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Overexpression of *aflR* Leads to Upregulation of Pathway Gene Transcription and Increased Aflatoxin Production in *Aspergillus flavus*

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The aflatoxin biosynthetic pathway regulatory gene, *aflR*, encodes a putative 47-kDa protein containing a zinc cluster DNA binding motif. It is required for the transcription of all of the characterized aflatoxin pathway genes in both *Aspergillus flavus* and *Aspergillus parasiticus*. The objective of this study was to examine the effects of *aflR* overexpression on temporal gene expression, aflatoxin production, and nitrate inhibition of aflatoxin biosynthesis in *A. flavus*. An inducible expression construct was made by fusing the coding region of *aflR* to the promoter region of the *A. flavus adh1* gene. This construct was transformed into *A. flavus* 656-2 (FGSC A1010), a strain mutated at the *aflR* locus. Strain 656-2 containing the *adh1*(p)::*aflR* construct had induced transcription of two early aflatoxin pathway genes, *nor-1* and *pksA*, and produced wild-type concentrations of aflatoxin in a temporal pattern similar to that of wild-type strains of *A. flavus*. Strains 656-2 and 86-10 (FGSC A1009) an aflatoxigenic strain, were transformed with a construct containing the constitutive promoter *gpdA* driving *aflR*. Transformants of these strains constitutively expressed *aflR*, *fas-1A*, *pksA*, *nor-1*, and *omtA* but did not constitutively produce aflatoxin. Strain 86-10 containing the *gpdA*(p)::*aflR* construct produced 50 times more aflatoxin than 86-10, but the temporal pattern of aflatoxin production was the same as for 86-10, and aflatoxin production was also induced by sucrose. The addition of 10 g of nitrate per liter to sucrose low salts medium inhibited aflatoxin production by both strain 86-10 and a transformant of 86-10 containing the *gpdA*(p)::*aflR* construct, indicating that nitrate inhibition of aflatoxin biosynthesis does not occur solely at the level of *aflR* transcription. These studies show that constitutive overexpression of the pathway transcriptional regulatory gene *aflR* leads to higher transcript accumulation of pathway genes and increased aflatoxin production but that the initiation of aflatoxin biosynthesis is not solely regulated by the transcriptional activities of the biosynthetic pathway.

Aspergillus flavus and *Aspergillus parasiticus* can colonize a number of substrates, and under favorable conditions these fungi can colonize the seed of plants in the field and produce aflatoxin. Of these two fungi, *A. flavus* is the more aggressive and is of major concern on corn, peanut, cottonseed, and tree nuts. Aflatoxins are a family of toxic and carcinogenic secondary metabolites. Aflatoxin B_1 is the predominant compound produced by these fungi and is also the most toxic and carcinogenic of all mycotoxins. More than 16 enzymatic steps are required for the conversion of the first stable polyketide intermediate to aflatoxin (2, 31). Several genes coding for these enzymes have been cloned and characterized, including *fas-1A* (19), *pksA* (8, 32), *nor-1* (10, 30), *avnA* (37), *avf-1* (24), *vbs* (27), *ver-1* (28), *omtA* (36), and *ord-1* (23) (Fig. 1).

The biosynthesis of aflatoxin is induced by simple sugars (6). The induction is associated with the transcriptional activation of the pathway genes *pksA*, *nor-1*, *ver-1*, and *omtA* (11, 29, 30, 32, 36) and the pathway regulatory gene, *aflR* (7, 33). Transcriptional activation of the pathway genes appears to be regulated by *aflR*. *A. flavus* 656-2, containing a mutated *aflR* locus, does not produce aflatoxin and does not have transcripts for the structural genes *nor-1* and *ver-1* (22). Transformation of this strain with a wild-type copy of *aflR* restores transcriptional activation of these genes and aflatoxin biosynthesis. Chang et al. (9) showed that nitrate inhibition of aflatoxin in *A. parasiticus* resulted in the transcriptional inhibition of *aflR*, *nor-1*, *ver-1*, and *omtA* and that an extra copy of *aflR* restored aflatoxin production and transcription of these pathway genes. Thus, in *A. flavus* and *A. parasiticus*, transcription of the aflatoxin pathway genes appears to be regulated solely by *aflR*. *Aspergillus nidulans* produces the polyketide sterigmatocystin by a pathway similar to the one for aflatoxin (3). A homolog of *aflR* present in *A. nidulans* appears to regulate gene transcription in this pathway as well. Yu et al. (38) showed that gene disruption of *A. nidulans aflR* prevented the accumulation of transcripts for pathway genes involved in sterigmatocystin biosynthesis. Thus *aflR* appears to be a transcriptional regulatory gene for the pathway genes involved in the biosynthesis of these two polyketides.

