Appl Microbiol Biotechnol (2001) 55:771–776
DOI 10.1007/s002530000524

ORIGINAL PAPER

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Absence of aflatoxin biosynthesis in koji mold (*Aspergillus sojae*)

Received: 10 August 2000 / Received revision: 16 September 2000 / Accepted: 29 September 2000 / Published online: 5 May 2001

Abstract Ten strains isolated from industrial soy sauce producing koji mold were identified as *Aspergillus sojae* and distinguished from *Aspergillus parasiticus* morphologically and physiologically. There was no detectable aflatoxin in any culture extracts of *A. sojae* strains. Strain 477 was chosen as a representative strain of industrial *A. sojae* for further molecular analysis. All enzymatic activities associated with the aflatoxin biosynthesis were not detected or negligible in strain 477 compared with that of the *A. parasiticus* strain. Southern analysis suggested that the genomic DNA of strain 477 contained aflatoxin biosynthetic pathway genes. In contrast, all industrial strains lacked detectable transcripts of *aflR*, the main regulatory gene for aflatoxin biosynthesis, under the aflatoxin-inducing condition. Our data suggest that defects in *aflR* expression cause the lack of expression of aflatoxin-related genes which results in the absence of aflatoxin biosynthesis in *A. sojae* strains.

Introduction

Aspergillus sojae is taxonomically classified in section *Flavi* along with *Aspergillus flavus*, *Aspergillus oryzae*, and *Aspergillus parasiticus* (Klich and Pitt 1988). While *A. flavus* and *A. parasiticus* are fungal contaminants of food and feed, and produce the potent carcinogen, aflatoxin, in contrast, *A. sojae* and *A. oryzae* are used not only for industrial enzyme production but also for fermented food production such as sake (rice wine), miso (bean paste), and shoyu (soy sauce) in eastern Asia. It is generally accepted that *A. sojae* and *A. oryzae* never produce aflatoxin under any culture conditions (Wei and Jong 1986). However, because of their taxonomical similarity to aflatoxin-producing fungi (Kurtzman et al. 1986), it is necessary and important to determine the cause of inability to produce aflatoxins by food-grade *Aspergillus* species.

While it is supposed that *A. sojae* is a non-aflatoxigenic domesticated derivative of *A. parasiticus* (Wicklow 1984), *A. sojae* can be practically distinguished from *A. parasiticus* by physiological [e.g., lack of growth in the presence of bleomycin (Klich and Mullaney 1989)] and/or morphological (Klich and Pitt 1988) characteristics. In addition, molecular biological techniques such as random amplification of polymorphic DNA (Yuan et al. 1995) have been applied successfully to distinguish *A. sojae* from *A. parasiticus*. Southern analysis revealed that some non-aflatoxigenic *A. sojae* strains retained two aflatoxin biosynthetic genes in their genome (Klich et al. 1995). Northern analysis showed that these strains lacked transcription of all aflatoxin-associated genes except for *aflR* and *uvm8* (Klich et al. 1997). These results suggested that the retardation of aflatoxin biosynthesis in *A. sojae* strains occurred at the level of transcription of aflatoxin-related genes other than *aflR*, which has been reported to encode a main transcriptional regulator of aflatoxin biosynthesis (Chang et al. 1993; Payne et al. 1993).

The aim of our study was to assess the safety level of koji mold strains used for producing fermented foods such as soy sauce and bean paste with regard to aflatoxin biosynthesis. We re-identified our laboratory strains from soy sauce koji mold as *A. sojae* and showed that the expression of aflatoxin-related genes, such as *pksA* (Chang et al. 1995a), *nor1* (Trail et al. 1994), and *aflR*, was not detected by enzymatic and transcriptional analysis.

