

タイプAトリコテセン生産菌のTri13によってコードされる シトクロムP450モノオキシゲナーゼはタイプBトリコテセンの 生合成経路でも機能する

組換えFusarium graminearumによる3-アセチルニバレノールの生産

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Production of 3-acetylnivalenol by transgenic *Fusarium graminearum* expressing *Tri13* of type A trichothecene-producer: participation of the encoded cytochrome P450 monooxygenase in type B trichothecene biosynthesis

Kazuyuki MAEDA^{1,2,3}, Takeshi TOKAI¹, Hinayo ICHIKAWA³, Naoko TAKAHASHI-ANDO⁴,
Nobuo OGURA², Katsuyoshi YONEYAMA², Minoru YOSHIDA¹ and Makoto KIMURA^{*1,3}

¹Chemical Genomics Research Group, Advanced Science Institute (ASI), RIKEN, 2-1
Hirosawa, Wako, Saitama 351-0198, Japan

²Department of Agriculture, Graduate School of Agriculture, Meiji University, 1-1-1
Higashimita, Kawasaki, Kanagawa 214-8571, Japan

³Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences,
Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

⁴Department of Applied Chemistry, Faculty of Science and Engineering, Toyo
University, Kujirai 2100, Kawagoe, Saitama 350-8585, Japan

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Abstract

Tri13 encodes a cytochrome P450 monooxygenase that catalyzes C-4 hydroxylation of some intermediates in the biosynthesis of type A and type B trichothecenes. This work demonstrated that *Tri13* (*FsTri13*) of *Fusarium sporotrichioides*, a typical type A trichothecene producer, can participate in the biosynthesis of type B trichothecenes. When *FsTri13* was expressed in *Fusarium graminearum* that produces 3-acetyldeoxynivalenol (3-ADON) as an end product, 3-acetylnivalenol (3-ANIV) was newly detected in the liquid medium. Although 3-ANIV can easily be prepared enzymatically from nivalenol (NIV) by using recombinant trichothecene 3-*O*-acetyltransferase (TRI101), there has been no conclusive report on the production of 3-ANIV in cultures of *Fusarium* strains. Possible biosynthetic pathway for production of 3-ANIV was suggested with regard to the function of *Tri8*, which is responsible for deacetylation of the side-chain *O*-acetyls in 3-ADON biosynthesis.

* Correspondence should be addressed to: Makoto Kimura

Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University,
Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan

Phone: (+81) 52-789-5744

Fax: (+81) 52-789-4087

E-mail: mkimura@agr.nagoya-u.ac.jp

Introduction

Trichothecenes are a group of toxic sesquiterpene metabolites with significant structural diversity and are produced by several taxonomically unrelated fungal genera¹⁾. Trichothecenes produced by *Fusarium* species are exclusively oxygenated at C-3 and are divided into two types based on the presence (*i.e.*, type B) or absence (*i.e.*, type A) of a keto group at C-8²⁾. Well-known examples of type A and type B trichothecenes include T-2 toxin produced by *Fusarium sporotrichioides* and nivalenol (NIV) produced by *Fusarium graminearum*, respectively. *Fusarium* type B trichothecenes are further divided into deoxynivalenol (DON)-type trichothecenes (including 3-*O*-acetyl and 15-*O*-acetyl derivatives) and NIV-type trichothecenes (including C-4 *O*-acetyl derivatives)^{3,4)}, which often threaten food and feed safety through contamination in agricultural products.

