Transcript of a homolog of \( \text{aflR} \), a regulatory gene for aflatoxin synthesis in \textit{Aspergillus parasiticus}, was not detected in \textit{Aspergillus oryzae} strains

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Received 13 July 1998; received in revised form 19 October 1998; accepted 21 October 1998

Abstract

Some strains of \textit{Aspergillus oryzae} were shown to have homologs of \( \text{aflR} \), a regulatory gene for aflatoxin synthesis in \textit{Aspergillus parasiticus}. Transcription of an \( \text{aflR} \) homolog was examined in six strains of \textit{A. oryzae} having the homologs, using polymerase chain reaction (PCR) coupled with reverse transcription. No PCR product was obtained when the RNA prepared from the \textit{A. oryzae} strains cultivated under aflatoxin-producing condition was used as template for amplification of the \( \text{aflR} \) cDNA. By contrast, a PCR product of the expected size was obtained with RNA from \textit{A. parasiticus} NIAH-26 processed by the same procedure. From genomic DNA of these strains, PCR products of the same size as above were obtained. Possible degradation of the \( \text{aflR} \) mRNA in the RNA preparation of the \textit{A. oryzae} strains was negligible, because the calmodulin transcript was detected by PCR from the same RNA samples. Thus, the \( \text{aflR} \) homologs in the non-aflatoxigenic \textit{A. oryzae} strains examined are not expressed even under aflatoxin-producing condition. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Aflatoxin synthesis; \( \text{aflR} \) homolog; \textit{Aspergillus oryzae}; Fungal toxin; Gene expression

1. Introduction

Aflatoxins are fungal secondary metabolites highly toxic and carcinogenic for several animal species including, probably, man [1]. They are produced by filamentous fungi, \textit{Aspergillus flavus} and \textit{Aspergillus parasiticus}, which belong taxonomically to \textit{Aspergillus} Section \textit{Flavi} [2]. Aflatoxin biosynthesis has been studied extensively in terms of enzymology and molecular biology: several genes encoding enzymes involved in aflatoxin biosynthesis and a gene (\textit{aflR}) responsible for regulation of the above genes have been characterized (reviewed by Woloshuk and Prieto [3]). The \textit{aflR} product is known to regulate the structural genes positively at the level of transcription [3].

The strains of \textit{Aspergillus oryzae} and \textit{Aspergillus parasiticus}
sojae are industrially important and are used traditionally in the production of several kinds of fermented foods such as sake (rice wine), miso (fermented soy bean paste) and soy sauce. These species belong to Aspergillus Section Flavi and are considered to be taxonomically similar to A. flavus and A. parasiticus. Strains of A. oryzae and A. sojae, however, do not produce aflatoxin [4,5].

In a previous study [6], we identified homologs of ver-1, a gene responsible for the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis in A. parasiticus, in some strains of A. oryzae and A. sojae. The transcript of these ver-1-homolog genes was, however, not detected under aflatoxin-producing condition. Recently, Klich et al. [7] showed that several strains of A. oryzae and A. sojae have homologs of aflR and omt-I, the aflatoxin biosynthesis genes. In the A. sojae species, three strains were revealed to have seven homologs of the aflatoxin biosynthesis genes, and two of the seven homologs, including aflR, were transcribed in two of the three strains, but the others were not transcribed [8]. Since the major strains of koji molds (those used in the production of fermented foods described above) are A. oryzae, investigations into the expression and regulation of those homolog genes for aflatoxin biosynthesis in A. oryzae strains should be given high priority.

Therefore we focused on the expression of the aflR-homolog genes in A. oryzae strains having these genes. Here we demonstrate that no transcripts of the aflR-homolog genes in the examined strains of A. oryzae were detected under the aflatoxin-producing condition, using polymerase chain reaction (PCR) coupled with reverse transcription.

### 2. Materials and methods

#### 2.1. Strains and growth conditions

A. oryzae strains IFO 30105, IFO 4202, IFO 4348, IFO 5240, RIB 23, RIB 333, and a strain of A. parasiticus, NIAH-26, were used. All of the A. oryzae strains have an aflR-homolog gene (K. Kusumoto, Y. Nogata and H. Ohta, submitted). That A. parasiticus NIAH-26 has the aflR gene was confirmed by PCR and restriction enzyme analysis of the PCR.

### Table 1
The oligonucleotide primers used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLF1</td>
<td>5'-TACAGTGTCCTCCAAGGGGATG-3'</td>
<td>172–192</td>
</tr>
<tr>
<td>AFLR1</td>
<td>5'-AGTAGGCATGCTGATCAT-3'</td>
<td>822–802</td>
</tr>
<tr>
<td>CMDF1</td>
<td>5'-GGTGGATGCGAGATCACCC-3'</td>
<td>268–274 followed by 401–413</td>
</tr>
<tr>
<td>CMDR1</td>
<td>5'-CCGATGAGGTCATGACAGTG-3'</td>
<td>721–702</td>
</tr>
</tbody>
</table>

*PVER11 and CMDF1 are forward primers, and PVER12 and CMDR1 are reverse primers. These primers are designed to amplify cDNA corresponding to the protein-coding regions for either aflR [11] or cmdA [12].