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Materials and methods

Fungal strains

All strains used in this study were originated from soy sauce koji and obtained from Noda Industrial Science Laboratory (NISL, Noda, Chiba, Japan) except for strain 477. These strains were deposited in the Centraal Bureau voor Schimmelcultures (CBS, Baan, Netherlands) and the American Type Culture Collection (ATCC, Manassas, Va.; see Table 1). Strain 477 of *A. sojae* is a high-protease mutant of IFO4241 and is currently used for soy sauce fermentation. Strains IFO 4082, and 30179 were used as typical *A. parasiticus* strains for morphology and aflatoxin production. NIAH-26 expresses all enzymatic activities involved in aflatoxin biosynthesis, though this is a non-aflatoxigenic mutant of SRRC 2999, which produces aflatoxin (Yabe et al. 1988). NIAH-26 was used to monitor the expression of genes for aflatoxin biosynthesis for the sake of safety.

Morphological and physiological characterization of *A. sojae* strains

To distinguish *A. sojae* from *A. parasiticus* with regard to morphological characteristics, we followed the methods of Klich and Pitt (1988). Conidial color on CYA medium was observed and conidial size was measured. Bleomycin tests were performed as previously described (Klich and Mullaney 1989). The conidiospores of each strain were inoculated to the modified minimal medium plates with or without 40 µg/ml bleomycin sulfate and then incubated for 6 days at 25°C. The diameter of the colonies was measured.

Detection of aflatoxin

Each fungal strain was grown on 50 ml of stationary YES aflatoxin-inducing liquid medium (2% yeast extract, 20% sucrose) at 25°C for 13 days (Abdollahi and Buchanan 1981). The koji mash was prepared as follows: 3 g of defatted soybeans, 3 g of crushed and puffed wheat seeds and 4 ml of water were mixed in a 150-ml Erlenmeyer flask. After sterilization by autoclaving for 60 min at 121°C, 10^9 conidiospores of each strain were inoculated into the mash, and the cultures were incubated at 30°C for 3 days. Pigments produced by fungi were extracted by mixing the culture with 50 or 100 ml of chloroform. Water could be added to improve mixing. After removing the debris using filter paper and centrifugation at 5,000 *g* for 15 min, the organic phase was collected and partially purified using Florisil column chromatography (Kamimura et al. 1985). High-performance liquid chromatography (HPLC) to determine aflatoxins in the extracts was then performed as described (Joshua 1993).

Enzymatic activities in extracts of koji mold

Aflatoxin B_1 and aflatoxin G_1 are enzymatically produced through the following biosynthetic pathway (Yabe et al. 1991a): acetate/malonate→ norsolorinic acid (NA)→ averantin (AVN)→ 5′-hydroxyaverantin (HAVN)→ averufin (AVR)→ versiconal hemiacetal acetate (VHA)→ versiconal (VHOH)→ versicolorin B (VB)→ versicolorin A (VA)→ demethylsterigmatocystin (DMST)→ sterigmatocystin (ST)→ *o*-methylsterigmatocystin (OMST)→ aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1). During this pathway there is a metabolic grid between VHA, versiconol acetate (VOAc), versiconol (VOH) and VHOH (Yabe et al. 1991a). To investigate the enzymes involved in aflatoxin biosynthesis, we quantified the enzymatic activities of nine reactions: (1) NA to AVN, (2) AVN to HAVN, (3) HAVN to AVR, (4) VHA to VHOH, (5) VHA to VOAc, (6) VHOH to VB, (7) DMST to ST, (8) ST to OMST, and (9) OMST to $AFB_1$and AFG_1. Among these intermediates VHOH was too unstable to isolate or quantify. Therefore, instead of measuring VHA→ VHOH conversion we quantified VOAc→ VOH conversion catalyzed by the same esterase (Yabe et al. 1991a). For the same reason, we used VHA as the substrate for the cyclase reaction converting VHOH to VB. VHA was converted to VHOH with the addition of excess porcine esterase and successively converted to VB by the cyclase (Yabe and Hamasaki 1993). The mycelia of strain 477 or NIAH-26 grown on YES medium were harvested after incubation for 4 days at 28°C then ground in the extraction buffer [0.2 M potassium phosphate (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 10% glycerol]. YEP medium (2% yeast extract, 20% peptone) was used as aflatoxin-non-inducing medium for comparison (Abdollahi and Buchanan 1981). The extracts were centrifuged at 20,000 *g* for 15 min at 4°C and the supernatant was collected as cell-free extracts, which was centrifuged again at 105,000 *g* for 90 min. The pellet was suspended in the extraction buffer as microsome fraction (Yabe et al. 1991b). Reaction mixture contained 60 mM potassium phosphate (pH 7.5), 10% glycerol, 60 µM of each substrate, and cell-free extract with 1.5 mg/ml final protein concentration (for reactions 1 and 3–9) or microsome fraction with 1.0 mg/ml final protein concentration (for reaction 2). The following were added to the appropriate reactions: 2 mM NADPH (reactions 1 and 2), 2 mM NAD (reaction 3), 2 mM NADPH and 100 ppm dichlorvos (reaction 5), 4.0 mg/ml porcine esterase (reac-