The objective of this study was to examine the regulation of aflatoxin biosynthesis by manipulating the transcription of *aflR*. Our hypothesis was that *aflR* is the sole regulator of aflatoxin biosynthesis. Hence we predicted that overexpression and misexpression of *aflR* should lead to overexpression and misexpression of aflatoxin pathway genes and, concomitantly, to altered aflatoxin accumulation in culture. We report the effect of overexpression and misexpression of *aflR* on transcript accumulation of pathway genes and aflatoxin accumulation.

MATERIALS AND METHODS

Strains, media, and growth conditions. *A. flavus* 656-2 (FGSC A1010) (*w leu-7 pyrG aflR*) (35) and 86-10 (*w arg-7 pyrG*) were used as the hosts for all transformations. Aflatoxigenic strain 86-10 (FGSC A1009) is a *pyrG* mutant of strain 86 (NRRL 60041 [16]) created by UV mutagenesis (19a). Strains were stored in glycerol at −80°C and cultured on potato dextrose agar (Difco Laboratories) for the production of conidia.

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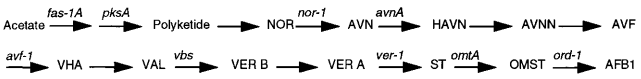

FIG. 1. Schematic pathway of aflatoxin B_1. Italics denote the pathway genes involved in the conversion of known pathway intermediates, designated by all uppercase letters. NOR, norsolorinic acid; AVN, averantin; HAVN, hydroxyaverantin; AVNN, averufanin; AVR, averufin; VHA, versiconal hemiacetal; VAL, versiconal; VER A, versicolorin A; VER B, versicolorin B; ST, sterigmatocystin; OMST, *O*-methylsterigmatocystin; AFB1, aflatoxin B_1.

For time course studies, cultures were grown by a modified resuspension technique (11). A conidial suspension was added to 10 ml of peptone mineral salts (PMS) (5) medium contained in 100- by 150-mm petri dishes to a final concentration of 10^5 conidia/ml. Mycelia were grown for 3 days at 28°C and resuspended onto fresh PMS or sucrose low salts (SLS) medium (34). At 6-h increments from the point of resuspension to 24 h, medium was collected from underneath the mycelial pads for enzyme-linked immunosorbent assay (ELISA) detection of aflatoxin concentration, and the mycelial pads were harvested for RNA extraction. ELISAs were performed as previously described (11).

All resuspension time course experiments were performed at least three times. Data points from all experiments are means of triplicate repetitions, and standard deviations are provided if above 5% of mean values. Continuous culture experiments were performed in duplicate for all graphical data represented.

Construction of plasmids. Plasmid pGAP18 (Fig. 2A), containing the *adh1* promoter (34), was constructed as follows. Plasmid pGAP14, containing a full-length *aflR* cDNA (22), was modified by the Kunkel method of site-directed mutagenesis (15) to include a *Pst*I site at the translational start codon of *aflR*. A 730-bp *Hin*dIII/*Pst*I fragment containing the functional promoter region of the *A. flavus adh1* gene (34) was subcloned immediately upstream from the open reading frame (ORF) of the *aflR* cDNA to create plasmid pGAP18. Transcription of the *adh1* promoter is induced in a profile similar to that of *aflR* (33).

Plasmid pGAP23 (Fig. 2B), containing the constitutive promoter from the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene, was constructed by PCR mutagenesis to create an *Nco*I restriction site over the translation start codon of *aflR* and a *Bam*HI site at the 3′ end of the *aflR* ORF. These restriction sites facilitated directional cloning of the *aflR* ORF immediately downstream of the *gpdA* promoter contained in pNOM102 (25). This construct utilizes the *A. nidulans trpC* terminator. Several investigators have demonstrated that *gpdA* promoter activity is strong and constitutive (25). pGAP23 was further modified by inserting the *pyr4* gene into this vector to eliminate the need for cotransformation; this plasmid was designated pGAP23-*pyr* (not shown).

