Analysis of Genomic Structure of an Aflatoxin Biosynthesis Homologous Gene Cluster in *Aspergillus oryzae* RIB Strains

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Abstract

To investigate non-aflatoxin-production of *A. oryzae* at the molecular level, an aflatoxin biosynthesis gene homolog cluster of RIB 40 was analyzed. Although most genes in the corresponding cluster exhibited from 97 to 99 % similarity to those of *Aspergillus flavus*, three genes shared 93 % similarity or less. In addition, although slight expression of *aflR*, positive transcriptional regulator gene, was detected in some *A. oryzae* strains having seven aflatoxin biosynthesis homologous genes, other genes related to aflatoxin production were not detected. RIB strains were mainly divided into group 1, having seven aflatoxin biosynthesis homologous genes (*aflT, nor-1, aflR, norA, avnA, verB*, and *vbs*), and group 2, having three homologous (*avnA*, *verB*, and *vbs*). Partial aflatoxin homologous gene cluster of RIB62 from group 2 was sequenced and compared with that of RIB40 from group 1. RIB62 showed a large deletion upstream of *ver-1* with more than half of the aflatoxin homologous gene cluster missing including *aflR*, a positive transcriptional regulatory gene. Adjacent to the deletion of the aflatoxin homologous gene cluster, RIB62 has a unique sequence of about 8kb and a telomere. Southern analysis of *A. oryzae* RIB strains with four kinds of probe derived from the unique sequence of RIB62 showed that all group 2 strains have identical hybridizing signals. Polymerase chain reaction with specific primer set designed to amplify the junction between *ver-1* and the unique sequence of RIB62 resulted in the same size of DNA fragment only from group 2 strains. Based on these results, we developed a useful genetic tool that distinguishes *A. oryzae* group 2 strains from the other groups' strains and propose that it might have differentiated from the ancestral strains due to chromosomal breakage.

Introduction

Taxonomically, the "food-grade" moulds *A. oryzae* and *A. sojae* have very close relationship with aflatoxin producer *A. flavus* and *A. parasiticus*, respectively. In general, it is thought that non-aflatoxigenic *A. oryzae* and

A. sojae were domesticated variant of *A. flavus* and *A. parasiticus*, respectively. Recently, cloning and functional analysis of many genes involved in aflatoxin biosynthesis from *A. flavus* and *A. parasiticus* have made it possible to approach molecular analysis of non-aflatoxigenic mechanism. The inability of *A. sojae* to produce aflatoxin is resulted from the defect in *aflR* homolog, while the reason for the non-production in *A. oryzae* is not yet known whether lack of essential genes in the metabolic pathway or some other reason. Furthermore, some strains of *A. oryzae* conserve almost whole homologous aflatoxin gene cluster. Using molecular techniques to clarify the non-production of aflatoxin in *A. oryzae* is important for the confirmation of safety in the use of this organism. This study has contributed to the understanding of the non-aflatoixigenicity in *A. oryzae*.

In this study, we tried to determine and analyze the aflatoxin homologous gene cluster in *A. oryzae* RIB40 and investigated expression of aflatoxin homologous genes in some *A. oryzae* strains with intact aflatoxin homologous gene cluster. In succession, we characterized the genomic structure of aflatoxin homologous gene cluster in 210 *A. oryzae* RIB strains and analyzed genomic structure of *A. oryzae* strains with a large deletion in aflatoxin homologous gene cluster.

Materials and Methods

Fungal strains

Two hundred and ten *Aspergillus oryzae* RIB strains were from the National Research Institute of Brewing (NRIB, Japan) culture collection. The mycological characteristics and the origin of strains used in this study are listed on the NRIB web site (http//www.nrib.go.jp/ken/asp/strain.html).

One *Aspergillus parasiticus* strain NFRI-95 was obtained from the culture collection of National Food Research Institute. *A. parasiticus* NFRI-95 was used to monitor the expression of genes for aflatoxin biosynthesis. *A. parasiticus* NFRI-95 is a UV-irradiated non-aflatoxigenic mutant of aflatoxigenic *A. parasiticus* SYS-4 (NRRL2999).

Preparation of fungal genomic DNA and RNA

All fungal strains were grown in 50 ml conical tubes containing 10 ml of DP medium (1% polypeptone, 2% dextrin, 0.5% KH_2PO_4, 0.1% $NaNO_2$, and 0.05% $MgSO_47H_2O$) for 3 days at 30 ℃ with rotary shaking at approximately 120 rpm. Genomic DNA was prepared from wet mycelia and extracted with phenol-chloroform and followed by ethanol precipitation. For the extract of total RNA, all fungal mycelia according to method of Lee et al. For extraction of total RNA, strains were grown on in 20 ml of YES (2% yeast extract, 20% sucrose, $ZnSO_47H_2O$ 0.001%, $CuSO_45H_2O$ at 30 ℃ for 2 days with rotary shaking at approximately 120 rpm. Total RNA was prepared from harvested mycelia with ISOGEN (Nippon Gene Co.) according to the manufacturer's instructions.