Table 1 *Aspergillus sojae* and *Aspergillus parasiticus* strains tested

	CBS designation	ATCC designation	Conidia		Colony color with age on CYA
			Surface	Diameter (µm)	
A. sojae					
NISL1478A	CBS100936	ATCC201946	Rough	5–8	Olive brown
NISL1478B	CBS101165	ATCC201938	Rough	5–8	Olive brown
NISL1777	CBS100932	ATCC201940	Rough	5–8	Olive brown
NISL1849	CBS100934	ATCC201939	Rough	5–8	Olive brown
NISL1905	CBS100931	ATCC201941	Rough	5–8	Olive brown
NISL1909	CBS100929	ATCC201942	Rough	5-8	Olive brown
NISL1920A	CBS100935	ATCC201943	Rough	5–8	Olive brown
NISL1939A	CBS100930	ATCC201944	Rough	5–8	Olive brown
NISL1939B	CBS100933	ATCC201945	Rough	5–8	Olive brown
Strain 477[a]			Rough	5–8	Olive brown
A. parasiticus					
IFO4082			Rough	3–6	Dark green
IFO30179			Rough	3–6	Dark green

[a] A high-protease mutant of IFO4241 which is currently used for soy sauce fermentation

tion 6), 10 μM S-adenosyl-methionine (SAM) and 4.0 mg/ml *N*-ethylmaleimide (reaction 7), 10 μM SAM (reaction 8), and 2 mM NADPH, 10.0 mg/ml liposome, and 20.0 mg/ml bovine serum albumin (reaction 9). The reaction mixtures (final volume, 50 μl) were incubated at 37°C (reactions 1 and 3–8), 30°C (reaction 2), or 24°C (reaction 9) for 1 h. After incubation, 75 μl of chloroform (reactions 1 and 6–9) or ethylacetate (reactions 2–5) were added to the mixture and shaken vigorously. The organic phase was collected and analyzed using HPLC as described previously (Yabe et al. 1991b, 1993, 1998, 1999; Yabe and Hamasaki 1993; Matsushima et al. 1994).

Southern and Northern analysis of aflatoxin biosynthesis-related genes in *A. sojae*

The DNA and RNA analysis were carried out basically following the methods of Maniatis et al. (1982). Fungal genomic DNA was extracted by using Sepa Gene (Sanko Pure Chemical, Tokyo, Japan). Each strain was grown in YES aflatoxin-inducing liquid medium at 30°C for 3 days and total RNA were extracted by using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The DNA fragments for probes were amplified from *A. parasiticus* NIAH-26 by PCR; the primers were 5′-GGT AAC AAC TGG GCC AAG GGT CAC-3′ and 5′-AGG CAT CCT GGT ACT GCT GGT ACT C-3′ for a 1.0-kb β-tubulin probe (Wu et al. 1996), 5′-GGC TGT CAA CTA CGG CTG AC-3′ and 5′-AAC AAG CAA CAA CAC GTT CC-3′ for a 1.0-kb *aflR* probe (Chang et al. 1995b), 5′-GTC GGG ACG CTC TTC ATC AAA CG-3′ and 5′-GCC CCG GGC ATG ATC GTA TC CAC CCA TCG-3′ for a 4.5-kb *pksA* probe (Chang et al. 1995a), and 5′-CCA GCA CGA TCA AGA GAG GCT CTC TAC GCC-3′ and 5′-GTG GAT AAC GAA GTG CCC CGA TGT AGT CTC C-3′ for a 0.9-kb *nor1* gene probe (Trail et al. 1994). The amplified DNA fragments were labeled with digoxigenin (DIG)-11-dUTP by using PCR DIG Probe Synthesis kit (Boehringer Mannheim, Mannheim, Germany). Southern and Northern hybridization procedures and the detection were carried out as described in the suppliers' manuals.

