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Nonfunctionality of *Aspergillus sojae aflR* in a Strain of *Aspergillus parasiticus* with a Disrupted *aflR* Gene

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***Aspergillus sojae* belongs to the *Aspergillus* section *Flavi* but does not produce aflatoxins. The functionality of the *A. sojae aflR* gene (*aflRs*) was examined by transforming it into an Δ*aflR* strain of *A. parasiticus*, derived from a nitrate-nonutilizing, versicolorin A (VERA)-accumulating strain. The *A. parasiticus aflR* gene (*aflRp*) transformants produced VERA, but the *aflRs* transformants did not. Even when *aflRs* was placed under the control of the amylase gene (*amyB*) promoter of *Aspergillus oryzae*, the *amy*(*p*)::*aflRs* transformants did not produce VERA. A chimeric construct containing the *aflRs* promoter plus the *aflRs* N- and *aflRp* C-terminal coding regions could restore VERA production, but a construct containing the *aflRp* promoter plus the *aflRp* N- and *aflRs* C-terminal coding regions could not. These results show that the *A. sojae aflR* promoter is functional in *A. parasiticus* and that the HAHA motif does not affect the function of the resulting hybrid AflR. We conclude that the lack of aflatoxin production by *A. sojae* can be attributed, at least partially, to the premature termination defect in *aflRs*, which deletes the C-terminal transcription activation domain that is critical for the expression of aflatoxin biosynthetic genes.**

Aspergillus sojae and *A. oryzae* are used in the production of enzymes and fermented foods and are taxonomically related to *A. parasiticus* and *A. flavus*. All four species belong to the *Aspergillus* section *Flavi*, which is commonly referred to as the *A. flavus* group. These species not only are morphologically similar but also share sufficient DNA sequence similarity that Kurtzman et al. (13) proposed that *A. parasiticus*, *A. oryzae*, and *A. sojae* be reduced to a varietal status. Geiser et al. (9) analyzed the genes involved in primary metabolism in strains of *A. flavus* and *A. oryzae* and concluded that *A. oryzae* is a species that evolved by domestication from one of the two *A. flavus* groups. Although the molecular evidence regarding the evolutionary origin of *A. sojae* is still inconclusive, it is generally agreed that *A. sojae*, which has never been isolated from the field, is a domesticated variant of *A. parasiticus* (29).

Neither *A. sojae* nor *A. oryzae* is known to produce aflatoxins (28), but homologues of several aflatoxin biosynthetic genes have been found in them (1, 12, 17). These genes apparently are not transcribed (16, 20, 27). The molecular mechanisms that prevent production of aflatoxins by *A. oryzae* isolates have been examined only recently. Kusumoto et al. (15) found that several strains of *A. oryzae* (groups 2 and 3) have partial deletions in the aflatoxin gene cluster. However, other *A. oryzae* strains (group 1) with intact aflatoxin gene clusters also were identified. Thus, the molecular mechanisms responsible for nontoxigenicity in *A. oryzae* appear to be diverse. Several studies have suggested that *A. sojae* isolates do not produce aflatoxins because of a defect in the aflatoxin pathway regulatory gene homologue, *aflR* (27). Most recently, Matsushima et al. (19) showed that *A. sojae aflR* (*aflRs*) does not elevate the production of aflatoxin precursors in a strain of *A. parasiticus* but that strains carrying an additional copy of *A. parasiticus aflR* (*aflRp*) do produce increased levels of these precursors.

The *A. parasiticus aflR* gene encodes AflR, a GAL4-type, zinc cluster transcriptional factor of 444 amino acids. AflR and its counterparts in *A. flavus* and *A. nidulans* are required for transactivation of the genes located in the aflatoxin and sterigmatocytin gene clusters (3, 4, 8, 21, 30, 32). The *A. sojae aflR* gene contains a 6-bp duplication and a substitution at position 1145 that results in a pretermination stop codon, TGA. The predicted *A. sojae* AflR thus has a distinct HAHA motif and is 62 amino acids shorter than the functional *A. parasiticus* AflR (19, 27).

In the present study, we constructed an *A. parasiticus* strain whose *aflR* gene was deleted. We used this strain to determine whether intact *A. sojae aflR*, the *A. sojae aflR* coding region fused to the strong inducible promoter of an amylase gene of *A. oryzae*, and *A. parasiticus aflR* containing the corresponding *aflR* carboxy-terminal coding region of *A. sojae* could complement the *aflR* deletion.

