***Fusarium graminearum* mycotoxins and their biosynthetic genes**

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Summary

The plant pathogenic fungus *Fusarium graminearum* (teleomorph: *Gibberella zeae*) is a crucial pathogen of major cereal crops and the fungus has taken on greater importance throughout the world. Taking advantage of rapid progress on fungal molecular techniques, we have studied biosynthetic pathways of harmful secondary metabolites of *F. graminearum* such as trichothecenes, zearalenone, and aurofusarin. We revealed that both *Tri13* and *Tri7* genes are required for the chemotype determination of trichothecenes for nivalenol and 4-acetyl-nivalenol production, respectively. We also constructed efficient chemotype determination system by utilizing disrupted *Tri7* gene sequences of deoxinivalenol producers. Forward and reverse genetic approaches enabled us to characterize the gene clusters responsible for the biosynthesis of two polyketide compounds, zearalenone and aurofusarin. Both gene clusters cover genes encoding polyketide synthases and transcription activators. We also characterized an ABC transporter, ZRA 1, related with zearalenone production and constructed a zearalenone conditional gene expression system from the microarray analyses. These results increased our understanding on *Fusarium* genetics and mycotoxicology. Our future works will focus on revealing the regulatory mechanisms of toxin production and biological functions of these secondary metabolites.

Key words : Aurofusarin, *Fusarium graminearum*, *Gibberella zeae*, Trichothecenes, Zearalenone

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Text

This proceeding paper summarizes our previous chemistry, genetics, and biology studies of cereal head blight fungus *Fusarium graminearum*. These studies were published in relating journals and increased our understanding on *Fusarium* genetics and mycotoxicology.

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1. A destructive cereal pathogen *Fusarium graminearum* in Republic of Korea

The filamentous fungus *Fusarium graminearum* (teleomorph: *Gibberella zeae*) is a destructive cereal pathogen that causes *Fusarium* head blight (FHB) of major cereal crops, such as wheat, barley, and rice and *Fusarium* ear and stalk rot in maize throughout the world¹⁾. Plant disease caused by this fungus leads to not only severe yield losses but also accumulation of mycotoxins such as trichothecenes [nivalenol (NIV) and deoxynivalenol (DON)] and zearalenone (ZEA) in the infected cereals which poses grave threats to the health of animals and humans²⁾.

An epidemic of FHB in Republic of Korea was firstly reported in 1963. The yield losses of cereals was about 80 to 100% in southern Korea, causing a serious social problem because of heavy economic losses and mycotoxicoses in humans and farm animals³⁾. Subsequent FHB epidemics have been occurring about every 10 years and mycotoxin contaminations of cereals were continuously occurred in Korea⁴⁻⁸⁾. Recent toxin survey of Korean rice and its by-products revealed that they were highly contaminated with trichothecenes and ZEA⁹⁾. Recently, FHB on wheat is much more frequently occurring in southern part of Korea because of warm and humid weather during the flowering season.

Population distribution of *F. graminearum* was closely dependent on regional differences in Korea. O'Donnell *et al*¹⁰⁾ described major *F. graminearum* clade as a lineage IV for Asia (Fig. 1 A). However, our in-depth population studies based on gene genealogy in Korea revealed that northern part were predominant with lineage II, III, IV, and VII on corn samples and lineage VI and VII on rice samples. And lineage VI was mainly recovered from rice and barley samples from southern provinces¹¹⁾. Strains with NIV chemotype commonly found in most of the rice and barley samples but DON chemotype strains were predominant in maize of Gangwon province (Fig. 1B)^{4, 8, 12)}.

Fig. 1. Distributions of *F. graminearum* strains. (A) Phylogeographic structures of *F. graminearum* strains by gene genealogy in the world based on previous phylogeographic study¹⁰⁾. (B) Population distribution of *F. graminearum* strains by gene genealogy and amplified fragment length polymorphism (AFLP) of Korea¹¹⁾. These figures were drawn based on the previous reports^{10,11)}.

