FEBS Letters 564 (2004) 126–130

FEBS 28280

Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*[1]

Jiujiang Yu*, Deepak Bhatnagar, Thomas E. Cleveland

U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, 1100 Robert E. Lee Boulevard, New Orleans, LA 70124, USA

Received 24 February 2004; revised 15 March 2004; accepted 16 March 2004

First published online 26 March 2004

Edited by Takashi Gojobori

Abstract An 82-kb *Aspergillus parasiticus* genomic DNA region representing the completed sequence of the well-organized aflatoxin pathway gene cluster has been sequenced and annotated. In addition to the 19 reported and characterized aflatoxin pathway genes and the four sugar utilization genes in this cluster, we report here the identification of six newly identified genes which are putatively involved in aflatoxin formation. The function of these genes, the cluster organization and its significance in gene expression are discussed.

Key words: Gene cluster; Pathway evolution; Aflatoxin; Mycotoxin; Secondary metabolite; *Aspergillus flavus*

1. Introduction

Aflatoxins are toxic and carcinogenic secondary metabolites produced primarily by the filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Due to a potential risk to livestock and human health [1–4], extensive research on aflatoxin biosynthesis has been conducted. In the last decade, significant progress has been made in deciphering the aflatoxin biosynthetic pathway and its genetic control [5–11]. The linkage between the *nor-1* and *ver-1* genes with the regulatory gene *aflR* in cosmid clones [12,13] and analysis of overlapping cosmid clones in *A. parasiticus* and *A. flavus* led to the discovery of the aflatoxin pathway gene cluster [6,10,11]. Currently, a total of 25 genes involved in aflatoxin biosynthesis has been identified which are clustered within a 70-kb DNA region in the chromosome in addition to the four sugar utilization genes (Fig. 1) [14]. The discovery of genes involved in aflatoxin formation significantly improved our understanding on the aflatoxin biosynthesis. The generally accepted aflatoxin biosynthetic pathway is as follows: acetate → → polyketide → norsolorinic acid (NOR) → averantin (AVN) → 5′-hydroxy-averantin (HAVN) → oxoaverantin (OAVN) → averufin (AVF) → versiconal hemiacetal acetate (VHA) → versiconal (VAL) → versicolorin B (VERB) → versicolorin A (VERA) → demethylsterigmatocystin (DMST) → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1); a branch point starting from VERB leading to aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2) formation via VERB → dihydrodemethylsterigmatocystin (DHDMST) → dihydrosterigmatocystin (DHST) → dihydro-*O*-methylsterigmatocystin (DHOMST) → AFB_2 and AFG_2. The genes, their enzymes and pathway involvement are summarized in Table 1. In this report, the completed sequence of the aflatoxin pathway gene cluster and the six newly identified putative aflatoxin pathway genes are presented. The cluster organization and its effect on cluster gene expression are discussed.

2. Materials and methods

2.1. Fungal strains and culture conditions

The completed aflatoxin gene cluster sequence was generated from *A. parasiticus* SRRC 143 (ATCC 56775) which produces AFB_1, AFB_2, AFG_1, and AFG_2. In order to investigate the position effect of aflatoxin pathway cluster genes on their expression, gene complementation experiments were carried out. The spontaneous *niaD* (nitrate reductase gene) mutant (named RHN1) derived from *A. parasiticus* SRRC 2043 (ATCC 62882) which is a field isolate that accumulates OMST and does not produce AFB_1, AFB_2, AFG_1, and AFG_2 [15], was used as the recipient strain in fungal transformation. The fungal transformation protocol used was as described previously [16]. Fungal strains were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA); PDA was also used for detection of aflatoxin production.