MATERIALS AND METHODS

Fungal strains and culture media. *A. parasiticus* CS10-N2 (*pyrG niaD ver-1 wh-1*) was derived from *A. parasiticus* CS10 (*pyrG ver-1 wh-1*) (24), which is deficient in the versicolorin A (VERA) reductase gene, *ver-1*, that is in part responsible for converting VERA to demethylsterigmatocystin. This mutant strain accumulates VERA and cannot use nitrate. An isogenic *aflRp* deletion strain of CS10-N2, CS10-N2Δ *aflR*, was used to study *aflRs* functionality. Potato

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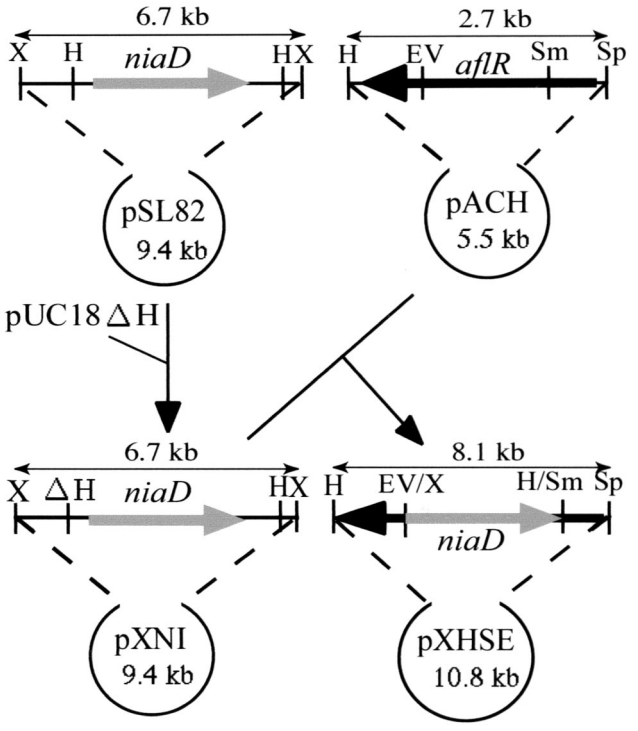

FIG. 1. Construction of the *aflR* disruption vector pHXSE. The *aflR* disruption vector pXHSE was constructed by ligating a 2.8-kb *Sac*I-*Hin*dIII fragment of pHX1 containing *A. parasiticus aflR* (3) into the corresponding sites in pUC18 to generate pACH. A 6.7-kb *Xba*I fragment of pSL82 (10) was subcloned into the *Xba*I site of a pUC18 derivative from which the *Hin*dIII site had been removed. Then, the *Hin*dIII site of a 6.7-kb *Xba*I fragment in the *niaD-niiA* intergenic region (2) was eliminated by partial digestion with *Hin*dIII and self-ligation, yielding pXN1, which contained only one *Hin*dIII site. The 6.7-kb *niaD*-containing *Hin*dIII-*Xba*I fragment of pXN1 was blunt ended with T4 DNA polymerase, ligated to pACH, pretreated with *Sma*I and *Eco*RV, blunt ended, and dephosphorylated to yield pXHSE. EV, *Eco*RV; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

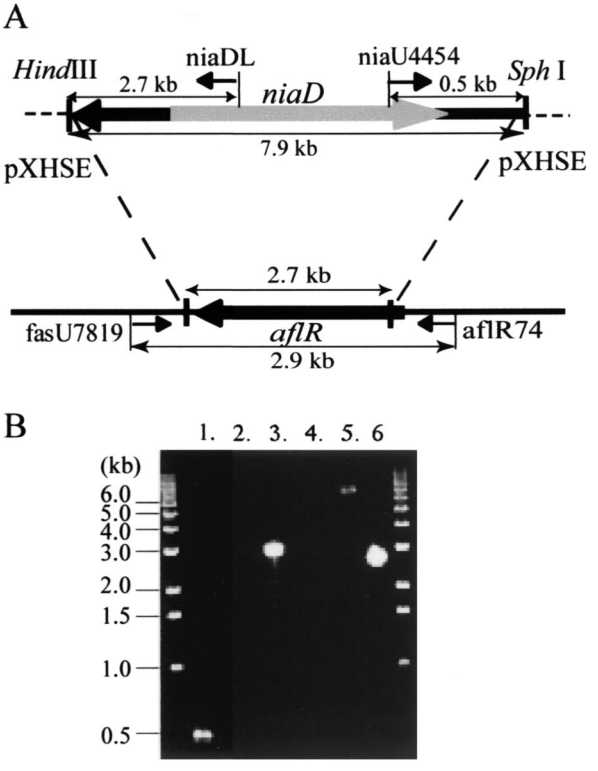

FIG. 2. Strategy and analysis of disruption of *aflR* of *A. parasiticus.* (A) Schematic representation of disruption of *aflR* in *A. parasiticus*. At the top is shown the replacement position of the *aflR* disruption plasmid pHXSE. The bottom of the figure shows the corresponding locus of *aflR* in *A. parasiticus*. Arrows show positions of oligonucleotide primers used for confirmation of *aflR* disruption. (B) Analysis of strains transformed with the *aflR* disruption plasmid. Insertion of *niaD* into *aflR* was confirmed by PCR with the primers fasU7819 and niaDL (lanes 1 and 2), aflR74 and niaU4454 (lanes 3 and 4), and fasU7819 and aflR74 (lane 5 and 6). Templates for PCR were prepared from CS10-N2 Δ*aflR* (lanes 1, 3, and 5) and CS10-N2 (lanes 2, 4, and 6).

dextrose agar (PDA) and potato dextrose broth (PDB), which permit aflatoxin production, were purchased from Difco (Detroit, Mich.). PDB supplemented with maltose (1% [wt/vol]) was used for the induction of the *A. oryzae* amylase gene promoter in the *aflR* overexpression experiments.