Tri13 on the trichothecene gene cluster encodes a cytochrome P450 monooxygenase (CYP) responsible for hydroxylation at C-4 of some intermediates of type A and type B trichothecenes^{5,6)}. This gene is a determinant of divergence between DON-producing fungi and others that produce type A or type B trichothecenes oxygenated at C-4. Function of this key gene was demonstrated by targeted deletion in both *F. sporotrichioides*⁶⁾ and *F. graminearum*⁵⁾, and by heterologous expression of NIV chemotype's *Tri13* in the DON-chemotype strain⁵⁾. However, it has not yet been reported whether *Tri13* participates in the biosynthesis beyond the boundary of type A and type B trichothecene producers, although we suggested the possibility of its function in our previous report⁷⁾. In this paper, we show that transgenic *F. graminearum* expressing *F.*

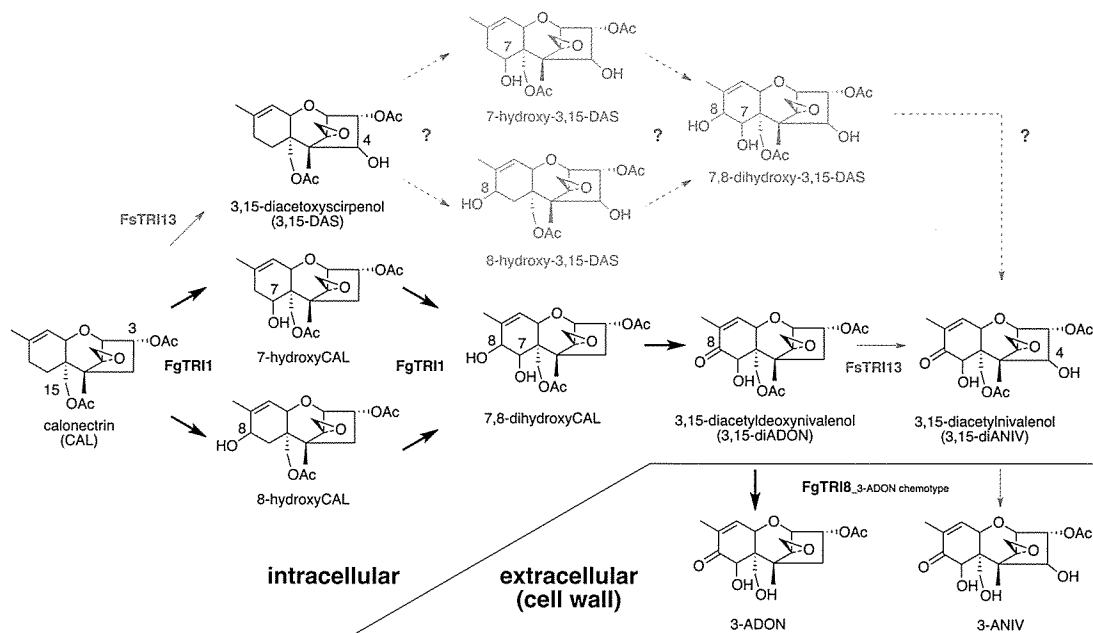


Fig. 1. Late biosynthetic pathway of 3-ADON-producing *F. graminearum* and its transformant expressing *FsTri13*. Red arrows indicate engineered pathways of the transgenic strain expressing *FsTri13*. There is no evidence for the operation of biosynthetic routes indicated by dots in the transgenic strain. Note the subcellular localization of FgTRI8, which differs from that of all other biosynthetic enzymes, including FgTRI13 and FgTRI1.

sporotrichioides Tri13 (*FsTri13*) produced an unnatural trichothecene 3-acetylivalenol (3-ANIV) (see Fig. 1), and then suggest possible biosynthetic pathway of this substance.

Materials and Methods

Fungal strains and culture conditions A hygromycin B resistant Tr-6-34 of *F. graminearum* F 15, a strain that accumulates 3-ADON in liquid culture⁷⁾, was used as a host for transgenic expression of *FsTri13*. For production of trichothecenes, transgenic strains were incubated in 100 ml of 1/2 × PD liquid medium (Potato Dextrose Broth from Difco Laboratories, in half-strength) at 28 °C for 4 days with reciprocal shaking. *F. sporotrichioides* NBRC 9955 was used for preparation of *FsTri13* cDNA.

