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Directed deletions in the aflatoxin biosynthesis gene homolog cluster of Aspergillus oryzae

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Abstract To investigate the structure of the aflatoxin gene cluster in Aspergillus oryzae, 39 strains belonging to this species were examined for the existence of pksA, fas-1A, aflR and vbs, and the results compared with those for ver-1 obtained previously. These five genes are involved in aflatoxin biosynthesis in Aspergillus parasiticus. The strains examined were categorized into three groups; group 1, having the five homologs; 2, having ver-1 and vbs; and 3, having vbs homologs. Long-PCR analysis of the regions between the five homologs in A. oryzae IFO 4135, coupled with Southern-hybridization analysis, shows that those homologs are clustered with the same arrangement as in A. parasiticus. These results suggest that directed deletions of the cluster occur in A. oryzae strains. The possible breakpoints of the deletions in the strains of group 2 and 3 were estimated.

Key words Aspergillus oryzae · Aflatoxin biosynthetic genes · Cluster · Deletion

Introduction

Aspergillus oryzae and Aspergillus sojae have traditionally been used as koji molds for fermented food and beverage production in east Asian countries. These species belong to Aspergillus Section Flavi. This group contains Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius, some strains of which produce aflatoxin, a strong natural carcinogen. On the other hand, strains belonging to A. oryzae and A. sojae do not produce aflatoxin (Yokotsuka et al. 1967; Manabe et al. 1968; Murakami 1971; Kusumoto et al. 1990), although these species are considered similar to A. flavus and A. parasiticus. All four species have been differentiated based on morphological and physiological characteristics (Raper and Fennel 1965; Murakami 1971). Recently, several molecular biological techniques have also been used in the classification. Kurtzman et al. (1986) reported a high degree of DNA homology among A. oryzae, A. sojae, A. flavus and A. parasiticus, and proposed that all four should be retained as separate species. By restriction fragment length polymorphism (RFLP) analysis, Klich et al. (1987) demonstrated the differentiation of A. oryzae and A. flavus using the SmaI restriction enzyme. Likewise, Gomi et al. (1989) demonstrated the differentiation of A. oryzae, A. flavus, A. sojae, and A. parasiticus by RFLP with Smal, and concluded that all four should be retained as separate species because of the importance of the clear differentiation of koji molds and aflatoxin producers, although they also recognized that these species are closely related. Yuan et al. (1995) used random amplified polymorphic DNA analysis to differentiate A. sojae and A. parasiticus, and also commented on the importance of proving the difference between the microorganisms used in the production of soy sauce from the aflatoxin producer. Geiser et al. (1998) analyzed 11 genes encoding the enzymes for primary metabolism in 31 strains of A. flavus and five strains of A. oryzae, and concluded that A. oryzae was a species that evolved
The biosynthetic pathway of aflatoxin in *A. flavus* and *A. parasiticus* has been extensively studied both biochemically and molecularly (reviewed by Minto and Townsend 1997; Woloshuk and Prieto 1998). A proposed biosynthetic pathway is: polyketide precursor → norsolorinic acid → averantin → 5′-hydroxyaverantin → averufin → versicolor hemiacetal acetate → versicolorin B → versicolorin A → sterigmatocystin → O-methylsterigmatocystin → aflatoxin B1. More than 17 steps of enzymatic conversion were also proposed during the pathway. Several genes have been cloned and appeared to be clustered (Fig. 1). The gene arrangement of the aflatoxin biosynthesis gene-cluster region in *A. parasiticus* was shown to be pksA, nor-1, fas-2A, fas-1A, aflR, adhA, aad (nor-4), ver-1A (functional homolog of ver-1), avs, avnA, verB, ord-2, omrA, cyp450, vbs, aflB and aflW in this order, as illustrated in Fig. 1 (Minto and Townsend 1997; Woloshuk and Prieto 1998). By finding more genes for aflatoxin biosynthesis, several research groups demonstrated that homologous genes were present in *A. oryzae* and *A. sojae*. Klich et al. (1995) examined two aflatoxin biosynthesis genes (aflR and omrA) in three strains of *A. oryzae*. Chang et al. (1995a) demonstrated the presence and sequence variability of aflR homologs in some *A. oryzae* and *A. sojae* strains. Kusumoto et al. (1996) and Kusumoto et al. (1998a) examined *A. oryzae* strains for the existence of ver-1, and found that some, but not all, had ver-1 homologs, although their transcripts were not detected. Klich et al. (1997) demonstrated the presence of seven homologs for the pathway genes in three strains of *A. sojae*, where some of those genes were transcribed. Watson et al. (1999) showed that two strains of *A. oryzae* and one strain of *A. sojae* had homologs of nor-1, ver-1, omrA and aflR, but no transcripts for any homologs were detected. *A. oryzae* involves the major strains of *koji* molds which are used in the food industry. Moreover, *koji* molds do not produce aflatoxin. Therefore the structure and role of the pathway cluster in *A. oryzae* should be of particular interest.

So far no information is available for the cluster structure of the pathway genes in *A. oryzae*, as well as in strains which did not have a ver-1 homolog, as studied previously (Kusumoto et al. 1998a). In the present study, we examined four aflatoxin biosynthesis genes (pksA, fas-1A, aflR and vbs) and compared the existence of ver-1 (Kusumoto et al. 1998a) in 39 strains of *A. oryzae*. The genes pksA (polyketide synthase for the synthesis of the polyketide precursor), fas-1A (fatty acid synthase for the synthesis of the polyketide precursor), ver-1 (ketoreductase for the conversion of versicolorin A), and vbs (oxidase/dehydrogenase for synthesis of versicolorin B) are considered to encode enzymes for conversion of the intermediates of aflatoxin synthesis, and aflR to encode for the regulatory protein for the expression of the other cluster genes. The pksA gene is located at the end of the pathway cluster, and vbs is close to the opposite end. The rest are located between pksA and vbs (Fig. 1). Thus we will roughly estimate the cluster structure from information on their existence. We also performed long-PCR analysis of the region between the homologs to examine the structure of the pathway gene cluster in *A. oryzae* IFO 4135.

**Fig. 1** Schematic presentation of the cluster region for aflatoxin biosynthesis genes in *A. parasiticus* and those of *A. oryzae* groups 1 through 3 proposed in this study. Open arrows indicate the genes and the direction of transcription. Shaded boxes are the probes for Southern hybridization. The region shown in the closed box is amplified with primers VBSF3 and VBSR3. Small arrows indicate the primers for long-PCR. Dotted lines indicate the DNA to be amplified by long-PCR, and their approximate sizes are shown. PF, FA, AV and VV are designation of the fragments used in the text. For the cluster in *A. oryzae*, only the homologs detected in this study are shown. The dotted lines in *A. oryzae* chromosome have not been analyzed in terms of structure.

**Materials and methods**

Fungal strains and growth

Among the *A. oryzae* strains tested, 27 were obtained from the Institute for Fermentation, Osaka (IFO), ten from the National Research Institute of Brewing (RIB), *A. oryzae* NFRI 1134 (IFO 30104), *A. oryzae* NFRI 1134 (ATCC 1011), and *A. parasiticus* SYS-4 (NRRL 2999) are stock strains of the National Food Research Institute. The growth and harvesting of mycelia were conducted according to Kusumoto et al. (1996).