2. Determination of trichothecene chemotypes

Trichothecene contamination is known to be related with feed refusal, vomiting, diarrhea, dermatitis, and hemorrhages in farm animals¹³⁾. As mentioned above, *F. graminearum* strains are divided into two chemotaxonomic groups according to the production of 8-ketotrichothecenes (Fig. 2A). While DON chemotype strains

Fig. 2. Chemotype determination of *F. graminearum* strains. (A) Chemical structure of trichothecenes. Functional groups of R 1 site of trichothecenes determine the chemotypes of *F. graminearum* strains. (B) Sequence variation of *Tri 7* and *Tri 13* between DON and NIV chemotype *F. graminearum* strains. Arrows mean the primers used for determination of chemotypes. The sequence comparisons were derived from the previous study¹⁹.

produce DON and acetyl-DONs such as 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON), NIV and 4-acetyl-NIV (4-ANIV, also known as fusarenon-X) are produced by NIV chemotype strains¹⁴. DON and NIV are the major 8-ketotrichothecenes found in Asian countries such as Korea and Japan and NIV is more frequently and highly present in cereals and exhibits greater toxicity than DON^{4, 15-17}. Since the NIV chemotype is of great concern and both chemotypes existed in Korea, we tried to develop an easy and efficient system for differentiating DON and NIV chemotypes.

At least 10 genes required for trichothecene biosynthesis are localized in a gene cluster and their biochemical roles were intensively studied in *F. sporotrichioides*. Several homologs of these genes have been previously characterized in *F. graminearum*, and the recent study revealed that different *Tri8* sequences determined whether *Fusarium* species produce 3-ADON or 15-ADON¹⁸. At 2001, we also built on the previous data of *F. sporotrichioides* to analyze the trichothecene biosynthesis gene clusters in the DON and NIV chemotypes of *F. graminearum* isolates¹⁹. We sequenced about 26-kb gene cluster from both DON and NIV producers and found that gene cluster of *F. graminearum* from both chemotypes contained 10 *Tri* gene homologs in the same order and transcriptional directions as those of *F. sporotrichioides* (Fig. 2B). However, *Tri7* and *Tri13* sequences were variable between two chemotypes. Especially *Tri7* gene open reading frame of DON producer carried several point mutations and insertion of tandem repeat sequences and the numbers of these repeats were different among the DON isolates. The primers which amplified this region of *Tri7* could be easily used for *F. graminearum* chemotype determination (Fig. 2B)¹⁹. Finally, we constructed an easy and efficient system for trichothecene chemotype determination in *F. graminearum* using PCR.

Supposing that *Tri7* and *Tri13* were the genetic determinants of the DON and NIV chemotypes in *F. graminearum* based on our previous sequence data, further genetic study was performed to clarify the function of *Tri7* and *Tri13* genes in *F. graminearum*²⁰. *Tri13* deletion mutant of NIV chemotype strain produced DON rather than NIV. And heterologous expression of *Tri13* alone or both of *Tri13* and *Tri7* of DON strain in NIV producer resulted in altered trichothecene production (Fig. 3). The results suggested that *Tri13* and *Tri7* function for oxygenation and acetylation of the C-4 oxygen during NIV and 4-ANIV production in *F. graminearum*²⁰.

Fig. 3. Total ion chromatograms of extracts of *F. graminearum* cultures. Strains or deletion or heterologous expression of genes are listed in left side of each chromatogram. $\Delta Tri7$, *Tri7* deletion; $\Delta Tri13$, *Tri13* deletion; +*Tri13*, heterologous gene expression of *Tri13* isolated from the NIV producer; +*Tri7*, *Tri13*, heterologous gene expression of both *Tri7* and *Tri13* isolated from the NIV producer. These chromatograms were derived from previous study with slight modifications²⁰.

3. Zearalenone biosynthesis and application of zearalenone for genetic studies

Zearalenone [ZEA, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)- β -resorcylic acid lactone] is a well-known polyketide metabolite produced by several *Fusarium* species¹³. ZEA contaminations are associated with estrogenic disorders in swine and laboratory animals such as rats and mice²¹. Together with trichothecene mycotoxins, ZEA was frequently occurred in cereal crops infected by *F. graminearum* and was also found in foodstuffs such as cornmeal, corn flakes, corn porridge, and beer¹³. Recently, positive relationship between environmental ZEA or zearalenol (ZOL) exposures and the development of central precocious puberty in females was reported²². However, the biological functions of ZEA in *F. graminearum* have not been characterized to date.

