induced to segregate into haploid sectors and screened for the desired phenotypes. Three haploid sectors derived from the 650-33 × 774 diploid had white conidial heads and required pyridoxine and uracil for growth. Two of these strains were designated 774-1 and 774-2, and both produced levels of aflatoxin similar to those of the parental strains (Table 1). Strain 774-2 was backcrossed with 650-33, and a diploid selected from this cross had green conidial heads and required uracil for growth. Aflatoxin produced by this diploid was 5 and 30 times that of the respective parental strains (Table 1), indicating that the *afl-2* allele of strain 650-33 was not transferred to strain 774-2. Six haploid sectors were obtained from the 650-33 × 827 diploid, which required lysine and uracil for growth and accumulated the aflatoxin biosynthetic pathway intermediate norsolorinic acid. Aflatoxin production by one of these six haploids, 827-40, was examined and found to be similar to that of the parent strains 827 and 650-33 (Table 1).

Transformation of *A. flavus*. The following isolates of *A. flavus* were found to be capable of being transformed with the *pyr-4* gene of *N. crassa*: 650-27, 650-29, 650-33, 650-34, 774-1, 774-2, and 827-40. Transformants were visible as growing hyphae after 24 h of incubation at 36°C. Two types of transformants were observed after 3 to 5 days of incubation. Type 1 colonies were fast growing and sporulated normally. Most type 2 transformants failed to grow past the initial growth observed after 24 h of incubation. Others exhibited significantly slower growth than the type 1 transformants, with the occasional production of one to five conidial heads. Conidia isolated from these heads were not capable of germination or growth without the addition of uracil. Transformation efficiency was quite variable between

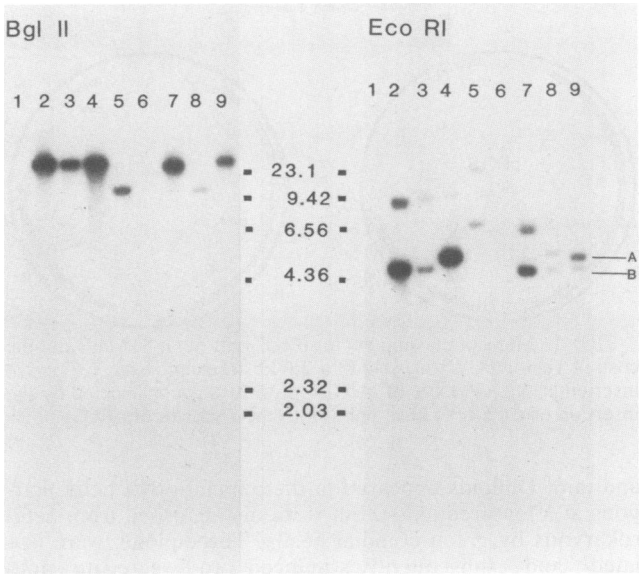

FIG. 2. Hybridization analysis of transformants of *A. flavus*. Total DNA isolated from *A. flavus* was digested with *Bgl*II or *Eco*RI. The restricted DNA (5 μg per lane) was electrophoresed through 0.7% agarose, transferred to nitrocellulose, and probed with ^{32}P-labeled pCW10. Lanes: 1, strain 650-27, not transformed; 2 and 3, 650-27 transformed with pRG3; 4 and 5, 650-27 transformed with pCW10; 6, strain 774-1, not transformed; 7 and 8, 774-1 transformed with pRG3; 9, 774-1 transformed with pCW10. Molecular weight markers in the center are λ DNA digested with *Hin*dIII. Lines denote the mobility of pCW10 (A) and pRG3 (B).

protoplast preparations. It was also variable between fungal isolates. Type 1 transformants were obtained from strains 650-27, 650-33, 774-1, and 774-2 at an average frequency of 20/μg of plasmid DNA. Strain 827-40 was transformed at a frequency of one type 1 transformant per μg of DNA. The frequency of type 2 transformants was estimated at 50 to 100 times that of type 1. The frequency for the other isolates was not determined. No difference was observed between the efficiency of transformation with pRG3 and pCW10. Also, no transformants were observed when plasmid DNA lacking the *pyr-4* gene was used.

Hybridization analysis of *A. flavus* transformants. Hybridization analysis of several type 1 transformants of *A. flavus* isolates 650-27 and 774-1 is shown in Fig. 2. No detectable similarity was observed between the *pyr-4* gene of *N. crassa* used as a probe and the orotidine-5′-phosphate decarboxylase gene of *A. flavus* (lanes 1 and 6). The observed hybridization of the probe with the DNA of the transformants therefore resulted from the presence of the vector DNA. When the DNA of the transformants was digested with an endonuclease (*Bgl*II) which does not restrict pRG3 or pCW10, single fragments of high molecular weight were detected. Failure to detect fragments of the size of the vectors (4.7 and 5.1 kilobases [kb]) indicates that the vectors integrated into the genome of the transformants and were not autonomously replicating. To determine whether tandem copies of vector had integrated into the transformants, we analyzed *Eco*RI-digested DNA because both vectors have only one *Eco*RI site. Transformants obtained with the vectors pRG3 (lanes 2, 3, 7, and 8) and pCW10 (lanes 4 and 9) clearly contained multiple copies of the vectors. The relative intensities of hybridization to the *Eco*RI-excised plasmid DNA (4.7 kb for pRG3 and 5.1 kb for pCW10) indicates that

the number of tandem copies varied between transformants. No 5.1-kb fragment was observed in the *Eco*RI-digested DNA of one transformant (lane 5), suggesting that the transformant contained only one copy of pCW10. Hybridization analysis of three transformants of 827 indicated a similar pattern of vector integration (data not shown).