*Numbers correspond to positions in the A. parasiticus aflR nucleotide sequence [11] for AFLF1 and AFLR1, or in the A. oryzae cmdA nucleotide sequence [12] for CMDF1 and CMDR1. For either case, the positions are relative to A of the initiation codon as +1.
A. parasiticus NIAH-26 is a non-aflatoxigenic mutant derived from the aflatoxigenic strain A. parasiticus SYS-4 (NRRL 2999). In strain NIAH-26, all enzymes responsible for the conversion of norsolorinic acid to aflatoxin are active under aflatoxin-producing condition [9]. Approximately $10^7$ conidia of each strain were inoculated into either 50 ml of SY liquid medium (6% sucrose
and 2% yeast extract) for aflatoxin production or 50 ml of PY liquid medium (4% polypeptone and 2% yeast extract) for aflatoxin non-production in a 200-ml Erlenmeyer flask, and grown at 28°C for 96 h on a rotary shaker (170 rpm).

2.2. Preparation of DNA

DNA of each strain was extracted from the wet mycelia after pulverization, according to Gomi et al. [10].

2.3. Preparation of RNA

The fungal mycelia were frozen in liquid nitrogen and pulverized with a pasteurized mortar and pestle. The fungal RNA was prepared with an RNeasy kit (Qiagen, Tokyo) according to the manufacturer’s instructions. The RNA was treated with DNase (Life Technologies, Tokyo), then with EDTA at 65°C to inactivate the DNase, and was stored at −80°C until use.

2.4. PCR technique

Reverse transcription of RNA and PCR were carried out with an RNA LA PCR kit (AMV) (Takara Shuzo, Kyoto) according to the manufacturer’s instructions. In PCR, reaction volume was 25 μl, the annealing temperature was set at 56°C, and 40 cycles (for cDNA) or 30 cycles (for DNA) of amplification were performed. Oligonucleotide primers used for amplification of aflR cDNA or aflR DNA (AFLF1 and AFLR1; Table 1) were designed according to the nucleotide sequences of aflR, which has no intron [11]. Those for calmodulin (cmdA) cDNA (CMDF1 and CMDR1; Table 1) were as described by Yasui et al. [12].

3. Results and discussion

The transcripts of aflR-homolog genes in the test strains of A. oryzae and A. parasiticus (used as positive control) were examined by PCR coupled with reverse transcription, using primers specific to the aflR coding region (AFLF1 and AFLR1; Table 1). Under aflatoxin-producing condition, only the RNA of A. parasiticus NIAH-26 gave a single PCR product of approximately 650 bp (Fig. 1A, lane 13), whose size was identical to that predicted from the nucleotide sequence of aflR (651 bp) [11], while no other RNAs (the six strains of A. oryzae, and A. parasiticus NIAH-26 cultured under aflatoxin-non-producing condition) could give any signal for the aflR cDNA (Fig. 1A, lanes 1, 3, 5, 7, 9, 11, and 15). Restriction analysis of the 650-bp PCR product using EcoRI, XhoI, Sau3AI and SalI showed the pattern predicted from the aflR sequence (data not shown). DNA was not contaminated in the RNA sample of strain NIAH-26 prepared in the aflatoxin-producing condition because no PCR products were obtained when the RNA sample without reverse transcription was used as template (Fig. 1A, lane 14). Therefore the condition for aflatoxin production used in this study was indeed suitable for inducing the expression of aflR in NIAH-26. Furthermore, when DNA from each strain was used as template, a PCR product of about 650 bp was obtained from all examined strains including A. oryzae (Fig. 1B, lanes 1-7). These results show that the condition and primers used in PCR were sufficient to amplify the cDNA or DNA of aflR and its homolog.

To confirm that the RNA of A. oryzae used above is not degraded, transcript of the calmodulin gene (cmdA) [12] was examined by PCR analysis, using cmdA-specific primers (CMDF1 and CMDR1; Table 1). From all of the RNA samples including those from A. oryzae, an amplified fragment of about 270 bp was obtained (Fig. 1C, lanes 1, 3, 5, 7, 9, 11, 13, and 15). This size is approximately the same as that predicted from the cDNA sequence (273 bp) [12]. These 270-bp fragments likely originated from the cmdA cDNA since no fragment was obtained when the RNA samples without reverse transcription were used as templates for PCR (Fig. 1C, lanes 2, 4, 6, 8, 10, 12, 14, and 16). This result shows that the RNA of A. oryzae is not degraded and is suitable for use as a template for first strand cDNA synthesis.

The findings obtained in this study demonstrated that transcripts of the aflR-homolog genes in the test strains of A. oryzae were not detected on PCR analysis, and imply that the aflR-homolog genes are not transcribed in the A. oryzae strains even under aflatoxin-producing condition. In our experiment, the
expression of aflR in A. parasiticus NIAH-26 was clearly detectable after incubation for 96 h, although Klich et al. [8] reported that in A. parasiticus it maximized at 24 h, and decreased thereafter. This observation shows that the sensitivity of PCR in this study was comparable to Northern blot analysis as described by Klich et al. [8]. As we described previously [6], 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 [13], it is reasonable to assume they do not express the ver-1-homolog genes because of lack of the aflR-homolog gene (in the 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 [11,14], these factors might affect the induction of the aflR expression. Evidence (K. Kusumoto, Y. Nogata and H. Ohta, submitted, also see reference [8]) suggests that several strains of A. oryzae and A. sojae have the entire gene cluster homologous to that of aflatoxin biosynthesis in A. parasiticus, while they do not produce aflatoxin. Klich et al. [8] demonstrated that two of three strains of A. sojae express homologs of aflR and fas-IA (a gene encoding fatty acid synthetase for initiation of aflatoxin biosynthesis [3]), but not the other five homolog genes (norA, nor-1, pksA, ver-1, and omn-1). 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.

References