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Indole-Diterpene Gene Cluster from *Aspergillus flavus*

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Aflatrem is a potent tremorgenic mycotoxin produced by the soil fungus *Aspergillus flavus* and is a member of a large structurally diverse group of secondary metabolites known as indole-diterpenes. By using degenerate primers for conserved domains of fungal geranylgeranyl diphosphate synthases, we cloned two genes, *atmG* and *ggsA* (an apparent pseudogene), from *A. flavus*. Adjacent to *atmG* are two other genes, *atmC* and *atmM*. These three genes have 64 to 70% amino acid sequence similarity and conserved synteny with a cluster of orthologous genes, *paxG*, *paxC*, and *paxM*, from *Penicillium paxilli* which are required for indole-diterpene biosynthesis. *atmG*, *atmC*, and *atmM* are coordinately expressed, with transcript levels dramatically increasing at the onset of aflatrem biosynthesis. A genomic copy of *atmM* can complement a *paxM* deletion mutant of *P. paxilli*, demonstrating that *atmM* is a functional homolog of *paxM*. Thus, *atmG*, *atmC*, and *atmM* are necessary, but not sufficient, for aflatrem biosynthesis by *A. flavus*. This provides the first genetic evidence for the biosynthetic pathway of aflatrem in *A. flavus*.

Aflatrem is a potent tremorgenic mycotoxin produced by the soil fungus *Aspergillus flavus* (17, 18). This compound is a member of a large, structurally diverse group of secondary metabolites known as indole-diterpenes that includes paspaline, paxilline, shearinines, paspalitrems, terpendoles, penitrems, lolitrems, janthitrems, and sulpinines (34). These metabolites all have a common structural core comprised of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from either tryptophan or a tryptophan precursor. Different patterns of prenylation, hydroxylation, epoxidation, acetylation, and ring stereochemistry around the basic indole-diterpene ring structure define this structural diversity (34). The mechanism by which aflatrem and related indole-diterpenes cause tremorgenicity in mammals is not well defined, but biochemical and clinical studies indicate that these effects are due in part to effects on receptors and to interference with neurotransmitter release in the central and peripheral nervous systems (40).

Very little is known about the pathways of indole-diterpene biosynthesis other than the origins of the indole and diterpene components (7, 10). Biosynthetic schemes based on the chemical identification of likely intermediates have been proposed, but until recently none of these steps had been validated by biochemical or genetic studies (25, 30).

Recently, a cluster of genes for paxilline biosynthesis was cloned from *Penicillium paxilli* (53). Key genes in this cluster include a GGPP synthase gene (*paxG*), a FAD-dependent monooxygenase gene (*paxM*), a prenyl transferase gene (*paxC*), and two cytochrome P450 monooxygenase genes, *paxP* and *paxQ*. The deletion of *paxG*, *paxM*, or *paxC* results in mutants that are defective in paxilline biosynthesis (53; B. Scott, L. McMillan, J. Astin, C. Young, A. Bryant, and E. Parker, unpublished results). PaxM and PaxC may catalyze the addition of indole-3-glycerol phosphate to GGPP and subsequent cyclization to form the first stable indole-diterpene, possibly paspaline (34). *paxP* and *paxQ* deletion mutants accumulate paspaline and 13-desoxypaxilline, respectively, suggesting that these compounds are the substrates for the corresponding enzymes (26). Thus, at least five genes are required for the biosynthesis of paxilline in *P. paxilli*.

Two other fungal gene clusters for diterpene biosynthesis have been reported, namely gibberellin production in *Fusarium fujikuroi* (44) and aphidicolin synthesis in *Phoma betae* (42). Like the paxilline biosynthesis cluster in *P. paxilli*, the gibberellin and aphidicolin gene clusters contain a GGPP synthase gene, named *ggs-2* and *Pbggs*, respectively. *P. paxilli* and *F. fujikuroi* also carry a second GGPP synthase gene, suggesting that the presence of two copies of GGPP synthase may be a molecular signature for diterpene biosynthesis (28, 53). Given that many genes for secondary metabolite biosynthesis in fungi are organized in clusters (23), the molecular cloning of GGPP synthases combined with chromosome walking could provide a rapid strategy for cloning new indole-diterpene gene clusters.

The objectives of this study were to (i) isolate GGPP synthases from *A. flavus* by using degenerate PCR, (ii) determine if one of the GGPP synthases is closely linked to homologs of the *P. paxilli pax* genes by performing chromosome walking, (iii) determine if the pattern of expression of these genes coincides with the onset of aflatrem biosynthesis, and (iv) test whether one of these genes can functionally complement a *P. paxilli pax* mutant. Our working hypothesis was that *A. flavus* contains two GGPP synthase genes, one of which is associated with a cluster of genes for indole-diterpene (aflatrem) biosynthesis. This work broadens our understanding of indole-diterpene metabolite biosynthesis in filamentous fungi and provides the first genetic evidence for the biosynthetic pathway of aflatrem in *A. flavus*.

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TABLE 1. Primers used for this study

Primer	Sequence (5′→3′)[a]
ggpps27	CAYMGIGGTCARGGTATGGA
ggpps28	TTCATRTAGTCGTCICKTATYTG
ggpps29	AACTTTCCYTCIGTSARGTCYTC
M13F	CCCAGTCACGACGTTGTAAAACG
M13R	AGCGGATAACAATTTCACACAGG
T7	TAATACGACTCACTATAGGG
Oligo-dT	GAGAGAATTCGGATCCTCTAGAG$(T)_{20}$
AF-93	*ACGGATCC*AGGAGTGGCGTTATCGTG
AF-94	*ACGGATCC*AATAGGAGAGGTTAGTCG
AF-tub-1	CTTCTTCATGGTTGGCTTCG
AF-tub-2	CTCTGATCTTCGCAAGTTGG
AF-tub-3	GGTGGAGGACATCTTGAGAC
AF-111	CCTTCTACGTCTCCATCCAG
AF-112	GTGATCTCCTTCTGCATACG
atmGF	TAGTCGTCACGGATCTGGAA
atmGR	CAAGTTCATTGTCGACCGAG
atmMF	GGCATCAATGGCATTATCC
atmMR	GGACAATGTATCGTGCAAGG
AF-78	AGGACAGGCTGAACATCATC
AF-79	GTTATCCGCTCTGAGATGTG
AF-80	CTGTCATAGACGTAACCTCC
AF-81	TCTCTTCGAGACATTGCAGC
atmCF	TCGGATATTATGTGGCGACC
atmCR	GTTGCCGCCTCTGTTGCCTT
AF-81	TCTCTTCGAGACATTGCAGC
AF-82	TTCAACCAGAGGAGCGAGTA
AF-106	CTTAGTCAGCCTCGCTATAC
AF-113	GATTACAAGGTGAGTGGAGC
AF-114	GCTCCACTCACCTTGTAATC
AF-115	AGAGGAAGTCCTACCGCTCA
AF-116	TGAGCGGTAGGACTTCCTCT
AF-117	GGATCAGAGCCGGTGAGACA
AF-9	GAAGACCGAGCACATTGA
AF-108	GTATGTCCTCTTGCATGTCC

