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Non-Aflatoxigenicity of Commercial *Aspergillus oryzae* Strains Due to Genetic Defects Compared to Aflatoxigenic *Aspergillus flavus*

Lin Tao and Soo Hyun Chung*

Department of Food and Nutrition, College of Health Science, Korea University, Seoul 136-703, Republic of Korea

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**Corresponding author*
Phone: +82-2-940-2854;
Fax: +82-2-941-7825;
E-mail: chungs59@korea.ac.kr

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Aspergillus oryzae is generally recognized as safe, but it is closely related to *A. flavus* in morphology and genetic characteristics. In this study, we tested the aflatoxigenicity and genetic analysis of nine commercial *A. oryzae* strains that were used in Korean soybean fermented products. Cultural and HPLC analyses showed that none of the commercial strains produced detectable amount of aflatoxins. According to the molecular analysis of 17 genes in the aflatoxin (AF) biosynthetic pathway, the commercial strains could be classified into three groups. The group I strains contained all the 17 AF biosynthetic genes tested in this study; the group II strains deleted nine AF biosynthetic genes and possessed eight genes, including *aflG*, *aflI*, *aflK*, *aflL*, *aflM*, *aflO*, *aflP*, and *aflQ*; the group III strains only had six AF biosynthetic genes, including *aflG*, *aflI*, *aflK*, *aflO*, *aflP*, and *aflQ*. With the reverse transcription polymerase chain reaction, the group I *A. oryzae* strains showed no expression of *aflG*, *aflQ* and/or *aflM* genes, which resulted in the lack of AF-producing ability. Group II and group III strains could not produce AF owing to the deletion of more than half of the AF biosynthetic genes. In addition, the sequence data of polyketide synthase A (*pksA*) of group I strains of *A. oryzae* showed that there were three point mutations (two silent mutations and one missense mutation) compared with aflatoxigenic *A. flavus* used as the positive control in this study.

Keywords: Commercial *Aspergillus oryzae*, non-aflatoxigenicity

Introduction

Aflatoxin (AF), a group of polyketide-derived furanocoumarins, was characterized after the acute hepatotoxic disease in turkeys (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal. The four major aflatoxins are called B_1, B_2, G_1, and G_2, based on their fluorescence (blue or green) under UV light and relative chromatographic mobility during thin-layer chromatography. AF B_1 is the most potent natural carcinogen known and is usually the major AF produced by toxigenic strains. The data on AF as a human carcinogen are far more dreadful than the data implicating it in acute human toxicities. Exposure to aflatoxins with the diet is considered as an important risk factor for the development of primary hepatocellular carcinoma, particularly in individuals already exposed to hepatitis B [2].

A. oryzae is an important fungus that has been used for centuries in the oriental food fermentation industry. By contrast, *A. flavus* is notorious for its production of aflatoxins, for causing aspergillosis in humans and animals, and as an opportunistic pathogen of animals and plants [22]. So far, *A. oryzae* is generally recognized as safe (GRAS). However, previous studies reported that *A. oryzae* is closely related to *A. flavus* in morphology and genetic characteristics [23]. *A. oryzae* and *A. flavus* belong to *Aspergillus* section *Flavi*. These two fungi are very similar in genome size and number of predicted genes: 36.7 Mb and 12,197 genes for *A. flavus*, and 36.7 Mb and 12,079 genes for *A. oryzae*. The high degree of sequence similarity in genes between the two fungi suggests that they may be ecotypes of the same species and that *A. oryzae* has resulted from the domestication of *A. flavus* [21].

The molecular biological elucidation of AF non-productivity in *A. oryzae* strains has important implications in terms of verifying the safety of the fermented products employing

those strains [12–14, 17, 20]. The molecular biological techniques, including analysis of rDNA internal transcribed spacer regions, restriction fragment length polymorphism, and hybridization with aflatoxin biosynthetic genes, are able to distinguish the *A. flavus/oryzae* group from the *A. parasiticus/sojae* group but cannot differentiate *A. oryzae* from *A. flavus* as a distinct species [5]. The recent studies of whole genomic sequencing and analysis of the aflatoxin biosynthetic cluster of *A. oryzae* and *A. flavus* can provide us with the difference between these two fungi [4, 11, 17, 21, 26].