Plasmid construction and expression analysis Based on the genomic DNA sequence of the region between *FsTri14* and *FsTri12* (accession number AB088350), the complete coding region of *FsTri13* from *F. sporotrichioides* NBRC 9955 was amplified by reverse transcription (RT)-PCR with primers 13-U (5'-ATGTTTCTCTCTCTTGGTCTAATA-3') and 13-D (5'-CTATGACGTTTTCAGATCGACTTT-3'), cloned into pCR/T7-TOPO (Invitrogen), and sequenced. For construction of pBF-*FsTri13* and pBF-*neo*, the *Bam*HI-*Hind*III fragment of the *FsTri 13* cDNA excised from pCRT 7 /NT-TOPO and the *Bgl*II-*Sma*I fragment of aminoglycoside phosphotransferase from pUCSV-*neo*⁸⁾ were inserted between *PtpC* and *TtrpC* of pBF101⁹⁾, respectively. Expression of *FsTri13* from this plasmid was confirmed by RT-PCR using primers 13TOPOATG (5'-ATGTTTCTCTCTCTTGGTCTAATAGTGC-3') and 13 TOPOTAG (5'-GAAAACACATGGATAAAT-CAGTTCG-3').

Metabolites 3-ANIV was prepared by enzymatic reaction with recombinant trichothecene 3-*O*-acetyltransferase (TRI101)¹⁰⁾, and herein used as an authentic standard. For extraction of trichothecene metabolites, four ml of *Fusarium* culture was mixed with an equal volume of ethyl acetate and the solvent layer was recovered by centrifugation. The ethyl acetate extract was then evaporated to dryness under nitrogen, and redissolved in an appropriate solvent before analysis.

In vitro deacetylase assay Mycelia were ground in a mortar in liquid nitrogen and suspended in 10 mM sodium citrate buffer containing 1.25 mM PMSF (phenylmethanesulfonyl fluoride). The mycelial suspension was further disrupted with ultrasonic disruptor, and then centrifuged at 4 °C for 15 min at 20,400 × *g*. The supernatant was filtered with Millex syringe filter unit (0.45 μm, Millipore) and used for the enzyme assay. The assay was initiated by the addition of the crude enzyme (equivalent to the extract from 60 mg of mycelia, wet weight) to a reaction mixture containing 20 μl of 1 M sodium citrate (pH 5.0) and 40 μg of trichothecenes in a total volume of 0.4 ml. The reaction mixture was incubated at 25 °C for 16 h. After the enzyme reaction, trichothecenes were extracted with equal volume of ethyl acetate, evaporated to dryness under nitrogen, redissolved in 15 μl of ethanol, and subjected to the thin layer chromatography (TLC) analysis.

TLC and GC-MS analyses For TLC analysis, trichothecenes redissolved in ethanol were developed on a TLC plate (Merck F₂₅₄ silica TLC) using ethyl acetate/toluene (3:1) as a solvent and visualized as described previously¹¹⁾. For GC-MS analysis, the evaporated extract was dissolved in 1 ml of trimethylsilyl (TMS)

reagent and incubated at ambient temperature for 15 min. After the chemical reaction, 0.6 ml of *n*-hexane was added to 0.4 ml of the solution containing the TMS trichothecenes. Aliquots of the supernatant were injected to a DB-5 fused silica column (J & W Scientific, CA) connected to the GC-MS system (Hewlett Packard HP6890), and monitored with a TIC (total ion current) mode as described previously¹²⁾.

Results and Discussion

FsTri13 of *F. sporotrichioides* NBRC 9955 and *FgTri13* of *F. graminearum* strain 88-1⁵⁾ was compared (Fig. 2A). The *FsTri13* contained two gaps of 12 bp and 18 bp in the central and 3'- portions of the coding region, respectively. In contrast, no gaps were found in the alignment of the other CYP hydroxylase genes involved in trichothecene biosynthesis (*i.e.*, *Tri4* and *Tri11*). Also, peptide sequence identity of *Tri13* gene between *F. graminearum* and *F. sporotrichioides* was 81 %, which was lower compared to that of most other functional *Tri* genes between these two species (86 % – 91 %)¹³⁾.