[a] I, inosine; M, A or C; K, G or T; R, A or G; S, C or G; and Y, C or T. Italic bases do not correspond to the *A. flavus* genomic sequence.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used for this study, XL-1 Blue (5), KW251 (Promega Corp., Madison, Wis.), and Top 10 (Invitrogen Corp., Carlsbad, Calif.), were grown on Luria-Bertani (LB) agar plates (38) supplemented, when necessary, with either ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

Fungal strains and growth conditions. *A. flavus* strain NRRL6541 (47) was obtained from Merck and Co. (Rahway, N.J.) and is maintained at Massey University as strain PN2280. Cultures were maintained on 2.4% potato dextrose agar (Difco, Spark, Md.) plates or as spore suspensions in 10% (vol/vol) glycerol at −80°C. Seed cultures of *A. flavus* for aflatrem production were started from an inoculum of approximately 5×10^6 spores in a 100-ml Erlenmeyer flask containing 25 ml of YEPGA medium (26) and were grown at 30°C for 2 days with agitation at 200 rpm. Two milliliters of seed culture was added to 50 ml of aflatrem production medium (see below), and the cultures were incubated in the dark without shaking at 30°C until they were harvested. The mycelium (approximately 1 g of fresh weight) was collected by filtration through Miracloth (Calbiochem Corporation, La Jolla, Calif.), washed with water, and blotted dry. Samples of 1 to 3 g (wet weight) were frozen in liquid nitrogen and stored at −80°C for the preparation of RNAs. The remainder of the mycelium was freeze-dried for indole-diterpene analysis. Cultures used for the preparation of genomic DNAs were grown under the same conditions as those used for the seed cultures except that CDYE medium (53) was used.

Aflatrem production medium contained the following ingredients per liter: 80 g of mannitol, 40 g of yeast extract, 10 g of Casamino Acids, 10 g of $CaCO_3$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 5H_2O$, 1 mg of $CuSO_4 \cdot 5H_2O$, and 0.8 mg of $CoCl_2 \cdot 6H_2O$, buffered to pH 6.0 with 10 g of 2-(*N*-morpholino)ethanesulfonic acid (MES).

P. paxilli cultures were grown and maintained as described previously (21, 26, 53).

Molecular biology. Genomic DNAs from *A. flavus* and *P. paxilli* were isolated from freeze-dried mycelia by the method of Möller et al. (29), Byrd et al. (6), or Yoder (51). Plasmid and cosmid DNAs were isolated by alkaline lysis (38) and were purified by use of either a Bio-Rad Quantum Prep plasmid miniprep kit (Bio-Rad Laboratories, Hercules, Calif.) or a Qiagen (Hilden, Germany) plasmid mini kit. PCR products amplified with *Taq* DNA polymerase (Roche, Penzberg, Germany) were routinely cloned into pGEM-T Easy (Promega) and transformed into *E. coli* XL-1 Blue. Genomic and cosmid digests were transferred to positively charged nylon membranes (Roche) by capillary transfer (41), and DNAs were fixed by UV cross-linking at 254 nm for 2 min. Filters were probed with either [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham, Buckinghamshire, United Kingdom)- or digoxigenin (DIG)-11-dUTP (Roche)-labeled probes. The labeling of DNAs was done by primed synthesis with the Klenow fragment and a High Prime kit (Roche) or by the PCR-mediated incorporation of DIG-11-dUTP (Roche). Hybridizations involving ^{32}P-labeled probes were carried out at 65°C overnight in Denhardt's solution as previously described (52). DIG hybridizations were carried out at 42°C in 50% formamide (DIG system user's guide for filter hybridization; Roche). The membranes were washed, and hybridization signals (phosphorescence) were detected by autoradiography as previously described (52) or by the use of a Fluoro image analyzer (model FLA-5000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

Nested PCR amplification of genomic DNAs. PCRs were carried out in a final volume of 25 μl containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), a 50 μM concentration of each deoxynucleoside triphosphate (dNTP), a 200 nM concentration of each primer, 0.5 U of *Taq* DNA polymerase (Roche), and 5 ng of genomic DNA. The first round of PCR was performed with the degenerate primer pair ggpps27 and ggpps29 (Table 1), followed by a second round with the degenerate primers ggpps27 and ggpps28 and a 1:100 dilution of the reaction mixture from the first round of PCR. The cycling conditions were as follows: 1 cycle of 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 45°C, and 1 min

at 72°C; and a final extension for 5 min at 72°C. Reactions were carried out in either a PC-960 or FTS-960 thermocycler (Corbett Research, Sydney, Australia). Degenerate primers were designed by using conserved sequences identified from an alignment of *Neurospora crassa* AL-3 (XP_326920), *Gibberella fujikuroi* GGS (Q92236), *P. paxilli* PaxG (AAK11531), *Saccharomyces cerevisiae* BTS1 (S60921), and *G. fujikuroi* GGS2 (CAA75568) polypeptide sequences.

Construction and screening of an *A. flavus* cosmid library. Genomic DNA (240 μg) isolated from *A. flavus* MF6421 (29) was partially digested with MboI (Roche) as described by Frischauf et al. (15) to maximize the yield of 35- to 40-kb DNA fragments. These DNAs were partially end-filled with dATP and dGTP by use of the Klenow fragment of DNA polymerase I (Roche) to generate 5′-GA protruding termini. Aliquots (3.6 μg) of this DNA were ligated in a 9:1 molar ratio of vector to insert with XbaI- and XhoI-digested pMOcosX (6.8 μg) (33). The XbaI termini were dephosphorylated with calf alkaline phosphatase (Roche) (0.5 U/μg of DNA), and the XhoI termini were partially end-filled by the incorporation of dCTP and dTTP to generate protruding 5′-TC ends by use of the Klenow fragment of DNA polymerase I (Roche). Half of the ligated mixture (~5 μg) was packaged by use of the Packagene system (Promega) according to the manufacturer's instructions (Packagene lambda DNA packaging system technical bulletin no. 005, Promega), the DNAs were transduced into the *E. coli* host strain KW251, and 2,700 ampicillin-resistant colonies were selected. Individual clones were transferred to 96-well microtiter plates containing LB medium supplemented with ampicillin and were amplified by growing at 37°C overnight. A copy of the cosmid library was maintained at −80°C in LB medium containing 4.4% glycerol (12).