AF biosynthesis is almost fully characterized and involves the coordinated expression of approximately 25 genes clustered in a 70 kb DNA region [28]. The genes involved in AF biosynthesis are clustered together on the fungal gene. AFLR, a translation product of the *aflR* gene in this cluster, is a positive transcriptional regulator that promotes transcription of AF biosynthetic genes [16]. *aflJ* (translation product is AFLJ), another transcriptional regulator, is also needed for AF biosynthesis [3, 18]. *A. oryzae* possesses the AF biosynthesis gene homolog cluster but does not produce aflatoxin [1, 11]. Recently, to reduce AF contamination on crops, a biocontrol strategy was applied to use non-aflatoxigenic *A. flavus* strains to competitively exclude field toxigenic *Aspergillus* species [7, 8, 19]. After molecular analysis of those strains, researchers found that they lacked the producing of aflatoxins owing to the single nucleotide mutations in the *pksA* gene [9].

In this study, we collected nine commercial strains of *A. oryzae* currently used in the soybean fermentation industry in Korea, and molecular analyses were conducted to provide the evidence of non-aflatoxigenicity of the strains for commercial use. For this, two regulatory genes (*aflR* and *aflJ*) and 15 structural genes in the AF biosynthetic pathway were analyzed to distinguish *A. oryzae* from aflatoxigenic *A. flavus*, based on the presence or absence of aflatoxin-specific DNA or mRNAs and *pksA* gene sequencing as well.

Materials and Methods

Fungal Strains

Commercial strains of *A. oryzae* M3, M5, M6, M7, M9, M11, M15, M17, and M18 were obtained from soybean fermentation companies in Korea. *A. flavus* KACC 2030 used as a positive control for AF production was supplied by Korean Agricultural Culture Collection (KACC) (Suwon, Korea). All the strains were inoculated onto Czapek Agar (MBcell, Seoul, Korea) slants and incubated at 25°C for 7 days, and then conidia were collected for the following experiments.

Determination of Aflatoxin Production

The AF production by fungal strains was determined using high-performance liquid chromatography (HPLC) (Dionex Ultimate 3000, Sunnyvale, CA, USA) [10]. A 0.1 ml volume of *A. oryzae* spore suspension (1×10^6 spores/ml) was inoculated into 50 ml of Potato Dextrose Broth (PDB) (MBcell) in a 250 ml Erlenmeyer flask at 25°C. After 14 days, the fungal culture broth was filtered through Whatman No. 1 filter paper (GE Healthcare Co., Buckinghamshire, UK), and 15 ml of the filtrate was then diluted with phosphate-buffered saline (pH 7.5) to 90 ml. The mixture was passed through a Whatman GF/A glass filter (GE Healthcare Co.), and 50 ml of the filtrate was loaded onto an immunoaffinity column (AflaTest, Vicam Co., Milford, MA, USA) at a flow rate of approximately 1 drop per second for clean-up. After washing the column with 10 ml of water at the same flow rate, aflatoxin was eluted with 2 ml of methanol. The eluate was evaporated at 40°C under a stream of N_2 until dry. The dry residue was derivatized by adding 200 µl of trifluoroacetic acid, and the mixture was left to stand for 30 min before it was diluted with 800 µl of acetonitrile-water (10:90 (v/v)). This derivatized sample was filtered through a 0.22 µm membrane filter, and the filtrate was used for HPLC analysis. Separation of AF B_1, B_2, G_1, and G_2 from the injected 50 µl of samples was carried out using a Waters Nova-Pack C18 column (150 × 3.9 mm, 5 µm; Milford, MA, USA). The mobile phase was acetonitrile-methanol-water (17:17:66 (v/v/v)) pumped at a constant flow rate of 0.5 ml/min. The quantitative determination of each AF was carried out using a fluorescence detector (excitation: 360 nm; emission: 440 nm).

Preparation of Genomic DNA for Polymerized Chain Reaction

Approximately 10^5 conidia were inoculated into 8 ml of PDB in a test tube. The tube was incubated at 25°C for 3 days with 120 rpm shaking, and then fungal genomic DNA was extracted from the lyophilized mycelia. Briefly, the ground lyophilized mycelia was mixed with lysis buffer (50 mM EDTA, 0.2% SDS, pH 8.5) and incubated at 65°C for 1 h, and then purification with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitation with isopropanol were conducted. Finally, the precipitation was washed with 70% ethanol and resuspended in 50 µl of TE solution (10 mM Tris-HCl, 0.1 mM EDTA).