A. flavus 656-2 was cotransformed with either pGAP18 or pGAP23 and a plasmid containing the *pyr4* gene of *Neurospora crassa*, which served as a selectable marker. Transformants complemented at the *pyrG* locus were further screened for complementation at the *aflR* allele by either *aflR* construct. *A. flavus* 86-10 was transformed with pGAP23-*pyr*, and transformants were selected directly for uracil prototrophy. Strain 86-10 also was cotransformed with pGAP25 and *pyrG* to create strain GAP25-1. Plasmid pGAP25 contains an *A. flavus ver-1* promoter fused to plasmid pZ-3. Plasmid pZ-3 contains a modified leader sequence derived from an *Aspergillus niger* glucoamylase gene (*glaA*). We have shown previously that glucuronidase (GUS) activity is correlated with *ver-1* promoter activity and aflatoxin accumulation in cultures of *A. flavus* transformants containing *ver-1*(p)::GUS (11). All fungal transformations were performed by a polyethylene glycol (molecular weight, 3,350) procedure as previously described (35).

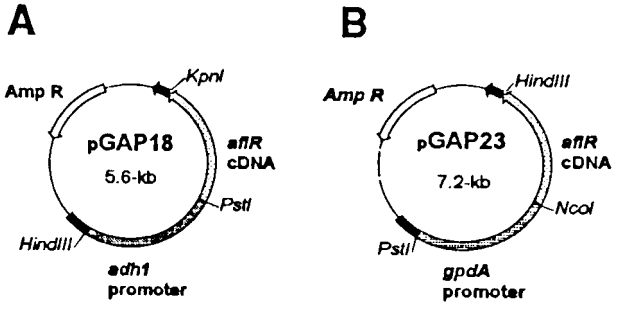

FIG. 2. (A) Plasmid pGAP18 contains the *adh1* promoter subcloned 5′ to the ORF of an *aflR* cDNA contained in a modified pGAP14 vector. pGAP14 (33) was modified to contain a *Pst*I site located over the translational start codon to facilitate directional cloning of the *adh1* promoter fragment with *Hin*dIII and *Pst*I restriction enzymes. (B) Plasmid pGAP23 contains a 1.8-kb *Eco*RI/*Hin*dIII fragment of the *gpdA* promoter fused in frame to an *aflR* cDNA from pGAP14. All plasmids derived in this study contain a pUC19 backbone.

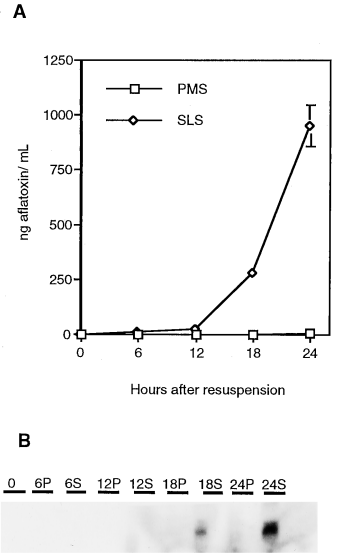

FIG. 3. Aflatoxin B_1 concentrations (A) and Northern blot analysis (B) for cultures of *A. flavus* 656-2/pGAP18 [*adh1*(p)::*aflR*]. Cultures were grown for 3 days on PMS medium and subsequently resuspended onto fresh PMS or SLS medium. Concentrations were determined at the time of resuspension and every 6 h for 24 h. Total RNA was extracted at all time points and hybridized with a ^{32}P-labeled DNA probe specific for *aflR*. P, PMS; S, SLS.

Isolation of RNA and Northern blot analysis. Total RNA was extracted from finely ground, lyophilized fungal mycelia with glass beads (200 μm) and by repeated acid phenol-chloroform extractions (33). After isopropanol precipitation, the resulting RNA pellets were resuspended in 1× HEPES-EDTA and quantified with a spectrophotometer. For Northern blot hybridization, 20 μg of total RNA was electrophoresed through a 1.2% agarose gel containing 1.5% formaldehyde, transferred to a nylon membrane (Zeta-Probe; Bio-Rad, Richmond, Calif.), and hybridized with ^{32}P-labeled DNA probes. For slot blot RNA analysis, 10 μg of total RNA was applied to each well, transferred to a nylon membrane, and hybridized with ^{32}P-labeled DNA probes. Equal loading of total RNA was confirmed by ethidium bromide staining (17).

Aflatoxin analysis. Culture filtrates at all time points (0, 6, 12, 18, and 24 h) were collected and quantitatively assayed for aflatoxin B_1. Aflatoxin concentrations were determined by an ELISA with aflatoxin B_1 antibodies (Sigma Chemical Co.).

Nitrogen studies. Continuous culture analysis was performed with SLS and SLS-NO_3 (10 g of $NaNO_3$ per liter added to SLS). Cultures were grown in shake culture (120 rpm) at 28°C, and culture filtrates were collected at the time of inoculation and every day for 6 days. At day 6, mycelia were collected for dry weight analysis, RNA extraction, and GUS analysis (11). pH values were taken for every time point assayed with an Orion Research Ionalyzer/510 pH meter.

