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Molecular Analysis of an Inactive Aflatoxin Biosynthesis Gene Cluster in *Aspergillus oryzae* RIB Strains†

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To help assess the potential for aflatoxin production by *Aspergillus oryzae*, the structure of an aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB 40 was analyzed. Although most genes in the corresponding cluster exhibited from 97 to 99% similarity to those of *Aspergillus flavus*, three genes shared 93% similarity or less. A 257-bp deletion in the *aflT* region, a frameshift mutation in *norA*, and a base pair substitution in *verA* were found in *A. oryzae* RIB 40. In the *aflR* promoter, two substitutions were found in one of the three putative AreA binding sites and in the FacB binding site. PCR primers were designed to amplify homologs of *aflT*, *nor-1*, *aflR*, *norA*, *avnA*, *verB*, and *vbs* and were used to detect these genes in 210 *A. oryzae* strains. Based on the PCR results, the *A. oryzae* RIB strains were classified into three groups, although most of them fell into two of the groups. Group 1, in which amplification of all seven genes was confirmed, contained 122 RIB strains (58.1% of examined strains), including RIB 40. Seventy-seven strains (36.7%) belonged to group 2, characterized by having only *vbs*, *verB*, and *avnA* in half of the cluster. Although slight expression of *aflR* was detected by reverse transcription-PCR in some group 1 strains, including RIB 40, other genes (*avnA*, *vbs*, *verB*, and *omtA*) related to aflatoxin production were not detected. *aflR* was not detected in group 2 strains by Southern analysis.

Koji molds, *Aspergillus oryzae* and *Aspergillus sojae*, have traditionally been used in the brewing industry for the production of sake, miso, and soy sauce. A history of safety (1) and nonproductivity of aflatoxin is well established for industrial strains, and *A. oryzae* is considered "generally recognized as safe" by the U.S. Food and Drug Administration (41). These fungi belong to the *Aspergillus* section *Flavi*, which includes *Aspergillus flavus* and *Aspergillus parasiticus*, some of which produce the procarcinogen aflatoxin. It is thought that *A. oryzae* and *A. sojae* are taxonomically differentiated from *A. flavus* and *A. parasiticus*, respectively (20, 21, 23, 35, 37). It has been demonstrated that most of the 25 identified genes clustered within a specific 70-kb region of the fungal genome are involved in aflatoxin biosynthesis (reviewed in references 3, 13, 22, 40, 45, 49, and 54). Among them, the *aflR* gene is known to encode a major transcriptional regulator of aflatoxin biosynthesis genes (6, 9, 19, 28, 47, 55). AflR binds to the consensus sequence 5′-TCGN$_5$CGR-3′ (16) found in the promoters of most of the aflatoxin biosynthesis genes (54), including *aflR* (14, 17). Putative binding sites (14, 17) for the transcription factors AreA (10, 34, 36), PacC (18), and FacB (43) have been identified in the *aflR* promoter.

For *A. sojae*, several studies (30, 31, 42) have suggested an inability to produce aflatoxin, because mutations have been found in the *aflR* homolog (46). On the other hand, Kusumoto et al. (25) reported that 39 strains of *A. oryzae* could be classified into three groups based on fragment analysis by a long PCR method targeting the aflatoxin biosynthesis gene homologs. Strains which belonged to groups 2 and 3 harbored deletions in the gene cluster. However, it is thought that group 1 strains have a nearly intact gene cluster, including an almost complete *aflR* gene.

Aflatoxin has not been detected in any *A. oryzae* cultures (24, 29, 33, 51). Therefore, it is thought that the aflatoxin gene homolog cluster in *A. oryzae* is not functional. It is important to prove at the molecular level that *A. oryzae* is incapable of producing aflatoxin in order to continue to use strains of this species with confidence in the food-processing industry.

In the present study, the complete sequence of the homologous aflatoxin biosynthesis cluster in *A. oryzae* RIB 40 was determined, and expression of cluster genes in *A. oryzae* strains was investigated. PCR primers were designed to examine the structure of the gene cluster in 210 *A. oryzae* strains, and these strains were classified based on amplification results.

MATERIALS AND METHODS

Fungal strains. Two hundred and ten *A. oryzae* RIB strains from the National Research Institute of Brewing (NRIB) (Higashi-Hiroshima, Japan) culture collection were used in this study. Information about the strains, including the isolation source and 20 mycological characteristics examined by Murakami (32), are available at the NRIB website (http://www.nrib.go.jp/ken/asp/strain.html).