Mitotic stability and radial growth. The mitotic stability and radial growth of two transformants (650-27-9 and 650-27-10) were examined. These transformants were chosen because one (650-27-10; Fig. 2, lane 5) appeared to contain only one copy of the cosmid pCW10 and the other (650-27-9; Fig. 2, lane 4) contained multiple copies in tandem. The analysis of colonies from 100 single conidia obtained from each of five consecutive transfers to medium containing uracil indicated that uracil-dependent individuals existed in the populations. As many as 20% of the colonies were uracil dependent. The continual transfer of the cultures to medium containing uracil, however, did not result in an increase in the number of uracil-dependent colonies. Little differences was detected in the radial growth between the two transformants. Four days after inoculation on medium lacking uracil, the radial growth of transformant 650-27-9 was 3.0 cm, while that of transformant 650-27-10 was 2.8 cm.

DISCUSSION

This is the first report of a transformation system for an aflatoxin-producing fungus. We achieved transformation of *A. flavus* protoplasts with both a plasmid and a cosmid vector containing the *pyr-4* gene of *N. crassa*. The *pyr-4* gene of *N. crassa* integrated into the genome of *A. flavus* and was sufficiently expressed to allow transformants to grow and sporulate on medium lacking uracil. The complex hybridization pattern observed when transformant DNA was digested with *Eco*RI (Fig. 2) suggests that the vectors integrated randomly into the genome of *A. flavus*. This type of integration has been reported for heterologous transformation systems in other fungi (4, 8, 10, 14, 22, 24, 28). As also observed in other fungal transformation systems, there was a tendency for multiple, tandem insertion of plasmid DNA into the genome of *A. flavus*. Such integration, however, does not appear to be required for expression of the gene because one transformant, chosen at random in this study, appeared to contain a single copy of pCW10. Copy number also did not affect growth of the two transformants tested. Further studies are in progress to quantify the level of orotidine-5′-phosphate decarboxylase in transformants with different copy numbers of vector DNA.

The cosmid vector appears to be relatively stable within the genome of *A. flavus*. There was no apparent shift in the population of uracil-requiring strains after five consecutive transfers to medium containing uracil. Conidia apparently do exist within the population of each strain that are incapable of growth without uracil; however, the number of uracil-dependent members of the population did not increase with consecutive transfers. It was not determined whether the loss of uracil independence resulted from the loss of the *pyr-4* gene.

The development of a genetic transformation system for *A. flavus* provides a new approach to studying aflatoxin biosynthesis. Genes involved in the biosynthetic pathway can be identified by their ability to complement strains of *A. flavus* with specific blocks in the pathway. Genomic libraries constructed in cosmid vectors, such as pCW10, are particularly useful in this type of study. The large DNA inserts (35 to 40 kb) attainable in cosmids increase the probability of the complete sequence of a particular gene residing in a single cosmid and decrease the number of clones needed in a library to represent the fungal genome.

A. flavus has several advantages over *A. parasiticus* for studies of aflatoxin biosynthesis. It has a well-described parasexual cycle, and there are 25 strains in the same heterokaryon compatibility group with distinct mutations disrupting aflatoxin biosynthesis (16–20). Studies conducted by K. E. Papa (16–20) indicated that the aflatoxin loci of these strains are all nonallelic and that all alleles are recessive in diploids except for the *afl-1* allele. There are also a total of 30 genes mapped to eight linkage groups in these strains, 12 of which affect aflatoxin biosynthesis. The ability to transfer the *pyr* locus from one strain of *A. flavus* to another through parasexual recombination, as demonstrated in this study, indicates the potential application for a transformation system with the *pyr-4* gene as a selectable marker in genetic studies in which the strains are in the same heterokaryon compatability group.

Aflatoxin contamination is a major health concern for both humans and animals. New approaches are needed to help elucidate the biosynthetic pathway and to develop strategies for reducing aflatoxin contamination. The system we described provides a new and expedient approach to studying the biosynthesis and regulation of aflatoxin. The transformation efficiency of 20 transformants per μg of DNA is adequate to screen a cosmid DNA library. Further, strains of *A. flavus* with distinct mutations disrupting aflatoxin biosynthesis are available, and these strains can be easily mutated at the *pyr* locus by parasexual recombination. We are currently using the system described here to identify genes involved in aflatoxin biosynthesis.

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