RESULTS

The *adh1*(p)::*aflR* construct (pGAP18) restores aflatoxin biosynthesis in a strain lacking a functional *aflR* locus. *A. flavus* 656-2 contains a mutated copy of *aflR* and does not produce aflatoxin (22). In a time course study (Fig. 3A), strain 656-2 transformed with the *adh1*(p)::*aflR* construct showed a profile of aflatoxin accumulation similar to that of wild-type strains and strain 656-2 transformed with a construct containing a wild-type copy of *aflR* (11, 22, 33). Aflatoxin appeared within 6 h after resuspension on SLS medium and peaked at 24 h. Only small amounts of aflatoxin were produced following resuspension onto PMS medium. The profile of *aflR* transcript accumulation also was similar to that observed previously for aflatoxigenic strains of *A. flavus* (22); *aflR* message could not be detected in cultures resuspended onto PMS medium and could be detected only in SLS-resuspended cultures during aflatoxin accumulation (Fig. 3B). Thus, the profiles of aflatoxin

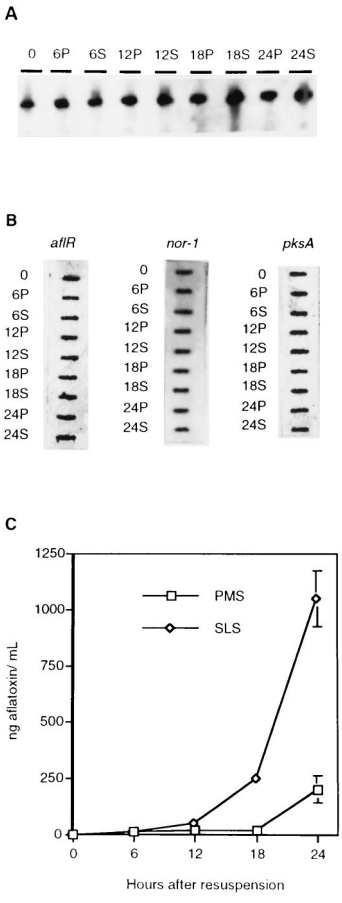

FIG. 4. Northern analysis of *aflR* (A), RNA slot blot analysis of *nor-1*, *pksA*, and *aflR* (B), and aflatoxin B_1 concentrations over time (C) for *A. flavus* 656-2/pGAP23 [*gpdA*(p)::*aflR*]. Cultures were grown for 3 days on PMS medium and subsequently resuspended onto fresh PMS or SLS medium. Concentrations were determined at the time of resuspension and every 6 h for 24 h. Total RNA was extracted and probed for *aflR*, *pksA*, and *nor-1* for all time points assayed. P, PMS; S, SLS.

and *aflR* accumulation, and the amount of aflatoxin produced by a transformant harboring the *adh1*(p)::*aflR* construct, were similar to those for wild-type strains containing a functional *aflR* and transformants of 656-2 containing a functional wild-type *aflR*.

Constitutive expression of *aflR* in 656-2 leads to altered expression of *nor-1* and *pksA*. Previous studies have shown that *nor-1* and *pksA* transcriptional activity increases dramatically during aflatoxin accumulation in culture (9, 29, 32). To determine if constitutive expression of *aflR* leads to constitutive pathway gene expression, we monitored the expression of two pathway genes, *nor-1* and *pksA*, in transformants of 656-2 with the *gpdA*(p)::*aflR* construct. All 656-2 transformants examined containing the *gpdA*(p)::*aflR* construct produced aflatoxin. Transcript accumulation determined by RNA blot analysis showed that constitutive expression of *aflR* (Fig. 4A) was accompanied by constitutive expression of otherwise-induced aflatoxin pathway genes *nor-1* and *pksA* (Fig. 4B). Transcripts of *aflR*, *nor-1*, and *pksA* were constitutively expressed in both PMS and SLS media. Surprisingly, even though transcripts for the pathway regulatory gene and at least two pathway genes were constitutively expressed in both PMS and SLS media, the profile of aflatoxin accumulation was similar to that of a wild-type strain. Aflatoxin appeared at 6 h and peaked at 24 h (Fig. 4C). Small quantities of aflatoxin were detected in PMS medium.