Preparation of fungal genomic DNA and RNA. All fungal strains were grown in DP medium, consisting of 1% peptone, 2% dextrin, 0.5% KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, for 3 days at 30°C. Genomic DNA was prepared from wet mycelia according to the method of Lee et al. (27). *A. oryzae* RIB 40, 81, 128, 176, 210, 515, 920, 1031, 1039, and 1401 and *A. parasiticus* NFRI-95, a UV-irradiated mutant of *A. parasiticus* SYS-4 (NRRL2999) (50), were grown on YES (2% yeast extract and 20% sucrose) liquid culture medium for secondary-metabolite production at 30°C for 2 days with shaking. Total RNA was prepared from harvested mycelia with ISOGEN (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions.

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† Supplemental material for this article may be found at http://aem.asm.org/.

TABLE 1. Primers

Primer[a]	Sequence	Use
aflR F	5′-TCAGTGTTTGTAGTGCTAGCG-3′	Probe for plaque hybridization
aflR R	5′-TCCTCAATCGAATCAACCACC-3′	
nor-1 F	5′-CGGACGAGGTCTCATTGAAGCTTT-3′	Probe for plaque hybridization and PCR amplification
nor-1 R	5′-ATCGATGATGAAGGCCGTGA-3′	
verB F	5′-GATGCACCATGACCTCATGCGTTA-3′	Probe for plaque hybridization, PCR amplification, and RT-PCR
verB R	5′-CACGGCAGCGTTATTGATCATCTC-3′	
aflR F2	5′-CCGGCGCATAACACGTACTC-3′	Probe for Southern hybridization and PCR amplification
aflR R2	5′-GGCGCTTGGCCAATAGGTTC-3′	
norB F	5′-AGTTGCGATCTGTAACACTGCTGA-3′	Subcloning for *norB* and *cypA* regions
cypA R	5′-GGAACGGGGTCAAGGATATAAGGG-3′	
aflT F	5′-GCACCAAATGGGTCTTTCTCGT-3′	PCR amplification
aflT R	5′-ATCCACGGTGAAGAGGGTAAGG-3′	
norA F	5′-GGCTGGAAAGGGGTAATGGG-3′	PCR amplification
norA R	5′-TCTTGCGACCCTCACGAGAA-3′	
avnA F	5′-AATCGCACCCAATGAGCTGTCT-3′	PCR amplification and RT-PCR
avnA R	5′-ATGGCCCGGGTTCTTTAGCAAC-3′	
vbs F	5′-TGCGAATGCTACGGCTCTCA-3′	PCR amplification and RT-PCR
vbs R	5′-CAACCGCCATCTCCTGGTCT-3′	
omtA F	5′-TATCTGGCCACGGCAGGTGA-3′	RT-PCR
omtA R	5′-GGGGCGACGAATGTCATGCT-3′	
aflR F3	5′-CAACCTGATGACGACTGATA-3′	RT-PCR and real-time Q-PCR
aflR R3	5′-ACAATCCTCGCCCACCATAC-3′	
β-tubulin F	5′-CCAAGAACATGATGGCTGCT-3′	RT-PCR and real-time Q-PCR
β-tubulin R	5′-CTTGAAGAGCTCCTGGATGG-3′	

[a] F and R indicate forward and reverse primers, respectively.

Primers, plaque hybridizations, and Southern hybridization analysis. PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The oligonucleotides used for PCR as probes for screening and for Southern analysis, for subcloning, and as primers for reverse transcription-PCR (RT-PCR) and real-time quantitative PCR (Q-PCR) and PCR amplification analysis of *A. oryzae* RIB strains are shown in Table 1. The amplified DNA fragments were labeled with digoxigenin-11-dUTP by using a PCR digoxigenin probe synthesis kit (Roche Diagnostics, Mannheim, Germany). Plaque, and Southern hybridization procedures were carried out according to the manufacturer's instructions.

Genomic-library construction and screening. *A. oryzae* RIB 40 was used for structure and sequence analysis of the aflatoxin biosynthesis gene homolog cluster. Genomic DNA from an *A. oryzae* RIB 40 genomic library was partially digested with SauIIIAI and then ligated to a λ fixII (XhoI-cut) partially filled-in vector (Stratagene, La Jolla, Calif.). *A. oryzae* RIB 40 in the λ vector was then packaged using a Gigapack III XL packaging extract (Stratagene). Screening was performed using PCR products as probes. The probes were regions of the *aflR*, *nor-1*, and *verB* open reading frames (ORFs) (Table 1). *Escherichia coli* P_{2392} was transfected with the phage mixture. Several positive genomic clones were isolated and digested with NotI in order to be sublconed into pBluescript. Sequence analysis of clones containing the largest genomic inserts was performed using the GPS-1 Genome Priming System (New England Biolabs, Inc.). Nucleotide sequence analysis of genomic DNA was performed using an ABI PRISM 310 or 3100 Avant Genetic Analyzer (Applied Biosystems Japan Ltd.).