Seventeen primer pairs previously designed for the specific amplification of AF biosynthetic genes [4, 6, 11, 24–26] were applied (Table 1). The PCR procedure was performed with 50 µl of reaction mixture containing 5.0 µl of 10× Taq buffer, 3.0 µl of 2.5 mM dNTP, 0.3 µl of Taq polymerse, 0.4 µl of each primer (SolGent, Deajeon, Korea), 39.9 µl of distilled water, and 1.0 µl of the DNA as template. Cycling parameters were 5 min at 94°C, 55°C (primer 3, 4, 5, 8, 10, 11, 14, 15, 16, and 18) or 60°C (primer 6, 7, 9, and 17) or 65°C (primer 1, 2, 12, and 13) for 35 cycles, 30 sec at 94°C, 60 sec at 55°C, 90 sec at 72°C, with a final extension at 72°C for 7 min in a DNA thermal cycler (Astec PC-708 Program Temp Control System, Tokyo, Japan). DNA fragments were separated in a 1.5% agarose gel electrophoresis.

Table 1. Primers, target genes, primer sequences, and expected PCR and RT-PCR product sizes.

No.	Primer code	Gene	Sequence (5′–3′)	PCR product size (bp)	RT-PCR product size (bp)
1	aflR-F	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1,034	1,034
	aflR-R		CCGTCAGACAGCCACTGGACACGG		
2	aflJ-5 f(F)	*aflJ*	CACCATGACCTTGACTGACCTAGAAACCTG	1,455	1,455
	aflJ-3(R)	*(aflS)*	TTAATATCGGTTGTCATCGTTATCCACACG		
3	Fas-1(F)	*aflA*	ATGGAAATGGCTCAGTTCGAATGG	350	350
	Fas-1(R)		AACCAACCGGTCTCCTGTTG		
4	pksA(F)	*aflC*	CACAGCCCAGTGACAGCTAC	399	399
	PksA(R)	*(pksA)*	ACTGCCACTTAGGGTGATGG		
5	Nor1-F	*aflD*	ACGGATCACTTAGCCAGCAC	990	812
	Nor1-R		CTACCAGGGGAGTTGAGATCC		
6	norA(F)	*aflE*	GGCTGGAAAGGGGTAATGGG	697	697
	norA(R)		TCTTGCGACCCTCACGAGAA		
7	n-c(F)	*aflF-aflU*	GTGCCCAGCATCTTGGTCCA	322	322
	n-c(R)	*(norB-cypA)*	AGGACTTGATGATTCCTCGTC		
8	AvnA-F	*aflG*	ATGCATCTTCCGCCCTTC	1,163	1,163
	AvnA-R		ATAGCGAGGTTCCAGCGTAA		
9	AdhA-F	*aflH*	ATCACGTCTGCTGCGATGTG	500	500
	AdhA-R		AGCAGCCATAGCCTTCTTCA		
10	AvfA-F	*aflI*	GGTCACATACGCTCTTCTCG	811	811
	AvfA-R		CACAACGCCGTCAACTACTG		
11	Vbs-F	*aflK*	GCTGTCAATCCGACCATCTT	1,980	1,897
	Vbs-R		GCAATCTTCTCAGCCAGAGC		
12	verB(F)	*aflL*	CACGGCAGCGTTATTGATCATCTC	715	700
	verB(R)		GATGCACCATGACCTCATGCGTTA		
13	Ver1-F	*aflM*	ATGTCGGATAATCACCGTTTAGATGGC	896	756
	Ver1-R		CGAAAAGCGCCACCATCCACCCCAATG		
14	OmtB(F)-F	*aflO*	GCCTTGACATGGAAACCATC	1,333	1,131
	OmtB(F)-R		CCAAGATGGCCTGCTCTTTA		
15	Omt1-F	*aflP*	GCCTTGCAAACACACTTTCA	1,490	1,210
	Omt1-R		AGTTGTTGAACGCCCCAGT		
16	Ord1(P)-F	*aflQ*	CGACTGTTGGCCTTTTCATT	1,088	1,088
	Ord1(P)-R		ATAGCGAGGTTCCAGCGTAA		
17	aflT(F)	*aflT*	GCACCAAATGGGTCTTTCTCGT	1,077	1,077
	aflT(R)		ATCCACGGTGAAGAGGGTAAGG		
18	Tub1-F	*tub1*	GCTTTCTGGCAAACCATCTC	1,498	1,198
	Tub1-R		GGTCGTTCATGTTGCTCTCA		

RNA Preparation for Reverse Transcriptional PCR

The tested strains were inoculated in the same conditions for DNA extraction. Total RNA was prepared using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Fifteen AF biosynthetic genes applied for the above conventional PCR (except *aflH* and *aflI*) were also used in the RT-PCR (Table 1). The housekeeping gene *tub1* coding β-tubulin was chosen and used as a system control for reverse transcription. Reverse transcription was performed using an Ominiscript Kit (Qiagen), and the PCR procedure was carried out as described above.