The library was screened by colony PCR with primers ggpps27 and ggpps28. The cycling conditions were as follows: 1 cycle of 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; and a final extension for 5 min at 72°C. Four cosmids, pSZ-17, pSZ-18, pSZ-19, and pSZ-20, contained the *atmG* gene and one, pSZ-21, contained *ggsA*.

Subcloning of pSZ-20 and pSZ-21 for sequencing. The cosmid pSZ-20 was subcloned into the PCR4Blunt-TOPO vector by use of a TOPO shotgun subcloning kit (Invitrogen) using 3.3 μg of cosmid DNA isolated by use of a Qiagen large construct kit. Clones (300) that failed to hybridize to DIG-11-dUTP-labeled pMOcosX DNA were arbitrarily selected for sequencing. Plasmid DNAs were isolated with a Bio-Rad Quantum Prep plasmid miniprep kit, and the ends were sequenced by the use of standard M13 forward and reverse sequencing primers (Table 1). The 7.3-kb BamHI fragment from pSZ-21 was subcloned into pBluescript II KS(+) (Stratagene) to generate pSZ-22, and this plasmid was used as a template to extend the sequence data previously derived from the 218-bp insert of pSZ-2.

RNA isolation and cDNA synthesis and analysis. RNAs were isolated from frozen mycelia by use of the TRIzol reagent (Invitrogen). mRNAs were isolated by oligo(dT) affinity chromatography with a Sigma (St. Louis, Mo.) GenElute mRNA miniprep kit and were quantitated by measurements of fluorescence with a RiboGreen RNA quantitation kit (Molecular Probes, Inc., Eugene, Oreg.). A denatured sample of mRNA was reverse transcribed by the use of Expand reverse transcriptase (Roche) in the presence of an oligo(dT) primer. Gene-specific amplifications of dilutions (1:10, 1:100, and 1:1,000) of the cDNA were carried out in a reaction volume of 20 μl containing 2.0 μl of 10× reaction buffer (Roche), a 50 μM concentration of each dNTP, a 200 nM concentration of each primer, and 0.5 U of *Taq* DNA polymerase (Roche). The cycling conditions were as follows: 1 cycle of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; and a final extension for 5 min at 72°C. The primer combinations (Table 1) used for cDNA analysis were as follows: atmGF-atmGR (introns 1, 2, and 3) for *atmG*; AF-79–AF-80 (intron 2), AF-79–atmMR (introns 2 and 3), and AF-80–atmMF (introns 1 and 2) for *atmM*; and atmCF–AF-82 (intron 2) and atmCR–AF-81 (intron 1) for *atmC*. These products were cloned into pGEM-T Easy and then sequenced.

For a cDNA analysis of *ggsA* transcripts, one-step reverse transcription-PCRs (RT-PCRs) were performed with SuperScript III with Platinum *Taq* (Invitrogen) and DNase I (Invitrogen)-treated mRNAs (5 to 50 ng) purified as described above under the reaction conditions described by the manufacturer. The cycling conditions were as follows: 1 cycle of 30 min at 55°C; 1 cycle of 2 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 60°C, and 90 s at 72°C; and a final extension for 5 min at 72°C. The primer (Table 1) combinations used were as follows: AF-106–AF-114, AF-113–AF-116 (intron 1), AF-115–AF-117 (intron 2), and AF-9–AF-108 (intron 2). These products were cloned into pGEM-T Easy (Promega Corp.) and then sequenced.

RT-PCR and Northern expression analysis. RT-PCR expression analysis was performed by use of a SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen). PCRs were carried out in a final volume of 25 μl containing 1.2 mM $MgCl_2$, a 200 μM concentration of each dNTP, a 200 nM concentration of each primer, 0.5 μl of reverse transcriptase-Platinum *Taq* mixture (Invitrogen), and 4 ng of mRNA from each time point. The primer (Table 1) combinations used were AF-tub1–AF-tub3 (spans intron 6) for *tub2* (β-tubulin), AF-111–AF-112 for *act1* (actin), atmGF–AF-78 (introns 2 and 3) for *atmG*, AF-79–AF-80 (intron 2) for *atmM*, and AF-81–atmCR (intron 1) for *atmC*. The cycling conditions were as follows: 1 cycle of 30 min at 50°C followed by 2 min at 94°C; 27 cycles of 30 s at 94°C, 60 s at 55°C, and 60 s at 72°C; and a final extension for 5 min at 72°C.

For Northern analysis, the total RNA (10 μg) from each time point was mixed with 3 μl of 10× MOPS buffer (38), 15 μl of formamide, 4.8 μl of formaldehyde (37%), 0.12 μl of ethidium bromide (10 mg/ml), and H_2O to a final volume of 30 μl. The RNA mixtures were denatured at 65°C for 15 min, loaded into a formaldehyde (6.2%)-agarose (1.2%) gel, and separated by electrophoresis for 4 to 5 h at 120 V. The denatured RNAs were transferred to Hybond N^+ (Amersham) membranes by capillary transfer, and the RNAs were fixed by UV cross-linking at 254 nm for 40 s. The filters were probed with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham)-labeled cDNA probes. Hybridizations were carried out at 68°C overnight in 10× Denhardt's solution. The filters were washed twice with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 68°C. The primer (Table 1) combinations used to generate the cDNA probes were AF-tub2–AF-tub3 for *tub2* (β-tubulin), AF-111–AF-112 for *act1* (actin), atmGF–atmGR for *atmG*, AF-79–AF-80 for *atmM*, and AF-81–atmCR for *atmC*.

Preparation of complementation construct. Plasmid pSZ-43 was constructed by ligating a 3,118-bp BamHI fragment containing 1,299 bp of the 5′ sequence, 1,645 bp of the coding sequence, and 174 bp of the 3′ sequence of *atmM* into pII99 (32). The 3,118-bp BamHI fragment was prepared by digesting a 3,134-bp PCR product that had been amplified by use of the primer set AF-93 and AF-94 with the pSZ-20 cosmid DNA as a template.