Reverse-transcription PCR

Approximately 20 μg total RNA was treated with 1 U of Dnase (Nippon Gene Co., Toyama, Japan) at 37 °C

for 15 min. Phenol-chloroform extraction of the treated total RNA was followed by ethanol precipitation and resuspension in 20 μl of nuclease free solution. The cDNA was prepared by transcription of 50 ng/μl of total with 0.5 μg of an oligo dT primer using the SuperScript™ II Rnase H⁻ reverse transcriptase (Invitrogen, USA) following the manufacturer's instruction. RT-PCR was performed in a 20 μl total volume containing: 15 μl Insert Check-Ready-Mix solution (Code No.:PIK-251, TOYOBO Co. Ltd., Osaka, Japan), 0.5 μM of each primer, and 3μl of the cDNA as template. Cycling parameters were as follows; 4 min at 94 °C and 25 cycles of 20 sec at 94 °C, 5 sec at 54 °C, 30 sec at 72 °C in a DNA thermal cycler (GeneAmp PCR systems 9700, Applied Biosystems).

Real-time Quantitative-PCR (Q-PCR)

We used real-time quantitative-PCR (Q-PCR) to compare the relative mRNA levels of *aflR* within *A. oryzae* group 1 strains, while using *A. parasiticus* NFRI-95 as a positive control strain. The real-time Q-PCR was performed using a GeneAmp 5700 sequence detection system (Applied Biosystems, USA) and QuantiTest SYBR Green PCR Kit (QIAGEN) as described in the manufacturers' manuals.

Results and Discussion

Sequence analysis of the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB40

We have shown that a number of mutations exist in the *aflR* promoter region and three ORFs (*aflT*, *norA*, and *verA*) within the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* RIB40 relative to the *A. flavus* sequence. These include deletions, frameshift mutations, and base pair substitutions (Fig. 1). In addition, a 1.5-kb deletion within the *norB-cypA* sequence was also detected, in agreement with observations made in other isolates of *A. oryzae* and in other *A. flavus* S isolates.

In aflatoxin biosynthesis, it is thought that products of *norA*, *nor-1*, and *norB* are involved in converting norsolorinic acid (NA) to averantin, which is an early step in aflatoxin biosynthesis. Disruption of *nor-1* has been reported to result in accumulation of NOR and in a corresponding and substantial decrease in aflatoxin B1. On the other hand, disruption of *norA* or *norB* did not severely influence aflatoxin production. It is thought that mutations of *A. oryzae* RIB40 *norA* and *norB* sequences do not contribute to non-production of aflatoxin in this strain.

The *aflT* gene, which encodes a major facilitator superfamily transporter, has been shown not to play a significant role in the production and secretion of aflatoxin. The partial deletion of the *aflT* sequence is thus presumed to have little share on the non-productivity of aflatoxin by *A. oryzae* RIB40. In addition to relatively low amino acid similarity found between *A. oryzae* and *A. flavus* (93%) homolog of *verA* (Table 2), which encodes a putative monooxygenase, a similar result (92%) was also found between *A. flavus* and *A. parasiticus* (data not shown).

As a result of sequencing analysis, three patterns of the *norB-cypA* region, 1.5-kb (98 strains) and 0.8-kb (5 strains) deletions and no deletion (19 strains) were confirmed in group 1 strains (data not shown).