Results

Identification of *Aspergillus* strains

The conidia of the tested koji mold strains showed brown with age and larger conidial diameter (5–8 μm) that were typical characteristics of *A. sojae*, whereas *A. parasiticus* strains showed dark-green and smaller (3–6 μm) conidia (Table 1). On modified minimal medium plates containing bleomycin, the colonies of *A. parasiticus* grew larger than 16 mm in diameter whereas soy sauce koji mold strains did not grow or formed only microcolonies without sporulation (Table 2). The soy sauce koji molds tested in this study were therefore distinguished from *A. parasiticus* on the basis of morphology and bleomycin sensitivity.

Determination of aflatoxin production

Table 3 shows the production of aflatoxins (B_1, B_2, G_1 and G_2) by *Aspergillus* strains in aflatoxin-inducing medium and in koji mash for soy sauce production. IFO4082 and IFO30179, aflatoxigenic strains of *A. parasiticus*, produced 2.5–1,380 ppb of aflatoxins in YES medium. These strains also produced aflatoxins in the koji mash. In contrast, no aflatoxins were detected by HPLC from YES medium grown soy sauce koji mold strains. They were also grown on koji mash to monitor the industrial process of soy sauce production. No aflatoxin was detected from koji mash with soy sauce koji mold strains cultured.

Table 2 Diameter (mm) of *A. sojae* and *A. parasiticus* colonies grown on modified minimal medium

	Bleomycin	
	+	–
A. sojae		
NISL1478A	0	26
NISL1478B	0	31
NISL1777	0	27
NISL1849	mc[a]	26
NISL1905	mc	30
NISL1909	0	23
NISL1920A	mc	37
NISL1939A	mc	36
NISL1939B	0	35
Strain 477	mc	37
A. parasiticus		
IFO4082	16	26
IFO30179	21	29

[a] Microcolony (<2 mm) without sporulation

Table 3 Aflatoxin production by *A. sojae* and *A. parasiticus* cultured on aflatoxin-conducive media (YES) or koji mash

Strains	YES media (ppb)				Koji mash (ppb)			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
A. sojae								
NISL1478A	–[a]	–	–	–	–	–	–	–
NISL1478B	–	–	–	–	–	–	–	–
NISL1777	–	–	–	–	–	–	–	–
NISL1849	–	–	–	–	–	–	–	–
NISL1905	–	–	–	–	–	–	–	–
NISL1909	–	–	–	–	–	–	–	–
NISL1920A	–	–	–	–	–	–	–	–
NISL1939A	–	–	–	–	–	–	–	–
NISL1939B	–	–	–	–	–	–	–	–
Strain 477	–	–	–	–	–	–	–	–
A. parasiticus								
IFO4082	24.1	2.5	314.7	22.5	190.2	6.6	2.3	27.5
IFO30179	352.9	29.6	1380	139.4	24.0	0.3	13.9	1.8

[a] Not detected

Enzymatic activities of koji mold for aflatoxin biosynthesis

To analyze the enzymatic activity of various steps of aflatoxin biosynthesis in koji mold, we incubated the precursor for each step with the cell-free extract from each fungus, and the pigments produced were analyzed using HPLC (Table 4). Strain 477 was chosen as the representative *A. sojae* strain for this analysis because it was typical of the industrial strains used for soy sauce fermentation.