Sequencing of *pksA* Gene

A part of the *pksA* gene encoding the polyketide synthase for aflatoxin biosynthesis was amplified from group I *A. oryzae* strains

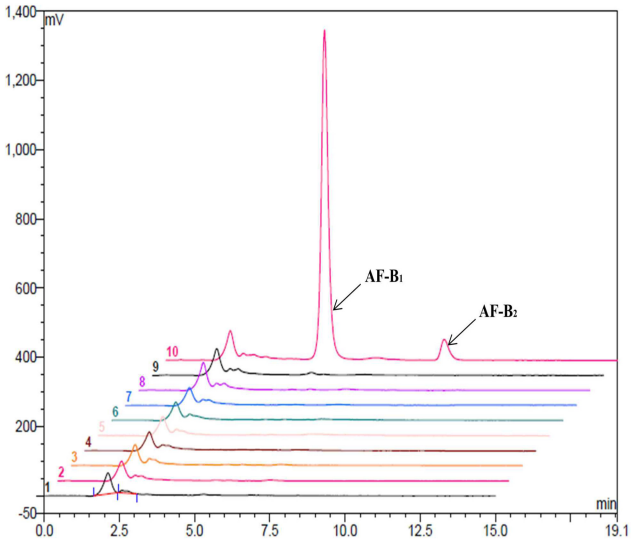

Fig. 1. Chromatogram of aflatoxin analysis of commercial *A. oryzae* strains and *A. flavus* KACC 2030: 1, *A. oryzae* M3; 2, *A. oryzae* M5; 3, *A. oryzae* M6; 4, *A. oryzae* M7; 5, *A. oryzae* M9; 6, *A. oryzae* M11; 7, *A. oryzae* M15; 8, *A. oryzae* M17; 9, *A. oryzae* M18; and 10, *A. flavus* KACC 2030.

Potato Dextrose Broth was used for the production of aflatoxin by each fungal strain. After 14 days of incubation at 25°C, the fungal culture filtrates were used for aflatoxin analysis.

and *A. flavus* KACC 2030 by PCR. The 399 bp amplicons were purified using an *EZWay* PCR Clean-up Kit (Komabiotech, Seoul, Korea) and sequenced by Solgent Co. Ltd (Daejeon, Korea).

Results

Aflatoxin Production

After extraction of PDB-culture filtrates with an immunoaffinity column, the eluate was analyzed by fluorescence detection with HPLC, which allowed the separation and identification of AF B_1, B_2, G_1, and G_2. None of the commercial *A. oryzae* strains (M3, M5, M6, M7, M9, M11, M15, M17, and M18) produced detectable aflatoxins, whereas *A. flavus* KACC 2030 strain produced AF B_1 (12.5 μg/ml) and AF B_2 (0.9 μg/ml) after 14 days of incubation (Fig. 1).

AF Biosynthetic Gene Profiles of Commercial *A. oryzae*

After screening 17 genes (*aflR, aflJ, aflA, aflD, aflE, aflF-aflU, aflG, aflH, aflI, aflK, aflL, aflM, aflO, aflP, aflQ, aflT*, and *pksA*) through PCR-gel electrophoresis, nine strains of *A. oryzae* were divided into three groups (Table 2). Group I, strains of M3, M6, M7, M9 and M11, contained all the 17 AF biosynthetic genes, including the regulatory genes *aflR* and *aflJ*; Group II, strains of M15, M17, and M18, had eight genes, including *aflG, aflI, aflK, aflL, aflM, aflO, aflP* and *aflQ*; Group III, only M5, possessed six genes, including *aflG, aflI, aflK, aflO, aflP* and *aflQ*. It was clear that group II and group III strains were unable to produce AF owing to the lack of more than half of the AF biosynthetic genes tested in this study. However, based on the PCR results, we could not prove the non-AF production by group I strains at the molecular level.

Table 2. Classification of commercial *A. oryzae* strains based on PCR amplification patterns of aflatoxin biosynthetic genes.

