Molecular and Genetic Studies of Fusarium Trichothecene Biosynthesis: Pathways, Genes, and Evolution

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Trichothecenes are a large family of sesquiterpenoid secondary metabolites of Fusarium species (e.g., F. graminearum) and other molds. They are major mycotoxins that can cause serious problems when consumed via contaminated cereal grains. In the past 20 years, an outline of the trichothecene biosynthetic pathway has been established based on the results of precursor feeding experiments and blocked mutant analyses. Following the isolation of the pathway gene Tri5 encoding the first committed enzyme trichodiene synthase, 10 biosynthesis genes (Tri genes; two regulatory genes, seven pathway genes, and one transporter gene) were functionally identified in the Tri5 gene cluster. At least three pathway genes, Tri101 (separated alone), and Tri1 and Tri6 (located in the Tri1-Tri6 two-gene cluster), were found outside of the Tri5 gene cluster. In this review, we summarize the current understanding of the pathways of biosynthesis, the functions of cloned Tri genes, and the evolution of Tri genes, focusing on Fusarium species.

Key words: biosynthesis gene cluster; fungal secondary metabolism; Fusarium graminearum ( Gibberella zeae ); sesquiterpene; trichothecene mycotoxins

Mycotoxins are fungal secondary metabolites toxic to animals. They often occur in agricultural products and threaten food safety. In terms of economic impact and scientific interest, trichothecenes are undoubtedly major mycotoxins (others being aflatoxins, fumonisins, and zearalenone). They are potent inhibitors of protein translation in eukaryotes and cause moldy-grain toxins in animals. Acute high-dose exposure in animals causes radiomimetic symptoms, including diarrhea, vomiting, leukocytosis, and gastrointestinal hemorrhage, with extremely high doses causing a shock-like syndrome ultimately resulting in death.

Several taxonomically unrelated fungal genera, including Fusarium, Trichothecium, Myrothecium, Stachybotrys, and certain saprophytic fungi, produce a diverse group of trichothecenes, non-volatile sesquiterpenes characterized by a 9,10-double bond and a 12,13-epoxide. To date, more than 200 trichothecenes have been reported. These are divided into four types (A–D) according to chemical structure. Type B trichothecenes are distinguished from type A by the presence of a keto group at C-8, and include the important trichothecenes deoxynivalenol (DON), nivalenol (NIV), and acetylated derivatives thereof (Fig. 1). Type C trichothecenes are a minor group of non-Fusarium trichothecenes containing an additional 7,8-epoxide. Type D

Abbreviations: 3-ADON, 3-acetyldeoxynivalenol; 15-ADON, 15-acetyldeoxynivalenol; 4-ANIV, 4-acetylnivalenol; CAL, calonectrin; CYP, cytochrome P450 monooxygenase; 4,15-DAS, 4,15-diacetoxyisocarpenol; 15-deCAL, 15-deacetylcalonectrin; DHC, 7,8-dihydroxycalonectrin; 3,15-diADON, 3,15-diacetyldeoxynivalenol; 4,15-diANIV, 4,15-diacetylnivalenol; DON, deoxynivalenol; EPT, 12,13-epoxytrichothec-9-ene; FHB, Fusarium head blight; 7-HCA, 7-hydroxycalonectrin; 8-HCA, 8-hydroxycalonectrin; HGT, horizontal gene transfer; 7-HIT, 7-hydroxyisotrichodermin; 8-HIT, 8-hydroxyisotrichodermin; isotrichodiol, 12,13-epoxy-9,10-trichoene-2α,11α-diol; isotrichotriol, 12,13-epoxy-9,10-trichoene-2α,3α,9β-triol; trichodiene; trichodiol, 12,13-epoxy-10,11-trichoene-2α,9β-diol; trichotriol, 12,13-epoxy-10,11-trichoene-2α,3α,9β-triol
trichothecenes, non-
Fusarium mycotoxins of a highly diverse group, contain a macrocyclic ring between C-4 and C-15. This increasingly important group includes satratoxins, roridins, and verrucarins (Fig. 1), which are detected in indoor environments as airborne Stachybotrys mycotoxins.8) Many of the toxic effects of trichothecenes on animals are now known to involve ribotoxic stress responses that activate mitogen-activated protein (MAP) kinases rather than a simple translational arrest.9,10) The resulting rapid deregulation of cell signaling provokes changes in downstream gene expression or induction of apoptosis.11) In plants, trichothecenes such as T-2 toxin also activate MAP kinases, cause changes in gene expression, and induce cell death.12) These elicitor-like activities of trichothecenes differ significantly among their chemotypic variants; e.g., DON does not show such activities at concentrations sufficient to cause translational arrest.

Trichothecenes (mostly type B) are often associated with Fusarium head blight (FHB), a devastating disease of wheat, barley, maize, and other important cereal crops caused by Fusarium graminearum and Fusarium culmorum.13) Infection and colonization of cereal heads by the FHB pathogens not only reduce crop yield but also lower grain quality with mycotoxins such as DON. Some F. graminearum strains concomitantly produce another mycotoxin, zearalenone, that shows estrogenic activity.14) For this estrogenic mycotoxin (mycoestrogen), a degradation gene was isolated from Clonostachys rosea15,16) and efficient detoxification systems have been established using transgenic technology.17–20) In contrast, there appear to be significant hurdles to the development of a similar strategy for trichothecenes.21)

Trichothecene mycotoxins are also known as phytoxins. For example, DON contributes to the virulence of F. graminearum during the infection of wheat plants.22,23) Although transgenic expression of the trichothecene 3-O-acetyltransferase gene confers resistance to DON in cereal plants,24) this strategy has not been successful in achieving a convincing and practical level of FHB resistance and mycotoxin prevention in the field.25,26)

Understanding the biological system of trichothecene

Fig. 1. Chemical Structure of Type A, Type B, Type C, and Type D Trichothecenes.