Nucleotide sequence analysis. Sequence data were assembled using ATGC software (Genetyx Co., Tokyo, Japan). In the cases of the *norB* and *cypA* genes, PCR products generated with primers *norB* F and *cypA* R (Table 1) were cloned directly using a Zero Blunt PCR Cloning kit (Invitrogen Corp., San Diego, Calif.). The aflatoxin biosynthesis gene homologs from *A. oryzae* RIB 40 and their deduced amino acid sequences were compared with those from *A. flavus* or *A. parasiticus* available in the NCBI nucleotide database. The accession numbers for the nucleotides used in the analysis are AY 510453 (complete sequence of the aflatoxin biosynthesis gene cluster from *A. flavus* AF70) (12, 15) and AY371490 (complete sequence of the aflatoxin biosynthesis gene cluster from *A. parasiticus* SU-1 [ATCC 56775]) (2, 54). Both strains are aflatoxin-producing fungi.

PCR analysis of the seven aflatoxin biosynthesis gene homologs. Amplification of the aflatoxin biosynthesis gene homologs was performed using Insert Check-Ready-Blue (Toyobo Co. Ltd., Osaka, Japan). PCR mixtures containing genomic DNA and primer mixtures were heated at 94°C for 4 min and then subjected to 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 55 ± 1°C (adjusted according to the primer's melting temperature) for 2 s, and extension at 74°C for 30 s. A 5-μl portion of each PCR product was electrophoresed in 1.0% agarose (Sigma-Aldrich, Inc., St. Louis, Mo.) in TAE buffer. The PCR products were visualized with UV light after the gels had been stained with ethidium bromide.

RT-PCR and real-time Q-PCR. cDNAs of all the samples were prepared by reverse transcription of 50 ng/μl of total RNA with 1 μl (0.5 μg/μl) of an oligo(dT) primer using SuperScript II RNase H reverse transcriptase (Invitrogen) following the manufacture's instructions. Primer pairs for *aflR*, *avnA*, *vbs*, *verB*, *omtA*, and β-tubulin (Table 1) were used for RT-PCR. The real time Q-PCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems) and QuantiTest SYBR Green PCR kit (QIAGEN) as described in the manufacturers' manuals. The real-time Q-PCR mixtures containing cDNA and each primer were heated at 95°C for 15 min and then subjected to 40 cycles consisting of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 72°C for 20 s. The primer pairs were designed for the specific amplification of the *aflR* gene. The housekeeping gene *tub1*, encoding β-tubulin, was chosen as a system control for reverse transcription. The set of primer sequences is shown in Table 1. Standard DNA for the calibration curve of the real-time Q-PCR was prepared using *A. oryzae* RIB 40 genomic DNA as a template. The real-time Q-PCR was carried out at three replicates per prepared cDNA sample, and the average data were normalized by mRNA accumulation of β-tubulin.

Nucleotide sequence accession numbers. The DDBJ accession numbers registered in this study are AB071288 (*aflR*, *aflJ*, *norA*, and *ver-1*), AB076803 (*aflT*, *pksA*, and *nor-1*), AB076804 (*avnA*, *verB*, *avfA*, and *omtB*), AB182368 (*norB-cypA* region), and AB196490 (complete sequences of the *A. oryzae* RIB 40 homologous aflatoxin biosynthesis genes).

RESULTS

Sequence analysis of the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB 40. The aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB 40 was cloned by plaque hybridization, and about 46 kb of sequence was determined. Recently, the complete *A. oryzae* RIB 40 genome sequence was determined by the Japanese *A. oryzae* genome consortium. Our sequence results were identical to those of the consortium. The structure of the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB 40 and the similarity of the deduced amino acid