Construction of the *aflR* disruption vector. We constructed *aflR* disruption vector pXHSE (Fig. 1). The 6.7-kb *niaD*-containing *Hin*dIII-*Xba*I fragment of pXN1 was blunt ended with T4 DNA polymerase, ligated to pACH, pretreated with *Sma*I and *Eco*RV, blunt ended again, and dephosphorylated to produce pXHSE. The *niaD*-selectable marker in pXHSE replaced a large segment of the *A. parasiticus aflR* coding region corresponding to amino acids 7 to 444 of AflR.

Generation of *aflR* disruption mutants. Plasmid pXHSE was linearized with *Hin*dIII and *Sph*I before transformation to release the portion derived from pUC18 (Fig. 2A). Transformation of *A. parasiticus* CS10-N2 was carried out by a protoplast-polyethylene glycol method as previously described (10). Czapek Solution (CZ) agar (Difco) supplemented with Cove's trace-element solution (7), 20 mM uracil, and 0.6 M KCl was used for the regeneration of protoplasts after transformation. To confirm the occurrence of *aflR* gene disruption, we performed PCRs with the following primers: fasU7819, 5′-GTCGGCCACGATGAACCGATCCTAT-3′; aflR74, 5′-CTTGCCATACCGGGTGATAGATCAT-3′; niaU4454, 5′-GCCGCCGAGGACGCATATCCGAAGA-3′; and niaDL, 5′-CTTGCCATACCGGGTGATAGATCAT-3′ (Fig. 2). Genomic DNA of *A. parasiticus* transformants for PCR was isolated from mycelia grown in PDB for 72 h at 30°C with a DNeasy Plant Mini Kit (Qiagen, Chatsworth, Calif.).

RT-PCR. Mycelia of *A. parasiticus* strains grown for 72 h at 30°C in PDB were harvested, blotted dry, and ground to a fine powder in liquid nitrogen with a mortar and pestle. Total RNA from 150 mg of mycelial powder per sample was prepared by using an RNeasy Total RNA Extraction Kit (Qiagen). The total RNA was treated with 1 to 2 U of RNase-free DNase at 37°C for 1 h to eliminate residual fungal DNA. The DNase-treated total RNA was purified with the RNeasy Total RNA Extraction Kit. Reverse transcription-PCR (RT-PCR) was carried out by using an Advantage RT-for-PCR Kit (Clontech, Palo Alto, Calif.) with an oligo(dT)18 primer. Primers flanking introns in aflatoxin genes were used to examine the presence of these gene transcripts in the total RNA samples. The *aflR* primers were 5′-CCGATTTCTTGGCTGAGT-3′ and 5′-TCCTCATCCACACAATCC-3′ (no intron). The *aflJ* primers were 5′-CTGGCTCCGTCAGCATCAGC-3′ and 5′-TATGCCATGATTATCGAGAC-3′ (intron 2). The *nor1* primers were 5′-TGCAAACTGATATGGGCGAC-3′ and 5′-GAACTGATCGAGCGAAAGCC-3′ (intron 3). The *omtA* primers were 5′-GTCACCATGATGGAGCATGA-3′ and 5′-GCGCAAACGCAGTTGTTGAACG-3′ (intron 4). Two sets of primers derived from the *A. parasiticus* β-tubulin gene sequence (31) were used as the positive controls to confirm successful RT-PCR of the total RNA samples. One set was β-E26F (5′-TACCTTCAGACCGGCCAGTG-3′) and β-E26R (5′-GCAGCCCTCAGCCTCGCGAC-3′), which encompassed exons 2 to 6. Another set was β-I6F (5′-TACCTCACCTGCTCTGCCAT-3′) and β-I6R (5′-GTGAACTCCATCTCGTCCAT-3′) (intron 6).

Quantitation of *aflR* gene expression by real-time PCR. The real-time PCR was performed in a two-step RT-PCR procedure with oligo(dT) as the extension primer. The first-strand cDNA was generated by RT-PCR with the SYBR Green RT-PCR kit (PE Biosystems [P/N 4306225], Foster City, Calif.). The thermal cycling parameters for the RT reactions are incubation at 25°C for 10 min, reverse transcription at 48°C for 30 min, and reverse transcriptase inactivation at 95°C for 5 min. Sample cDNAs were used in three serial dilutions: 1 μl straight, followed by 10e and 100-fold dilutions. The primer pair of *A. parasiticus* β-actin