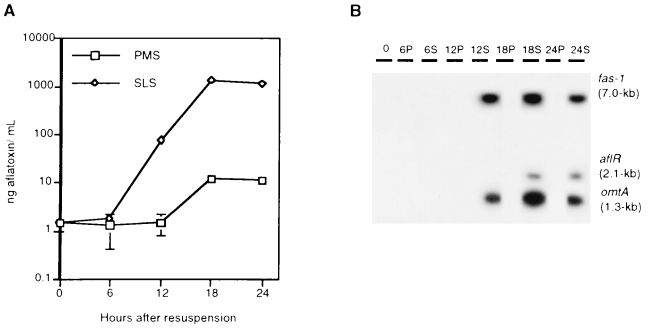

FIG. 5. Aflatoxin B_1 concentrations (A) and Northern blot analysis (B) over time of *A. flavus* 86-10. Cultures were grown for 3 days on PMS medium and subsequently resuspended onto fresh PMS or SLS medium. Concentrations were determined at the time of resuspension and every 6 h for 24 h. Total RNA was extracted and probed for *aflR*, *fas-1A*, and *omtA* for all time points assayed. P, PMS; S, SLS.

Constitutive expression of *aflR* under the transcriptional control of a glyceraldehyde-3-phosphate dehydrogenase promoter leads to constitutive pathway gene transcription and increased aflatoxin production in *A. flavus* 86-10. In contrast to strain 656-2, *A. flavus* 86-10 contains a functional *aflR* gene and produces wild-type concentrations of aflatoxin (Fig. 5A). Aflatoxin appeared in cultures of strain 86-10 within 6 h after resuspension on SLS, and peak concentrations occurred within 18 h. Much less aflatoxin was produced on PMS medium. Northern blot analysis (Fig. 5B) showed the presence of *aflR* transcripts at 18 and 24 h and *fas-1A* and *omtA* transcripts at 12, 18, and 24 h after resuspension onto SLS medium.

Transformants of 86-10 harboring the *gpdA*(p)::*aflR* construct exhibited a temporal profile of aflatoxin accumulation (Fig. 6A) similar to that of the parent strain and strain 656-2 transformants that harbor the *adhl*(p)::*aflR* construct (Fig. 4A) in that maximal concentrations could be observed 18 to 24 h after resuspension on SLS medium (Fig. 6A). However, strain 86-10 with the *gpdA*(p)::*aflR* construct produced aflatoxin concentrations several times higher than those produced by the parental strain 86-10 in all comparative experiments conducted (Fig. 4 and 7 versus 6 and 8). The maximum aflatoxin concentration produced by the 86-10 *gpdA*(p)::*aflR* transformant was

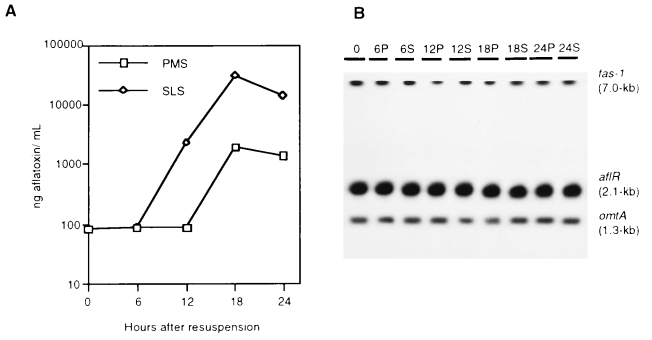

FIG. 6. Aflatoxin B_1 concentrations (A) and Northern blot analysis (B) over time of *A. flavus* 86-10/pGAP23 [*gpdA*(p)::*aflR*]. Cultures were grown for 3 days on PMS medium and subsequently resuspended onto fresh PMS or SLS medium. Concentrations were determined at the time of resuspension and every 6 h for 24 h. Total RNA was extracted and probed for *aflR*, *fas1-A*, and *omtA* for all time points assayed. P, PMS; S, SLS.

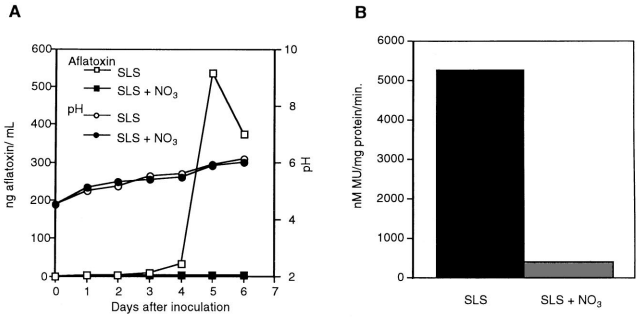

FIG. 7. (A) Aflatoxin concentrations of *A. flavus* 86-10 GAP25 (*ver-1*::GUS) grown continuously for 6 days in either SLS or SLS–10 g of NO_3 per liter. (B) GUS activity at day 6 reflects *ver-1* promoter activity in both media. MU, methylumbelliferone.