In the trichothecene skeleton, there are five positions at which side chains can be added (C-3, C-4, C-15, C-7, and C-8). The trichothecenes are collectively divided into type A (a single bond at C-8), type B (keto at C-8), type C (an epoxide at C-7,8), and type D (a macrocyclic ring between C-4 and C-15) on the basis of chemical structure.
production (including its pathways, enzymes, genes, regulation, and evolution) is of great importance as a model of fungal secondary metabolism. In the long term, such knowledge not only helps in the development of practical control strategies, but should also translate into future applications in the metabolic engineering of sesquiterpenes. In this review, we summarize progress and perspectives in molecular and genetic studies of trichothecone biosynthesis, focusing on *Fusarium* species.

I. Pathways for the Biosynthesis of Trichothecone Mycotoxins

Classified into the chemical group sesquiterpene, trichotheccenes have a skeleton derived from farnesyl pyrophosphate (FPP), which is synthesized by the condensation of isopentenyl pyrophosphate (IPP), an isoprene unit, with its isomer dimethylallyl pyrophosphate, followed by the repeated condensation of IPP with the resulting prenyl pyrophosphate, geranylpyrophosphate. FPP is a common intermediate in protein prenylation and in the biosynthesis of sterol, ubiquinone, dolichol, and various secondary metabolites. Cyclizations of a single acyclic FPP give many different carbon skeletons, which has provoked considerable interest in studies of natural sesquiterpene products. In the pathway producing trichothecenes and related mycotoxins (e.g., sambucinol, apotrichodiol), the first committed intermediate was identified as trichodiene (TDN).

1. Cyclization of FPP to TDN (first cyclization)

In earlier days, natural product chemists used *Trichothecium roseum* as an experimental mold to elucidate the isoprenoid pathways, because the fungus produces both sesqui- and diterpene metabolites, whose structures are well understood. Indeed, trichothecin (Fig. 1) was the first sesquiterpene whose biosynthesis was investigated, using [2-14C]mevalonate. The distribution of the label in the product was later revised because the wrong structure of trichothecin was determined at that time. In the 1970s, Nozoe and Machida reported the isolation of alicyclic hydrocarbon TDN (Fig. 2) as a natural product of *T. roseum*. In a feeding experiment with tritiated TDN, they demonstrated a precursor role of this bicyclic compound in the biosynthesis of trichothecin. Extensive studies (some with erroneous conclusions) to elucidate the stereochemistry of the enzymatic cyclization of FPP followed and after much controversy, a sequence from mevalonate via all-trans-FPP and nerolidyl pyrophosphate to TDN was found to be operative by Cane’s group (Fig. 2). Since the mid-1980s, *Fusarium* species have been used for biosynthesis studies by several groups. Compared to *Myrothecium* or *Trichothecium* trichothecenes, *Fusarium* trichotheccenes are unique in that C-3 is occupied with a hydroxyl or an acetyl; e.g., 3-acetyl-deoxynivalenol (3-ADON) and T-2 toxin produced by *F. culmorum* and *F. sporotrichioides* respectively. The outline of the biosynthetic route was found to be the same as that of *T. roseum*, based on the pattern of
incorporation of $[1^{-13}C]$- and $[2^{-13}C]$acetate into 15-acetyldeoxynivalenol (15-ADON) in a feeding experiment with *F. graminearum*. By specifying the location of $[15-2H_2]$ and $[13^{-13}C]$ (trichothecene numbering) in 3-ADON from the labeled precursor TDN, Zamir's group and Greenhalgh's group respectively rigorously proved TDN to be an intermediate of trichothecene biosynthesis. In this way, the common early biosynthetic intermediates of trichothecenes were identified in distantly related fungal genera, *Trichothecium* and *Fusarium*.

2. Oxygenation steps after TDN

Desjardins and co-workers in the USDA-ARS (United States Department of Agriculture, Agricultural Research Service, Peoria, IL) demonstrated that $^{18}O$ molecular oxygen is incorporated into oxygen at position 1, 12, 13-epoxide, and hydroxyl groups at C-3, C-4, C-8, and C-15 of T-2 toxin in *F. sporotrichioides*. This result indicates the involvement of cytochrome P450 monooxygenases (CYPs) in the oxygenation reactions. In further support of this, CYP inhibitors ancymidol and xanthotoxin inhibited T-2 toxin production and caused the concomitant accumulation of a non-oxygenated precursor TDN in *F. sporotrichioides*.

The oxygenation steps after TDN are sequential and not random (Fig. 3). They were studied extensively using *Fusarium* species by the research groups of the USDA, Hesketh, and Zamir. Several oxygenated derivatives of TDN were isolated as candidates for intermediates (listed below in order of year of publication).

- **Trichotriol** (12,13-epoxy-10,11-trichoene-2,3,9-triol) and **trichodiol** (12,13-epoxy-10,11-trichoene-2,9-diol). An earlier study with *F. sporotrichioides* had already identified two natural metabolites, trichotriol and trichodiol, as oxygenated TDN derivatives. Since non-enzymatic cyclization of trichotriol to isotrichodermol was observed in an NMR tube containing CDCl$_3$, trichotriol was hypothesized to be a post-TDN intermediate in the biosynthesis of Fusarium trichothecenes.

