Koji Molds and Mycotoxin Production Genes

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Abstract

Our group found that some strains, but not all, of *A. oryzae* and *A. sojae* have homologs of *ver-1*, a gene essential for aflatoxin biosynthesis. We compared the nucleotide sequences of *ver-1* homologs in *A. oryzae*, *A. sojae*, *A. flavus* and *A. parasiticus*, and that the homologs in *A. oryzae* and *A. sojae* exhibited an extremely high degree of sequence identity with that of *A. flavus* and *A. parasiticus*. Transcripts of the homologs in *A. oryzae* and *A. sojae* are not detected. Moreover, we examined the expression of the *aflR*-homolog genes in *A. oryzae* strains having this gene, which encodes a regulatory protein for other aflatoxin synthesis genes, and demonstrated that no transcripts of the *aflR*-homolog genes in the examined strains of *A. oryzae* were detected under the aflatoxin producing condition. Considering the above observations together with the information from other research groups, non-aflatoxigenic *A. oryzae* and *A. sojae* basically have some homologs of aflatoxin synthesis genes. Thus, they are possibly domesticated, differentiated from aflatoxin producer evolutionally. Nevertheless, their transcripts are not found in any of the strains and some homologs are not found in the examined strains. Therefore these species do not produce aflatoxin because the synthesis genes are neither transcribed nor existent, probably due to mutations during repetitive use as domesticated strains in manufacturing fermented foods. Further characterization of the regulatory mechanism for aflatoxin production might resolve why *koji* molds having the homologs do not produce aflatoxin.

Key words: *Aspergillus oryzae*, *Aspergillus sojae*, aflatoxin, synthesis gene, gene expression

Introduction

The Genus *Aspergillus* involves industrially important fungi which produces enzymes used in food industry (i.e. amylase, protease, etc.). In East Asia including Japan, traditionally we have a benefit in use of *Aspergillus* species to produce fermentation foods (rice wine, soy sauce, miso). On the other hand, some groups of *Aspergillus* species produce a variety of mycotoxins and contaminates crops to give a huge economical loss to the society. This presentation focuses on the recent progress of biochemical and molecular biological approach of aflatoxin (AF) biosynthesis, and also organisms belonging to *Aspergillus* Section *Flavi* which produce AF, and its relation to aflatoxin non-producers.

Focuses

AF is a potent carcinogen produced by some strains of *Aspergillus flavus*, *A. parasiticus*

and *Aspergillus nomius*. Although AF is produced by neither any known strain of *Aspergillus oryzae* nor *Aspergillus sojae*¹⁻⁴, these organisms are considered similar to the AF-producer *A. flavus* and *A. parasiticus* in microbial classification. *A. oryzae* and *A. sojae* include strains which are used as koji molds in the production of rice wine (sake), soy sauce, miso *etc.* Because of widespread industrial use as koji molds, differentiation of aflatoxin-producers from non-producer strains is critically important. All four species are differentiated primarily based on morphological and physiological characteristics^{3,5}. These involve diameter, color, texture of the colonies, size and texture of conidia, and conidiophore structure. In addition, several molecular biological techniques are used in the classification. Kurtzman *et al.*⁶ reported a high degree of DNA homology among *A. oryzae*, *A. sojae*, *A. flavus*, and *A. parasiticus* and proposed that the four are varieties of the same species. Klich and Mullaney⁷ or Gomi *et al.*⁸ used restriction fragment length polymorphism analysis to classify these species. Yuan *et al.*⁹ used random amplified polymorphic DNA analysis to differentiate *A. sojae* and *A. parasiticus*. Geiser *et al.*¹⁰ analyzed 11 genes encoding the enzymes for primary metabolism in 31 strains of *A. flavus* and 5 strains of *A. oryzae*, then concluded that *A. oryzae* was a species that had evolved by domestication from one of the two groups of *A. flavus*.

Besides the above studies, the series of research to clarify the biosynthetic pathway of aflatoxin are ongoing. This project is being performed mainly by several research groups in USA and Japan genetically and biochemically, and in at least eighteen steps concerning on the pathway, eight kind of genes for the enzymes were characterized (reviewed by 11). *aflR*, the regulatory gene for the biosynthetic pathway was also characterized. Moreover, the pathway genes and *aflR* are found to be a cluster on an about 60 kb DNA region. The gene arrangement of the aflatoxin biosynthesis gene cluster region in *A. parasiticus* is so far cleared as *pksA*, *nor-1*, *fas-2A*, *fas-1A*, *aflR*, *aflJ*, *adhA*, *aad (norA)*, *ver-1*, *aflS*, *avnA*, *verB*, *ord-2*, *omtA*, *cyp450*, *vbs*, *aflB* and *aflW* in this order^{11,12}. Most of these genes are known to be essential to aflatoxin biosynthesis.