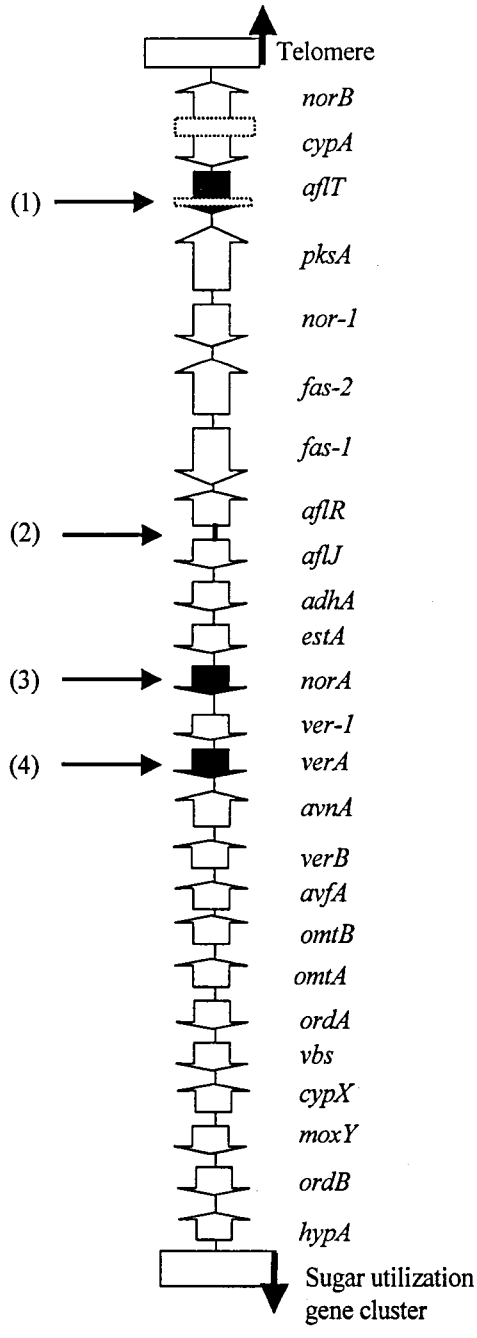

Fig. 1. Structure of the aflatoxin biosynthesis gene homolog cluster in *Aspergillus oryzae* RIB40. Boldface black arrows indicate genes encoding deduced polypeptides with less than 93 % amino acid similarity to those of *A. flavus* (arrow length is not proportional to gene size). Dotted boxes indicate deletions. The boldface line indicates mutations in a recognized consensus sequence within a promoter region. The numbered horizontal arrows indicate specific mutations: 1, a 257-bp deletion resulting in loss of one of 14 putative transmembrane regions; 2, base substitutions in consensus sequences for putative AreA and FacB binding sites; 3, a frameshift mutation resulting in a truncation; 4, amino acid substitutions.

The *aflR* sequences of 19 no-deletion strains were consistent with that of *A. sojae*. Assuming that the 19 no-deletion strains are now classified as *A. sojae*, 95 % of group 1 strains have the deletion structure of 1.5-kb in the *norB-cypA* region. It seems that 1.5-kb-deletion strains are the majority of *A. oryzae* group 1 strain. Strains having 1.5-kb- and 0.8-kb-deletions are also present in *A. flavus*. On the other hand, both *A. parasiticus* and *A. sojae* have no deletion. The structures of the *norB-cypA* regions support the hypothesis that *A. oryzae* and *A. sojae* are differentiated from *A. flavus* and *A. parasiticus*, respectively.

Table 1. Alignment analysis of predicted polypeptides encodes by aflatoxin biosynthesis gene homologs in *A. oryzae* RIB40

Gene	Identity[a] (%)	Length	Gene	Identity[a] (%)	Length
norB and *cypA*	Not possible[b]	Not possible[b]	*verA*	93	493
aflT	87	513(509)[c]	*avnA*	98	495
pksA	99	2109	*verB*	97	500
nor-1	98	271	*avfA*	98	282
fas-2	99	1679(1671)	*omtB*	98	386
fas-1	99	1888	*omtA*	98	418
aflR	99	444	*ordA*	98	528
aflJ	99	438	*vbs*	99	643
adhA	98	278	*cypX*	97	508
estA	99	314	*moxY*	99	487
norA	93	315(388)	*ordB*	99	266
ver-1	99	262	*hypA*	97	481(498)

[a] Amino acids sequence data for *A. flavus* AF70 were obtained from the NCBI nucleotide database.

[b] Because the putative start codon was not found in *A. oryzae* RIB40 and *A. flavus* AF70, an alignment analysis could not be performed.

[C] Length of amino acids in *A. flavus* described in parenthesis.

RT-PCR and real-time Quantitative-PCR of the aflatoxin biosynthesis pathway genes homologs in group 1 strains

The amino acid similarity of the deduced polypeptide encoded by *aflR* from *A. oryzae* RIB40 and that from *A. flavus* was 99 % (Table 2), and AflR binding motifs present on the promoters of genes in the cluster were completely conserved, except for one intergenic region between *norB* and *cypA*. From the results of RT-PCR (Fig. 2) and real-time Q-PCR (Fig. 3), which are more sensitive than Northern blot analysis, the transcription level of *alfR* in group 1 strains is much lower than compared to that of *A. parasiticus* NFRI-95.