Strains	Amplified genes by PCR	Number of strains (Incidence)	Classification
M3, M6, M7, M9, M11	*aflR, aflJ, aflA, aflC, aflD, aflE, aflF-aflU aflG, aflH, aflI, aflK aflL, aflM, aflO, aflP, aflQ, aflT,*	5 (55.5%)	Group I
M15, M17, M18	*aflG, aflI, aflK, aflL aflM, aflO, aflP, aflQ*	3 (33.3%)	Group II
M5	*aflG, aflI, aflK, aflO, aflP, aflQ*	1 (11.1%)	Group III

AF Biosynthetic Gene Expression of Group I *A. oryzae*

RT-PCR analysis was carried out to evaluate the AF biosynthetic gene expression of group I *A. oryzae* using the 15 pairs of primers that were used for the AF biosynthetic gene profiles mentioned above, except two primers for *aflH* and *aflI*. The primer pairs for the amplification of *aflH* and *aflI* gene expression could not generate their positive signals with aflatoxigenic *A. flavus* KACC 2030 in preliminary tests (data not shown).

The results showed that after the expression analysis of the 15 genes, M3, M6, and M7 strains generated 12 positive signals (*aflR, aflJ, aflA, aflD, aflE, aflF-aflU, aflK, aflL, aflO, aflP, aflT*, and *pksA*) (Table 3). Strains of M9 and M11 generated 14 positive signals (*aflR, aflJ, aflA, aflD, aflE, aflF-aflU, aflG, aflK, aflL, aflO, aflP, aflQ, aflT*, and *pksA*). Consequently, M3, M6 and M7 strains were incapable of producing aflatoxin owing to the lack of expression of *aflG, aflM*, and *aflQ*; M9 and M11 strains were unable to produce AF owing to the lack of expression of the *aflM* gene. It was reported that the *aflG* gene represented one of the early steps in the AF biosynthetic pathway, encoding a cytochrome P450 monooxygenase that converted averantin to 5P-hydroxy-averantin [27]. The research also showed that *aflM*

Table 3. Expression of aflatoxin biosynthetic genes by commercial *A. oryzae* and *A. flavus* KACC 2030.

Strains	Aflatoxin production	Gene expression															
		aflR	*aflJ*	*aflA*	*aflC*	*aflD*	*aflE*	*aflF-alfU*	*aflG*	*aflK*	*aflL*	*aflM*	*aflO*	*aflP*	*aflQ*	*aflT*	*tub1*
A. oryzae																	
M3	§—	+	+	+	+	+	+	+	§—	+	+	§—	+	+	§—	+	+
M6	§—	+	+	+	+	+	+	+	§—	+	+	§—	+	+	§—	+	+
M7	§—	+	+	+	+	+	+	+	§—	+	+	§—	+	+	§—	+	+
M9	§—	+	+	+	+	+	+	+	+	+	+	§—	+	+	+	+	+
M11	§—	+	+	+	+	+	+	+	+	+	+	§—	+	+	+	+	+
A. flavus KACC 2030																	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

In aflatoxin production, +: afltoxin production; §—: no aflatoxin production by HPLC.
In gene expression, +: amplification signal present; §—: amplification signal absent by RT-PCR.

and *aflQ* lay at the end of the biosynthetic pathway of aflatoxins: *aflM* was required for the conversion of versicolorin A to demethylsterigmatocystin; *aflQ* was involved in the conversion of *O*-methylsterigmatocystin to AF B_1 (and AF G_1) and of dihydrodemethylsterigmatocystin to AF B_2 (and AF G_2).

Determination of Mutations of *pksA* Gene of Group I *A. oryzae*

A gene sequencing for the 0.4 kb partial *pksA* gene region of group I *A. oryzae* and *A. flavus* KACC 2030 strains was performed. The sequence data showed that group I strains had three point mutations compared with *A. flavus* KACC 2030 (Fig. 2): T to C point mutation at nucleotide position 303, C to T point mutation at position 315, and G to A point mutation at position 325, respectively. The first two point mutations at positions 303 and 315 were silent mutations, which respectively induced the asparagine to asparagine codon and the arginine to arginine codon. The point mutation at nucleotide position 325, referring to a missense mutation, changed from GAG to AAG, resulting in an amino acid change within the polypeptide from glutamic acid to lysine.