60,000 ng/ml (Fig. 6A), whereas 656-2 *adh1*(p)::*aflR* produced 1,000 ng/ml (Fig. 3A) and 656-2 *gpdA*(p)::*aflR* produced 1,200 ng of aflatoxin per ml (Fig. 4C). The aflatoxin profile of 86-10 *gpdA*(p)::*aflR* differed in another important aspect; relatively high concentrations of aflatoxin were detected on PMS medium at the time of resuspension, and aflatoxin concentrations continued to increase in PMS medium in a pattern similar to the one that occurred in SLS medium (Fig. 6A). Compared to the parental strain, *gpdA*(p)::*aflR* transformants of 86-10 produced at least 100 times more aflatoxin in PMS medium; the concentrations produced on PMS were comparable to that of wild-type aflatoxin production in SLS medium (Fig. 5A and 6A). Despite upregulation of the pathway, aflatoxin production was still at least 10-fold greater on SLS than on PMS for all strains tested. The ratio of aflatoxin concentrations (SLS/PMS ratio) during time points of maximum aflatoxin production (18 or 24 h postresuspension) ranged from 1,000 for 656-2 *adh1*(p)::*aflR* to 50 for 656-2 *gpdA*(p)::*aflR*, 100 for 86-10, and 50 for 86-10 *gpdA*(p)::*aflR*.

As expected from our results with 656-2 *gpdA*(p)::*aflR*, strain 86-10 *gpdA*(p)::*aflR* abundantly produced constitutive amounts of *aflR* transcripts (Fig. 6B) as detected by Northern blot analyses. Furthermore, the transcriptional profile of aflatoxin pathway genes *fas-1A* (19) and *omtA* (36) showed that they appeared to be constitutively produced in 86-10 *gpdA*(p)::*aflR* (Fig. 6B). This transcriptional profile differs from those of the parent strain 86-10 (Fig. 5B) and wild-type *A. flavus* 3357 (33) in that in the parent strain *aflR* and the corresponding aflatoxin pathway genes are induced in culture only during aflatoxin biosynthesis and their regulated expression correlates with aflatoxin accumulation (Fig. 5B).

Thus transformants of 86-10 containing the *gpdA*(p)::*aflR* overexpression construct constitutively produced *aflR*, *fas-1A*, and *omtA* transcripts and elevated concentrations of aflatoxin. Higher levels of aflatoxin were also produced on the otherwise nonconducive PMS medium. However, the induction profile for aflatoxin biosynthesis appeared very similar to that of the wild-type strain, and SLS medium remained a more conducive medium for aflatoxin biosynthesis than PMS.

Nitrate inhibition of aflatoxin accumulation occurs in the presence of upregulated *aflR* transcription. Several researchers have previously shown that nitrate inhibits the accumulation of aflatoxin and pathway intermediates (1, 9, 14, 21). Chang et al. (9) showed that in *A. parasiticus*, *aflR* transcript accumulation and aflatoxin production are suppressed in a nitrate-containing medium and that an extra copy of *aflR* relieves both the inhibition of aflatoxin biosynthesis and the suppression of *aflR* transcription compared to a wild-type *A. parasiticus* strain. If nitrate repression occurs solely at the level of *aflR* transcription, then overexpressing *aflR* should also relieve nitrate inhibition of aflatoxin biosynthesis.

We compared aflatoxin production in two strains of *A. flavus* 86-10. Strain 86-10 *gpdA*(p)::*aflR* was compared with GAP25-1, a transformant of 86-10 containing a *ver-1*(p)::GUS reporter construct. The two strains were cultured continuously for 6 days on either SLS or SLS-NO_3 (10 g of $NaNO_3$ per liter) medium. For all strains, SLS supported the highest levels of aflatoxin production in all experiments conducted in this study (Fig. 3A, 4C, 5A, and 6A). In strain 86-10 *ver-1*::GUS, SLS-NO_3 did not support significant aflatoxin production (Fig. 7A). The maximum concentration of aflatoxin produced on SLS-NO_3 by the strain was 0.5% of that produced on SLS. The *gpdA*(p)::*aflR* transformant produced more aflatoxin than GAP25-1 (Fig. 7A versus 8A) in both SLS and SLS-NO_3, but the addition of nitrate to the medium resulted in a 20-fold (day 5) decrease in aflatoxin levels compared to *gpdA*(p)::*aflR* cultures grown in SLS alone. Northern blot analysis of strain 86-10 *gpdA*(p)::*aflR* confirmed that *aflR*, *aflJ*, and *omtA* transcripts were produced abundantly in both SLS and SLS-NO_3 (Fig. 8B). Thus, elevated *aflR* transcription did not relieve NO_3 inhibition. A reduction in *ver-1* promoter activity was observed in strain GAP25-1 cultured on SLS-NO_3 (Fig. 7B), supporting previous observations that nitrate inhibition of aflatoxin biosynthesis is correlated with a decrease in pathway gene transcription. Overexpression of the pathway-specific regulatory gene, *aflR*, served to transcriptionally activate all corresponding pathway genes examined but failed to alleviate the repressive effects of nitrate on aflatoxin biosynthesis.