Table 4 Enzymatic conversion of various intermediate during the aflatoxin biosynthetic pathways by extract of *A. sojae* strain 477 and *A. parasiticus* NIAH-26 grown on YES medium

Substrate	Product	Strain 477 (pmol/mg/min)	NIAH-26[b] (pmol/mg/min)
NA	AVN	–[c]	47.2
AVN	HAVN	–	77.2
HAVN	AVR	–	281
VOAc	VOH	38.0	131
VHA	VOAc	–	281
VHOH(VHA)[a]	VB	–	140
DMST	ST	–	14.5
ST	OMST	–	46.7
OMST	AFB_1	–	2.88
	AFG_1	–	0.0117

[a] VHOH was produced in the reaction mixture as the substrate from VHA by addition of excess porcine esterase and successively converted to VB by the cyclase
[b] The enzymatic activity was described as the amount of product from 60 µM substrate per protein content per minute in 50 µl reaction mixture
[c] Not detected; see Materials and methods for abbreviations

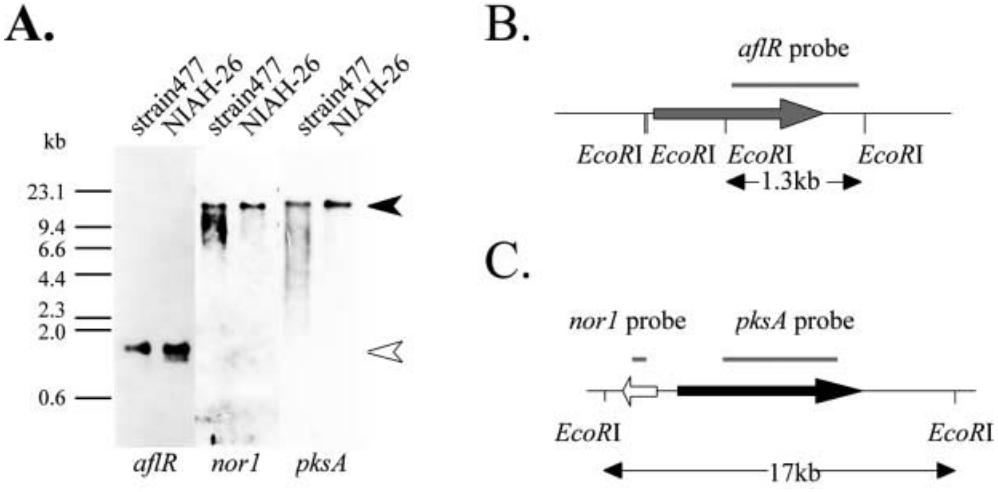

Fig. 1A–C Aflatoxin biosynthesis-related genes in the genomic DNA of *Aspergillus sojae*. **A** Southern analysis was carried out for genomic DNA of strain 477 with aflatoxin-related genes. Each lane contained 10 µg of *Eco*RI-digested genomic DNA. Hybridization with probes for *aflR*, *nor1*, and *pksA* showed the expected hybridization at 1.3 kb (*open arrowhead*) and 17 kb (*solid arrowhead*) in both *A. sojae* strain 477 and *A. parasiticus* NIAH-26. **B** The location of *aflR* gene probe on the physical map. The *aflR* probe hybridizes to a 1.3-kb *Eco*RI fragment. The coding region of *aflR* was indicated as a *gray arrow*. **C** The location of the *pksA* and *nor1* gene probes on the physical map. Both the *nor1* and *pksA* probes hybridize to 17-kb *Eco*RI fragments. The coding region of *nor1* is shown as an *open arrow*. The coding region of *pksA* is indicated as a *solid arrow*

The cell-free extract from NIAH-26 cultured in YES medium showed the enzymatic activities for all steps in the aflatoxin biosynthetic pathway that were tested in this study. On the other hand, the cell-free extract from *A. sojae* strain 477 had no detectable activity for any of the enzymes involved in the aflatoxin biosynthetic pathway that we tested except for the esterase activity converting VOAc to VOH (Table 4). Using cell-free extracts of strain 477, 38.0 pmol/mg per min (cultured in YES) or 37.2 pmol/mg per min (cultured in YEP) of VOH were produced from VOAc. Meanwhile by using cell-free extracts of NIAH-26, 131 pmol/mg per min (cultured in YES) or 43.2 pmol/mg per min (cultured in YEP) of VOH were produced from VOH. The esterase activity of strain 477 cultured in YES medium did not increase compared with that in YEP aflatoxin-non-inducing medium. The esterase activity of NIAH-26, however, increased in YES medium in accordance with aflatoxin-inducing conditions. We concluded that this esterase activity of strain 477 was due to nonspecific enzymatic activity and was different from the enzyme that was induced in NIAH-26 cultured in YES medium.