Previous chemical studies have proposed that ZEA is derived from acetate through the polyketide synthesis pathway²³ and ZOL is a precursor for ZEA²⁴. Polyketide synthesis pathway is mediated by polyketide synthases (PKSs) and fungal type I PKSs are multifunctional proteins including all of the functional domain required for the formation of full-length polyketides²⁵. Complete genome sequencing of *F. graminearum* has allowed for genome-wide PKSs functional studies^{26,27}. We focused on non-reducing PKSs and firstly found that PKS13 and PKS4 were required for ZEA production²⁸. Other ZEA biosynthetic genes also are located in a gene cluster like trichothecene cluster genes (Fig. 4.). *ZEB1* encoding isoamyl alcohol oxidase is required for conversion from ZOL to ZEA. *ZEB2* is a transcriptional regulator and required for the activation of other cluster genes²⁸.

To investigate other genes involved in ZEA biosynthesis, we performed microarray analyses in ZEA-deficient and ZEA-enhanced conditions. *ZEB2* deletion mutant was used for ZEA-deficient condition

Fig. 4. Genetic organization of the 50 kb DNA region near *PKS13*. The arrow and arrowhead represent a putative gene and its transcriptional direction, respectively. Genes identified as required for ZEA biosynthesis in *F. graminearum* are indicated by filled arrows. Each DNA region of contigs in the *F. graminearum* genome databases is indicated by a thick bar with the contig number indicated above. Below table represents sequence similarities of the genes located at a cluster of *PKS* genes. These figure and table were in the previous report²⁸.

and starch-glutamate (SG) medium supplemented with ZEA was used for ZEA-enhanced condition^{28,29}. In this study, we found a putative ABC transporter (*ZRA1*) which was under positive regulation of the *ZEB2* gene and up-regulated in ZEA-enhanced condition. Although *ZRA1* was not directly associated with ZEA export in *F. graminearum*, deletion and overexpression of *ZRA1* gene resulted in reduced and enhanced ZEA production, respectively (Fig. 5 A). However, localization of *ZRA1* in plasma membrane and vacuoles still indicated possible roles of *ZRA1* as a transporter (Fig. 5B).

Owing to the advanced molecular techniques such as targeted gene deletion, overexpression, and gene fusion to green fluorescent protein (GFP), many molecular studies have been performed to uncover the mechanisms related to pathogenicity, toxin production, and sexual development in *F. graminearum*. However, a conditional gene expression system for the studies of essential genes or heterologous gene expression was

Fig. 5. ZEA production of *ZRA1* mutants and localization of *ZRA1*-GFP. (A) ZEA production in wild-type, *ZRA1* deletion mutant, and *ZRA1* overexpression mutant strains. ZEA was extracted from culture filtrates of starch-glutamate (SG) liquid medium. (B) Cellular localization and expression of *ZRA1*-GFP in hyphae from SG liquid medium. Sampling time (day after inoculation) is indicated on the top. Arrows indicate plasma membrane localized *ZRA1*-GFP. Scale bars = 10 μ m. WT, wild-type strain GZ3639; *zra1*, transgenic GZ3639 strain with *ZRA1* deletion; OZRA1, *ZRA1* overexpressed strain DAI, days after inoculation. These data were from the previous study on *ZRA1* gene with slight modifications²⁹.

not available in this fungus. In the previous microarray data²⁹⁾, we additionally got a new insight for genetic manipulation study³⁰⁾. We isolated a zearalenone response gene (*ZEAR*) which was highly expressed after ZEA treatment in microarray of ZEA-enhanced condition. *ZEAR* transcripts were dramatically increased after 1 h of ZEA treatment and that was dependent on ZEA concentration (Fig. 6A). When *GFP* was fused with promoter of *ZEAR* (*Pzear*), ZEA concentration-dependent increase in GFP expression was shown (Fig. 6B). We also confirmed that this inducible promoter system was applicable in other *Fusarium* species including *F. oxysporum* and *F. verticillioides*³⁰⁾. This was the first report of an inducible promoter in *F. graminearum* and our recent genetic study actually utilized this conditional gene expression system to prove the lethality of gene deletion³¹⁾.

Fig. 6. Inducible expression of *Pzear* with ZEA treatment. (A) Analysis of *ZEAR* mRNA expression level. Total RNA of the *F. graminearum* wild-type strain was extracted from complete medium (CM) cultures grown for 1, 2, 4, and 8 h after ZEA treatment. An ethidium-bromide-stained gel is shown as a loading control. (B) *Pzear*-GFP expression. Conidia were incubated for 1 h in CM supplemented with ZEA. These data were come from the previous report with slight modifications³⁰⁾.