- **Isotrichotriol** (12,13-epoxy-9,10-trichoene-2a,3a,11α-triol), 11α-hydroxytrichodiene, and three oxygenated TDN derivatives. To provide a tool for studies of trichothecene biosynthesis, Beremand (USDA) generated *F. sporotrichioides* mutants blocked in their ability to synthesize T-2 toxin by UV mutagenesis. Using these blocked mutants, McCormick and co-workers in the USDA identified five new trichothecene-related alicyclic compounds, as follows (see Fig. 3): isotrichotriol (4), 12,13-epoxy-9,10-trichoene-2a,3a,8α,11α-tetraol (8α-hydroxyisotrichotirol), 12,13-epoxy-9,10-trichoene-2a,3a,8β,11α-tetraol (8β-hydroxyisotrichotirol), and 12,13-epoxy-9,10-trichoene-2a,3a,11α,16-tetraol (16-hydroxyisotrichotirol) from a MB2972 mutant (tri$^{3^-}$), and 11α-hydroxytrichodiene from a MB5493 mutant (tri$^{4^-}$). In feeding experiments with MB5493 (tri$^{4^-}$), isotrichotriol (4), as well as trichotriol and its 9α-epimer, were converted to T-2 toxin. In contrast, the other three new metabolites of MB2972 (tri$^{3^-}$) did not prompt production of T-2 toxin by MB5493 (tri$^{4^-}$). The precursor role of the above tetra-oxygenated TDN isomers suggests the presence of a common transient intermediate in the major biosynthetic pathway of trichothecenes (see section I-4 below).
Isotrichodiol (12,13-epoxy-9,10-trichoene-2α,11α-diol). Hesketh’s group identified isotrichodiol (3; Fig. 3), a product of three oxygenation reactions of TDN, in a *F. culmorum* culture treated with a large amount of TDN. An approach such as the feeding of an excess of a substrate can be used to stimulate accumulation of biosynthetic intermediates that are otherwise undetected or present in trace amounts (see also identification of tricyclic intermediates by Zamir’s group in section I-5 below). Perhaps the last oxygenation giving isotrichodiene (4) is the rate-limiting step among the four consecutive steps of oxygenation of TDN in the early biosynthetic pathway. When [14C]isotrichodiol (3) was fed to the fungus, the 14C-label was efficiently incorporated into 3-ADON. This result indicates that isotrichodiene (3) is a biosynthetic intermediate of trichothecenes.

12,13-Epoxy-9,10-trichoene-2α-ol. Zamir’s group took advantage of kinetic pulse-labeling techniques, coupled with radiolabel feeding experiments, in an attempt to identify earlier biosynthetic intermediates of 3-ADON in *F. culmorum*. They discovered a less-oxygenated TDN derivative, 12,13-epoxy-9,10-trichoene-2-ol (without knowledge of the stereochemistry at C-2), and proved it to be a precursor to trichotheccenes by demonstrating efficient incorporation of the [14C]-labeled into 3-ADON. These results also suggest that 11α-hydroxytrichodiene, identified in *F. sporotrichioides* mutant MB5493 (tri4’), was a shunt metabolite produced by the action of CYP unrelated to trichothecene biosynthesis.

2α-hydroxytrichodiene. On the basis of the observation that TDN was not subject to epoxidation by the cell-free system of *F. culmorum*, which is capable of mediating the epoxidation of C-12,13 of 9β,10β-epoxytrichodiene [a synthetic inhibitor of trichothecene biosynthesis that causes accumulation of isotrichodiol (3)], Hesketh and coworkers speculated that the epoxidation was not the first step in the biosynthetic pathway after TDN. Based on this and the previous results, the sequence of oxygenation steps after TDN was proposed to proceed by C-2 hydroxylation [2α-hydroxytrichodiene (1; Fig. 3) → C-12,13 epoxidation (12,13-epoxy-9,10-trichoene-2α-ol (2; Fig. 3) → C-11 hydroxylation [isotrichodiol (3)] → C-3 hydroxylation [isotrichodiene (4)]]. Although 2α-hydroxytrichodiene (1) was not isolated at that time. Later, Zamir and co-workers isolated this postulated intermediate (1) from *F. culmorum*. They also provided rigorous proof of the operation of this biosynthetic route by feeding experiments with chemically synthesized radiolabeled compounds, 2-hydroxytrichodiene [2α- (1) and 2β-], 12,13-epoxytrichidiene, and 12,13-epoxy-9,10-trichoene-2-ol [2α- (2) and 2β-].

3. Isotrichodermin is the first “trichotheccene” in the biosynthesis of *Fusarium* trichothecenes

By a kinetic pulse-labeling experiment and subsequent follow-up studies, Zamir’s group has rigorously proven that isotrichodermin (ITD) (7; Fig. 4), a natural product of *F. culmorum*, shows all the marks of a true intermediate in the biosynthesis of 3-ADON, viz., isolation from the culture, an appearance and disappearance consistent with the time course of the biosynthesis, and incorporation of the label into the product with correct stereochemistry. By radiolabel feeding experiments, they also showed that 12,13-epoxytrichothec-9-ene (EPT; Fig. 4) is not metabolized into 3-ADON by *F. culmorum*. Around the same time, the USDA research group isolated isotrichodermin (6; Fig. 4) from MB2972 (tri3”) and demonstrated the intermediacy of this earlier intermediate in the biosynthesis of T-2 toxin in feeding experiments with *F. sporotrichioides* MB5493 (tri4”). This mutant was able to use neither EPT nor trichodiol to resume biosynthesis of T-2 toxin.

EPT is an unsubstituted basic trichotheccene skeleton, and in terms of chemical structure, its transformation into isotrichodermin requires only the introduction of one hydroxyl at C-3 of the trichotheccene skeleton. The finding that EPT was not biosynthetically transformed into *Fusarium* trichothecenes clearly indicates that EPT was not the branching intermediate between C-3 unoxygenated and oxygenated trichothecenes. In the biosynthesis of *Fusarium* trichothecenes, C-3 hydroxylation must therefore precede the second cyclization, and isotrichodermin is the first “trichotheccene.”

4. Formation of isotrichodermin from any of the three tetra-oxygenated TDN isomers (second cyclization)

As mentioned in section I-2, the USDA researchers found that isotrichodiene (4), its isomer trichodiene, and the 9α-epimer of trichotriol, might be precursors of T-2 toxin. The conversion of isotrichodiene (4) to isotrichodermin (6) was shown to proceed non-enzymatically in acidic conditions, although the reaction was not rapid enough to feature in a biosynthetic pathway. Since the transient formation of trichotriol was observed during the acid-catalyzed chemical conversion [i.e., the appearance and disappearance of trichotriol coincided with the disappearance of isotrichodiene (4) and the appearance of isotrichodermin (6) respectively], they proposed an intermediate role for trichotriol in the second cyclization. Although the *Fusarium* culture was sufficiently acidic (pH 4.5) to prompt cyclization after the incubation period of 7 d, it is difficult to incorporate this two-step reaction in the biosynthetic pathway without enzymatic intervention.
only slightly by trichotriol. Based on these results, trichodiol and trichotriol were considered to be artifacts rather than true intermediates, and the second cyclization was proposed to proceed via isotrichodiol (3), isotrichotriol (4), and a transient allylic carbocation intermediate (5, Fig. 4).