Aflatoxin biosynthesis-related genes in genomic DNA from *A. sojae* strain 477

Genomic DNAs from NIAH-26 and strain 477 were digested with *Eco*RI and analyzed by Southern hybridization with *aflR*, *pksA* and *nor1* probes. The digestion of NIAH-26 genomic DNA with *Eco*RI would generate a 1.3-kb fragment including *aflR* and a 17-kb fragment including both *pksA* and *nor1*. A 1.3-kb band of the *aflR* and 17-kb bands of the *pksA* or *nor1* were detected in the genomic DNA of strain 477 as well as NIAH-26 (Fig. 1A). Therefore, the genomic DNA of strain 477, which is not aflatoxigenic, contains homologs for three aflatoxin biosynthesis-related genes.

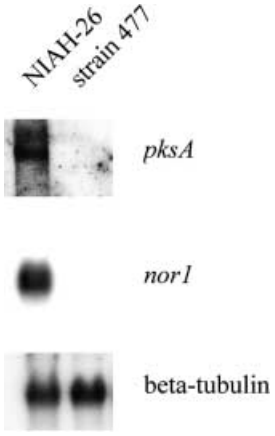

Fig. 2 Northern analysis of aflatoxin biosynthesis-related mRNA in *A. sojae* strain 477. Both *A. parasiticus* NIAH-26 and *A. sojae* strain 477 were grown in YES media for 3 days, then Northern analysis was performed. Each lane contained 10 μg of total RNA. For a positive control, a β-tubulin probe was used. No *pksA* or *nor1* transcripts were detected in strain 477, whereas NIAH-26 showed expression of these aflatoxin biosynthesis-related genes

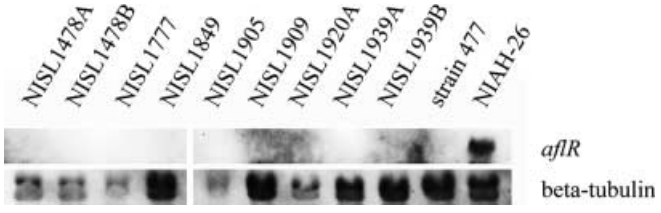

Fig. 3 Northern hybridization of the *aflR* transcript from *A. sojae* strains. Ten micrograms of total RNA was loaded in each lane, β-tubulin was used as a positive control. *A. parasiticus* NIAH-26 only showed specific hybridization to the *aflR* probe, whereas no transcripts were detected in soy sauce koji molds (*A. sojae*)

The transcripts of aflatoxin-related genes in *A. sojae* strain 477

To analyze the transcription of aflatoxin biosynthesis-related genes in NIAH-26 and strain 477, Northern analyses were performed for *pksA* and *nor1* (Fig. 2). Unlike NIAH-26, no transcripts were detected in the total RNA of strain 477 cultured in aflatoxin-inducing YES medium.

To examine the transcription of *aflR*, total RNAs from various strains of soy sauce koji mold were analyzed by Northern analysis. As shown in Fig. 3, no *aflR* transcripts were detected from soy sauce koji molds including strain 477 cultured in aflatoxin-inducing YES medium whereas *A. parasiticus* NIAH-26 showed an *aflR*-specific hybridization. Therefore, none of the koji molds examined here did transcribed *aflR*.

Discussion

We examined koji molds used for soy sauce production after distinguishing them from *A. parasiticus* with regard to physiological, morphological, and molecular biological characteristics. The olive conidial color with age and larger conidial diameters of these strains were typical of *A. sojae* strains and were markedly different from those described for *A. parasiticus* strains (Table 1; Klich and Pitt 1988). Bleomycin is known to inhibit the growth of *A. sojae* molds whereas it scarcely affects the growth of *A. parasiticus* strains (Klich and Mullaney 1989). We showed that the soy sauce koji mold strains were sensitive to bleomycin whereas *A. parasiticus* molds were resistant to it (Table 2). Their morphological and physiological characteristics indicated that the soy sauce koji mold strains tested in this study belonged to *A. sojae*. The analysis of aflatoxin production showed that none of these strains produced aflatoxins in aflatoxin-inducing liquid medium or in koji mash, which is used for food production (Table 3). It should be emphasized that all koji molds tested in this study were non-aflatoxigenic, classifiable into *A. sojae* and were readily distinguishable from *A. parasiticus* species.