4. Revealing aurofusarin biosynthetic pathway

Aurofusarin and rubrofusarin are PKS compounds and responsible for golden yellow and red color of fungal cultures of *F. graminearum* and *F. culmorum*^{32,33}. Aurofusarin is a dimeric naphthoquinone which is toxic to poultry and frequently found in *Fusarium* infected crops^{34,35}. In addition, rubrofusarin is known to have antimycobacterial and antiallergic activities and this compound is phytotoxic to some weeds³⁶⁻³⁸.

We have tried several forward genetic approaches to reveal the biological studies of *F. graminearum* during last 10 years. Restriction enzyme-mediated integration (REMI) mutagenesis was the most successful and we previously generated about 35,000 REMI transformants of *F. graminearum*. We further characterized genes related to vegetative growth, asexual and sexual reproduction, toxin production, and virulence³⁹⁻⁴⁷. Among them, we found a laccase gene (*GIP1*) disrupted REMI mutant which was defected in red pigment production⁴⁷. *GIP1* gene was located in a gene cluster which includes 30-kb region and 10 genes. We additionally identified a gene encoding fungal type I PKS (*PKS12*) in the gene cluster and found that both *GIP1* and *PKS12* is required for aurofusarin biosynthesis in *F. graminearum* (Fig. 7A)⁴⁷.

One of the cluster genes (*GIP2*) encoded putative transcription factor that carries Zn(II)₂Cys₆ binuclear cluster DNA-binding domain commonly found in transcription factors of fungal species (Fig. 7A). Deletion, complementation, and overexpression of *GIP2* gene demonstrated that this transcriptional factor is a key regulator for aurofusarin biosynthesis. Similar with ZEB2 transcription factor in zearalenone biosynthesis, *GIP2* also closely involved in activation of other genes in an aurofusarin biosynthetic gene cluster⁴⁸. We also suggested that aurofusarin production is negatively correlated with vegetative growth and ZEA biosynthetic pathway is negatively regulated by the accumulation of aurofusarin in this fungus⁴⁹.

For better understanding on aurofusarin biosynthesis, we deleted all of the aurofusarin cluster genes and revealed that *GIP7*, *GIP8*, and *GIP3* are additionally required for the completion of aurofusarin production in *F. graminearum*^{50,51}. Further in-depth biochemical study performed by Frandsen *et al.*⁵² revealed that a new compound YWA1 is produced during an aurofusarin biosynthetic pathway and *GIP6* /*AurZ* is required for transition from YWA1 to nor-rubrofusarin⁵². And Frandsen *et al.*⁵² additionally characterized *AurS* is a member of extracellular enzyme complex with *GIP1*, *AurF*/*GIP8*, and *AurO*/*GIP3*.

5. Conclusion and future works

Since *Fusarium* mycotoxins pose a great health treats to humans and animals and they are frequently detected in major cereals worldwide, continuous efforts for reducing the mycotoxin contamination are necessary. We have been revealed the biosynthetic pathways of ZEA, trichothecenes, and aurofusarin in head blight fungus *F. graminearum*. Our comprehensive results on mycotoxins have increased our understanding on *Fusarium* genetics and mycotoxicology. However, regulatory mechanisms of toxin production and biological functions of these secondary metabolites were rarely known to date and our future works will focus on these topics.

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Fig. 7. Aurofusarin gene cluster and function of each gene in aurofusarin biosynthesis. (A) Molecular organization of the putative aurofusarin biosynthesis cluster in *F. graminearum*. This cluster was found in contig 1.116 from the *F. graminearum* genome databases. Numbers and arrows indicate possible ORFs and transcriptional directions, respectively. Nucleotide positions of the contig are indicated on the thick vertical bar. (B) HPLC chromatograms of pigments in *F. graminearum* wild-type and transgenic strains with the deletion of aurofusarin biosynthetic genes and the predicted pathway from the acetyl-CoA to aurofusarin in aurofusarin biosynthesis. The hydroxyl group of primary product produced by PKS12, nor-rubrofusarin, is converted to a methyl group by GIP7 resulting rubrofusarin. After oxidation by GIP8 and dimerization of monomers by GIP1, aurofusarin is synthesized as a result of the dehydrogenation by GIP3. The order of reactions by GIP1, GIP3, and GIP8 is not clear. Nor, nor-rubrofusarin; Aur, aurofusarin; Rubro, rubrofusarin. These data were in our previous reports^{47,48,50)}

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