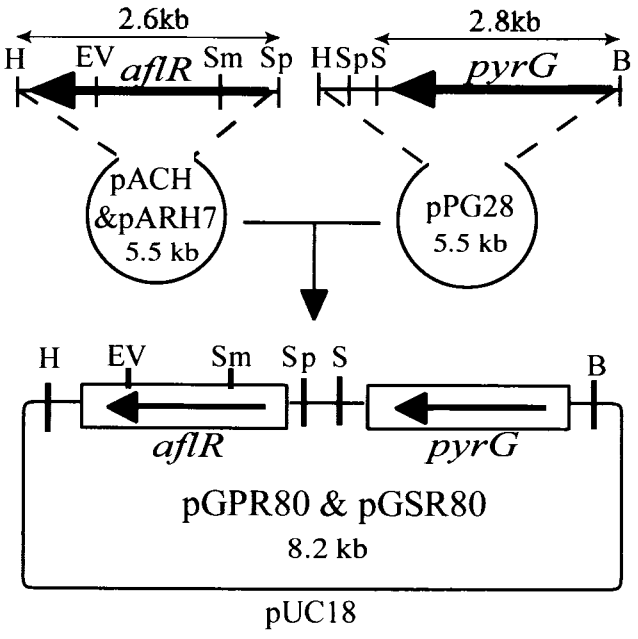

FIG. 3. Vectors for introducing *aflR* into *A. parasiticus*. pGPR80 and pGSR80 contain an *aflR* fragment from *A. parasiticus* and *A. sojae*, respectively. B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I.

gene was used as a positive control for normalization. No template control (NTC) was used as the negative control. The *aflR* primer pair used was the forward primer 5′-TCCGCCATCTTTTCTCACCA-3′ and the reverse primer 5′-CCGAATTCCGAATCGACTGTTA-3′. The β-actin primers were as follows: forward, 5′-CCGACCGTATGCAGAAGAA-3′; reverse, 5′-ACTGCCCTTGCT CCCTCTTCCATGAA-3′. The *aflR* primers used in the real-time PCR experiment were designed to not amplify the transcript of second copy *aflR*. Real-time PCR was performed by using the GeneAmp 5700 Sequence Detection System (PE Biosystems). The thermal cycling parameters consisted of an initial heating at 95°C for 10 min, denaturing at 95°C for 15 s, and annealing and extension at 60°C for 1 min, with an amplification of 40 cycles. The relative quantitation values of *aflR* transcripts were obtained by using the comparative Ct method (ΔΔCt) as described elsewhere (*Relative Quantitation of Gene Expression* [User Bulletin No. 2]; PE Biosystems) (22).

Northern blot analysis. A total of 5 μg of total RNA was fractionated in a 0.4 M formaldehyde–1.2% agarose gel and transferred to a GeneScreen Plus membrane (DuPont NEN Research Products, Boston, Mass.). The membrane was probed with aflatoxin gene DNA probes prepared with a DIG High Prime DNA Labeling and Detection Kit (Roche, Indianapolis, Ind.).

Vectors for *aflR* genetic complementation. Vectors containing the *A. parasiticus pyrG*-selectable marker and the complete *aflR* genes *of A. parasiticus* and *A. sojae* were constructed for genetic complementation experiments. A 2.8-kb *Bam*HI-*Sal*I fragment from pBZ5, which contains the *A. parasiticus pyrG* gene (23), was cloned into pUC18 to give pPG28. The 2.6-kb *Hin*dIII-*Sph*I fragment of pACH containing *A. parasiticus aflR* and the corresponding 2.6-kb *Hin*dIII-*Sph*I fragment of pARH7 containing *A. sojae aflR* (19) were ligated to the *Hin*dIII-*Sph*I-digested pPG28; the resulting constructs were designated pGPR80 and pGSR80, respectively (Fig. 3).

Fusion vectors for forced expression of the *aflRs* and *aflRp* coding regions. Vectors containing the *aflR* coding regions of *A. parasiticus* and *A. sojae* placed under the control of the *A. oryzae* TaKa-amylase A gene (*amyB*) promoter (11) were constructed for the *aflR* overexpression experiments. An *Sph*I site was introduced at the initiation codon of *aflR* in pGPR80 and pGSR80 by using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif.). A 600-bp fragment containing the *amyB* gene promoter was amplified from the genomic DNA of *A. oryzae* RIB40 by using the following PCR primers: AoAmy5′-SP (5′-GAACTACGTGGAATGCATGCTGTTTTGATC-3′) and AoAmy3′-SP (5′-ACCACGCGACCAGCATGCATGCCTTATGTG-3′) (with the *Sph*I site underlined). The PCR fragment was digested with *Sph*I and inserted into the *Sph*I site of pGPR80 and pGSR80; the resulting constructs were designated pGAmP and pGAmS, respectively. Sequences of the promoter and C-terminal region of *aflR* in each vector were confirmed.

Chimeric vectors for determining the region responsible for the defect in *A. sojae aflR*. The 1.9-kb *Bgl*II-*Hin*dIII fragment containing the carboxyl-terminal coding region (amino acids 164 to 444) of *aflRp* in pGPR80 was swapped with the corresponding region of *aflRs* in pGSR80 to give two chimeric constructs, pNSCP1 and pNPCS2, respectively (Fig. 4). Regions around the *Bgl*II site in each vector were sequenced and confirmed.

Analysis of VERA production in transformants. Fungal transformants were grown in PDB at 30°C for 3 days. Aflatoxin intermediates extracted from mycelia with acetone and chloroform (4:1 [vol/vol]) were separated by silica gel thin-layer chromatography with an ether-methanol-water (96:3:1 [vol/vol/vol]) solvent system as previously described (6).