2.2. Genomic DNA sequencing and gap closing between cluster genes

A. parasiticus cosmid clones harboring aflatoxin pathway genes were screened previously [10]. These overlapping cosmid clones forming a consensus aflatoxin pathway gene cluster were used as DNA templates for sequencing and for gap closing by a polymerase chain reaction (PCR) strategy. Sequencing by primer waking was performed to identify additional open reading frames (ORFs) in the gene cluster involved in aflatoxin formation. Primer pairs were made specific to the coding region of these genes covering the intergenic regions for gap closure. The resulting PCR products were sequenced directly.

2.3. Coding sequence and intron identification by reverse transcriptase (RT)-PCR

Total RNA was isolated from 48-h mycelia of *A. parasiticus* SRRC 143 by Qiagen RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). First strand cDNA was synthesized by Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA) and used as the template

*Corresponding author. Fax: (1)-504-286 4419.
E-mail address: jiuyu@srrc.ars.usda.gov (J. Yu).

[1] The nucleotide sequence data in *A. parasiticus* reported here are available in the NCBI GenBank database under the accession number AY371490.

Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5′-hydroxy-averantin; OAVN, oxoaverantin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; ST, sterig- matocystin; DHDMST, dihydrodemethylsterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00327-8

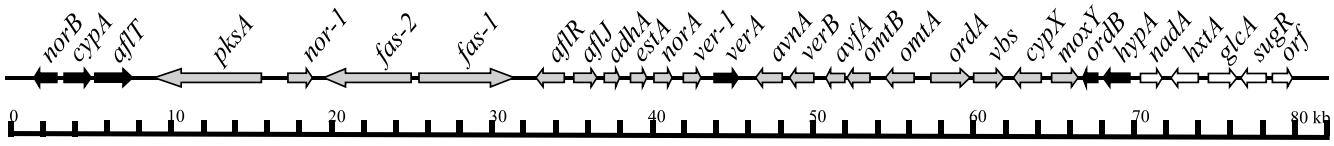

Fig. 1. Schematic representation of the aflatoxin pathway gene cluster. The 25 aflatoxin pathway cluster genes including the four sugar utilization genes (*nadA*, *hxtA*, *glcA* and *sugR*) and an additional ORF (*orf*) in *A. parasiticus* are presented. The 19 previously reported genes are presented in gray arrows; the six newly identified genes are presented in solid arrows; and the four sugar utilization genes and the additional ORF are presented in unfilled arrows. Arrows indicate the direction of gene transcription. The names of the genes are labeled on top of each gene.

in PCR. The PCR amplification was carried out as described earlier [14]. The resulting cDNA fragments were sequenced directly. Intervening sequences (introns) were located by comparison of the genomic DNA to cDNA sequences. Sequence analysis and amino acid prediction/translation have been performed using PC/Gene program (IntelliGenetics, Inc., Mountain View, CA, USA). All primers were made with a DNA Synthesizer (Applied Biosystems, Model 380A).

3. Results and discussion

3.1. Identification of the six new ORFs

By chromosome walking, we obtained extended sequence data on both the 5′ end to the *pksA* and the 3′ end to the *moxY* genes of the established gene cluster [10] in *A. parasiticus* strain SRRC 143. Blast search to the GenBank database identified three new ORFs on the 5′ end to the *pksA* gene, named *norB*, *cypA* and *aflT*, and two new ORFs on the 3′ end to the *moxY* gene, named *ordB* and *hypA*. The sequencing gaps in intergenic regions of the reported genes have been closed by direct sequencing of PCR products. Coding sequences and introns were confirmed and verified with corresponding cDNA derived by RT-PCR. After joining the gaps, an additional ORF was identified between *ver-1* and *omtB*, named *verA*, due to its homology to the *ver-1* gene (Fig. 1). These newly identified ORFs were characteristic of putative aflatoxin pathway genes. A typical AflR-binding motif has been identified in the 5′ untranslated regions (UTRs) of all of these genes, a potential evidence for aflatoxin formation under *aflR* regulation [17]. On the contrary, no AflR-binding motif was identified in the UTRs of the four sugar utilization genes adjacent to the aflatoxin gene cluster [14]. More importantly, these new genes were found to be expressed under aflatoxigenic growth conditions in the *A. flavus* EST database, indicating possible functional involvement in aflatoxin synthesis (Yu, unpublished data).