***P. paxilli* transformation and molecular analysis of transformants.** Protoplasts of a *paxM* deletion mutant (LMM-100) were prepared as previously described (52), except that 10 mg of Glucanex (Chemcolour Industries, Auckland, New Zealand)/ml was used to digest the cell walls, and the mycelium was gently shaken (100 rpm) overnight at 30°C. The protoplasts were transformed with circular pII99 or pSZ-43 by the method of Vollmer and Yanofsky (46) as modified by Itoh et al. (21). Transformants were selected on RG medium (26) containing Geneticin (Invitrogen) at a final plate concentration of 150 μg/ml. Single spores of the resulting transformants were subcultured and maintained on potato dextrose agar supplemented with Geneticin at the same concentration.

Indole-diterpene analysis. Thin-layer chromatography and reverse-phase high-performance liquid chromatography (HPLC) analyses of *A. flavus* and *P. paxilli* indole-diterpenes were performed as described previously (26). For HPLC, eluted products were analyzed by UV detection at either 230 or 280 nm and were compared with the elution times of paxilline and aflatrem standards. Samples were quantitated with respect to known concentrations of paxilline (30) or aflatrem (Merck and Co.). The sensitivity of detection was approximately 50 to 100 ng.

DNA sequencing. DNA fragments were sequenced by the dideoxynucleotide chain termination method (39) using Big-Dye (version 3) chemistry with oligonucleotide primers (Sigma Genosys) for pBluescript II KS(+), pGEM-T Easy, and PCR4Blunt-TOPO (M13F, M13R, and T7) or for *A. flavus* sequences. The products were separated in an ABI Prism 377 sequencer (Perkin-Elmer Corp., Foster City, Calif.).

Bioinformatics. Sequence data were assembled into contigs by the use of Sequencher, version 4.1 (Gene Codes), and were analyzed with the Wisconsin Package, version 9.1 (Genetics Computer Group, Madison, Wis.). Sequence comparisons were performed at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/) by using the Brookhaven (PDB), SWISSPROT and GenBank (CDS translation), PIR, and PRF databases, employing algorithms for both local (BLASTX and BLASTP) and global (FASTA) alignments (1, 2, 36). BLASTP analyses were also performed by use of the Whitehead fungal sequence database (http://www-genome.wi.mit.edu/cgi-bin/annotation/fgi/blast_page.cgi).

The pSZ-20 and *ggsA* sequence contigs were annotated and analyzed with MacVector 7.2 (Accelrys Inc., San Diego, Calif.).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *ggsA* and *pSZ-20* sequences are AY559848 and AY559849, respectively.

RESULTS

GGPP synthase sequences in *A. flavus*. An alignment of fungal GGPP synthase polypeptide sequences identified three highly conserved motifs within domains III and V that flanked

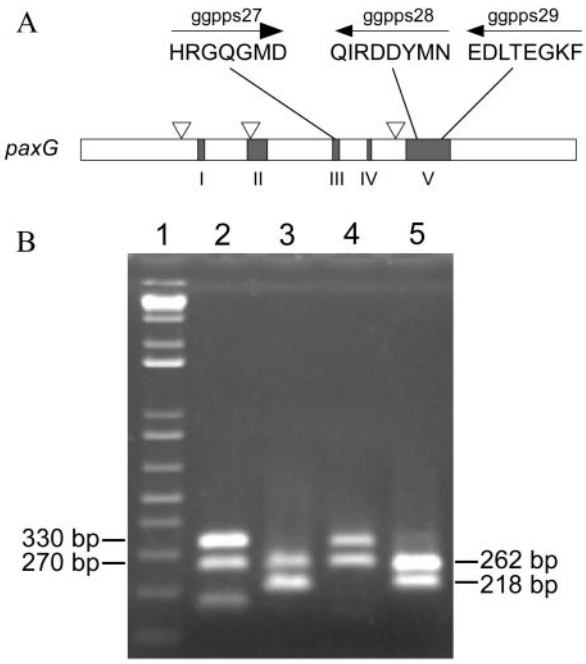

FIG. 1. *A. flavus* contains two GGPP synthases. (A) *P. paxilli paxG* gene map showing conserved domains (I to V), intron positions (inverted triangles), and consensus polypeptide sequences used to design degenerate primers to amplify the region between domains III and V. (B) PCR products amplified with degenerate primers ggpps27 and ggpps29 (lanes 2 and 4) and ggpps27 and ggpps28 (lanes 3 and 5) from genomic DNAs of *P. paxilli* (lanes 2 and 3) and *A. flavus* (lanes 4 and 5). The products shown in lanes 3 and 5 were from nested PCR amplifications of 1/100 dilutions of the products shown in lanes 2 and 4, respectively. Lane 1 contains a 1-kb DNA ladder (Invitrogen).

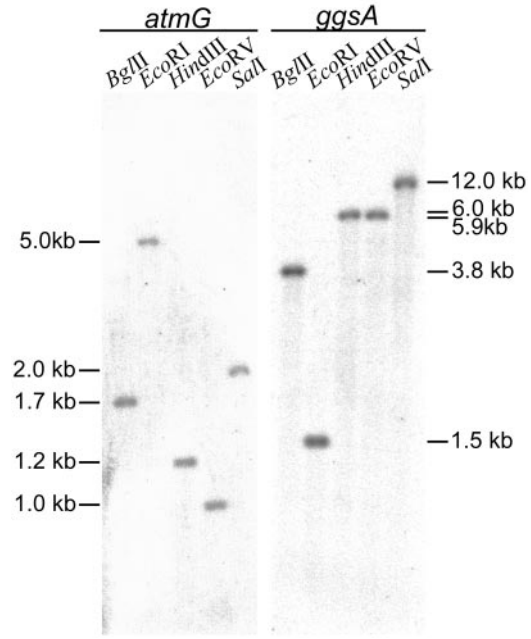

FIG. 2. Southern analysis of *A. flavus* genomic DNA probed with *atmG* and *ggsA*. The autoradiograph shows Southern blots of *A. flavus* genomic DNA (1 μg) digested with five different restriction enzymes and probed with ^{32}P-labeled 262-bp *atmG* and 218-bp *ggsA* probes. The numbers on the left and right correspond to the sizes of the restriction fragments that hybridized to the probes.