RESULTS

Generation of *A. parasiticus aflR*-disrupted strains. We used a double-crossover strategy with a linearized vector that contained the *A. parasiticus niaD* gene as a selectable marker to disrupt the *A. parasiticus aflR* gene (Fig. 2A). Transformation

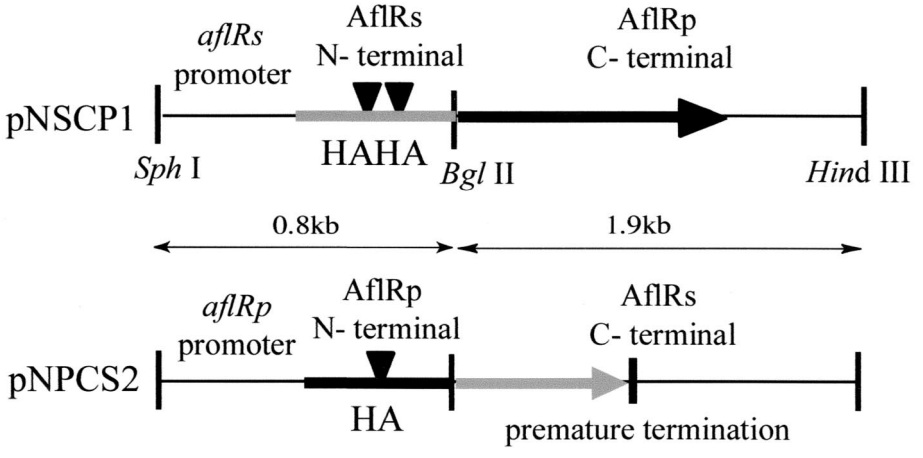

FIG. 4. Construction of chimera *aflR* between *A. sojae* and *A. parasiticus*. *aflRs* and *aflRp* were swapped at the *Bgl*II site. pNSCP1 consisted of the *aflRs* promoter, AflRs N-terminal domain, and AflRp C-terminal domain. pNPCS2 consisted of the *aflRp* promoter, AflRp N-terminal domain, and AflRs C-terminal domain.

TABLE 1. Introduction of *A. sojae aflR* into an *A. parasiticus aflR*-disrupted strain

Introduced plasmid containing *aflR*[a]	No. of transformants	
	VERA production	Total
Intact[a]		
pGSR80 (*A. sojae aflR*)[b]	0	12
pGPR80 (*A. parasiticus aflR*)	8	15
Amylase gene promoter-*aflR* fusion[a]		
pGAmS (*A. sojae aflR*)[b]	0	20
pGAmP (*A. parasiticus aflR*)	10	20
Chimera *aflR* between *A. sojae* and *A. parasiticus*[a,c]		
pNSCP1	8	20
pNPCS2	0	20

[a] Differences between the data are significant according to the Fisher's exact test ($P < 0.05$).
[b] pGSR80 and pGAmS are replicated twice, and no VERA production was seen.
[c] pNSCP1 indicates a plasmid containing the *aflRs* promoter, the AflRs N-terminal moiety, and the AflRp C-terminal moiety. pNPCS2 indicates a plasmid containing the *aflRp* promoter, the AflRp N-terminal moiety, and the AflRs C-terminal moiety.

of *A. parasiticus* CS10-N2 with pXHSE digested with *Hin*dIII and *Sph*I produced more than 500 transformants on CZ regeneration plates in a single experiment. One hundred randomly selected transformants were screened for VERA production on PDA plates. About 10% of these transformants did not accumulate the bright yellow VERA on the PDA plates, which suggested that the *aflR* gene in these transformants had been disrupted. Disruption of *aflR* was confirmed by PCR with the *niaD* internal primers (niaU4454 and niaDL) and the *aflR* external primers (fasU7819 and aflR74) (Fig. 2A). When genomic DNA of two nonpigmented transformants was amplified, primer pair niaU4454-aflR74 gave a 0.5-kb PCR fragment, and primer pair niaDL-fasU7819 gave a 3.0-kb PCR fragment (Fig. 2B, lanes 1 and 3). In contrast, no PCR fragments were obtained from *A. parasiticus* CS10-N2 genomic DNA (Fig. 2B, lanes 2 and 4). Moreover, primer pair fasU7819-aflR74 yielded an 8.2-kb PCR fragment from the genomic DNA of the nonpigmented transformants and a 2.9-kb PCR fragment from the CS10-N2 genomic DNA (Fig. 2B, lanes 5 and 6). These results indicate that the *niaD* marker had been inserted into the *aflR* locus through a double-crossover event in the nonpigmented transformants. One of the transformants, CS10-N2 Δ*aflR*, was used in the subsequent genetic complementation experiments.

Complementation of *A. parasiticus* CS10 Δ*aflR* with intact *aflR* of *A. sojae* and *A. parasiticus*. To determine whether *A. sojae aflR* (*aflRs*) could complement the genetic defect of *A. parasiticus aflR* (*aflRp*), we transformed pGSR80 or pGPR80 (Fig. 3) into *A. parasiticus* CS10-N2 Δ*aflR*. Of the 12 *aflRs* (pGSR80) transformants examined, none produced VERA on the PDA plates, whereas 8 of 15 *aflRp* (pGPR80) transformants produced VERA (Table 1).