3.1.1. The norB *gene.* The first ORF identified in the cluster consists of 1146 nucleotides encoding a polypeptide of 382 amino acids without introns. Although there is no significant sequence homology at DNA level, this polypeptide shares 68% amino acid similarity to an aryl alcohol dehydrogenase encoded by a *norA* gene, which is putatively involved in the conversion of NOR to AVN [18]. So this gene was tentatively named *norB*. Analysis of its 5′ UTR revealed an AflR-binding motif (TCGn5CGA) for transcriptional activation of aflatoxin pathway genes at position −113. A gene named *nor-1*, encoding a ketoreductase for the conversion of NOR to AVN, was cloned and characterized [19,20] in *A. parasiticus*. It is interesting that both the *norB* and *norA* genes have no significant sequence homologies to the *nor-1* gene at both nucleotide and

Table 1
Aflatoxin pathway cluster genes

Gene name	Enzyme	Function in the pathway
fas-2 (*hexA*)	FASα subunit	acetate → polyketide
fas-1 (*hexB*), *uvm8*, *fas1*, *fas-1A*	FASβ subunit	acetate → polyketide
pksA, *pksL1*	PKS	acetate → polyketide
nor-1	reductase	NOR → AVN
norA, *aad*, *adh-2*	NOR reductase/dehydrogenase	NOR → AVN
norB	dehydrogenase	NOR → AVN
avnA, *ord-1*	P_{450} monooxygenase	AVN → HAVN
adhA	alcohol dehydrogenase	HAVN → OAVN, averufanin, AVF
avfA, *ord-2*	oxidase	AVF → VHA
estA	esterase	VHA → VAL
vbs	VERB synthase	VAL → VERB
verB	desaturase	VERB → VERA
ver-1	dehydrogenase/ketoreductase	VERA → DMST
verA	monooxygenase	VERA → DMST
omtB or *dmtA* (*mtI*)	*O*-methyltransferase B or *O*-methyltransferase I	DMST → ST and DHDMST → DHST
omtA, *omt-1*	*O*-methyltransferase A or *O*-methyltransferase II	ST → OMST and DHST → DHOMST
ordA, *ord-1*	oxidoreductase/P_{450} monooxygenase	OMST → AFB_1 and AFG_1, DHOMST → AFB_2 and AFG_2
aflR, *apa-2*, *afl-2*	transcription activator	pathway regulator
aflJ	transcription enhancer	pathway regulator
aflT	transmembrane protein	this study
cypA	P_{450} monooxygenase	this study
cypX	P_{450} monooxygenase	this study
moxY	monooxygenase	this study
ordB	monooxygenase/oxidase	this study
hypA	hypothetical protein	this study

amino acid levels even though the three genes are possibly involved in the conversion from NOR to AVN. In attempts to disrupt the *norB* gene, no NOR-accumulating mutant was generated due possibly to the three genes for the same bioconversion step in aflatoxin synthesis. This may help explain the phenomenon that the NOR-accumulating mutants generated by disruption of either *nor-1* or *norA* gene are always leaky and produce some aflatoxins or next step intermediate [12,21]. The *nor-1* gene homolog in *Aspergillus nidulans* is *stcE* [22]. No *norB* gene homolog is identified in the ST gene cluster [22]. Functional elucidation of the *nor-1*, *norA* and *norB* genes is under investigation through double deletion strategy.