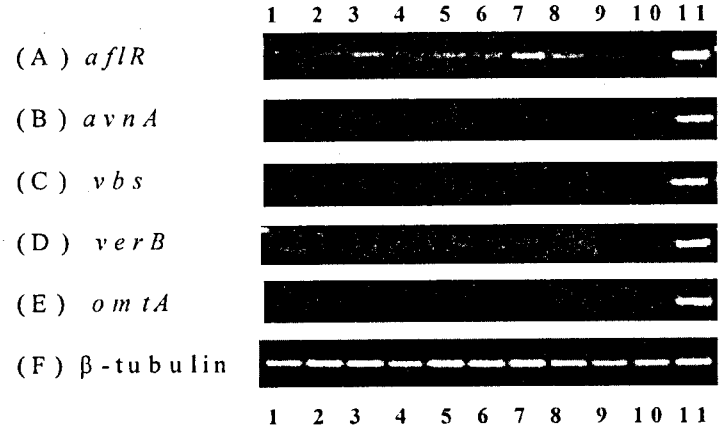

Fig. 2. Gel eletrophoretic analysis of RT-PCR products using primers for the indicated genes. Strains were grown in YES medium for 2 days at 30°C. Lanes: 1, *A. oryzae* RIB40; 2, RIB81; 3, RIB128; 4, RIB176; 5, RIB210; 6, RIB515; 7, RIB920; 8, RIB1031; 9, RIB1039; 10, RIB1401; 11, *parasiticus* NFRI-95.

Expression of *aflR* by real-time RT-PCR and RT-PCR is also detected in *A. sojae* strains, which have been proven nonaflatoxigenic, and *A. flavus* strains, which do not produce aflatoxin. In addition, both *aflR* mRNA and its protein were present in non-aflatoxin-producing strains of *A. flavus*, *A. sojae*, and *A. oryzae*, however mRNA accumulation of *omtA*, an aflatoxin biosynthetic pathway structural gene, was not detected in any of these strains. Therefore, it is thought that the reason for the lack of expression of *avnA, vbs, verB*, and *omtA* genes is a lower transcription level of the regulatory gene, *aflR*. However, it is possible that translation is not performed even if *aflR* is expressed slightly or AflR is degraded. Further work is needed to investigate these possibilities. In any case, it is obvious that group 1 strains cannot produce aflatoxin, because *avnA, verB, vbs*, and *omtA* genes were not expressed, which are necessary for aflatoxin production. In particular, *avnA* is considered essential to produce aflatoxin. The lack of expression of these genes in group 1 strains proves that *A. oryzae* does not produce aflatoxin. Furthermore, in the *A. oryzae* RIB40 *aflR* promoter, base substitutions were found in putative AreA and FacB binding sites related to utilization of nitrogen and carbon sources, respectively. However, it seems unlikely that base substitutions in those putative binding sites alone are the root cause for the non-productivity of aflatoxin by *A. oryzae*. At present, it is extremely difficult to determine a cause for non-productivity from the structural analysis.

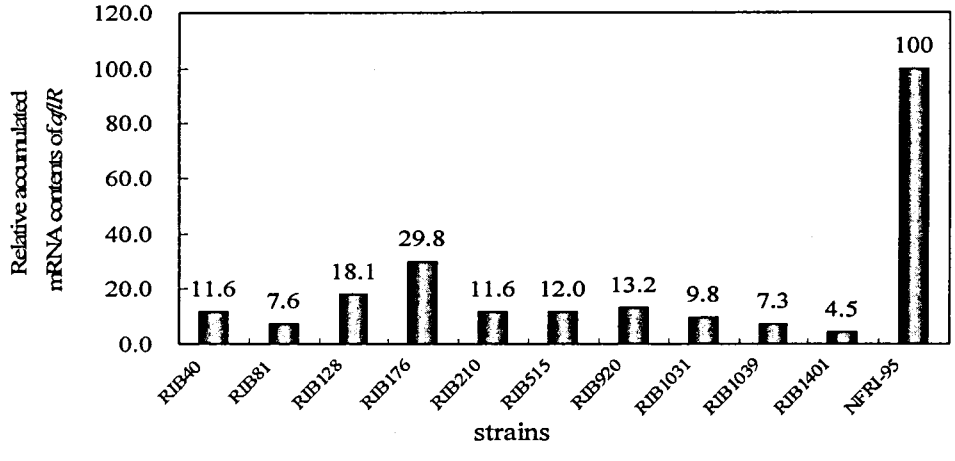

Fig. 3. Relative mRNA contents of *aflR* accumulation in *A. oryzae* group 1 strains and *A. parasiticus* NFRI-95.

Despite the importance and interest in secondary metabolism, very little is known its molecular regulation. In addition, the molecular mechanisms responsible for the loss of aflatoxin production in nonaflatoxigenic *A. flavus* isolates are not well understood. Recently, a series of studies have shown that *aflR* expression is

regulated by G-protein/cAMP/protein kinase A - mediated signaling. The *laeA* gene, which is involved in *aflR* expression, has been described. In succession, further work is needed to clarify non-aflatoxigenic mechanism of *A. oryzae* through various approaches of possibility that non-cluster genes may cause non-productivity of aflatoxin.