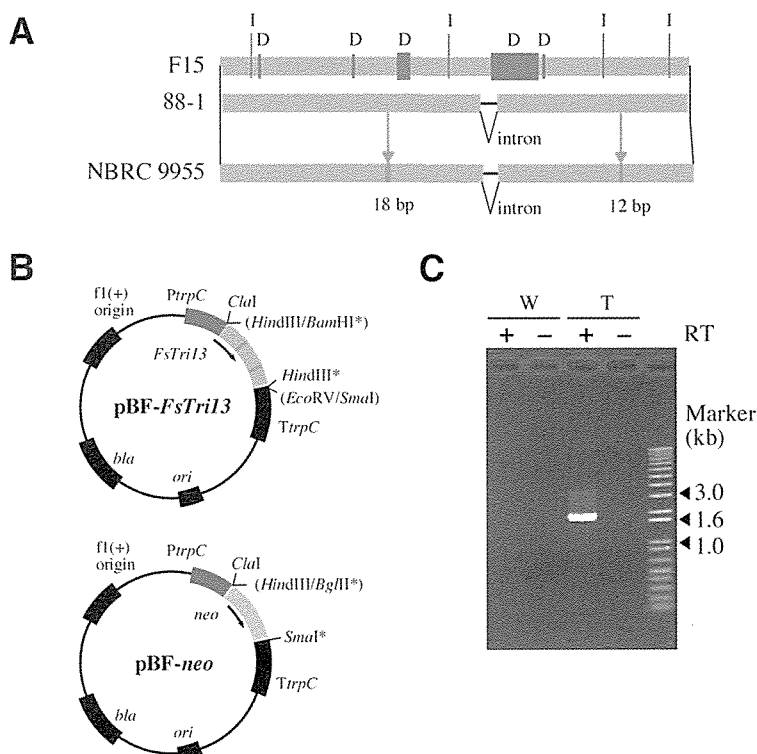


Fig. 2. Transgenic expression of *FsTri13* in *F. graminearum*. A. Comparison of the coding regions of *Tri13* from *F. graminearum* strains F15 and 88-1, and *F. sporotrichioides* NBRC 9955. The CLUSTAL W program was used to align nucleotide sequences of *FgTri13* (from strains F-15 and 88-1) and *FsTri13* (from strain 9955). Red bars on strain F15 *FgTri13* indicate insertions (I) or deletions (D) that inactivate *FgTri13*. Green bars on strain 9955 *FsTri13* indicate insertions of 18 bp and 12 bp. B. Structure of vectors used for transformation. Asterisks indicate the restriction sites derived from the above *FsTri13* and *neo* fragments; restriction sites in parentheses divided by slash were connected together after blunting both ends. C. RT-PCR analysis of Tr-6-34⁷⁾ (W) that constitutively transcribes *FgTri6* and the double transformant Tr-6-34-32 (T) that constitutively transcribes both *FgTri6* and *FsTri13*. RT-minus negative controls (-) are also shown next to the RT-PCR samples (+).

To confirm the function of *FsTri13*, we constructed pBF-*FsTri13* (Fig. 2B); the *Bam*HI-*Hind*III fragment of the *FsTri13* cDNA excised from pCRT7/NT-TOPO was inserted between *P*trpC and *T*trpC of pBF101⁹. This vector was used to co-transform Tr-6-34 of *F. graminearum* F 15⁷ with a neomycin resistance vector pBF-*neo* (Fig. 2B); pBF-*neo* contains the *Bgl*II-*Sma*I fragment of aminoglycoside phosphotransferase (*neo*) gene from pUCSV-*neo*⁸ between *P*trpC and *T*trpC of pBF101. Following PEG-CaCl₂ treatment of the protoplasts, transformants were selected with 50 µg/ml of G418. By screening with RT-PCR, we obtained a transformant Tr-6-34-32 that constitutively transcribed *FsTri13* in the liquid medium (Fig. 2C).