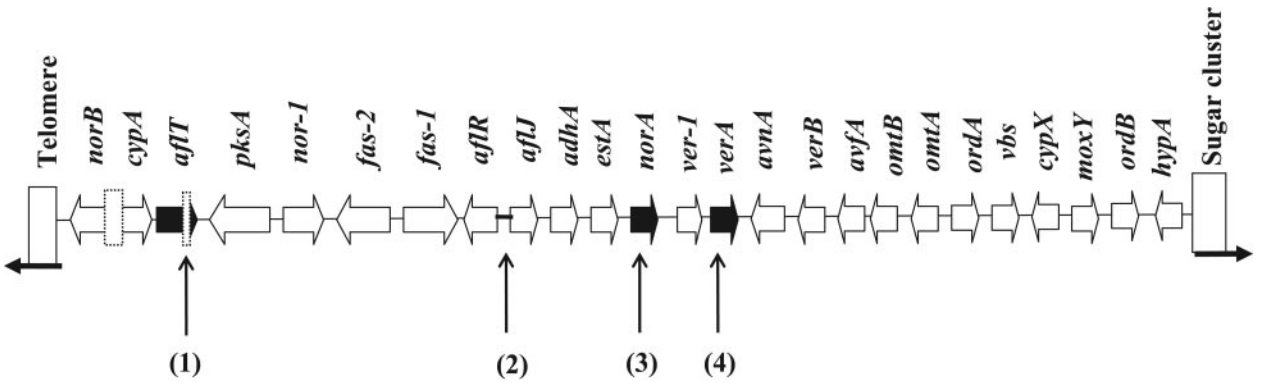

FIG. 1. Structure of the aflatoxin biosynthesis gene homolog cluster in *Aspergillus oryzae* RIB 40. Boldface black arrows indicate genes encoding deduced polypeptides with less than 93% amino acid similarity to those of *A. flavus* (arrow length is not proportional to gene size). Dotted boxes indicate deletions. The boldface line indicates mutations in a recognized consensus sequence within a promoter region. The numbered vertical arrows indicate specific mutations: 1, a 257-bp deletion resulting in loss of 1 of 14 putative transmembrane regions; 2, base substitutions in consensus sequences for putative AreA and FacB binding sites; 3, a frameshift mutation resulting in a truncation; 4, amino acid substitutions.

sequence for each gene were compared to those of *A. flavus* and *A. parasiticus* (Fig. 1 and Table 2). The distribution of the genes within the cluster in *A. oryzae* RIB 40 and the directional alignment of each are the same as in *A. flavus* (15) and *A. parasiticus* (52). While most of the amino acid sequences in the corresponding cluster genes from *A. oryzae* RIB 40 exhibited from 97 to 99% similarity to those from *A. flavus*, three genes (*aflT*, *norA*, and *verA*) shared 93% similarity or less.

TABLE 2. Alignment analysis of predicted polypeptides encoded by aflatoxin biosynthesis gene homologs in *A. oryzae* RIB 40 (% amino acid identity[a])

Gene	% Amino acid identity[a]	
	A. flavus	*A. parasiticus*
norB	Not possible	Not possible[b]
cypA	Not possible	Not possible
aflT	87	88
pksA	99	99
nor-1	98	96
fas-2	99	98
fas-1	99	98
aflR	99	97
aflJ	99	98
adhA	98	98
estA	99	96
norA	93	93
ver-1	99	99
verA	93	94
avnA	98	95
verB	97	96
avfA	98	86
omtB	98	94
omtA	98	97
ordA	98	96
vbs	99	98
cypX	97	97
moxY	99	94
ordB	99	92
hypA	97	93

[a] Amino acid sequence data for *A. flavus* AF 70 and *A. parasiticus* SU-1 (ATCC 56775) were obtained from the NCBI nucleotide database.
[b] Because the putative start codon was not found in *A. oryzae* RIB 40 and *A. flavus* AF 70, an alignment analysis could not be performed.

The *aflR* gene encodes a major transcriptional regulator of the aflatoxin biosynthesis genes (6, 9, 19, 28, 47, 55). At least one copy of the AflR binding motif (5′-TCGN$_5$CGR-3′) (16) is present in the promoters of the *A. parasiticus* aflatoxin biosynthesis genes, including *aflR* itself (54). AflR binding motifs in *A. oryzae* RIB 40 were conserved almost completely, except in the *norB* and *cypA* intergenic region, in which a deletion was recognized relative to *A. parasiticus* (15). Five distinct types of protein binding sites are predicted in the *aflR* promoter: three copies of the AflR motif itself, two PacC sites, three AreA sites, one AbaA site, and one FacB site (14, 17). The *A. oryzae* RIB 40 *aflR* promoter was compared to that of *A. flavus*. Two substitutions (italicized) were found in one of the three putative AreA binding sites (HGATAR → AGAT*G*G) which regulate nitrogen source utilization (10, 34, 36) and in the FacB binding site (GCAACGAAAAGGGC → GCAACGAAAAG GG*T*). *facB* is a positively acting regulatory gene involved in acetate induction (43).