Considering the co-occurrence of the three tetra-oxygenated TDN isomers, the proposed transient carbocation intermediate (5) appears to be a common pathway intermediate. This unstable compound (5) undergoes either irreversible cyclization to isotrichodermol (6) by intramolecular attack of the C-2 hydroxyl to C-11 or reversible epimerization to trichotriol and its 9α/C11-epimer by regiospecific attack of a water nucleophile at C-9 (but not at C-11, due to the presence of a bulky five-membered ring) (see Fig. 4). Since the Fusarium culture is not acidic enough rapidly to promote the cyclization of isotrichotriol (4) to isotrichodermol (6), the major biosynthetic route may involve a direct enzymatic cyclization. However, a gene for this second cyclization step is not present in the cluster of genes involved in the biosynthesis of trichothecenes in Fusarium species (section II). It is possible that a locally high concentration of isotrichotriol (4) on the endoplasmic reticulum (ER) membrane greatly enhances progression of the forward chemical reaction even under mildly acidic conditions.

5. Formation of calonectrin (CAL) from isotrichodermol

Before the USDA group studied the pathway of trichothecene biosynthesis using blocked mutants, Greenhalgh and coworkers had already isolated a series of minor metabolites from large-scale cultures of trichothecene-producing Fusarium species in an effort to identify candidate biosynthetic intermediates. All such trichothecene-related compounds had a trichothecene structure with a hydroxyl or acetyl at C-3; these included ITD (7), 7-hydroxyisotrichodermin (7-HIT), 8-hydroxyisotrichodermin (8-HIT), 8-ketoisotrichodermin, 15-deacetylcalonectrin (15-deCAL; 8), CAL (9), 3-deacetylcalonectrin, 3,15-dideacetylcalonectrin, 7-hydroxycalonectrin (7-HCA; 10a), 8-hydroxycalonectrin (8-HCA; 10b), 7,8-dihydroxycalonectrin (DHC; 11), 8-ketoicalonectrin, 8-keto-15-deacetylcalonectrin, and 3, 15-diacetyldeoxynivalenol (3,15-diADON). Although the authors did not investigate the precursor roles of these minor natural metabolites, it is reasonable to assume that some of these metabolites are biosynthetic intermediates of 3-ADON (see Fig. 5).

In the 1990s, Zamir’s group identified five of the above trichothecene-related metabolites, 15-deCAL (8), CAL (9), 7-HIT, 8-HIT, and 7-HCA (10a), by feeding an excess of labeled ITD (7) to F. culmorum. Except for 7-HCA (10a), found in an extremely limited amount, the [14C] label on ITD (7) was equally incorporated into...
these minor metabolites; also, they proved to behave like transient intermediates in a kinetic pulse-labeling experiment. Re-feeding of the deuterated derivatives of these compounds demonstrated efficient incorporation of the $^2\text{H}$ label into the expected positions on 3-ADON. In this way, the four metabolites were shown to meet all the criteria of true intermediates. This implies that random oxygenation at C-7, C-8, and C-15 is responsible for the conversion of ITD (7) to 7-HIT, 8-HIT, and 15-deCAL (8), all of which participate in the biosynthesis along metabolic grids (Fig. 5). Nevertheless, a major pathway from ITD (7) to 3-ADON probably involves 15-deCAL (8) and CAL (9), because these intermediates showed greater incorporation of the $^{14}\text{C}$ label into 3-ADON than those of 7-HIT or 8-HIT. It should be noted that the presence of the intermediate CAL (9) implies the occurrence of a C-15 deacetylation step later in the 3-ADON production pathway.

Although biosynthetic intermediates of the minor branched pathways (such as 7-HIT and 8-HIT) were identified, a proposed essential intermediate isotrichodermol (6) was never isolated as a natural product by the kinetic pulse-labeling experiment or from the large-scale Fusarium cultures. This compound was somehow isolated as a minor metabolite of a F. sporotrichioides MB2972 (tri3') mutant blocked at a later step in the biosynthesis (see section 1.3 above). Later, we reported possible reasons why isotrichodermol (6) escaped detection in the sensitive kinetic pulse-labeling experiments: the toxicity of isotrichodermol (6), the first biosynthetic intermediate with a trichothecene skeleton, must immediately be masked by acetylation for the self-protection of the toxin-producing Fusarium species. This implies that the enzymatic conversion of isotrichodermol (6) to ITD (7) is least likely to be rate-limiting in the biosynthesis of 3-ADON. With the availability of Tri101 encoding trichothecene 3-O-acetyltransferase, McCormick and coworkers identified isotrichodermol (6) as the major metabolite of the Tri101' targeted gene disruption mutant of F. sporotrichioides.

Based on the above results, the major biosynthetic route after isotrichodermol (6) was found to proceed via C-3 acetylation (ITD; 7) → C-15 hydroxylation (15-deCAL; 8) → C-15 acetylation (CAL; 9), although other minor routes appear to exist, as found in the 3-ADON-producing F. culmorum.

6. Pathway for the biosynthesis of trichothecenes after CAL

After the formation of CAL (9), a diversity of trichothecene structures (e.g., type A or B, DON- or NIV-type) arises. Historically, 3-ADON has been used in studies of the late steps of biosynthesis by chemical approaches. In feeding experiments with $^{14}\text{C}$isotrichodiol, Hesketh and coworkers observed incorporation of the label into possible transient intermediates: 15-deCAL (8), CAL (9), 7-HCA (10a), and DHC (11), in the biosynthetic pathway to 3-ADON. Based on the identification of 15-deacetyl-7,8-dihydroxycalanectrin as a natural product and subsequent follow-up feeding experiments in F. culmorum, they proposed the following order: ITD (7) → 15-deCAL (8) → CAL (9) → 7-HCA (10a) → DHC (11) → 15-deacetyl-7,8-dihydroxycalanectrin → 3-ADON. However, whether 15-deacetyl-7,8-dihydroxycalanectrin is indeed a major pathway intermediate remains to be determined by characterizing the substrate specificity of the C-15 deacetylase of the 3-ADON-producing strain.