Grouping of *A. oryzae* RIB strains by PCR amplification patterns.

Based on the *A. oryzae* RIB40 sequence, we examined the aflatoxin gene homolog cluster in 210 *A. oryzae* RIB strains. Seven homologous aflatoxin biosynthesis genes (*aflT, nor-1, aflR, norA, avnA, verB,* and *vbs)* were selected to cover sequences throughout the cluster. PCR primers specifically amplifying these genes were designed and used to detect their presence.

As shown in Table 2, 210 strains were mainly classified into groups 1 and 2.Group 1, in which amplification of all seven genes was confirmed, including RIB40. Group 2 strains were amplified three genes, *vbs, verB,* and *avnA*. It is possible that the breakpoint within the cluster of group 2 strains would be near the *ver-1* gene. Nine strains (4.3 %) in which at least *vbs* was amplified were classified into group 3. Two strains (0.9 %) that could not be classified into group 1, 2, and 3 were called "others". Most RIB strains (94.8 %) were classified into groups 1 and 2.

Table 2. Classification of *A. oryzae* RIB strains based on PCR amplification patterns of aflatoxin biosynthesis gene homologs

Classification	Aflatoxin biosynthesis genes amplified by PCR[a]	No. of strains	Ratio (%)
Group 1	*aflT, nor-1, aflR, norA, avnA, verB, vbs*	122	58.1
Group 2	*avnA, verB, vbs*	77	36.7
Group 3	*verB, vbs* or *vbs*	9	4.3
Others	No shared patterns	2	0.9

[a] Amplification of seven homologous aflatoxin biosynthesis genes was examined.

Deletion of a large part of the aflatoxin biosynthesis gene homolog cluster, including *aflR*, was detected in 40 % of the RIB strains (groups 2 and 3). The deletion of *aflR* in these strains was confirmed by Southern blotting analysis (data not shown). Furthermore, 60 % of the RIB strains originating from tane koji (the mold starter for making koji), used in sake, soy sauce, and miso production, belong to group 2 (data not shown). PCR amplification pattern of group 3 strains revealed two types. One of them have only *vbs,* the other have *verB* and *vbs* homolog(s). Therefore, we assumed that the deleted region of the aflatoxin biosynthesis gene homolog cluster in group 3 strains is various. Since two strains showed no shared patterns might have misclassifications, we did not classify into groups 1 to 3.

Although the deletions in the aflatoxin gene homolog cluster of *A. oryzae* appeared to be approximately

three types, those of *A. flavus* showed more diverse patterns. In addition, the deletions of the aflatoxin gene homolog cluster in *A. oryzae* and *A. flavus* extend toward the end region in the opposite direction to sugar utilization gene cluster.

In *A. flavus*, fall in aflatoxigenic potentials during subcultureing and its nonrestoration can possibly be associated with the regulation of toxin elaborating genes. It was suggested that aflatoxin production seems to be one of adaptations, such as competition and stressed conditions, in nature. This acquired character gradually fades out when the fungus is transferred to culture media, which are nutritionally rich without any competition for food. Therefore, we assume that this deletion, found in such a large number of *A. oryzae* strains, may have been caused by a long history of use in the brewing industry or may be suitable for life under brewing conditions. In addition, it is necessary to develop molecular biological methods for distinction of these two groups and this method and mycological characters may be applied to intraspecific classification together. In addition, we proved that *A. oryzae* groups 2 and 3 strains were absolutely lack aflatoxigenic ability at molecular level. It is safe for the food fermentation and enzyme industry to use these strains with faith.

Nucleotide sequence of the aflatoxin biosynthetic homologous gene cluster in *Aspergillus oryzae* RIB62

DNA sequencing of partial aflatoxin homologous genes (middle of *avnA* to downstream of telomere) in *A. oryzae* RIB62 belonging to group 2 was performed and compared with that of *A. oryzae* RIB40, which had been determined by our previous results and also by the *A. oryzae* project. As shown in Fig. 4, RIB40 contained almost all of the aflatoxin biosynthetic homologous genes, while RIB62 had a large deletion of this gene cluster. About 8 kb of sequence in RIB62, which did not exist in RIB40 cluster, extends from –300 bp upstream of the *ver-1* homologue and connected to the repeat sequence TTAGGGTCAACA, which was reported to be an *A. oryzae* telomeric sequences. This 8-kb sequence in RIB62 did not match any known nucleotide sequences in the *A. oryzae* RIB40 genome database. After a BLASTX search, several parts of the sequence were found to be partially identical to various known genes with low identity of less than 60% (data not shown). The *ver-1* upstream sequences up to near –300 bp from start codon of *ver-1* were highly conserved, however upstream sequences from –300 bp were quite different from each other (data not shown).