RT-PCR analyses of *aflR* gene expression in CS10 Δ*aflR* and its *aflRs* transformant. RT-PCR analysis performed with the first-strand cDNA prepared from DNase-treated, 72-h total RNA resulted in the amplification of *aflR*, *aflJ*, *nor1*, and *omtA*

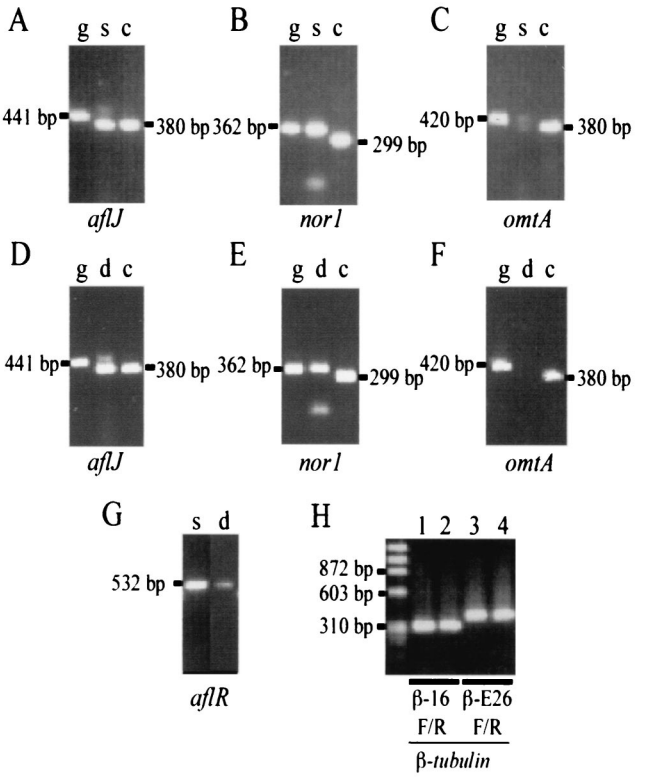

FIG. 5. Profile of aflatoxin gene transcripts of CS10Δ*aflR* and its *aflRs* transformant detected by RT-PCR. (A to F) Lane g, genomic DNA of CS10-N2 (control); lane c, cDNA of CS10-N2 (control); lane s, cDNA of CS10-N2 *aflRs*-S8 (pGSR80); lane d, cDNA of CS10-N2 Δ*aflR*. (A and D) *aflJ* unprocessed (expected size of fragment, 441 bp), *aflJ* processed (380 bp). (B and E) *nor1* unprocessed (362 bp) and *nor1* processed (299 bp). (C and F) *omtA* unprocessed (42 0bp) and *omtA* processed (380 bp). (G) *aflR* (532 bp). (H) β-Tubulin gene (control). The processed sizes of the transcripts by primers β-I6F/R and β-E26F/R are 346 and 382 bp, respectively. Lane 1, β-I6F/R, CS10-N2 Δ*aflR*; lane 2, β-I6F/R, CS10-N2 *aflRs*-S8; lane 3, β-E26F/R, CS10-N2 Δ*aflR*; lane 4, β-E26F/R, CS10-N2 *aflRs*-S8.

(Fig. 5A to G) from CS10-N2 Δ*aflR*, its *aflRs* transformant S8, and CS10-N2, which has a functional *aflRp*. This process was successful because only processed size transcripts of the *A. parasiticus* β-tubulin gene (Fig. 5H) were amplified from the first-strand cDNA. If β-tubulin gene transcripts were processed, primers βI6F/R and βE26F/R yielded 346- and 382-bp fragments, respectively, but if left unprocessed they yielded 462- and 691-bp fragments. Moreover, no PCR fragment was obtained from the DNase-treated total RNA. Thus, the template RNA was not contaminated by carryover genomic DNA. The processed sizes of the *aflJ* transcripts were amplified from cDNA of CS10-N2, CS10-N2Δ*aflR*, and CS10-N2*aflRs*-S8 (Fig. 5A and D). On the other hand, only the unprocessed size of the *nor1* transcript was recovered from CS10-N2Δ*aflR* and CS10-N2*aflRs*-S8 (Fig. 5B and E). The *omtA* transcripts were detected at low levels in CS10-N2 *aflRs*-S8 (Fig. 5C) but not at all in CS10-N2Δ*aflR* (Fig. 5F). RT-PCR analysis detected *aflR* gene transcripts in both CS10-N2Δ*aflR* and CS10-N2 *aflRs*-S8 that had *aflRs* integrated at the *pyrG* locus (Fig. 5G). However, the levels of aflatoxin gene transcripts expressed in CS10-N2

TABLE 2. Relative expression of *Aspergillus sojae aflR* to *A. parasiticus aflR*

Ratio type	Relative expression (mean ± SE)[a] in:			
	CS10N2	CS10N2 Δ*aflR*	CS10N2 *aflRp*-P8	CS10N2 *aflRs*-S8
Ratio to CS10N2 *aflRs*-S8	1.82 ± 1.11	0.00	2.51 ± 1.28	1.00
Ratio to CS10N2	1.00	0.00	1.47 ± 0.53	0.68 ± 0.32

[a] The values are based on three determinations. Steady-state mRNA levels were determined by real-time RT-PCR from total RNA prepared from 48-h-old mycelia grown in PDB medium supplemented with uracil. CS10N2 is the parent of CS10N2 Δ*aflR*. CS10N2 Δ*aflR* is an *aflR*-disrupted strain. CS10N2 *aflRp*-P8 is CS10N2 Δ*aflR* complemented with pGPR80 (*A. parasiticus aflR*). CS10N2 *afls*-S8 is CS10N2 Δ*aflR* complemented with pGSR80 (*A. sojae aflR*).