Discussion

Kusumoto *et al.* [15] studied 39 strains of *A. oryzae* and found those strains could be classified into three groups according to the existence of five genes (*aflR, aflA, aflK, aflM,* and *pksA,*): In group I, almost all of the AF biosynthetic genes were preserved, containing 24 strains (61.5% of examined strains); in group II (28.2%), more than half of the AF biosynthetic genes (including *aflR*) were missed; in group III (10.2%), the AF biosynthetic genes were almost lost. Similarly, Tominaga *et al.* [26] analyzed the existence of seven AF biosynthetic genes (*aflR, aflD, aflE, aflG, aflK, aflL,* and *aflT*) and found that the 210 strains of *A. oryzae* tested were still classified into the three groups (58.1%, 36.7%, and 4.3%, respectively). We studied the existence of 17 AF biosynthetic genes with nine commercial *A. oryzae* strains and got a consistent result with Kusumoto *et al.* and Tominaga *et al.*; the nine *A. oryzae* strains in this study were

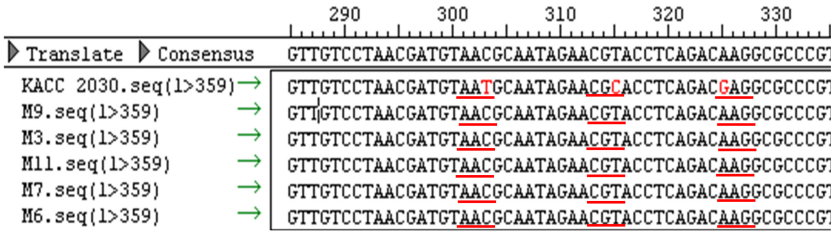

Fig. 2. Comparison of the *pksA* gene of group I *A. oryzae* strains (M3, M5, M6, M7, M9, and M11) with aflatoxigenic *A. flavus* KACC 2030.
The base sequences of 1–158 and 335–359 were omitted.

also divided into three groups and similar proportions (55.5%, 33.3%, and 11.1%, respectively). Group II and group III strains evidently could not produce AF owing to their gene deletions in the AF biosynthetic pathway. The group I strains, however, possessed intact AF biosynthetic genes, but still needs molecular evidence to explain their non-AF production. It is very important to have molecular evidence to prove that group I *A. oryzae* strains are incapable of producing aflatoxins and safe for commercial use.

Scherm *et al.* [24] suggested that RT-PCR could be efficiently used to distinguish between AF-producing and non-producing strains of *A. flavus* and *A. parasiticus by* measuring the presence of *aflD*, *aflO*, and *aflP* transcripts. In this paper, we studied the expression of two regulatory genes (*aflR* and *aflJ*) and 13 structural genes (*aflA*, *aflD*, *aflE*, *aflF-aflU*, *aflG*, *aflK*, *aflL*, *aflM*, *aflO*, *aflP*, *aflQ*, *aflT*, and *pksA*), and found that group I *A. oryzae* strains were not capable of AF production owing to the lack of expression of one gene (*aflM*) or three genes (*aflG*, *aflM*, and *aflQ*). The results proved the safety of group I *A. oryzae* strains in the molecular level of AF production. Furthermore, it also supported that RT-PCR was an effective method to distinguish *A. oryzae* from aflatoxigenic *A. flavus*.

Ehrlich and Cotty [9] reported that the *pksA* gene from an isolate of non-aflatoxigenic *A. flavus* had a G to A point mutation (position 192) compared with a strain of aflatoxigenic *A. flavus*, which induced a TGA stop codon that caused truncation of the resulting polyketide synthase. In our result, there was no stop codon present in the *pksA* gene of the *A. oryzae* strains, but depending on the open reading frame of the *pksA* gene, we found three positions of point mutations: two silent mutations and one missense mutation. The missense mutation was found to result in an amino acid change from glutamic acid to lysine. Whether the *pksA*-translated protein structure change of this missense mutation affects the production of AF is still not clear and needs to be studied in the future.

In summary, molecular approaches based on the presence or absence of AF-specific DNA or mRNA were employed in an attempt to distinguish nine commercial strains of *A. oryzae* and aflatoxigenic *A. flavus* KACC 2030 in this study. PCR experiments using 17 genes in the AF biosynthetic pathway differentiated four of the nine strains of *A. oryzae* from aflatoxigenic *A. flavus*, and the other five strains needed RT-PCR for the expression of the aflatoxigenic genes. One (*aflM*) or three genes (*aflG*, *aflM*, and *aflQ*) were not expressed in the five *A. oryzae* strains, which had all the 17 genes tested. Our results show the molecular evidence of non-aflatoxigenicity of the *A. oryzae* strains used commercially in Korea.

Acknowledgments

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