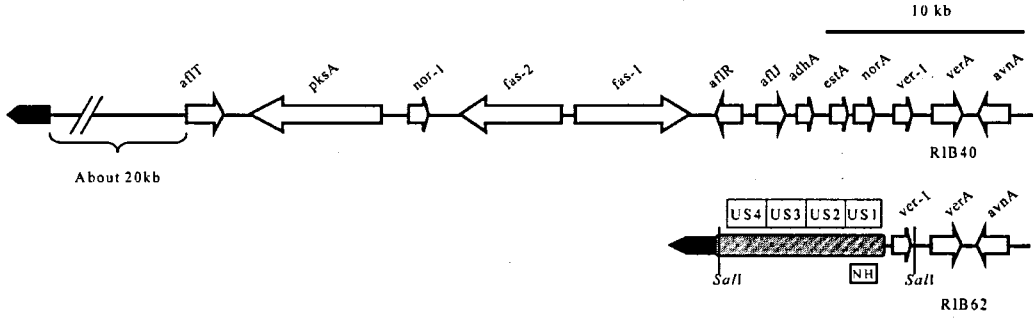

Fig. 4. Schematic representation of the partial aflatoxin biosynthesis homologous gene cluster in *A. oryzae* RIB40 and RIB62. Upper and lower gene cluster are from RIB40 and RIB62, respectively. Open arrows indicate the genes and the direction of transcription. The striped box indicates the region of the unique sequence and the black box indicates repeats of telomeric sequence of *A. oryzae*. The boxes above (US1-4) and below (NH, "no-hit" probe) the unique sequence indicate the location of the probes used in genomic and chromosomal Southern blot analysis. Two vertical lines indicate the *Sal*I restriction enzyme site.

Southern blot analysis of *A. oryzae* RIB strains with the unique sequence probes synthesized from RIB62

Based on DNA sequence analysis, it was confirmed that *A. oryzae* RIB62 did not possess more than half of the aflatoxin homologous gene cluster upstream of *ver-1* (Fig. 4). Consequently, it was confirmed that group 2 strains lack aflatoxin-producing ability because of the large deletion of the aflatoxin biosynthetic homologous gene cluster including *aflR*, which is an aflatoxin biosynthetic regulatory gene. From the results of no homology of the nucleotides in the 8-kb unique sequence between RIB62 and RIB40 (data not shown), it was assumed that the RIB40 orthologous sequence of the unique sequence in RIB62 might have been lost from the genome of RIB40 after the differentiation from a common ancestor. Furthermore, differences in the degree of similarity of the region from *avnA* homolog to breakpoint between RIB62 and RIB40 (data not shown) might show that RIB62 and RIB40 originated from different secondary *A. oryzae* ancestors. However, further work on its cluster and unique sequence is necessary to clarify the differentiation mechanism of *A. oryzae*.