In RIB 40, a 257-bp deletion at the C-terminal coding region of the *aflT* gene, which encodes a major facilitator superfamily transporter in *A. parasiticus* and *A. flavus* (11), was found, and this deletion caused a lack of the last 1 of 14 putative transmembrane regions. A frameshift mutation was found in a C-terminal coding region, resulting in a stop codon. The *norA* gene may be involved in the conversion of norsolorinic acid (NA) to averantin (7). The deduced polypeptide encoded by the predicted *A. oryzae* RIB 40 *norA* was 73 amino acids shorter than in *A. flavus* or *A. parasiticus*. Amino acid substitutions were found only in the *verA* gene product, which may encode a monooxygenase (52). Thirty of 453 amino acids (compared to the *A. flavus verA* gene product) were identified as substitutions, although no deletions or frameshift or nonsense mutations were found in the nucleic acid sequences of the *verA* gene.

A 1.5-kb deletion of nucleic acid sequences was observed between *norB* and *cypA* relative to these genes in *A. parasiticus*, which may encode an arylalcohol dehydrogenase and a cytochrome P450-type monooxygenase, respectively, as reported by Ehrlich et al. (15). This deletion is thought to result in loss of the start codons in both genes and an intergenic region.

TABLE 3. Classification of *A. oryzae* RIB strains based on PCR amplification patterns of aflatoxin biosynthesis gene homologs

Classification	Aflatoxin biosynthesis genes amplified by PCR[a]	No. of strains[b]	Ratio (%)
Group 1	*aflT*, *nor-1*, *aflR*, *norA*, *avnA*, *verB*, *vbs*	122	58.1
Group 2	*avnA*, *verB*, *vbs*	77	36.7
Group 3	*verB*, *vbs* or *vbs*	9	4.3
Others	No shared patterns	2	0.9

[a] Amplification of seven homologous aflatoxin biosynthesis genes was examined.

[b] A total of 210 *A. oryzae* RIB strains were used in this study, details of which are available at the NRIB website described in Materials and Methods.

Classification of *A. oryzae* RIB strains by PCR amplification. Based on the *A. oryzae* RIB 40 sequence, we examined aflatoxin gene homolog clusters in 210 *A. oryzae* RIB strains. Seven homologous aflatoxin biosynthesis genes (*vbs*, *verB*, *avnA*, *norA*, *aflR*, *nor-1*, and *aflT*) were selected to cover sequences throughout the cluster. PCR primers which specifically amplified these genes were designed and used to detect their presence.

As shown in Table 3, 210 strains were classified into groups 1, 2, and 3 and others. The PCR amplification patterns of each group are shown in Fig. 2. (The grouping list is shown in Table S1 in the supplemental material, and the amplification patterns of all examined strains are listed in Fig. S1A to D in the supplemental material). Group 1, in which amplification of all seven genes was confirmed, contains 122 RIB strains (58.1% of examined strains), including RIB 40 (Fig. 2A). Seventy-seven strains (36.7%) belonged to group 2, characterized by the amplification of only three genes, *vbs*, *verB*, and *avnA*, found in half of the cluster (Fig. 2B). It is possible that the breakpoint within the cluster of group 2 strains would be near the *ver-1* gene, as described by Kusumoto et al. (25). Nine strains (4.3%) in which at least *vbs* was amplified were classified into group 3 (Fig. 2C1 and C2). Two strains (0.9%) that could not be classified into group 1, 2, or 3 were called "others." Most RIB strains (94.8%) were classified into groups 1 and 2.