TLC of the liquid culture extracts of the transformant Tr-6-34-32 revealed production of a new metabolite (see Fig. 3 A; marked by an arrow). *R_f* value of this metabolite was the same as that of the 3-ANIV standard. This substance was further analyzed by GC-MS analysis; a TIC chromatogram of the TMS derivatives identified a new peak in the Tr-6-34-32 extract (see Fig. 3B; marked by an arrow in the lower panel), whose mass spectrum was identical to that of the TMS 3-ANIV standard (Fig. 3 C). These results clearly indicated that *FsTri13* functions to produce 3-ANIV in *F. graminearum*. The CYP encoded by *FsTri13* may

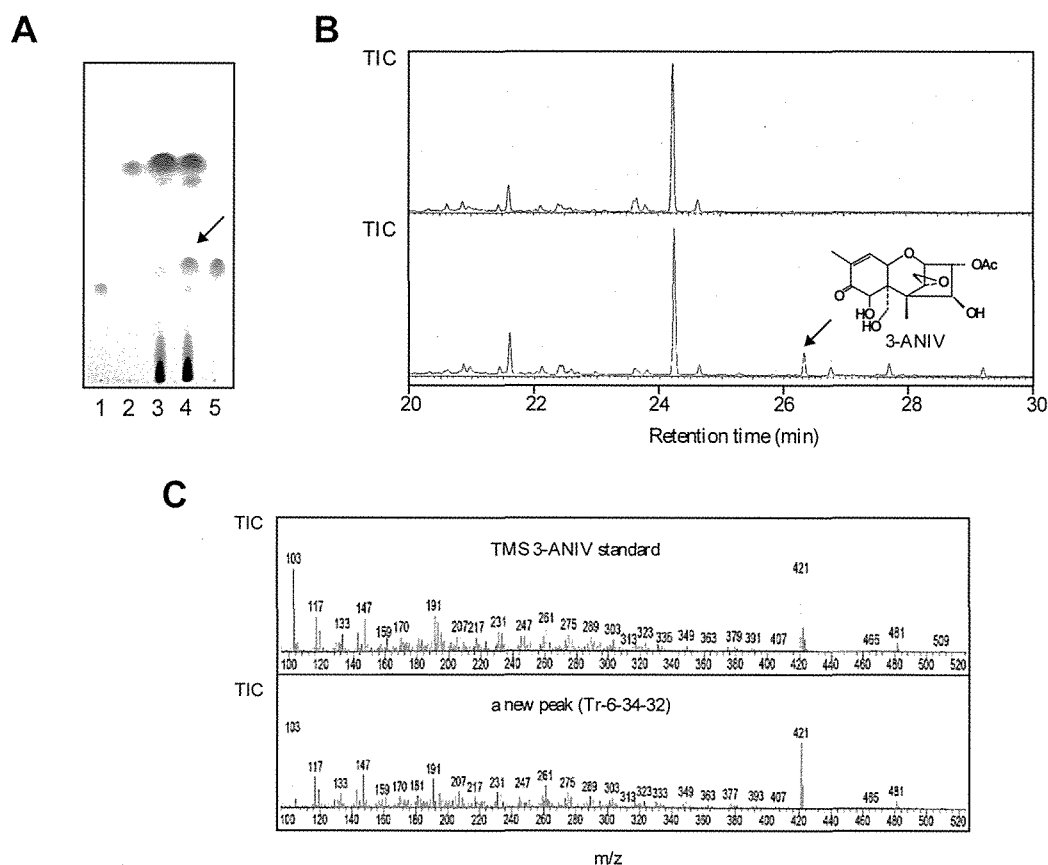


Fig. 3. Production of 3-ANIV by transgenic *F. graminearum* expressing *FsTri13*. A. TLC of the culture extracts 4 days after inoculation of Tr-6-34 (lane 3) and Tr-6-34-32 (lane 4). Thirty µg of DON, 3-ADON, and 3-ANIV standards were loaded on lanes 1, 2, and 5, respectively. B and C. GC-MS of the culture extracts 4 days after inoculation (each equivalent to 4 ml) of Tr-6-34 (upper panel) and Tr-6-34-32 (lower panel) strains. C. Mass spectra obtained for the TMS ethers in the GC-MS analysis. TMS 3-ANIV standard (upper panel) and a new peak (marked by an arrow in Fig. 3B) of Tr-6-34-32 showed the same spectra.

catalyze C-4 hydroxylation of some intermediate(s) in the engineered type B trichothecene biosynthetic pathway, although the intermediate(s) that served as the substrate(s) of this enzyme remain to be clarified.