an intron in *P. paxilli paxG*, but not *ggs1*, and that were suitable for the design of degenerate primers (Fig. 1A). Nested PCR amplification of genomic DNAs from *P. paxilli* and *A. flavus* with primers ggpps27 and ggpps29 and then with primers ggpps27 and ggpps28 resulted in two products from each reaction (Fig. 1B). Products of 330 and 270 bp (lanes 2 and 4) were obtained after the first round of amplification, and products of 262 and 218 bp (lanes 3 and 5) were observed after the nested amplification. The *P. paxilli* products corresponded to sequences of the GGPP synthase genes *ggs1* (the smaller band) and *paxG* (the larger band) (53). The difference in size was due to an intron in *paxG*. The two *A. flavus* PCR products (lane 5) each displayed approximately 50% amino acid sequence identity to the *P. paxilli* GGPP synthase genes. Southern blot analysis confirmed that each sequence was unique and present as a single copy in the genome (Fig. 2). The larger, 262-bp PCR product was predicted to contain an intron and, by analogy with the sequences from *P. paxilli*, was proposed to be the copy required for aflatrem biosynthesis and was designated *atmG* (aflatrem GGPP synthase). The smaller, 218-bp product was designated *ggsA* (*A. flavus* GGPP synthase) and, as described below, appears to be a pseudogene.

From an *A. flavus* cosmid library constructed in pMOcosX (33), 2,700 clones were screened by PCR, and five of them, pSZ-17 to pSZ-21, carried *atmG* or *ggs1*. Cosmids pSZ-17 to pSZ-20 contained overlapping fragments, but cosmid pSZ-21 had a unique restriction pattern (data not shown). The 262-bp *atmG* probe hybridized to 2.4-kb BamHI and 5.0-kb EcoRI fragments of cosmids pSZ-17 to pSZ-20 but did not hybridize with pSZ-21. Conversely, the 218-bp *A. flavus ggsA* product hybridized to a 7.3-kb BamHI fragment of pSZ-21 but did not hybridize with the other four cosmids (data not shown).

Organization of genes for indole-diterpene biosynthesis in *A. flavus*. We sequenced the *atmG*-containing cosmid pSZ-20 and identified a potential gene cluster (Fig. 3) with high sequence similarity to the *P. paxilli pax* genes, which are required for the biosynthesis of the indole-diterpene paxilline. These genes included *atmG* (AF111), *atmC* (AF112), and *atmM* (AF113), which are orthologs of *paxG*, encoding a GGPP synthase, *paxC*, encoding a prenyltransferase (cyclase), and *paxM*, encoding a FAD-dependent monooxygenase (Table 2), respectively. Overall, 16 putative genes, denoted AF101 to AF116, were identified within this 38.9-kb sequence (Fig. 3).

atmG has three introns whose positions and phase are the same as those found in *P. paxilli paxG*. The first two are also shared with *F. fujikuroi ggs-2* (Fig. 4). *atmG* is predicted to encode a polypeptide of 340 amino acids with an unmodified molecular mass of 38.5 kDa (Table 2) that has 49% identity (64% similarity) to PaxG from *P. paxilli*. AtmG contains the five conserved domains found in all prenyl diphosphate synthases (8), including the highly conserved aspartate-rich motifs, DDXXD and DDXXN/D, of domains II and V that are the proposed binding sites for the isopentenyl diphosphate and allyl isoprenoid substrates (Fig. 4).

atmC contains two introns whose positions and phase are the same as those found in *paxC*. *atmC* is predicted to encode a

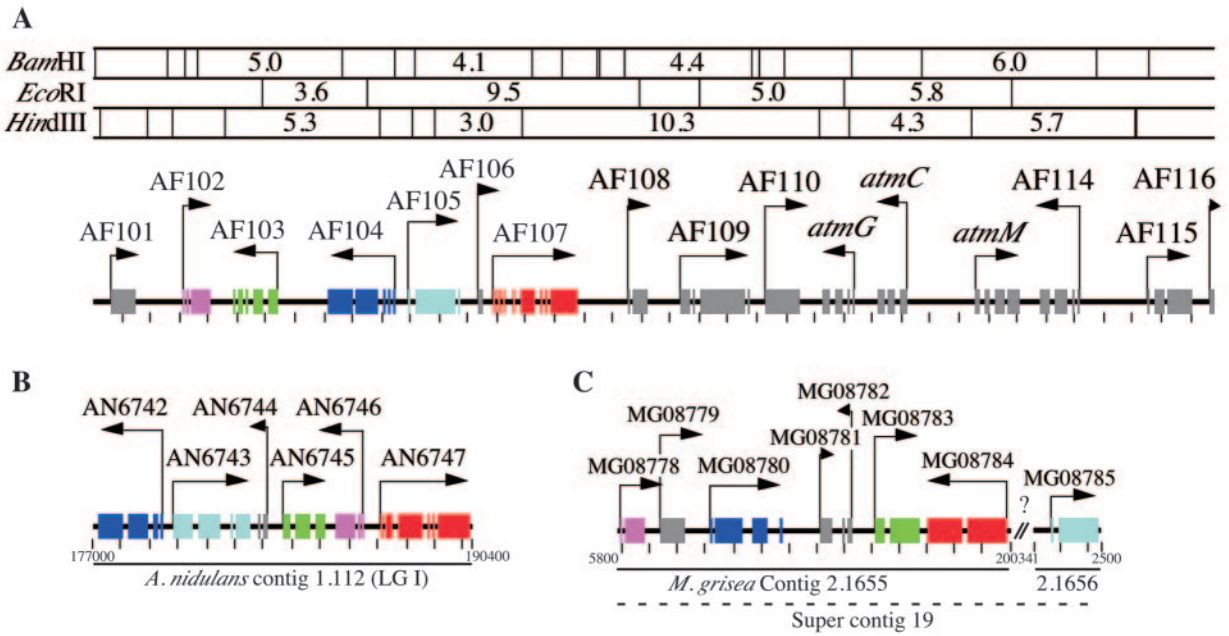

FIG. 3. Physical gene map of *A. flavus atm* locus and conserved synteny with *A. nidulans* and *M. grisea*. (A) Physical map of *A. flavus* insert DNA from cosmid pSZ-20 showing restriction enzyme sites for BamHI, EcoRI, and HindIII and the sizes of fragments of >3 kb. The 16 putative genes identified within this 38,895-bp sequence are labeled AF101 to AF116. Orthologs of *P. paxilli* indole-diterpene biosynthetic genes are labeled as *atm* (aflatrem biosynthesis) genes in accordance with *A. nidulans* nomenclature, as described by Bennett and Lasure (4). Exons of each gene are indicated by blocks, and the arrows indicate the direction of transcription. Tick marks are at 1-kb intervals. Syntenous regions for the *A. flavus atm* locus are shown for *A. nidulans* (B) and *M. grisea* (C). Exons with the same color are orthologous.

polypeptide of 334 amino acids with an unmodified molecular mass of 38.1 kDa (Table 2). This polypeptide contains the five conserved domains found in other prenyl diphosphate synthases (8) and has 55% identity (70% similarity) to PaxC.

atmM has three introns and is predicted to encode a polypeptide of 479 amino acids with an unmodified molecular mass of 53.9 kDa (Table 2). AtmM has 52% identity (67% similarity) to PaxM from *P. paxilli*. AtmM and PaxM both contain four highly conserved motifs, the dinucleotide binding domain (48), an ATG motif (45), a GD motif (11), and a G helix. These motifs suggest a modified Rossman fold, which is used by many flavoproteins to bind FAD.