DISCUSSION

The basic assumption that determined the design of the experiments in this study was that *aflR* is the transcriptional regulator of the aflatoxin biosynthesis genes. If this is true, altered expression of *aflR* transcription should lead to altered transcription of the pathway genes. Further, if aflatoxin biosynthesis is regulated only by *aflR*, altered transcription of *aflR* should lead to altered timing and accumulation of aflatoxin and production of aflatoxin under nonconducive conditions.

This hypothesis is based on previous research showing that *aflR* is required for the transcription of the pathway genes *nor-1* and *ver-1* in *A. flavus* (22) and that an additional copy of *aflR* in *A. parasiticus* leads to increased aflatoxin production and elevated transcript accumulation of *nor-1*, *ver-1*, and *pksA*

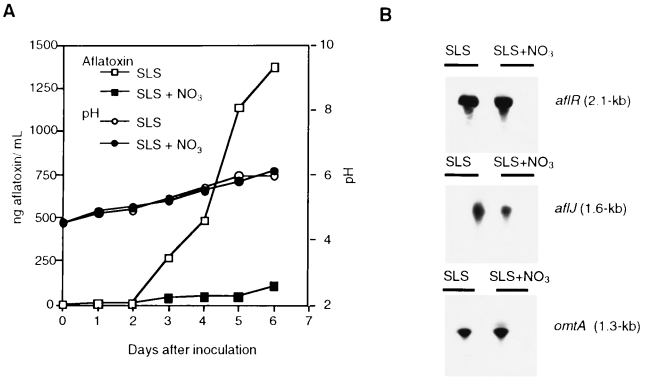

FIG. 8. (A) Aflatoxin concentrations of *A. flavus* 86-10/pGAP23 [*gpdA*(p)::*aflR*] grown continuously for 6 days in either SLS or SLS–10 g of NO_3 per liter. (B) Northern blot analyses performed on tissue harvested at day 6 show high levels of *aflR*, *aflJ*, and *omtA* transcription in both media.

(9). Additionally, Yu et al. (38) have shown that the *A. nidulans aflR* is required for the transcription of the pathway genes leading to sterigmatocystin production.

To test our hypothesis, we used two fungal gene promoters that differed in the timing and magnitude of their corresponding transcriptional activities. For regulated induction of aflatoxin biosynthesis, we used the promoter region of the *A. flavus* alcohol dehydrogenase *adh1* gene, whose transcription has been shown to follow that of aflatoxin biosynthesis in culture (34). We showed that transformation of 656-2, an *A. flavus* strain lacking a functional copy of *aflR*, with this construct [*adh1*(p)::*aflR*] was sufficient to activate pathway gene transcription and restore regulated aflatoxin biosynthesis. Further, the profile of aflatoxin accumulation (Fig. 3A) was similar to that of a wild-type strain (33). Thus, regulated transcription of pathway genes and aflatoxin accumulation was associated with the regulated expression of *aflR*.

In order to manipulate the timing and magnitude of *aflR* expression, we transformed strains 656-2 and 86-10 with a construct that constitutively expressed high levels of *aflR* transcript. As predicted, transformants of 656-2 and 86-10 containing the *gpdA*(p)::*aflR* construct showed constitutive expression of *aflR* and of the pathway genes, *fas-1A*, *pksA*, *nor-1*, and *omtA*. Transcripts for these genes were present in conducive as well as nonconducive media for aflatoxin production. Although we did not examine all of the genes in the pathway, others (9, 29) have shown that the pathway genes are coordinately regulated, and we would expect all of the pathway genes to be regulated similarly. These data argue that altered *aflR* expression leads to a corresponding altered expression of the aflatoxin pathway genes.

While overexpression of *aflR* in 86-10 led to increased aflatoxin production on medium conducive for aflatoxin production, it also led to increased aflatoxin production in nonconducive medium. Maximum aflatoxin production by 86-10 *gpdA*(p)::*aflR* was 50-fold greater than that for 86-10. Strain 86-10 *gpdA*(p)::*aflR* did produce elevated concentrations of aflatoxin in PMS medium, but SLS supported at least 50 times more aflatoxin.