3.1.2. The cypA *gene.* Adjacent to *norB* a second ORF, encoding a polypeptide of 498 amino acids, was identified. RT-PCR experiments demonstrated that there are four introns (63, 50, 70, and 76 bp respectively) within the coding region. Blast search identified significant homologies to cytochrome P_{450} type monooxygenase enzymes in the GenBank database. On the amino acid level, there is 38% sequence identity to Tri4 gene product, another P_{450} monooxygenase involved in trichothecene biosynthesis in *Fusarium sporotrichioides* [23]. A typical heme-binding motif of cytochrome P_{450} monooxygenase near the C-terminus beginning at amino acid 447, a P_{450} E-x-x-R motif at amino acids 355–358, and a protein transfer groove A/G-G-x-D/E-T-T/S at amino acids 297–302 have been identified. This putative ORF was named *cypA* for a cytochrome P_{450} enzyme. Sequence analysis identified a typical AflR-binding motif at position −176 in the UTR. Expression studies by RT-PCR experiments showed that the transcript was detected only under aflatoxin-conducive conditions [8,24] and not on non-conducive medium (peptone medium, data not shown) [24]. These observations support the possible involvement of this gene in aflatoxin biosynthesis. Based on the putative function of this enzyme and the enzymatic requirement for G-group toxin biosynthesis from OMST to AFG_1 and DHOMST to AFG_2, this *cypA* gene is most likely involved in G-group toxin formation in aflatoxin biosynthesis.

3.1.3. The aflT *gene.* Located between *cypA* and *pksA*, an ORF named *aflT* (AF268071, Chang et al., unpublished data) was identified. The *aflT* gene encodes a putative protein consisting of 514 amino acids. There are five introns within the coding region as confirmed by RT-PCR. Amino acid analysis identified 14 putative transmembrane domains. Blast search demonstrated homologies to many genes encoding multidrug resistance proteins or toxin transporters [25]. A deviated AflR-binding motif (TCGn5CGC) was identified in the UTR of *aflT* at position −320. However, the *aflT* gene was demonstrated to be not under the *aflR* and *aflJ* regulation (Chang et al., unpublished data) as the aflatoxin pathway genes are. Disruption of this gene does not affect aflatoxin formation and secretion (Chang, personal communication).

3.1.4. The verA *gene.* Between the *ver-1* and *avnA* genes, an ORF encoding a polypeptide of 492 amino acids with one intron was identified in *A. parasiticus* SRRC 143. A typical AflR-binding motif was identified in the UTR at position −238. Sequence analysis indicated that this gene is a homolog of *stcS* in *A. nidulans* involved in ST synthesis (formerly named *verB*) [26], a cytochrome P_{450} type monooxygenase which is involved in the conversion of VERA to DMST. Disruption of *stcS* resulted in the accumulation of VERA. Thus, the new ORF was named *verA* for its possible function in converting VERA to DMST in aflatoxin biosynthesis, even though no significant sequence homology was found to the *ver-1* gene, which encodes a ketoreductase required for the conversion of VERA to DMST [27].

3.1.5. The ordB *gene.* Adjacent to *moxY*, another ORF was found. It encodes a polypeptide of 266 amino acids with significant homology to putative NADH-flavin reductase, oxidases or oxidoreductases in the GenBank database. Notably 54% amino acid identity and 68% similarity to the *stcQ* gene in the ST gene cluster in *A. nidulans* [22]. No intron has been identified in the coding region. A typical AflR-binding motif in the UTR was located at position −145. We tentatively named it *ordB* due to its possible function as an oxidoreductase in aflatoxin synthesis.

3.1.6. The hypA *gene.* The last ORF that is potentially involved in aflatoxin synthesis was identified between the *ordB* and the sugar cluster gene *nadA*. It encodes a polypeptide of 495 amino acids with 96% homology to a hypothetical protein from *Aspergillus oryzae* and 50% homology to a hypothetical protein from *Neurospora crassa*. And so it is tentatively named *hypA*. There are two introns identified in the coding region. A typical AflR-binding motif is identified at position −124 in the 5′ UTR. A highly conserved motif is located between amino acid positions 365 and 505, a typical nicotinate phosphoribosyltransferase (NAPRTase) enzyme family. This family is related to quinolinate phosphoribosyltransferase. No homologous gene is present in the ST gene cluster and the putative polypeptide sequence does not reveal any functional domains.