In feeding experiments with $^2\text{H}_3$, 4β,15-$^2\text{H}$ITD (deuterated on the ring and C-3 acetyl), Zamir and coworkers observed that the labeled C-3 acetyl was mostly lost during its metabolism to 3-ADON. They speculated that subsequent C-3 deacetylation and repeated
C-3 acetylation are included at some point in the above pathways in the production of 3-ADON (Fig. 5).

Phylogenetically close *Fusarium* species produce various trichothecenes with different substitution patterns of functional groups at C-3, C-4, C-7, C-8, and C-15. In fact, the substrate specificity of certain late biosynthetic enzymes differs between type A and type B trichothecene producers (our unpublished results), and several biosynthetic routes operate along metabolic grids rather than a single pathway. Partly due to the difficulties of preparing labeled derivatives of all intermediates, chemical procedures were not used to determine the biosynthetic grids. Instead, the availability of gene manipulation techniques and trichothecene pathway genes has opened alternative ways to study the late steps in the biosynthesis of trichothecenes.

II. Trichothecene Biosynthesis Genes of *F. sporotrichioides* and *F. graminearum*

Hohn and coworkers in the USDA pioneered molecular genetic studies of trichothecene biosynthesis. They purified TDN synthase, the first committed enzyme in the pathway, and isolated the *Tri5* gene (formerly designated *Tox5*) encoding this enzyme from *F. sporotrichioides*. A cosmid transformation vector containing *Tri5* complemented the T-2 toxin-deficient mutants MB2972 (*tri3*), and MB5493 (*tri4*), but not MB1716 (*trie*). This result suggested that at least some genes involved in trichothecene biosynthesis (*Tri* genes) are clustered, as is often the case with genes for the biosynthesis of antibiotics in fungi. This finding offered a theoretical basis for a search for other *Tri* genes around *Tri5*. Subsequently a gene cluster (designated the *Tri5* gene cluster) was identified in *F. sporotrichioides* (containing *FgTri* genes) and *F. graminearum* (containing *FgTri* genes).

1. Trichothecene biosynthetic pathway, transport, and regulatory genes in the *Tri5* gene cluster

The functions of the cluster *Tri* genes were determined initially using *F. sporotrichioides* by the USDA group, and later using *F. graminearum* by the USDA group, Lee’s group, and our group. Cosmid clones containing *Tri5* were used in the cloning of other *Tri* genes. Overall, the structure of the *Tri5* gene cluster and the function of *Tri* genes are conserved between *F. sporotrichioides* and *F. graminearum*. The *Tri5* gene cluster’s evolution in type B trichothecene producers was discordant with the phylogeny inferred from genes outside the cluster, and a novel form of balancing selection was suggested to explain the discordance.

In the *Tri5* gene cluster of T-2 toxin- and 4-acetyl-nivalenol (4-ANIV)-producing strains, there are 11 other *Tri* genes, *Tri4*, *Tri6*, *Tri3*, *Tri1*, *Tri12*, *Tri7*, *Tri10*, *Tri13*, *Tri8*, *Tri9*, and *Tri14*, which are upregulated with the onset of trichothecene biosynthesis (Fig. 6). Among these cluster genes, *Tri9* is a gene whose coding region (comprising 43 amino acid residues) was identified by comparative analysis of *F. graminearum* and *F. sporotrichioides* cDNAs, but the contribution of *Tri9* to the biosynthesis is not clear. *Tri4* is not needed for production of trichothecenes on media, but this gene has been reported to be necessary for high virulence and the production of toxin during pathogenesis in wheat tissues. The features of the other 9 *Tri* genes are described below (listed in order of date of publication).

*Tri4* (encoding a multifunctional oxygenase responsible for conversion of TDN to isotrichotriol). *Tri4* was originally isolated as a 7.1-kb genomic DNA fragment that complemented the MB5493 (*tri4*−) mutant of *F. sporotrichioides*. Removal of three introns revealed a single open reading frame that codes for a protein showing significant similarity to members of the CYP superfamily. Its expression paralleled the time course of trichothecene production. Because of its relatively low amino acid sequence similarity to other CYP members, it was grouped into a distinct family designated CYP58. The *Tri4* gene is not needed for high virulence and the production of trichothecenes, and instead accumulated TDN. This suggests that *Tri4* is responsible for the first C-2 hydroxylation step (see section I-2 above).

As to the subsequent oxygenation steps leading to isotrichotriol (4), we tried to identify uncloned *Tri* genes by conducting a comprehensive search of CYP genes using the whole genome DNA sequence database of *F. graminearum*. However, none of the functionally unidentified CYP genes in the genome database, which are induced to express under toxin-producing conditions and are unique to toxin-producing strains, was related to trichothecene biosynthesis. This raised another possibility, that the function of *Tri4* is not restricted to a single hydroxylation step at C-2, but that *Tri4* encodes a multifunctional oxygenase responsible for the three subsequent steps of 12,13-epoxidation, C-11 hydroxylation, and C-3 hydroxylation.

While our search for the genes for the above oxygenation steps was in progress, McCormick and coworkers found that transgenic *Fusarium verticillioideae* (a trichothecene non-producer) expressing *F. graminearum* *Tri4* converted exogenously added TDN to ITD (7), and concluded that *Tri4* is a multifunctional CYP that catalyzes all the oxygenation steps before the formation of the trichothecene skeleton. We fed each bicyclic precursor to trichothecene non-producing mutants of *F. graminearum* with a *Tri4*+ and *Tri4*− genetic background (the *Tri5*− and *Tri4*− targeted gene disruption mutants respectively), and obtained unambiguous evidence that *Tri4* indeed encodes an enzyme responsible for the four consecutive oxygenations steps: TDN → 2α-hydroxytrichodiene (1) → 12,13-epoxy-9,10-trichone-2α-ol (2) → isotrichodiol (3) → isotrichotriol (4). Since these oxygenation steps are indispensable to build up the toxic trichothecene skeleton, *Tri4* can be
a target with which to develop highly specific inhibitors of trichothecene biosynthesis.