We were surprised to find that constitutive expression of *aflR* did not change the characteristic temporal induction profile of aflatoxin accumulation common to wild-type strains or strain 656-2 transformed with *adh1*(p)::*aflR*. Even though transcripts for the pathway genes were present constitutively in both 656-2 and 86-10 transformants containing the *gpdA*(p)::*aflR* construct, aflatoxin accumulation in the medium followed an induction profile similar to that observed for wild-type strains. These data argue that transcriptional regulation of the biosynthetic pathway by *aflR* is not the only mechanism involved in the regulation of aflatoxin biosynthesis.

Overexpression of *aflR* also failed to overcome nitrate inhibition of aflatoxin biosynthesis. Consistent with the findings of others (9, 14, 36), we observed a strong inhibitory effect of nitrate on aflatoxin biosynthesis. The addition of nitrate to SLS resulted in greater than 99% reduction in aflatoxin biosynthesis by strain 86-10. Despite the high levels of transcripts for *aflR*, *pksA*, and *aflJ* (a gene involved in aflatoxin biosynthesis whose function is not known), strain 86-10 *gpdA*(p)::*aflR* showed greater than 90% reduction in aflatoxin production when grown on media with elevated nitrate concentrations. Chang et al. (9) showed that the addition of a single extra copy of genomic *aflR* relieved nitrate inhibition of aflatoxin biosynthesis in *A. parasiticus* SU-1. They argued that the extra copy of *aflR* relieved nitrate inhibition by overcoming a *cis*-acting negative regulator in the nitrogen control circuit. If this hypothesis is true, it should have been possible to relieve nitrate inhibition of the aflatoxin pathway by overexpressing AflR with the *gpdA*(p)::*aflR* construct. Our data indicate that nitrate exerts a repressive effect on aflatoxin biosynthesis even in the presence of a constitutively transcribed *aflR*. The data do not argue against transcriptional inhibition by nitrate, but they argue that the effect is not solely on the *aflR* promoter. Our data also support the hypothesis set forth by several investigators that the regulation of aflatoxin biosynthesis by nitrate is a complex phenomenon (1, 9, 13, 14, 17, 20, 36). It is clear in our studies that overexpression of *aflR* does not totally alleviate nitrate inhibition. This study does show that the nitrate effect is not related to pH differences in the media, as the addition of nitrate did not appreciably change the pH of the medium.

At this time, it is not clear why *gpdA*(p)::*aflR* did not lead to a dramatic increase in aflatoxin production in strain 656-2. It seems unlikely that the presence of a functional *aflR* in 86-10 could explain the difference observed between transformants of 656-2 and 86-10, especially since both transformants displayed much greater *aflR* transcript levels than wild-type strains. The nature of the *aflR* mutation in 656-2 has not been characterized. It is possible that a mutated form of AflR that may prevent upregulation of aflatoxin biosynthesis is being produced by this strain. At this time, it is difficult to formulate an explanation of the mechanism by which this may occur. It is more conceivable that strain 656-2 is not as physiologically active as 86-10 and may not be capable of producing excessive amounts of aflatoxin despite elevated pathway gene transcription. Strains of *A. parasiticus* have been shown to vary in aflatoxin production independently of *aflR* copy number (18).

The findings in this study are consistent with *aflR* being a transcriptional activator of aflatoxin biosynthesis. From our data, however, we must conclude that transcriptional activation of the pathway is not the only requirement for the initiation of aflatoxin biosynthesis. The mechanism of this regulation is not known, but we provide two lines of evidence that it is mediated through fungal metabolism. Under normal conditions, transcription of the pathway requires specific conducive conditions, for example, the presence of simple sugars. If the regulation of pathway transcription is bypassed by the constitutive transcription of *aflR*, the induction profile for aflatoxin is the same as in wild-type strains. This argues that additional medium-dependent factors are required for initiation of aflatoxin biosynthesis. The second line of evidence is from our nitrate study. In this study, the addition of a single compound affects the biosynthesis of aflatoxin, and we now know that it is not at the level of *aflR* transcription. Several studies have suggested that the physiology of the fungus plays an important role in the regulation of aflatoxin biosynthesis (4–6, 26). Recent evidence for the complex regulation by media is supported by the observation in *A. niger* that intracellular cyclic AMP levels were associated with the initial sucrose levels in the media (12). Thus the regulation of aflatoxin biosynthesis is more complicated than previously considered and does not solely involve the transcription activation of the pathway.

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