Due to the putative functions of the *cypA*, *ordB* and *hypA* genes and their AflR-binding signatures in the promoter regions, it is tempting to postulate that these genes may be involved in G-group aflatoxin formation since additional enzymes are required for AFG_1 and AFG_2 formation as suggested [16,28,29].

3.2. The 25 genes or ORFs represent a well-defined aflatoxin pathway gene cluster

The completed 70-kb DNA sequence containing the 25 genes represents a well-defined aflatoxin pathway gene cluster (Fig. 1). On average, about a 2.8-kb genomic DNA region contains one gene. Among these there are three large genes of about 5–7 kb each for the fatty acid synthase α (FASα, 5.8 kb), β (FASβ, 5.1 kb) subunits and the polyketide synthase (PKS, 6.6 kb). Excluding these three large genes, the average size of the other 22 genes is about 2 kb. In the 5′ end of the cluster sequence, an approximate 2-kb DNA region with no identifiable ORF was located. Recent sequence data of the upstream regions in several *A. parasiticus* and *A. flavus* strains confirmed the notion that the *norB* gene marks the end of the aflatoxin pathway gene cluster in the 5′ end (Ehrlich, personal communication). The 3′ end of this gene cluster is delineated by a well-defined sugar utilization gene cluster consisting of four genes [14].

3.3. Positional effects of aflatoxin pathway genes

The chromosomal arrangement of the aflatoxin pathway genes in the cluster may have biological advantage in terms of gene expression in the genome. The phenomenon of positional effect was observed [30] when integrating a *ver-1A*-GUS reporter construct and a *nor-1*::GUS plus *pyrG* construct [31], in which gene expression was markedly reduced in compari-