Tri6 (encoding a zinc finger transcription factor). Tri6 was originally identified as a 651-bp open reading frame (ORF) during the sequencing of an upstream region of Tri5 in F. sporotrichioides. It is an intron-less gene with expression very similar to that of Tri4. Targeted disruption of Tri6 results in a T-2 toxin non-producing mutant, in which mRNA expression of Tri5 and Tri4 was markedly reduced or eliminated.

TRI6 contains an acidic activation domain in the N-terminal half of the protein and three regions showing some similarity to known Cys2His2 zinc finger motifs at the C-terminus. Together with the result of reporter gene activation in Saccharomyces cerevisiae by the GAL4-TRI6 hybrid protein, Tri6 was found to be a positive regulator of Tri genes. Subsequently, Hohn and coworkers determined the TRI6-binding sequence to be TNAGGCCT by dividing the Tri5 promoter region into short fragments and assessing each for its ability to bind to in vitro synthesized TRI6. This consensus sequence is found upstream of other Tri genes in F. sporotrichioides and F. graminearum. Further gel retardation analysis with the

promoter regions of Tri3 and Tri4 revealed the minimum sequence required for binding TRI6 to be YNAGGCC. Tri3 (encoding 3-acetyltrichothecene 15-O-acetyltransferase). Tri3 was originally isolated as a 4.0-kb genomic DNA fragment that complemented the MB-2972 (tri3/C0) mutant of F. sporotrichioides. Its transcriptional regulation was similar to that of other Tri genes, such as Tri4 and Tri5. Although TRI3 did not show significant similarity to known proteins in GenBank, it had the HxxxDG sequence (where x is any amino acid residue), which is conserved among several other acetyltransferases. Targeted disruption of Tri3 resulted in an accumulation of 15-deCAL and 3,15-dideacetylcalonectrin rather than T-2 toxin or its C-15 deacetylated derivative. This result indicates that Tri3 encodes 15-deCAL 15-O-acetyltransferase, and that C-15 acetylation is essential for efficient progression of subsequent oxygenation at C-4 and C-8 in the biosynthesis of T-2 toxin. Recombinant TRI3 acetylated C-15 of 3-acetyltrichothecenes (e.g., 3-ADON and 3-ANIV), but 3-hydroxytrichothecenes (e.g., DON and NIV) were very poor substrates (our unpublished results).

Tri11 (encoding ITD C-15 hydroxylase). Tri11 was originally identified as a 2.9-kb stretch of the genomic
DNA of *F. sporotrichioides* that hybridized to a 2-kb band on a blot of a trichothecene-producing-stage RNA, which is enriched in the mRNA of *Tri* genes.\(^8\) Sequencing of genomic DNA and cDNA identified four introns. Sequence similarity analysis of *Tri11* protein suggested that it is the first member of a new CYP family, CYP65A1. Targeted disruption of *Tri11* blocked the biosynthesis of T-2 toxin and resulted in the accumulation of ITD (7), 8-HIT, 8-hydroxyisotrichodermol, and a trace amount of 3,4,8-trihydroxytrichothecene.\(^8\) This result suggests that *Tri11* encodes a putative gene in the biosynthesis gene cluster, like *Tri10* in *F. graminearum*, \(9\) or *Tri12* in *F. sporotrichioides*. \(^{10}\) While the promoter region lacks a TRI6-binding consensus sequence, the coding region contains the sequence. The targeted gene disruption mutant did not produce trichothecenes, implying that *Tri10* is essential in production of trichothecenes.

Unexpectedly, *Tri10* expression was dramatically increased by insertion of a vector into the *Tri5*-to-*Tri10* intergenic region, that is, by extension of the distance between the *Tri5* terminator and *Tri10* promoter. Overexpression of *Tri10* increased toxin production through transcriptional activation of *Tri6* and *Tri6*-mediated transcription of other *Tri* genes (including those outside the *Tri5* gene cluster), and the genes encoding enzymes for FPP synthesis in the isoprenoid primary metabolic pathway; e.g., acetyl-CoA acetyltransferase (EC 2.3.1.9), hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5), malonate kinase (EC 2.7.1.36), and FPP synthase (EC 2.5.1.10).\(^{11}\) The regulatory control of *Tri10* extends beyond the genes essential for trichothecene biosynthesis, and upregulates unrelated genes designated *Ibt* (influenced by *Tri10*: *gen*), which includes homologues of aspartyl protease, cellulase, and fumarate reductase genes.\(^{12}\)

The expression level of *Tri10* is elevated in an *F. sporotrichioides* *Tri6* mutant. Perhaps a consensus TRI6-binding site located in the coding region of *Tri10* causes TRI6-dependent downregulation of *Tri10* expression by interfering with its transcription, implying the existence of a negative feedback loop that includes *Tri6*. Alternatively, extension of the *Tri6*-to-*Tri10* region (the core region of the *Tri5* gene cluster) by insertion of the *Tri6* disruption vector\(^{13}\) might cause deregulation of *Tri* gene expression as observed in the above (*Tri5*-to-*Tri10* intergenic region)\(^{14}\) and other (*Tri6*-to-*Tri5* intergenic region)\(^{15}\) cases.

*Tri13* (encoding 3-acetyltrichothecene 4-O-acetyltransferase). *Tri13* was identified upstream of *Tri12* as a region whose translated sequence shows significant similarity to CYP. A comparison of the nucleotide sequence of the *Tri5* gene cluster in T-2 toxin-producing *F. sporotrichioides* and 15-ADON-producing *F. graminearum* indicated that *FgTri13* of the latter strain is not functional due to frameshift mutations in its coding region.\(^{16}\) This observation suggests that DON biosynthesis in *F. graminearum* arose by loss-of-function mutations from the ancestral trait of NIV production.