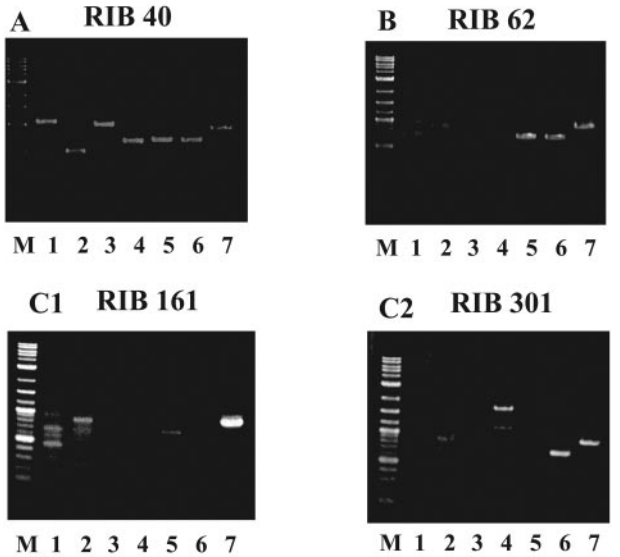

FIG. 2. PCR amplification patterns of aflatoxin biosynthesis genes in *A. oryzae* RIB strains. Two hundred and ten *A. oryzae* RIB strains were classified into four groups. (A) Group 1; amplification of all seven genes was confirmed. (B) Group 2; amplification of only *avnA*, *verB*, and *vbs* was confirmed. (C1) Group 3; amplification of only *vbs* was confirmed. (C2) Group 3; amplification of *vbs* and *verB* was confirmed. Lanes: M, marker; 1, *aflT*; 2, *nor-1*; 3, *aflR*; 4, *norA*; 5, *avnA*; 6, *verB*; 7, *vbs*.

Furthermore, the *norB-cypA* regions, in which a 1.5-kb deletion has been recognized in *A. oryzae* RIB 40, of all group 1 strains were examined. Among other group 1 strains, 97 strains had the same 1.5-kb deletion as observed in *A. oryzae* RIB 40, 5 strains had a 0.8-kb deletion, and 19 strains had no deletion (*A. parasiticus* type) (the deletion sizes of the *norB-cypA* regions in group 1 strains are shown in Table S1 in the supplemental material). We also examined other *Aspergillus* strains. Among four *A. flavus* strains, two strains had 1.5-kb deletions and two others had 0.8-kb deletions. No deletions were found in 4 *A. parasiticus* or in 22 *A. sojae* strains. In 19 *A. oryzae* strains which had complete *norB-cypA* regions, *aflR* was sequenced, because these strains were thought to be strains of *A. sojae.* The sequences in all 19 strains were found to be consistent with those of *A. sojae* (30, 31, 42, 46, and data not shown).

RT-PCR and real-time Q-PCR of the aflatoxin biosynthesis pathway gene homologs in group 1 strains. All 210 *A. oryzae* strains used in this study were proved by Murakami (32, 33) not to produce aflatoxin. Kusumoto et al. (26) reported that *aflR*, a gene involved in regulation of aflatoxin synthesis in *A. flavus* and *A. parasiticus*, was not expressed in *A. oryzae* strains. In order to determine whether the aflatoxin biosynthesis pathway gene homolog cluster was functional in *A. oryzae* RIB 40 and another nine group 1 strains selected at random, expression of *aflR*, *avnA*, *vbs*, *verB*, and *omtA* was tested by RT-PCR in cultures grown in YES medium. As a positive control, *A. parasiticus* NFRI-95 was used. This strain is an NA-accumulating mutant (50) that produces an orange pigment, so that its presence serves as a marker for aflatoxin production. Expression of β-tubulin was examined as a positive control for the genes.

Slight expression of *aflR* was detected in some group 1 strains, including RIB 40 (Fig. 3A). All *avnA* (53), *vbs* (39), *verB* (3, 48), and *omtA* (4) genes related to aflatoxin production were not detected in group 1 strains examined in this experiment (Fig. 3B to E). On the other hand, expression of all examined genes was confirmed in *A. parasiticus* NFRI-95. Further, real-time Q-PCR was performed in order to compare the transcription levels of *aflR* between those group 1 strains and *A. parasiticus* NFRI-95 quantitatively. Assuming that the transcription level in *A. parasiticus* NRFI-95 was 100, that in *A. oryzae* RIB 40 was 11.6. Almost the same transcription levels were confirmed in the other nine group 1 strains (data not shown).

Southern analysis of the *aflR* gene in group 2 strains. The presence of the *aflR* gene region in group 2 isolates could not be detected by PCR amplification of genomic DNA. The presence of the *aflR* gene in strains of this group was examined instead by Southern analysis using the *aflR* ORF as a probe, because of the possibility of mutations in the annealing sites of the *aflR* primers (Fig. 4). In *A. oryzae* RIB 40, the *aflR* gene was detected as the predicted 5-kb band. On the other hand, the *aflR* gene was not detected in any of the 77 strains of group 2