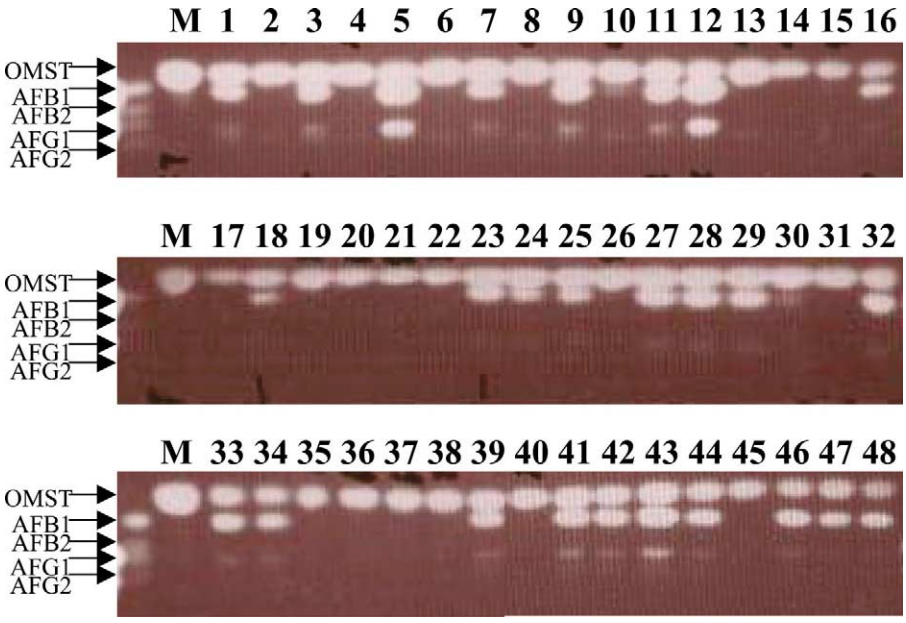

Fig. 2. Positional effect of gene integration. The survived colonies on the screening medium were putative *ordA* gene complemented transformants. Aflatoxins and other metabolites were extracted from 3-day-old fungal mycelia culture in aflatoxin supportive medium by a standard procedure of acetone/chloroform extraction as described earlier [32]. The extracted metabolites were assayed on a thin-layer chromatography (TLC) plate (catalog no. 7001-04; 20 cm×20 cm silica gel; J.T. Baker, Inc.) in EMW (ether:methanol:water, 96:3:1, v/v/v) and TEA (toluene–ethyl acetate–acetic acid, 50:30:4, v/v/v) solvent systems. One fourth (20 μl) of the total extracted metabolites from each sample was loaded. The conversion of AFB_1 and AFB_2 from OMST in SRRC 2043 was observed in lanes 1, 3, 5, 7, 9, 11, 12, 16, 18, 23–25, 27–29, 32–34, 39, 41–44, 46–48 when the *ordA* gene was inserted in the aflatoxin gene cluster. When the *ordA* gene was inserted in the *niaD* locus it was not expressed and no aflatoxin was produced in lanes 2, 4, 6, 8, 13–15, 17, 19–22, 26, 31, 35–38, 40, 45. Lane M: OMST produced in the recipient strain *A. parasiticus* RHN1. The location of gene integration was confirmed by gene-specific PCR amplification (data not shown).

son to native gene expression. In studying the relationship of the physical location of aflatoxin pathway cluster genes and their expression, gene complementation experiments were performed. After transformation of the OMST-accumulating mutant, *A. parasiticus* RHN1 with a functional copy of the *ordA* gene construct from *A. flavus*, we complemented the non-functional *ordA* gene in the *A. parasiticus* recipient strain RHN1. Out of 92 transformants obtained, 56 (61%) of them were confirmed to convert a significant amount of OMST to AFB_1 and AFG_1 and smaller amounts to AFB_2 and AFG_2, while 36 (39%) produced only OMST (Fig. 2 shows 48 of the 92 colonies assayed). When the functional *ordA* gene construct was integrated into the defective *ordA* site in the aflatoxin gene cluster as confirmed by Southern blot and PCR detection (data not shown), the *ordA* gene was expressed and aflatoxins produced (Fig. 2, lanes 1, 3, 5, 7, 9, 11, 12, 16, 18, 23–25, 27–29, 32–34, 39, 41–44, 46–48). For those colonies where the *ordA* gene construct was integrated into the marker gene *niaD* site (a selectable marker), the *ordA* gene was not expressed, consequently no aflatoxins were produced (Fig. 2, lanes 2, 4, 6, 8, 12, 14, 15, 17, 19–22, 26, 31, 35–38, 40, 45). We, however, noticed that some of the colonies produced trace amounts of AFB_1 and AFG_1 (Fig. 1, lanes 10 and 30, about 4% of total transformed colonies). It is possible that the *ordA* gene construct might have been integrated into neither the *ordA* site nor the *niaD* site, instead randomly integrated somewhere else in the genome (integration location unconfirmed). It could be that the *niaD* gene is less stringent in its expression in either *niaD* site or in the *ordA* site. However, the aflatoxin pathway gene, such as *ordA*, is expressed only within the aflatoxin gene cluster. Previous observations [30,31] and our experiment consistently demonstrated that chromosomal location is important for aflatoxin pathway gene expression in *A. parasiticus*. The mechanism for such transcriptional regulation is under investigation. It is noteworthy that the rate of conversion from OMST to aflatoxins in the complemented transformants is only 50% compared with wild-type strain *A. parasiticus* 143 (Fig. 2, lane M). The limiting factor(s) might lie in the *A. parasiticus* SRRC 2043 itself due, possibly, to other defect(s) or selectable marker (*niaD*) interference.