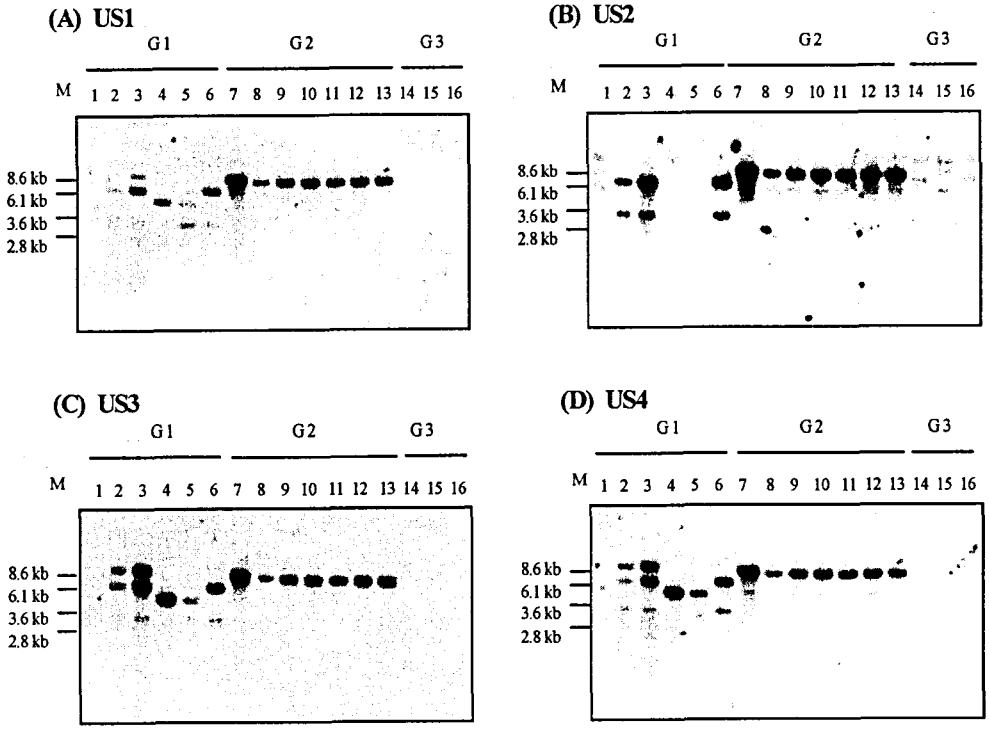

Fig. 5. (A)-(D) Southern blot analysis of *A. oryzae* strains with four kinds of probe for the unique sequence. M, DNA molecular weight marker VII, Digoxigenin-labeled (Roche); lanes 1 to 6, RIB strains belonging to group 1 are 40, 176, 311, 435, 920, and 1039; lanes 7 to 13, RIB strains belonging to group 2 are 62, 144, 171, 406, 430, 915, and 1370; lanes 14 to 16, RIB strains belonging to group 3 are 143, 301, and 326. The group number is described above the lane number.

From the results of Southern blot analysis (Fig. 5), we estimated that unique sequence of group 2 strains might be present in group 1 strains with partial differences, and are located on one or two chromosome(s). In addition, we assumed that the unique sequence might have originated from the ancestor strain of group 1, and group 3 and some group 1 strains like RIB40 lost the unique sequence after they were differentiated from a common ancestor, although further work is needed to clarify this.

Polymerase chain reaction amplification of *A. oryzae* RIB strains with a set of primers for distinguishing group 2 strains

To confirm the structure of the region flanking the partial aflatoxin homologous gene cluster in *A. oryzae* group 2 strains, we investigated the pattern of PCR amplification in 210 *A. oryzae* RIB strains with a set of primers, "no-homology"-F and -R, designed to amplify between *ver-1* and the unique sequence. The oligonucleotide primer for the *ver-1* side, "no-homology"-F, was common to both RIB 40 and RIB62, while that of the unique sequence side, "no-homology"-R, was derived from RIB62. From the results of PCR with this set of primers, a fragment of about 1 kb was amplified from all group 2 strains (Fig. 6, a subset of the total data) and none of strains from other group generated PCR products. Therefore, it is possible to distinguish group 2 strains from other group strains with this set of primers.

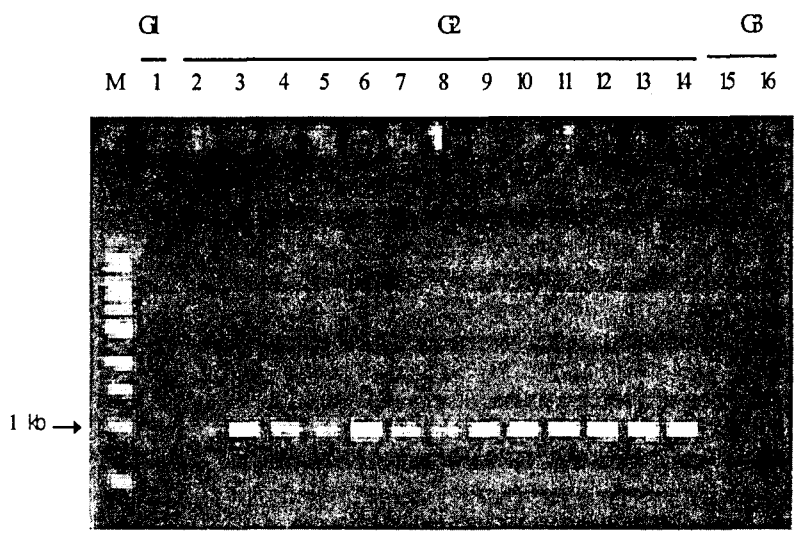

Fig. 6. PCR amplification of *A. oryzae* RIB strains with group 2 specific primers. M, 1 kb DNA ladder (Biolabs Inc New England); lane 1, RIB40 belonging to group 1; lanes 2 to 14, RIB strains belonging to group 2 are 62, 67, 72, 127, 142, 144, 151, 158, 163, 171, 179, 180, and 202; lanes 15 and 16, RIB strains belonging to group 3 are 301 and 326.