Δ*aflR* and the *afiRs* transformant were not detectable by conventional Northern blot analysis (data not shown).

Quantitation of *aflR* gene expression by real-time PCR. To quantify the level of *aflR* expression, a real-time PCR experiment was carried out. The parental strain of CS10-N2 Δ*aflR* contains a partially duplicated aflatoxin gene cluster (26). To avoid the detection of transcript from the second copy *aflR*, we used PCR primers that did not amplify the transcript from the second copy *aflR*. The data from each sample treatment were normalized against that for the β-actin positive control. The experimental data are accurate with minimum errors, as demonstrated both by the positive control (β-actin) and the NTC and by the dissociation curve. Due to the disruption of the *aflR* gene in the CS10N2 Δ*aflR* mutant, no *aflR* transcription was detected. The reaction threshold of the Δ*aflR* strain was at ca. 33 cycles, i.e., approximately the same as for the NTC. The level of transcription of *aflR* in the nondisrupted strain (CS10N2) is about two-thirds (100 versus 147%) the level of that for the *A. parasiticus aflR*-complemented strain (CS10N2 *aflR p*-P8) (Table 2). The *A. sojae aflR* in the *A. parasiticus* background is expressed well enough to more than half of the level of the nondisrupted strain.

Overexpression of *aflRs* and *aflRp* in *A. parasiticus* CS10-N2 Δ*aflR*. To examine whether overexpression of *aflRs* could complement the *aflR* mutation in CS10-N2 Δ*aflR*, we constructed pGAmP and pGAmS, in which the *aflRp* and *aflRs* coding regions, respectively, were placed under the control of the *A. oryzae* amylase gene promoter. We chose 20 colonies each of pGAmP and pGAmS transformants at random and transferred them onto PDA plates with or without 1% maltose, which is required for the induction of the amylase gene promoter. Half of the pGAmP (*aflRp*) transformants activated aflatoxin biosynthetic genes, as judged by VERA production on PDA. VERA production by the transformants was increased by the addition of maltose to the PDA. None of the pGAmS (*aflRs*) transformants produced VERA on either medium. Promoter function of pGAmS was confirmed by using a chimera vector, exchanging the 1.9-kb *Bgl*II-*Hin*dIII fragment containing the carboxyl-terminal region of *aflRs* in pGAmS for that of *aflRs* in pGAmP. About half of the transformant with the chimera vector produced VERA on PDA (data not shown).

Effect of the *aflRs* promoter, the HAHA motif, and truncation of the carboxy-terminal 62 amino acids on the function of *A. sojae* AflR. There are three differences in the *aflR* genes of *A. sojae* and *A. parasiticus*. One difference is a nucleotide substitution of G for A at position −132 in the *aflR* promoter (1). There also are two differences between the predicted AflR proteins of *A. sojae* and *A. parasiticus*: a duplication of His-Ala and a nonsense mutation give change in the coding region of *aflRs*, resulting in an AflR protein in *A. sojae* that is 62 amino acids shorter than the protein from *A. parasiticus* (27). To examine the effect of these differences on the functions of *aflRs* and AflRs, we constructed two chimeras (Fig. 4). pNSCP1 consisted of the *aflRs* promoter and encoded the AflRs N-terminal moiety plus the AflRp C-terminal moiety. pNPCS2 consisted of the *aflRp* promoter and encoded the AflRp N-terminal moiety and the AflRs C-terminal moiety. Forty percent of the CS10-N2 Δ*aflR* transformants transformed with pNSCP1 showed transactivation of the aflatoxin biosynthetic genes, based on VERA production. However, pNPCS2 could not complement the *aflR* mutation of CS10-N2 Δ*aflR*.

DISCUSSION

In *A. parasiticus* and *A. flavus*, AflR is the only known transcriptional regulator of aflatoxin-related genes that result in aflatoxin production. *aflR* homologues are found in non-aflatoxin-producing *A. sojae*, which is used industrially in food fermentation, but transcription of these homolgues is not detectable (20, 27). Determining the cause of transcriptional loss of *aflR* in *A. sojae* is important because this fungus is used to produce products that are routinely used for human consumption.

The C-terminal domain of active AflR from *A. parasiticus* or *A. flavus* is the transcription-activating domain of this protein (5). The AflR homologue from *A. sojae* (AflRs), if expressed, lacks this domain (27) and has little or no transcriptional activation activity (19). Introduction of an extra copy of *aflR* in an *A. parasiticus* wild-type strain increased aflatoxin production (3), but the introduction of *aflRs* had no discernible effect on the level of aflatoxin production.