Acknowledgements: The authors acknowledge contributions by scientists who were significantly involved in the discoveries of the aflatoxin pathway genes in *A. parasiticus* and *A. flavus*. Those include: Drs. Perng-Kuang Chang, Kenneth C. Ehrlich, Jeffrey W. Cary, Gary A. Payne, John E. Linz, Charles P. Woloshuk, Christopher D. Skory, Frances Trail, Joan W. Bennett, Kimiko Yabe, and Craig A. Townsend. We are grateful for the technical assistance of Leslie Scharfenstein and secretarial help of Janell Becker.

References

[1] Bennett, J.W. Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia, (1987) Mycopathologia 100, 3–5.
[2] Bennett, J.W. and Klich, M. Mycotoxins, (2003) Clin. Microbiol. Rev. 16, 497–516.
[3] Bhatnagar, D., Yu, J. and Ehrlich, K.C. Toxins of filamentous fungi, (2002) Chem. Immunol. 81, 167–206.
[4] Lancaster, M.D., Jenkins, F.P. and Phillip, J.M. Toxicity associated with certain samples of groundnuts, (1961) Nature 192, 1095–1096.
[5] Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. Molecular genetic analysis and regulation of aflatoxin biosynthesis, (2003) Appl. Microbiol. Biotechnol. 61, 83–93.
[6] Cleveland, T.E., Cary, J.W., Brown, R.L., Bhatnagar, D., Yu, J., Chang, P.-K., Chlan, C.A. and Rajasekaran, K. Use of biotechnology to eliminate aflatoxin in preharvest crops, (1997) Bull. Inst. Compr. Agric. Sci. Kinki Univ. 5, 75–90.