Targeted gene disruption of *FsTri13* in *F. sporotrichioides* resulted in accumulation of 8-HCA (\(10\)), 8-hydroxy-3-deacetylcalonectrin, and 4-deoxy T-2 toxin. This indicates that *FsTri13* is required for the addition of the C-4 oxygen to CAL (9), and that this oxygenation is not needed for subsequent steps (the addition of a C-8
hydroxyl and esterification of the hydroxyl by an isovalerate) to proceed in the biosynthesis of T-2 toxin (see Fig. 7). Heterologous expression of FgTri13 from the NIV-type strain in a DON-producing strain conferred the ability to produce NIV-type trichothecenes, demonstrating that FgTri13 is a determinant of DON- and NIV-producing chemotypes of F. graminearum.54) While FsTRI13 is able to hydroxylate several trichothecene intermediates [e.g., CAL (9), 3, 15-diADON], the substrate specificity of FgTRI13 appears to be limited to type B trichothecenes (our unpublished results).

Tri8 (encoding trichothecene C-3 deacetylase). Tri8 was originally identified as an intron-less gene in a comparative analysis of the Tri5 gene cluster of F. graminearum and F. sporotrichioides.70) It shows similarity to lipases, and it demarcates an end of the Tri5 gene cluster.75,76) Targeted gene disruption experiments with both species indicated that Fusarium Tri8 encodes an esterase responsible for deacetylation of acetyl at C-3 of the trichothecene skeleton.99) Deacetylation assays using crude cell extracts of F. graminearum (15-ADON producer) and F. sporotrichioides revealed that TRI8 efficiently deacetylated C-3 of trichothecenes that have an acetyl at C-15 [e.g., 3-acetyl T-2 toxin, CAL (9), 3, 15-triacetylscirpenol, 3,7,15-triacetyldeoxynivalenol, 3,8-diacetyldeoxynivalenol], but that 15-hydroxytrichothecenes [e.g., 15-deCAL (8), ITD (7)] were poor substrates.99) In a 3-ADON-producing strain of F. graminearum, a TRI6-binding consensus sequence is not found in the Tri8 promoter region. The FgTri8 gene of this strain is constitutively expressed when the fungus is grown on both trichothecene producing- and non-producing media, but the recombinant FgTRI8 protein did not show C-3 deacetylase activity.76)

2. Tri genes outside the Tri5 gene cluster
As described above, there are seven or fewer pathway Tri genes in the Tri5 gene cluster of Fusarium species. Outside this gene cluster, there are at least three pathway Tri genes. The first-cloned non-cluster gene is Tri101,69) which is moderately conserved between F. graminearum and F. sporotrichioides.100) Other pathway Tri genes isolated later include Tri1 and Tri16, which are located in a two-gene cluster (the Tri1-Tri16 two-gene cluster) distinct from the Tri5 gene cluster.101) However, Tri genes for C-15 deacetylation and C-8 oxidation, steps specific to type B trichothecene biosynthesis, remain to be identified. In addition to these pathway genes, a gene designated Tri15 has been found to be upregulated by Tri10. Tri15 encodes a putative transcription factor with two Cys2His2 zinc finger motifs near the amino-terminal end,102) but this gene is not controlled by Tri6 and is not necessary for toxin production in F. sporotrichioides102) or F. graminearum (our unpublished results). Therefore, it may be more appropriate to define Tri15 as Ibt genes96) rather than as Tri genes. The features of Tri genes identified outside the Tri5 gene cluster are described below.

Tri101 (encoding trichothecene 3-O-acetyltransferase). Tri101 was originally identified as a cDNA that encodes resistance to T-2 toxin when expressed in Schizosaccharomyces pombe.60) This gene has a TR16-binding consensus sequence in its promoter region. Recombinant Tri101 is able to acetylate the C-3 of various Fusarium trichothecenes, including T-2 toxin, DON, and 4,15-DAS. Targeted disruption of Tri101 in F. sporotrichioides is not lethal, and the Tri101 mutant accumulates isotrichoermoderol (6) (and small amounts of 3,15-diacontylecalonectrin and 3-deacetylcalonectrin) rather than T-2 toxin.70) This demonstrates that Tri101 is responsible for the conversion of isotrichoermoderol to ITD (7) in the pathway and that the C-3 acetyl is necessary for progression to the next step. Feeding experiments with a Tri101 mutant of F. sporotrichioides revealed that 3-hydroxytrichothecenes [e.g., isotrichoermoderol (6), 3,15-diacontylecalonectrin, 4,15-DAS] are not metabolized along the biosynthetic pathway, while 3-acetyltrichothecene intermediates [e.g., ITD (7), 15-deCAL (8)] are efficiently converted to T-2 toxin.70) This indicates that TRI101 is an integral enzyme in the progression of the biosynthetic steps in addition to its role in self-protection.

In both F. sporotrichioides and F. graminearum, Tri101 is located between the phosphate permease (pho5) and UTP-ammonia ligase (ura7) genes, indicating that this unique pathway gene occurs alone, separated from all other Tri genes in the Tri5 gene cluster.100,103) Nevertheless, a TRI6-binding sequence is found in the promoter region of Tri101 in both F. graminearum and F. sporotrichioides. Consistent with this structural feature, expression of Tri101 is under the regulatory control of Tri10 and Tri6.95,96)

Tri1 (encoding 3-acetyltrichothecene C-8 hydroxylase in F. sporotrichioides and CAL C-7/C-8 hydroxylase in F. graminearum). Tri1 (formerly Toxl) was the first gene involved in the production of trichothecenes to be defined through a genetic analysis of UV-induced F. sporotrichioides mutants blocked in T-2 toxin production.53) The Tri1− mutant strain MB1716 accumulates 4,15-DAS and lacks C-8 hydroxylation activity. Using Tri10-deleted and -overexpressing strains of F. sporotrichioides, Beremand’s group screened a cDNA library of the Tri10-overexpressing strain. As a candidate for FsTril (which is assumed to be expressed under the regulatory control of Tri10), they obtained a new CYP gene containing the TR16-binding consensus sequence in the promoter region.101) This new CYP gene proved to be FsTril, because targeted gene disruption led to the accumulation of 4,15-DAS and transgenic expression in the Tri1− mutant strains (e.g., MB1716) restored production of T-2 toxin. FsSTRI1 appears to accept ITD (7), CAL (9), and 3,15-diacontylecalonectrin as substrates, because C-8 oxygenated-trichothecenes were detected in Tri1I− (only in trace amounts),90) Tri13−98) and Tri7−79) targeted disruption mutants respectively. A homologue of FsSTRI1 is present in another type A
trichothecene-producer, *Fusarium sambucinum*, but no homologue was detected on a Southern blot of *F. graminearum*.