Calonecristin (CAL) is known to serve as a substrate of FsTRI13 enzyme in T-2 toxin biosynthesis. CAL is also a major intermediate in the biosynthesis of type B trichothecenes, which implies that at least a part of this intermediate is converted to 3,15-diacetoxyscirpenol (3,15-DAS) (Fig. 1). However, involvement of 3,15-DAS in the engineered pathway to 3-ANIV is not certain since substrate specificity of CYP encoded by *F. graminearum* *TriI* (*FgTriI*) has not yet been clarified¹⁴. It may also be possible that 3,15-diacetyldeoxynivalenol (3,15-diADON), the last precursor of 3-ADON biosynthesis, serves as a substrate of FsTRI13. If this is the case, the expected product, 3,15-diacetylnivalenol (3,15-diANIV), must be deacetylated at C-15 to yield the 3-ANIV. In support of this possibility, crude cell extracts of the 3-ADON producer was able to remove the 15-*O*-acetyl from 3,15-diANIV, although with much less efficiency compared to 3,15-diADON (Fig. 4). The slow deacetylation rate may be attributed to a low catalytic efficiency of *FgTri8*_3-ADON chemotype, the extracellular esterase of the 3-ADON producer, against C-15 of 3,15-diANIV (Fig. 1). In fact, 3,15-diANIV is a trichothecene not included in the biosynthetic pathway of the 3-ADON producer while 3,15-diADON serves as an authentic substrate of the *TRI8*_3-ADON chemotype enzyme¹⁵. In any case, the presence of C-3 acetyl in the trichothecenes produced by the transgenic 3-ADON producer suggests such functions of *Tri8* in the genetically modified biosynthetic pathway (see Fig. 1).

A previous report claimed identification of 3-ANIV from *Fusarium*-inoculated wheat head with mass spectral data only¹⁶. The authors speculated structure of the unidentified compound as 3-ANIV from the fragmentation pattern and abundance of each fragment ion, but standard sample of 3-ANIV was not used for the structure determination. No further structural characterization was reported by the same group. There is also a report describing possible production of 3-ANIV by Brazilian *Fusarium* strains¹⁷, but its identification is based on the mass spectral data of the previous paper¹⁶. To our knowledge, there has been no confirmative evidence for the production of 3-ANIV by wild-type *Fusarium* strains. The situation does not disagree with our model of biosynthetic pathway of NIV¹, in which 3-ANIV does not exist as a pathway intermediate due to the strong C-3 deacetylase activity of NIV chemotype's *TRI8* enzyme.

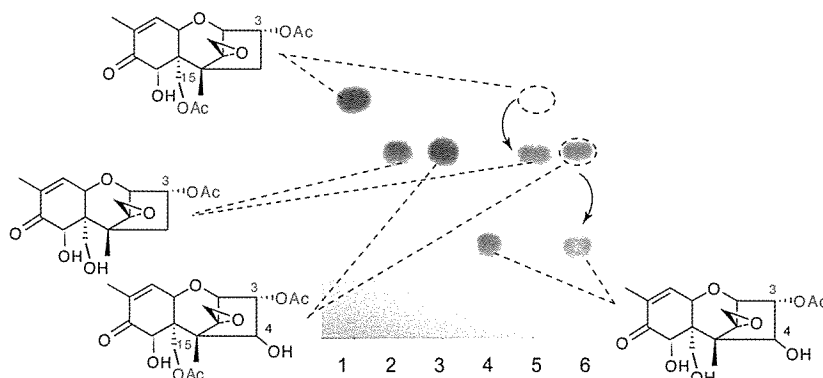


Fig. 4. Deacetylation at C-15 of trichothecenes by crude cell extracts of the 3-ADON producer. Lane 1: 3,15-diADON standard (30 µg); lane 2: 3-ADON standard (30 µg); lane 3: 3,15-diANIV standard (30 µg); lane 4: 3-ANIV standard (30 µg); lane 5: 3,15-diADON incubated with the crude cell extract; lane 6: 3,15-diANIV incubated with the crude cell extract. An arrow on arc indicates conversion of a substrate (dotted circle) to a product.