From the results of both Southern blot analysis (Fig. 5) and PCR amplification pattern (Fig. 6), it was estimated that all group 2 strains conserved the unique sequence and sequences between deleted region of the aflatoxin biosynthetic homologous gene cluster and the unique sequences. Therefore, it is possible to distinguish group 2 strains from other groups' strains by PCR with a set of specific primers. This method may be a useful tool to distinguish group 2 strains from other group strains. Group 2 strains are commonly used in the fermentation industries and further knowledge on this strain provided in this study is expected to build on the sense of security and safety in its use.

Among the fungi belonging to *Aspergillus* Section *Flavi*, translocation of the chromosome occurred in *A. flavus* and a partial duplication of the complete aflatoxin gene cluster occurred in *A. parasiticus*. In yeast, a model for nonreciprocal translocation of chromosomes caused by the repair of telomere loss was proposed. This process, which is called break-induced replication, requires homologous regions between different chromosomes. Consequently, spontaneous telomere loss destabilizes chromosomes and may eventually lead to chromosome loss and may be lethal. It is interesting to note that it appears that the aflatoxin gene cluster of RIB40 was similar to that of RIB62, which is located at the end of the chromosome. In addition, it was estimated that the unique sequence, which located at flanking region of deleted homologous aflatoxin gene cluster in group 2 strains, and aflatoxin gene homolog cluster of group 1 strains are located at same chromosome (Fig. 7). Although the cause is unknown, we assume that *A. oryzae* group 2 strains might have differentiated from an ancestral *A. oryzae* strain, which has the complete homologous aflatoxin biosynthetic gene cluster, by accidental breakage of chromosome and/or perhaps by restoration of telomere. Further research on their clusters and together with group 3 strains may help in clarifying the mechanism of the cluster deletion.

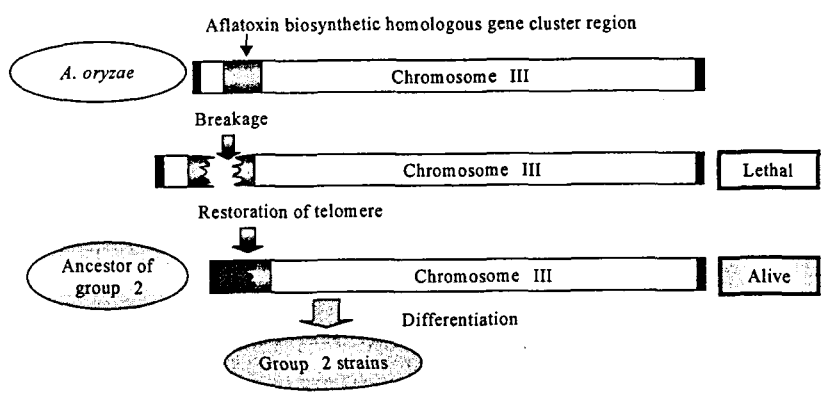

Fig. 7. Model for the differentiation of *A. oryzae* group 2 strains. The black boxes located at ends of the chromosome indicate telomere.

Although it is extremely difficult to determine the reason for the non-aflatoxigenicity of *A. oryzae* from the analysis of the genomic structure, this dissertation may provide basic molecular information for the profound approaches. In succession, further research on *aflR* protein activity or other related signal transduction pathway and the deleted aflatoxin biosynthesis gene homolog cluster of group 2 strains together with group 3 strains may help in clarifying mechanism of the cluster deletion and differentiation.

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Main Scientific Publication	Lee, Y.H, Cho Y.J and Kim H.D. 2002. Comparison in cultural characteristics according to pot diameter and substrate weight in pot cultivation of *Pleurotus ostreatus.* Korean J. Mycol 30(1): 18-22 Lee, Y.H, Cho Y.J and Kim H.D. 2002. Effect on mycelial growth and fruit body development according to additives and mixing ratio in pot cultivation of *Pleurotus ostreatus.* Korean J. Mycol 30(2): 104-108 Lee, Y.H, Cho Y.J, Kim H.D and Chi J.H. 2002. Comparison in mycelial grwoth and fruit body development according to sterilization condition of substrate in pot cultivation of *Pleurotus ostreatus.* Korean J. Mycol 30(2): 99-103 Mihoko Tominaga, Yun-Hae Lee, Risa Hayashi, Uiji Suzuki, Osamu Yamada, Kazutoshi Sakamoto, Kuniyasu Gotoh, Osamu Akita. 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. Applied and Environmental Microbiology 72(1): 484-490 Yun-Hae Lee, Mihoko Tominaga, Risa Hayashi, Uiji Suzuki, Kazutoshi Sakamoto, Osamu Yamada, Osamu Akita. 2006. *Aspergillus oryzae* strains with a large deletion of the aflatoxin biosynthesis homologous gene cluster differentiated by chromosomal breakage. Appl. Microbiol. Biotechnol. p.339-345 (in press)		