To the right of *atmM* are three genes that encode a putative polytopic membrane protein (AF114), a cytochrome P450 monooxygenase (AF115), and a partial sequence for an acyltransferase (AF116). AF114 is predicted to be an integral membrane protein with seven transmembrane domains. Adjacent to the AF114 gene is a gene encoding a putative cyto-

TABLE 2. Sequence and bioinformatic analysis of genes within the *A. flavus* pSZ-20 cosmid insert

Gene/ORF or ORF	Size (aa)	Putative Function	Top functionally characterized BLASTP match			
			Protein	E-value	Organism	Reference or accession no.
AF101	315	Unknown				
AF102	299	Dioxygenase	DodA	4e−06	*Portulaca grandiflora*	CAE45178
AF103	472	Dehydrogenase	Sim15	2e−06	*Streptomyces antibioticus*	19
AF104	694	Monooxygenase	SalA	6e−22	*Acinetobacter* sp. strain ADP1	22
AF105	591	Acyl esterase	GACAAP	7e−17	*Bacillus laterosporus*	3
AF106	87	Unknown	BinA	6e−13	*Aspergillus nidulans*	AJ011295
AF107	819	Transcription factor	RegA	6e−79	*Aspergillus fumigatus*	CAE46958
AF108	214	Dehydrogenase	P22	2e−05	*Gallus gallus*	54
AF109	785	Zn_2Cys_6 transcription factor	Hap1	0.002	*Saccharomyces cerevisiae*	37
AF110	408	Monooxygenase	SalA	1e−15	*Acinetobacter* sp. strain ADP1	22
atmG	340	Geranylgeranyl diphosphate synthase	PaxG	2e−97	*P. paxilli*	53
atmC	334	Prenyltransferase	PaxC	1e−109	*P. paxilli*	53
atmM	479	Monooxygenase	PaxM	1e−139	*P. paxilli*	53
AF114	378	Polytopic membrane protein	Pth	3e−27	*Blumeria graminis*	AF329397
AF115	508	Cytochrome P450	Cyp8b1	3e−13	*Mus musculus*	16
AF116[a]	75	Acyl transferase				

[a] AF116 is truncated at the 3′ end in pSZ-20, and therefore only a partial sequence was studied.

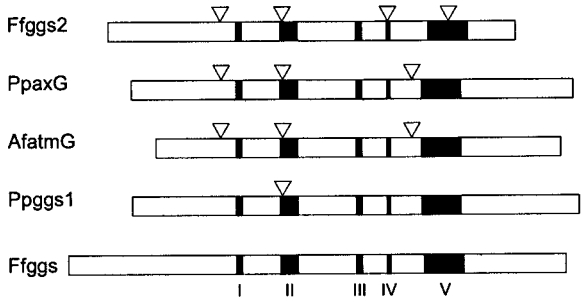

FIG. 4. Comparison of gene structure of *A. flavus* GGPP synthase gene with those of other fungal GGPP synthase genes. Gene structures are given for *F. fujikuroi* primary (Ffggs) and secondary (Ffggs2), *P. paxilli* primary (Ppggs1) and secondary (PppaxG), and *A. flavus atmG* (AfatmG) GGPP synthases. The positions of introns (inverted triangles) and conserved domains I to V (shading) are identified.

chrome P450 monooxygenase, AF115, that contains the classical signature motifs of cytochrome P450 enzymes, including a heme-binding domain (FGSSPHICPGRHFA) (20), but that does not appear to be an ortholog of either PaxP (E value of 2e−16) or PaxQ (E value of 7e−10), which are cytochrome P450 enzymes that are required for paxilline biosynthesis by *P. paxilli* (26). The best match (1e−111) is to a hypothetical protein, AN4117 from *Aspergillus nidulans*. AF115 (partial sequence) has 41 to 43% similarity to trichothecene 3-*O*-acetyltransferases from *Gibberella* and *Fusarium* spp. as its most closely related polypeptides.

Immediately to the left of *atmG* are three genes that are predicted to encode a dehydrogenase (AF108), a transcription factor (AF109), and a FAD-dependent monooxygenase (AF110). AF109 appears to be a transcription factor of the classical $Zn(II)_2Cys_6$ binuclear cluster type, with its best match being a hypothetical protein from *Magnaporthe grisea* (MG02408; E value, 5e−49). AF110 also has the conserved motifs that are indicative of a modified Rossman fold, but it does not appear to have an ortholog in the *pax* gene cluster.

Further to the left of these three genes is a set of six genes (AF102 to -107) with synteny to orthologs from *A. nidulans* and *M. grisea*, although the gene order and proposed transcriptional orientation vary among species (Fig. 3). The *A. nidulans* (AN6746) and *M. grisea* (MG08778) orthologs of AF102 share 71 and 69% identity, respectively, with AF102. AF103 is predicted to be a dehydrogenase and has orthologs in the simocyclinone biosynthetic gene cluster of *Streptomyces antibioticus* (19).

AF104 is a third FAD-dependent monooxygenase found in the *atm* region (Table 2), with four introns and E values of 0 and 2e−38 for the *A. nidulans* (AN6742) and *M. grisea* (MG08780) orthologs, respectively. AF104 does not appear to have an ortholog in the *pax* gene cluster. Although AF106 has a very small open reading frame (encoding 87 amino acids), the predicted polypeptide shares significant similarity with the C terminus of *A. nidulans* AN6743 and with *A. nidulans* BinA, with the latter being defined as functional by the isolation of an expressed sequence tag (27). Thus, *A. nidulans* AN6743 appears to be split into two genes in *A. flavus*, corresponding to AF105 and AF106.