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フザリウム属菌に劇感染した国産秋まき小麦の製粉画分におけるニバレノール分布

シャリフ Md. ホッセン¹, 吉田めぐみ², 中川博之¹, 長嶋 等¹, 岡留博司¹, 中島 隆^{2, 3}, 〇久城 真代^{1, 3}
(¹(独)農研機構 食総研, ²同機構 九州研, ³同機構 本部)

国産秋まき小麦の製粉過程におけるフザリウム属マイコトキシン・ニバレノールの動態を解析した。ニバレノール濃度が異なる2種類の小麦子実を用いてそれぞれ試験製粉を行い、6つの粉画分（プレーキ粉：1B, 2B, 3B, ミドリングス粉：1M, 2M, 3M）と2つの外皮画分（大フスマと小フスマ）を得た。また可食部となる上質粉は1B, 1M, 2Bおよび2Mより、また末粉は3Bおよび3Mより作製した。上記4種類の試料（上質粉, 末粉, 大フスマおよび小フスマ）についてHPLC-UV法によりニバレノール含量を分析した。その結果、ニバレノール濃度が異なる2種類の小麦子実試料ともに製粉画分におけるニバレノールの分布は類似のパターンを示した。

キーワード：ニバレノール, 加工, 小麦粉, 製粉, HPLC-UV

タイプAトリコテセン生産菌の *Tri13* によってコードされるシトクロム P450 モノオキシゲナーゼはタイプBトリコテセンの生合成経路でも機能する：組換え *Fusarium graminearum* による 3-アセチルニバレノールの生産

前田一行^{*1,2,3}, 東海武史^{*1}, 市川雛代^{*3}, 安藤直子^{*4}, 小倉信夫^{*2}, 米山勝美^{*2}, 吉田稔^{*1}, 木村真^{*1,3}

^{*1} 理研 ASI, ケミカルゲノミックス研究グループ (351-0198 埼玉県和光市広沢 2-1)

^{*2} 明治大学大学院 農学研究科 (214-8571 神奈川県川崎市多摩区東三田 1-1-1)

^{*3} 名古屋大学大学院 生命農学研究科 (464-8601 愛知県名古屋市千種区不老町)

^{*4} 東洋大学 理工学部 応用化学科 (350-8585 埼玉県川越市鯨井 2100)

Tri13 はタイプ A およびタイプ B 生産菌においてそれぞれのトリコテセン経路中間体の C-4 位の水酸化を担うシトクロム P450 モノオキシゲナーゼをコードする。本研究では, *Fusarium sporotrichioides* の *Tri13* (*FsTri13*) がタイプ B トリコテセンの生合成経路でも機能する証拠を示す。 *FsTri13* を 3-アセチルデオキシニバレノール (3-ADON) を生産する *Fusarium graminearum* に導入し発現させたところ, 新規代謝物 3-アセチルニバレノール (3-ANIV) が培養液から検出された。3-ANIV は組換えトリコテセン 3-O-アセチルトランスフェラーゼ (*TRI101*) を用いてニバレノール (NIV) を特異的にアセチル化することで簡単に調製できるが, *Fusarium* 菌株そのものを用いて 3-ANIV を生産できることを示した報告はこれまでなかった。遺伝子改変を施したこの 3-ADON 生産菌の 3-ANIV 生合成経路について, 側鎖 O-アセチルの脱アセチル化に関わる *Tri8* 遺伝子の機能と関連づけて議論する。

キーワード：C-4 位水酸化, C-15 位脱アセチル化, *Fusarium graminearum*, *Fusarium sporotrichioides*, *Tri8*, タイプ B トリコテセン