[7] Minto, R.E. and Townsend, C.A. Enzymology and molecular biology of aflatoxin biosynthesis, (1997) Chem. Rev. 97, 2537–2556.
[8] Payne, G.A. and Brown, M.P. Genetics and physiology of aflatoxin biosynthesis, (1998) Annu. Rev. Phytopathol. 36, 329–362.
[9] Yabe, K. (2002) Pathway and genes of aflatoxin biosynthesis, in: Microbial Secondary Metabolites: Biosynthesis, Genetics and Regulation (Fierro, F. and Francisco, J., Eds.), pp. 227–251, Research Signpost, India.
[10] Yu, J., Chang, P.-K., Cary, J.W., Wright, M., Bhatnagar, D., Cleveland, T.E., Payne, G.A. and Linz, J.E. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*, (1995) Appl. Environ. Microbiol. 61, 2365–2371.
[11] Yu, J., Chang, P.-K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P. and Bennett, J.W. Clustered pathway genes in aflatoxin biosynthesis, (2004) Appl. Environ. Microbiol. 70, 1253–1262.
[12] Skory, C.D., Chang, P.K. and Linz, J.E. Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis, (1993) Appl. Environ. Microbiol. 59, 1642–1646.
[13] Trail, F., Mahanti, N., Rarick, M., Mehigh, R., Liang, S.H., Zhou, R. and Linz, J.E. Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway, (1995) Appl. Environ. Microbiol. 61, 2665–2673.
[14] Yu, J., Chang, P.-K., Bhatnagar, D. and Cleveland, T.E. Cloning of sugar utilization gene cluster in *Aspergillus parasiticus*, (2000) Biochim. Biophys. Acta 1493, 211–214.
[15] Chang, P.-K., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Bennett, J.W., Linz, J.E., Woloshuk, C.P. and Payne, G.A. Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis, (1993) Appl. Environ. Microbiol. 59, 3273–3279.
[16] Yu, J., Chang, P.-K., Cary, J.W., Ehrlich, K.C., Montalbano, B., Dyer, J.M., Bhatnagar, D. and Cleveland, T.E. Characterization of the critical amino acids of an *Aspergillus parasiticus* cytochrome P450 monooxygenase encoded by *ordA* involved in aflatoxin B_1, G_1, B_2, and G_2 biosynthesis, (1998) Appl. Environ. Microbiol. 64, 4834–4841.
[17] Ehrlich, K.C., Montalbano, B.G. and Cary, J.W. Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*, (1999) Gene 230, 249–257.
[18] Cary, J.W., Wright, M., Bhatnagar, D., Lee, R. and Chu, F.S. Molecular characterization of an *Aspergillus parasiticus* dehydrogenase gene, *norA*, located on the aflatoxin biosynthesis gene cluster, (1996) Appl. Environ. Microbiol. 62, 360–366.
[19] Chang, P.-K., Skory, C.D. and Linz, J.E. Cloning of a gene associated with aflatoxin B1 biosynthesis in *Aspergillus parasiticus*, (1992) Curr. Genet. 21, 231–233.
[20] Trail, F., Chang, P.-K., Cary, J. and Linz, J.E. Structural and functional analysis of the *nor-1* gene involved in the biosynthesis of aflatoxins by *Aspergillus parasiticus*, (1994) Appl. Environ. Microbiol. 60, 4078–4085.
[21] Cary, J.W., Barnaby, N., Ehrlich, K.C. and Bhatnagar, D. Isolation and characterization of experimentally induced, aflatoxin biosynthetic pathway deletion mutants of *Aspergillus parasiticus*, (1999) Appl. Microbiol. Biotechnol. 51, 808–812.
[22] Brown, D.W., Yu, J.-H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H. and Leonard, T.J. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*, (1996) Proc. Natl. Acad. Sci. USA 93, 1418–1422.
[23] Hohn, T.M., Desjardins, A.E. and McCormick, S.P. The Tri4 gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis, (1995) Mol. Gen. Genet. 248, 95–102.
[24] Adye, J. and Mateles, R.I. Incorporation of labeled compounds into aflatoxins, (1964) Biochim. Biophys. Acta 86, 418–420.
[25] Pitkin, J.W., Panaccione, D.G. and Walton, J.D. A putative cyclic peptide efflux pump encoded by the TOX4 gene of the plant-pathogenic fungus *Cochiobolus carbonum*, (1996) Microbiology 142, 1557–1565.
[26] Keller, N.P., Segner, S., Bhatnagar, D. and Adams, T.H. *stcS*, a putative P-450 monooxygenase, is required for the conversion of versicolorin A to sterigmatocystin in *Aspergillus nidulans*, (1995) Appl. Environ. Microbiol. 61, 3628–3632.
[27] Skory, C.D., Chang, P.-K., Cary, J. and Linz, J.E. Isolation and characterization of a gene from *Aspergillus parasiticus* associated with the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis, (1992) Appl. Environ. Microbiol. 58, 3527–3537.
[28] Yabe, K., Matsushima, K., Koyama, T. and Hamasaki, T. Purification and characterization of *O*-methyltransferase I involved in conversion of demethylsterigmatocystin to sterigmatocystin and of dihydrodemethylsterigmatocystin to dihydrosterigmatocystin during aflatoxin biosynthesis, (1998) Appl. Environ. Microbiol. 64, 166–171.
[29] Yabe, K., Nakamura, M. and Hamasaki, T. Enzymatic formation of G-group aflatoxins and biosynthetic relationship between G- and B-group aflatoxins, (1999) Appl. Environ. Microbiol. 65, 3867–3872.
[30] Liang, S.-H., Wu, T.S., Lee, R., Chu, F.S. and Linz, J.E. Analysis of mechanisms regulating expression of the *ver-1* gene, involved in aflatoxin biosynthesis, (1997) Appl. Environ. Microbiol. 63, 1058–1065.
[31] Chiou, C.H., Miller, M., Wilson, D.L., Trail, F. and Linz, J.E. Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*, (2002) Appl. Environ. Microbiol. 68, 306–315.
[32] Dutton, M.F. Enzymes and aflatoxin biosynthesis, (1988) Microbiol. Rev. 52, 274–295.