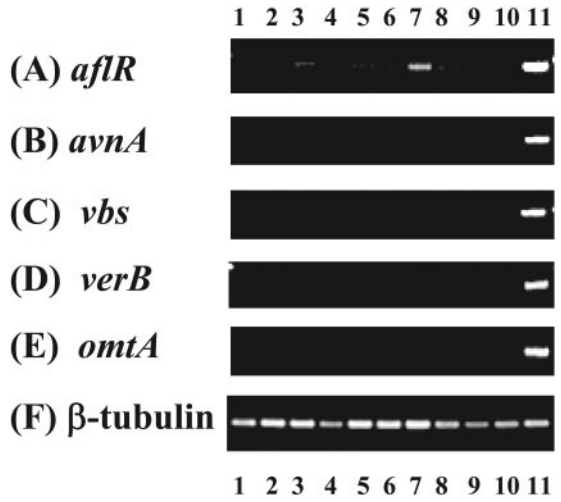

FIG. 3. Gel electrophoretic analysis of RT-PCR products using primers for the indicated genes. Strains were grown in YES medium for 2 days at 30°C. Lanes: 1, *A. oryzae* RIB 40; 2, RIB 81; 3, RIB 128; 4, RIB 176; 5, RIB 210; 6, RIB 515; 7, RIB 920; 8, RIB 1031; 9, RIB 1039; 10, RIB 1401; 11, *A. parasiticus* NFRI-95.

(Fig. 4; other data not shown). This result demonstrates that the group 2 *A. oryzae* RIB strains lack the *aflR* gene.

DISCUSSION

We have shown that a number of mutations exist in the *aflR* promoter region and in three ORFs (*aflT*, *norA*, and *verA*) within the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB 40 relative to the *A. flavus* sequence. These include deletions, frameshift mutations, and base pair substitutions (Fig. 1 and Table 2). In addition, a 1.5-kb deletion within the *norB-cypA* sequence was also detected, in agreement with observations made in other isolates of *A. oryzae* and in other *A. flavus* S isolates (15). In aflatoxin biosynthesis, it is thought that products of *norA* (7), *nor-1* (44, 56), and *norB* (52) are involved in converting NA to averantin, which is an early step in aflatoxin biosynthesis (49, 54). Disruption of *nor-1* has been reported to result in accumulation of NA and in a corresponding and substantial decrease in aflatoxin B1 (44). On the other hand, disruption of *norA* (7) or *norB* (52) did not severely influence aflatoxin production. It is thought that mutations of *A. oryzae* RIB 40 *norA* and *norB* sequences do not contribute to nonproductivity of aflatoxin in this strain.

The *aflT* gene, which encodes a major facilitator superfamily transporter, has been shown not to play a significant role in the production and secretion of aflatoxin (11). The partial deletion of the *aflT* sequence is thus presumed to have little share on the nonproductivity of aflatoxin by *A. oryzae* RIB 40. In addition to relatively low amino acid similarity found between *A. oryzae* and *A. flavus* (93%) or *A. parasiticus* (94%) homologs of *verA* (Table 2), which encodes a putative monooxygenase (52), a similar result (92%) was also found between *A. flavus* and *A. parasiticus*. The deduced amino acid similarity of cluster genes between *A. flavus* and *A. parasiticus* was relatively low compared to that between *A. oryzae* and *A. flavus* (data not shown).

As a result of the sequencing analysis of the *norB-cypA* region, three patterns of 1.5-kb (98 strains) and 0.8-kb (5 strains) deletions and no deletion (19 strains) were confirmed in group 1 strains. The *aflR* sequences of 19 no-deletion strains were consistent with that of *A. sojae*. Assuming that the 19 no-deletion strains are now classified as *A. sojae*, 95% of group 1 strains have the deletion structure of 1.5-kb in the *norB-cypA* region. It seems that 1.5-kb-deletion strains are the majority of *A. oryzae* group 1 strains. Strains having 1.5- and 0.8-kb deletions are also present in *A. flavus* (15). On the other hand, both *A. parasiticus* (15) and *A. sojae* have no deletion. The structures of the *norB-cypA* regions support the hypothesis that *A. oryzae* and *A. sojae* are differentiated from *A. flavus* and *A. parasiticus*, respectively.