It is difficult to separate weak activity attributable to AflRs from background expression from the original copy of *aflRp.* Moreover, the complete functional loss of AflRp in the host strain while keeping other *aflR*-related signal transduction intact is important for detecting weak transcriptional activity and for sensitive monitoring of AflR activity. In addition, *aflR*-deficient strains obtained by UV mutagenesis were not suitable for this experiment because this form of mutagenesis could cause other lesions that could adversely affect the outcome. To avoid background activity from wild-type *aflRp*, we created *aflR*-disrupted strains by homologous recombination and examined AflRs functionality in an *A. parasiticus* Δ*aflR* strain that allowed us to monitor AflR activity.

We constructed an *aflR*-disrupted strain from an *A. parasiticus* VERA-accumulating mutant (24) to assess the function of *aflRs* in vivo. VERA is a precursor of aflatoxins; the mutant does not produce any aflatoxin (33). The parent strain, CS10-

N2, accumulated VERA, and colonies turned bright yellow on PDA plates. Inactivation of genes in the aflatoxin biosynthetic pathway by the *aflR* disruption prevented the creation of VERA, and colonies of the Δ*aflR* strains remained white. Thus, the Δ*aflR* strains were suitable for *aflR* complementation tests because of their distinguishable phenotype.

A. parasiticus strains have two copies of *aflR* (18, 26, 27). One is located outside the aflatoxin gene cluster and is inactivated by functional loss resulting from amino acid substitution or insufficient transcription (J. W. Cary, K. C. Ehrlich, M. S. Wright, P.-K. Chang, and D. Bhatnagar, unpublished data). Disruption of the active copy, which is located in the aflatoxin gene cluster, eliminates detectable AflR function from cells. Using PCR, we confirmed that the *aflR* disruption mutation occurred at the active *aflR* locus in the aflatoxin gene cluster by double-crossover recombination (Fig. 2A). Real-time PCR did not detect transcript from the first copy *aflR* of CS10-N2 Δ*aflR* (Table 2). So the transcript detected by the RT-PCR experiment was derived from the second copy of *aflR* in CS10-N2 Δ*aflR* (Fig. 5G). *aflJ*, *nor1*, and *omtA* were also detected by RT-PCR in both CS10-N2 Δ*aflR* and *aflRs* transformants, but these strains did not produce VERA, and conventional Northern blot analysis could not detect the transcription of *nor1*, *ver1*, or *omtA* (data not shown). These results suggest that RT-PCR detected the basal-level transcription (25) of genes not activated by functional AflR.

The *aflR* disruption could be complemented, and these strains could produce VERA after integration of *aflRp* (pGPR80) at the *pyrG* locus (data not shown) but not by the integration of *aflRs* (pGSR80) in the same locus. The transcription level of *aflRs* in strain CS10N2 *aflRs*-S8, which has *aflRs* in its *pyrG* site, is more than half of that of CS10N2 (Table 2). These results suggest that the failure of *aflRs* transformants to produce VERA was not caused by gene silencing mediated by a positional effect.

DNA sequence information suggests that *aflRs* will produce a truncated protein (AflRs) compared to AflRp. Using our chimeric constructs we showed that the *aflRs* promoter is functional, that the HAHA motif in the AflRs N-terminal domain is probably not involved in the functional loss of AflRs activity, and that the loss of the 62-amino-acid sequence from the C terminus of AflRs is probably responsible for the observed loss of activity.

Matsushima et al. (20) reported that without the expression of *aflRs* there was no production of aflatoxin in *A. sojae* in spite of the presence of the toxin pathway genes in this fungus. However, we detected transcription of *aflRs*, i.e., that the level is equivalent to that of *aflRp* (CS10-N2), in an *A. parasiticus* background. The transcription of *aflR* and other aflatoxin-related genes (*aflJ*, *nor1*, and *omtA*) was also detected by RT-PCR in the *A. parasiticus* Δ*aflR* strain. Thus, the lack of a functional AflRs is insufficient to explain the complete repression of *aflRs* and other aflatoxin-related genes in *A. sojae* (20, 27). Kusumoto et al. (14, 16) reported that they did not detect several aflatoxin gene transcripts from *A. oryzae*, whose *aflR* does not have nonsense mutations as does *aflRs* (16, 27). Therefore, we conclude that the nonsense mutation in AflR is a reason for the nonproduction of aflatoxin in *A. sojae* but that other factors might also be involved. Factors involved in the complete repression of *aflRs* in *A. sojae* are still unknown.

It is generally accepted that *A. sojae* and *A. oryzae* never produce aflatoxins under any culture conditions (28). We conclude that the lack of aflatoxin production by *A. sojae* can be attributed, at least in part, to the premature termination defect in *aflRs*, which deletes the C-terminal transcription activation domain that is critical for the expression of aflatoxin biosynthetic genes. The lack of *A. sojae aflR* expression in the *A. sojae* genetic background coupled with its relatively high expression in an *A. parasiticus* genetic background indicates that an additional defect(s), in addition to the premature termination of the *aflR* transcript, may occur in the industrial strain *A. sojae* and reduce even further the potential ability of these strains to synthesize aflatoxins.

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