We measured the enzymatic activities associated with various steps of aflatoxin biosynthesis in strain 477, an industrial strain used for soy sauce production, to investigate whether absence of aflatoxin production in koji molds was due to a single defect in any step in aflatoxin biosynthesis or the loss of whole pathway. The cell-free extract from strain 477 did not show meaningful enzymatic activities required for aflatoxin biosynthesis (Table 4). Therefore, the loss of aflatoxin biosynthesis in strain 477 is not because of single defect in any particular steps in the sequence of aflatoxin-related enzymes but because of the absence of expression of the all enzymes needed for aflatoxin biosynthesis.

The presence of the aflatoxin pathway genes, *aflR*, *pksA* and *nor1* in genomic DNA of *A. sojae* strain 477 was confirmed by Southern analysis (Fig. 1). In addition, PCR analysis revealed that these genes exist in other koji molds (data not shown). Similar results have been reported previously for various *A. sojae* strains (Klich et al. 1995). Therefore, *A. sojae* fungi may typically have homologs of genes involved in aflatoxin biosynthesis.

However, the mere presence of such homologs does not signify that *A. sojae* strains are potently aflatoxigenic. Strain 477 lacked transcripts of the aflatoxin biosynthesis-related genes (Fig. 2). Moreover, all soy sauce koji molds tested in this study lacked transcription of *aflR* gene even when they were grown in YES aflatoxin-inducing medium (Fig. 3). In aflatoxigenic *Aspergillus* species, *aflR* is a main transcriptional regulatory gene for aflatoxin biosynthesis, and its deficiency causes the loss of expression of aflatoxin biosynthesis-related genes, leading to lack of aflatoxin production (Chang et al. 1993, 1995b; Payne et al. 1993). Therefore, we conclude that the loss of *aflR* expression in *A. sojae* is likely responsible for the lack of transcription of other aflatoxin-related genes, which leads to the inability to produce aflatoxin in all the *A. sojae* strains tested, including strain 477. Interestingly, *A. oryzae* strains are also lacking *aflR* transcription (Kusumoto et al. 1998b) regardless of the presence or absence of the aflatoxin gene cluster (Kusumoto et al. 1998a, 2000). As the *aflR* sequence of *A. oryzae* is identical to that of aflatoxigenic fungi (Watson et al. 1999), it is suggested that there is an unknown defect in signal transduction that activates transcription of *aflR* in *A. oryzae*. The same defect may exist in *A. sojae* and cause the retardation of the transcription of *aflR*, which causes the lack of aflatoxin biosynthesis.

In contrast, Klich et al. (1997) reported that transcripts of *aflR* occurred in two strains of *A. sojae* although no other aflatoxin-related genes were expressed. Watson et al. (1999) reported that strains of *A. sojae* had a nonsense mutation in *aflR* gene that would result in truncated AflR protein by 62 amino acid. These findings suggest that mutations in the *aflR* gene contribute to the loss of its functionality or that there is another defect in the signal transduction system mediated by AflR protein. The absence of aflatoxin biosynthesis in *A. sojae* might be due to multiple defects in the expression of genes involved in aflatoxin biosynthesis. Further investigations into the function of AflR are in progress.

Here we show that the ten tested soy sauce koji strains do not produce aflatoxin due to loss of expression of enzymatic activities of the aflatoxin biosynthetic pathway, which is due to loss of transcription of *aflR* gene. The *aflR* gene is considered as essential element for expression of aflatoxin biosynthesis and loss of *aflR* transcription would lead to the loss of expression of all genes involved in aflatoxin biosynthesis at transcription level or at enzyme level as we showed. These multiple inhibitions of aflatoxin biosynthesis strongly support the safety of these strains for food production.

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