AF107 belongs to a class of transcriptional activators, including the proteins encoded by *regA* from *Aspergillus fumigatus* (accession number CAE46958), *CMR1* from *Colletotrichum lagenarium* (43), and *PIG1* from *M. grisea* (43), that contain both Cys_2His_2-type zinc finger and $Zn(II)_2Cys_6$ binuclear cluster DNA binding motifs. Both motifs are present in the N-terminal region of AF107, with the Cys_2His_2 zinc finger motif being present in two copies.

Molecular analysis of *ggsA*. A 7.3-kb BamHI fragment of pSZ-21 containing *ggsA* was subcloned into pBluescript II KS(+) to generate pSZ-22, which was used as a template to extend the sequence data previously derived from the 218-bp insert in pSZ-2. This sequence (*ggsA*) contained the five conserved domains found in other prenyl diphosphate synthase genes (8) and had significant identity to *paxG* (E value of 5e−40). RT-PCR confirmed that *ggsA* is transcribed and has three exons and two introns. The position of intron 1 is identical to that found in *P. paxilli paxG* and *F. fujikuroi ggs-2* (Fig. 4). Intron 2 is in the same position as intron 4 from *F. fujikuroi ggs-2*. However, a detailed analysis of the *ggsA* genomic and cDNA sequences failed to identify a putative methionine start codon. Furthermore, a frameshift mutation resulting in a premature translation stop codon was also identified, indicating that *ggsA* is a nonfunctional gene.

***atm* gene expression and aflatrem biosynthesis.** Total RNAs were isolated from mycelia harvested at different times during the growth of cultures (Fig. 5). The steady-state levels of mRNA for *atmG*, *atmC*, and *atmM* increased dramatically with the onset of aflatrem biosynthesis, at approximately 72 h postinoculation (Fig. 5). In contrast, the steady-state levels of *tub2* (β-tubulin) and *act1* (actin) were relatively constant. The same expression pattern was found when the samples were analyzed by semiquantitative RT-PCR. Thus, an increased expression of *atmG*, *atmC*, and *atmM* is coincident with the onset of aflatrem biosynthesis.

Expression of *A. flavus atmM* in *P. paxilli*. Plasmid pSZ-43, containing a wild-type copy of *atmM*, was introduced into protoplasts of a *paxM* deletion derivative of *P. paxilli*, strain LMM-100. Twenty arbitrarily selected Gen^r and Hyg^r transformants were purified by subculturing of single spores and then were analyzed by PCR to confirm the correct genetic background. With a primer set specific for *paxM*, 3.0-kb products were amplified from LMM-100, LMM-100/pII99, and six LMM-100/pSZ-43 transformants, and a 2.3-kb fragment was amplified from the wild type (results not shown). Thus, all six transformants had an LMM-100 background. With a primer set specific for *atmM*, 3.1-kb products were amplified from pSZ-43, an *A. flavus* wild-type strain, and six transformants, but no product was obtained from the *P. paxilli* wild-type, LMM-100, or LMM-100/pII99 (results not shown).

Nine of 10 LMM-100/pSZ-43 transformants produced a metabolite with the same R_f as authentic paxilline (results not shown). No paxilline was found in extracts from LMM-100 and LMM-100/pII99 cells by either thin-layer chromatography or reverse-phase HPLC analysis (Fig. 6). Extracts of LMM-100/pSZ-43 (Fig. 6C) accumulated an indole-diterpene that eluted at the same retention time (6.8 min) as authentic paxilline (Fig. 6D). No indole-diterpenes were detected in extracts of LMM-100 (Fig. 6B). Thus, the *A. flavus atmM* gene can functionally replace the *P. paxilli paxM* gene.

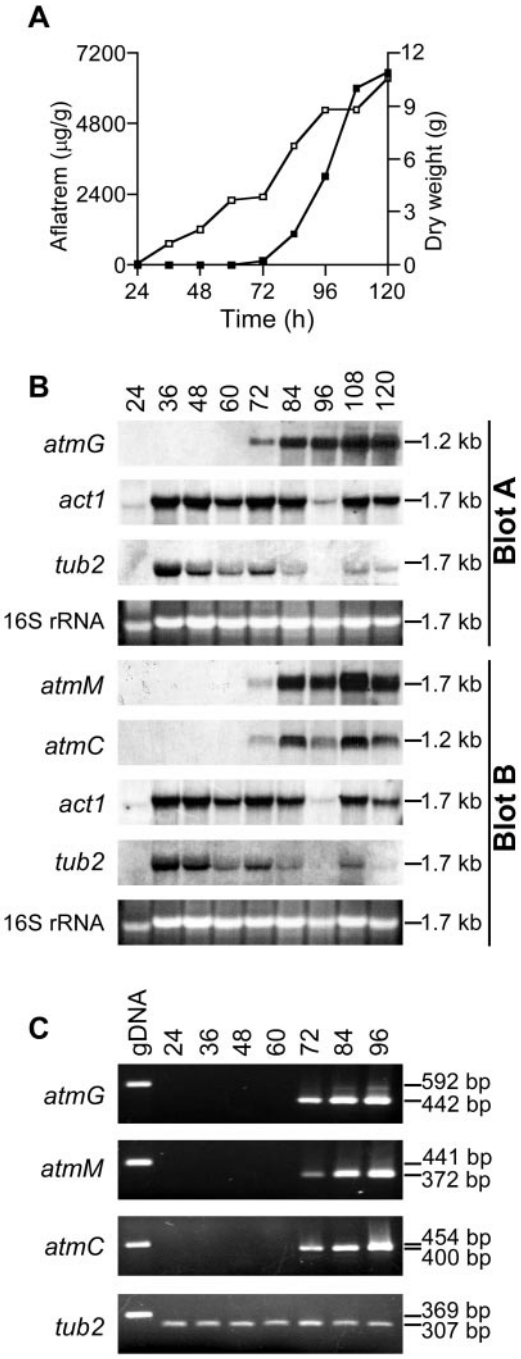

FIG. 5. Gene expression analysis. (A) Growth of *A. flavus* showing changes in dry weight (□) and aflatrem production (■). (B) Autoradiographs of Northern blots of *A. flavus* total RNAs (10 μg) probed with ^{32}P-labeled 568-bp *atmG*, 400-bp *atmC*, 372-bp *atmM*, 562-bp *act1*, and 358-bp *tub2* cDNAs. The ethidium bromide-stained 16S rRNA region of each gel is shown for comparison. The numbers on the right correspond to the sizes of the hybridized transcripts by comparison to a set of RNA standards (Promega). (C) RT-PCR analysis from 24 to 96 h, with mRNA as the template together with the genomic product (gDNA). Fragment sizes are indicated in base pairs.