The amino acid similarity of the deduced polypeptide encoded by *aflR* from *A. oryzae* RIB 40 and that from *A. flavus* was 99% (Table 2), and the AflR binding motifs present on the promoters of genes in the cluster (54) were completely conserved, except for one intergenic region between *norB* and *cypA*. From the results of RT-PCR (Fig. 3) and the real time Q-PCR, which are more sensitive than Northern analysis, the transcription level of *aflR* in group 1 strains is extremely low compared to that of *A. parasiticus* NFRI-95. Expression of *aflR* by real-time RT-PCR or RT-PCR is also detected in *A. sojae* strains, which have been proven nonaflatoxigenic (8), and *A. flavus* strains, which do not produce aflatoxin (38). It is thought that the reason for the lack of expression of *avnA*, *vbs*, *verB*, and *omtA* genes is a lower transcription level of the regulatory gene, *aflR*. However, it is possible that translation is not performed even if *aflR* is expressed slightly or AflR is degraded.

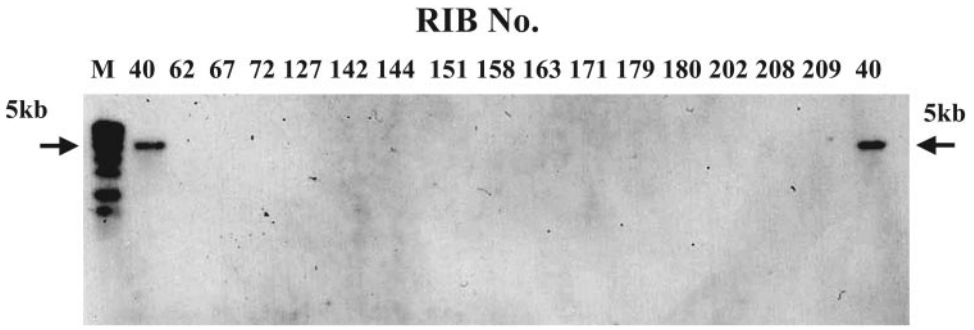

FIG. 4. Southern blot analysis of group 2 *A. oryzae* RIB strains using an *aflR* ORF probe. Lane numbers correspond to RIB numbers. Genomic DNA of group 2 strains was digested with PstI, and *A. oryzae* RIB 40 was used as a positive control.

Further work is needed to investigate these possibilities. In any case, it is obvious that group 1 strains cannot produce aflatoxin, because *avnA*, *verB*, *vbs*, and *omtA* genes, which are necessary for aflatoxin production, could not be confirmed by RT-PCR. In particular, *avnA* is considered essential to produce aflatoxin (53). The lack of expression of these genes in group 1 strains proves that *A. oryzae* does not produce aflatoxin. Furthermore, in the *A. oryzae* RIB 40 *aflR* promoter, base substitutions were found in putative AreA (17) and FacB (14) binding sites related to utilization of nitrogen (10, 34, 36) and carbon sources (43), respectively. However, it seems unlikely that base substitutions in those putative binding sites alone are the root cause for the nonproduction of aflatoxin by *A. oryzae*. At present, it is extremely difficult to determine a cause for nonproduction from the structural analysis. In addition, the *laeA* gene, which is involved in *aflR* expression, has recently been described (5), demonstrating the possibility that noncluster genes may cause nonproductivity of aflatoxin by *A. oryzae*.

Deletion of a large part of the aflatoxin biosynthesis gene homolog cluster, including *aflR*, was detected in 40% of the RIB strains (groups 2 and 3). The deletion of *aflR* in these strains (group 2) was confirmed by Southern analysis (Fig. 4). Furthermore, 60% of the RIB strains originating from tanekoji (the mold starter for making koji), used in sake, soy sauce, and miso production, belong to group 2 (data not shown). Group 3 strains amplify *vbs* at least, and few amplified patterns were confirmed. "Others" were strains which could not be classified into groups 1 to 3 and which may have misclassifications. Our examination focused on groups 1 and 2 in this study, because almost all RIB strains were classified in these two groups. However, group 3 and other strains need to be analyzed in detail and separately. This is the first report that has analyzed the aflatoxin biosynthesis gene homolog cluster in a large number of *A. oryzae* strains. We suggest that this deletion, found in such a large number of *A. oryzae* strains, may have been caused by a long history of use in the brewing industry or may be suitable for life under brewing conditions.

To our knowledge, this report is the first detailed characterization of the aflatoxin biosynthesis pathway gene homolog cluster in *A. oryzae*. While we have shown that the genes in the cluster in *A. oryzae* RIB 40 are not functional and that group 2 strains cannot produce aflatoxin, a determination of the cause cannot be made based on the structural analysis alone in the case of group 1 strains. Continued industrial use of group 2 strains appears to pose no risk of potential aflatoxin production. Attempts to develop easy methods to select group 2 strains are in progress.

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