The gene cluster of *A. flavus* was also characterized and found that the gene arrangement is the same as that of *A. parasiticus*¹³. One of either pathway genes in *A. flavus* and *A. parasiticus* complement its function each other and show high nucleotide and amino acid sequence homology.

With *aflR* as a probe, DNA of each species belonging to *Aspergillus* Section *Flavi* were analyzed by Southern blotting. The result was that each one strain of *A. oryzae* and *A. sojae* have homologs of *aflR*¹⁴. This is the first report of the existence of the homolog of aflatoxin biosynthetic gene in non-toxigenic *A. oryzae* and *A. sojae*. Later differentiation of *A. oryzae*, *A. flavus*, *A. sojae* and *A. parasiticus* due to the difference of the sequence for *aflR* homologs was reported¹⁵.

Klich *et al.*¹⁶ performed Southern blot analysis of each three strains of *A. oryzae* and *A. sojae* with *aflR* and *omtA* (a gene encoding *O*-methylsterigmatocystin methyl transferase) as probes, and showed that three strains of *A. oryzae* do not have *aflR*, one do not have *omtA*, and three strains of *A. sojae* have *aflR* and *omtA* homologs. Later the above three strains of *A. sojae* were reported to have *norA*, *nor-1*, *fas-1A*, *pksA*, and *ver-1* homologs¹⁷.

The existence of the homolog of *ver-1*, a gene essential for aflatoxin biosynthesis¹⁸, was reported in each two strains of *A. oryzae* and *A. sojae*¹⁹. Part of the sequence of the homologs (about 660 bp) was examined and was compared with that of *A. parasiticus*, then it was found that the identity between sequences was over 90%, but there is no fingerprint to

distinguish species. Moreover, the deduced amino acid sequences showed higher identity each other, and only one to two amino acid residues differed in *A. oryzae*, no difference in *A. sojae*²⁰. The observation of a smaller number of amino acid changes than nucleotide changes among the homologs is because most of the nucleotide changes in *ver-1* homologs occur at either the third nucleotide in each codon or within the intron. The ratio of strains having *ver-1* homolog in 48 strains was 83%²⁰.

As mentioned above, there are several reports about the homologs of aflatoxin biosynthetic genes in *A. oryzae* and *A. sojae*. Then how about its expression in aflatoxin non-producers? Klich *et al.*¹⁷) analyzed the transcripts of the homologs mentioned above in three strains of *A. sojae* by Northern analysis. The transcripts of *aflR* and *fas-1A* homologs reported to detect in two strains, but no transcript of *norA*, *nor-1*, *pksA*, *ver-1*, and *omt-1* were detected¹⁷). PCR analysis coupled with reverse transcription detect no transcript of *ver-1* homologs in each two strains of *A. oryzae* and *A. sojae*²⁰). In the same manner, six strains of *A. oryzae* which have *aflR* homologs were examined and the expression of the homologs was not detected²¹).

As we described previously²⁰), *ver-1*-homolog genes were not expressed in two strains of *A. oryzae* (IFO 30104 and IFO 30105). As the expression of *ver-1* requires *aflR*, it is reasonable to assume they don't express the *ver-1*-homolog genes because of lack of the *aflR*-homolog gene (in case of *A. oryzae* IFO 30104, data not shown) or absence of expression of the *aflR*-homolog gene (*A. oryzae* IFO 30105, this study). The reason why the *aflR*-homolog gene is not expressed in *A. oryzae* IFO 30105 and the other *A. oryzae* strains examined in this study might be a deficiency in the induction mechanism of *aflR*. As aflatoxin production is regulated by several factors including growth stage and nutritional conditions, these factors might effect the induction of the *aflR* expression. Klich *et al.*¹⁷) demonstrated that two of three strains of *A. sojae* express homologs of *aflR* and *fas-1A* (a gene encoding fatty acid synthetase for initiation of aflatoxin biosynthesis), but not the other five homolog genes (*norA*, *nor-1*, *pksA*, *ver-1*, and *omtA*). The reason for this might be that expression of these five homolog genes requires factors other than *aflR* in aflatoxin production. Further characterization of the regulatory mechanism for aflatoxin production might resolve the difference between aflatoxin producers and non-producers.

Conclusion

Considering the above observations together with the information from other research groups, non-aflatoxigenic *A. oryzae* and *A. sojae* basically have some homologs of aflatoxin synthesis genes. Thus, they are possibly domesticated, differentiated from aflatoxin producer evolutionally. Nevertheless, their transcripts are not found in any of the strains and some homologs are not found the examined strains. Therefore these species do not produce aflatoxin because the synthesis genes are neither transcribed nor existent, probably due to mutations during repetitive use as domesticated strains in manufacturing fermented foods. Further characterization of the regulatory mechanism for aflatoxin production might resolve why *koji* molds having the homologs do not produce aflatoxin.

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