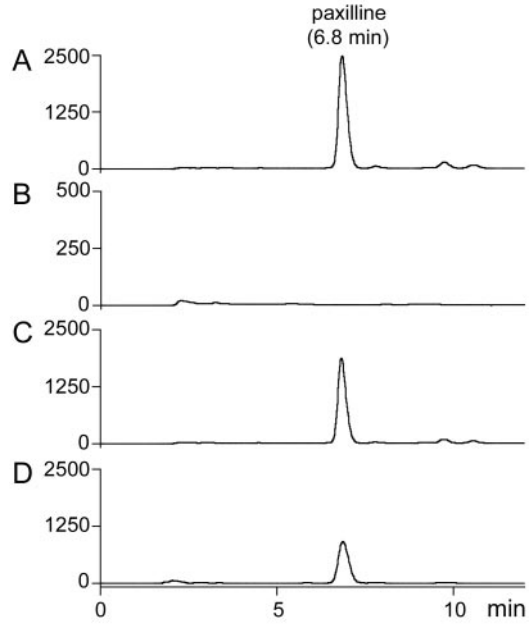

FIG. 6. HPLC analysis of indole-diterpenes from extracts of a *P. paxilli paxM* mutant containing the *A. flavus atmM* gene. (A) Wild type. (B) LMM-100 (*paxM* mutant). (C) LMM-100 containing *atmM* (pSZ-43). (D) Paxilline standard (5 μg). The units on the *y* axis are milliabsorbance units at 230 nm, and the retention time on the *x* axis is given in minutes.

DISCUSSION

The putative indole-diterpene gene cluster from *A. flavus* that we have cloned may be responsible for aflatrem biosynthesis. This gene cluster contains three genes, *atmG*, *atmM*, and *atmC*, that are up-regulated at the onset of aflatrem biosynthesis and that have functional orthologs in the paxilline biosynthesis gene cluster of *P. paxilli* (53), although the arrangement of the genes on the chromosome is not conserved across species. The *A. flavus* cluster is the second indole-diterpene gene cluster to be characterized for a filamentous fungus.

atmG probably encodes a GGPP synthase and is presumed to catalyze the first step in the biosynthesis of aflatrem, i.e., the synthesis of GGPP. A second coding sequence for a GGPP synthase in *A. flavus* appears to be nonfunctional. Thus, AtmG presumably provides GGPP for aflatrem biosynthesis as well for other GGPP metabolic requirements, such as the C-20 prenylation of proteins (13, 24, 50).

Deletions of *paxG*, *paxM*, and *paxC* in *P. paxilli* result in mutants with a paxilline-negative phenotype. No identifiable indole-diterpene intermediates have been identified in these strains, suggesting that *paxG*, *paxM*, and *paxC* are involved in very early steps in the pathway. We have been unable to make targeted replacements of any of the *atm* genes due to a lack of sufficient numbers of protoplasts from *A. flavus* strain NRRL6541. Procedures that we have used successfully with *P. paxilli* (21) and *Epichloë* endophytes (31) as well as previously described protocols for *A. flavus* (35, 49) were all unsuccessful (unpublished results). Strains of *A. flavus* with either a *tan* or *white* genotype have been successfully induced to form protoplasts (35, 49), suggesting that the pigments produced by strain

NRRL6541 may inhibit protoplast formation. A hyaline mutant was also needed to obtain protoplasts from *Venturia inaequalis* (14). *Agrobacterium*-mediated transformation (9) has been successful for some filamentous fungi and might circumvent the present difficulties.

Our working model for paxilline biosynthesis in *P. paxilli* is that PaxM and PaxC are required for the epoxidation and cyclization of GGPP and the addition of indole-3-glycerol phosphate to form the first stable indole-diterpene, possibly paspaline (34). By analogy, we propose that AtmM and AtmC catalyze the same early reactions in aflatrem biosynthesis, since *atmM* is a functional homolog of *paxM* and can complement a *paxM* mutant of *P. paxilli*. *paxP* and *paxQ*, which encode cytochrome P450 enzymes, are required for paxilline biosynthesis, and similar modifications to the paspaline skeleton are predicted to be required for the generation of aflatrem. However, only a single P450 gene, AF115, is known to be closely linked to the core *atmG*, *atmC*, and *atmM* genes, and it does not appear to be a functional ortholog of either *paxP* or *paxQ*. Orthologs of *paxP* and *paxQ* may be further to the right of AF116. Other modifications to the paspaline skeleton in the biosynthetic pathway to aflatrem include oxidation and acetal formation at C-7 and prenylation of the C-20 carbon of the indole group. Candidate genes for the former are AF104 and AF110, both of which encode putative FAD-dependent monooxygenases of a type predicted to be capable of making this type of biochemical modification. If the break in conserved synteny with *A. nidulans* and *M. grisea* between AF107 and AF108 defines the left-hand boundary of the *atm* cluster, then AF110 is the best candidate for this function.

In summary, we predict that at least eight genes are required for the biosynthesis of aflatrem, including *atmG*, *atmC*, *atmM*, two genes encoding cytochrome P450 enzymes, one FAD-dependent monooxygenase gene, a dehydrogenase gene, and a prenyltransferase gene. Further genetic analyses of the genes identified here and of their adjacent genes will help us to elucidate the pathway for aflatrem biosynthesis. Comparisons with the steps required for paxilline biosynthesis in *P. paxilli* should elucidate the basic biochemistry and genetics of this important group of secondary metabolites.

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ADDENDUM IN PROOF

A recent analysis of *A. flavus* EST sequences (53a) identified a GGPP synthase sequence (accession number CO146826) which is neither *atmG* nor *ggsB*, suggesting the presence of three GGPP synthase genes in *A. flavus*. Further analysis of our data showed that the 262-bp *atmG* and 218-bp *ggsA* nested products (Fig. 1B, lane 5) originate from the upper 330-bp product of the first-round PCR (Fig. 1B, lane 4). Sequence analysis of the lower 270-bp product (Fig. 1B, lane 4) identified a GGPP synthase sequence that corresponds to CO146826. This gene, designated *ggsB*, is proposed to be the GGPP synthase for primary metabolism.

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