From *F. graminearum*, McCormick and co-workers cloned *FgTri1* using expressed sequence tag (EST) libraries of *F. graminearum* grown under trichothecene-producing conditions. While *Tri* genes in the *Tri5* gene cluster are highly conserved between *F. sporotrichioides* and *F. graminearum* (e.g., 87% total protein sequence identity between *FsTRI4* and *FgTRI4*, and 90% between *FsTRI11* and *FgTRI1*), *Tri1* is much more divergent between these *Fusarium* species (e.g., 59% identity between *FgTRI1* and *FgTRI3*). The *FgTri1* target gene disruption mutant (generated from the 15-ADON-producing *F. graminearum* strain) accumulated CAL (9) and 3-deacetylcalonectrin, which are oxygenated neither at C-7 nor at C-8, and did not add any hydroxyl at C-7 of exogenously added 8-hydroxy-3-deacetylcalonectrin. In addition, *F. verticillioides* expressing *FgTri1* converted exogenous ITD (7) to 7-HIT and 8-HIT, and CAL (9) to 7-HCA (10a), 8-HCA (10b), DHC (11), and 3,15-diADON, all of which are known as minor natural metabolites of trichothecene-producing *Fusarium* species (see section I-5 above). These results indicate that *FgTRI1* catalyzes hydroxylation of both C-7 and C-8 of ITD (7) and CAL (9), and that either position is oxygenated earlier in the biosynthesis of type B trichothecenes (see Fig. 5).

*Tri16* (encoding an acyltransferase that catalyzes the formation of ester side groups in trichothecenes). *Tri16* was originally identified in the Tri1-Tri16 two-gene cluster as a putative acyltransferase gene during sequencing of the region downstream of Tri1 in *F. sporotrichioides* (Fig. 6). As in the case of other *Tri* genes, *Tri16* was not expressed in the Tri10*−* or Tri16*−* mutant, and was overexpressed in the Tri10-overexpressing strain. Targeted gene disruption of *Tri16* resulted in production of neosolaniol (NEO) and a secondary level of 4,15-DAS, but not other minor metabolites. Since the original *F. sporotrichioides* strain primarily accumulates T-2 toxin along with low levels of 4,15-DAS and NEO, and trace amounts of 8-propionyl-neosolaniol and 8-isobutyryl-neosolaniol, *Tri16* proved to encode an acyltransferase that catalyzes the formation of ester side groups at C-8. In *F. graminearum*, a pseudogene of *Tri16* was found adjacent to *FgTri1*, but in the upstream region and oriented in the opposite direction.

### III. Evolutionary History of *Tri* Genes

Genes that specifically contribute to secondary metabolism are often found in a cluster. Horizontal gene transfer (HGT) is one possible mechanism for the acquisition of a cluster of genes for the biosynthesis of toxins in fungi. The HGT of fungal nuclear genes has been suggested for the T-toxin biosynthesis gene cluster of *Cochliobolus heterostrophus*, the HC-toxin biosynthesis gene cluster of *C. carbonum*, and the AK-toxin biosynthesis gene cluster of *Alternaria alternata*. The experimental basis supporting HGT is that the genes for the biosynthesis of toxins are present only in a particular strain or isolate of a species and are completely absent from others with a near-isogenic background.

#### I. Comparative analyses of regions surrounding *Tri* genes in both trichothecene-producing and non-producing *Gibberella* species

In contrast to the scenario proposed for the acquisition of fungal toxin biosynthesis genes, there has been no circumstantial evidence of HGT of *Tri* genes because there are no near-isogenic non-producing strains for comparison. The genomic diversity of trichothecene-producing and non-producing species makes it difficult to trace the numerous evolutionary events that happened to the ancestral *Fusarium* species long ago. However, if genes not involved in the biosynthesis (e.g., housekeeping genes) surrounding *Tri* genes are found in a region of synteny (or colinearity) between trichothecene-producing and non-producing fusaria, it is possible to gain insight into the evolution of *Tri* genes. Hence we cloned the regions surrounding (1) *Tri10* and (2) the *Tri5* gene cluster from *F. graminearum* and examined to determine whether a microsyntenic region exists in the genome of trichothecene-non-producing fusaria belonging to the same teleomorph genus *Gibberella*.

With regard to the flanking non-biosynthetic genes identified at the *Tri10* locus (pho5 and ura7), we were able to demonstrate that they comprise a region of microsynteny among *Gibberella* species. Unexpectedly, the non-producing *Gibberella*, such as *Fusarium oxysporum* and *Fusarium fujikuroi*, had a dysfunctional copy of *Tri10* in the *pho5-to-ura7* microsyntenic region. This result suggests that HGT is not the cause of the isolated occurrence of *Tri10* in the genome of trichothecene-producing *Gibberella* species, and that the non-producer’s *Tri10* in the microsyntenic region was inactivated by mutations accumulated during evolution.

In contrast to the identification of the *pho5-to-ura7* microsyntenic region, which ruled out HGT of *Tri10* in *Gibberella* species, the non-biosynthetic genes that demarcate the ends of the *Tri5* gene cluster were not mapped to a single locus in *F. oxysporum*. Due to the lack of colinearity for these genes between *F. graminearum* and *F. oxysporum*, we are not able to exclude the possibility that *F. oxysporum* has a pseudo- (or inactivated) cluster of *Tri* genes. Evidence supporting HGT of the *Tri5* gene cluster to ancestors of trichothecene-producing *Gibberella* might be obtained as more fungal genome sequences become available for comparison.
2. Pathway Tri genes in ascomycete not producing trichothecenes

Generally, microorganisms possess certain xenobiotic-metabolizing activities. The same holds true for Fusarium species not producing trichothecenes, which have an enzyme capable of metabolizing trichothececine intermediates. For example, F. verticillioides metabolizes exogenously added 4,15-DAS and readily produces 3,15-diacetoxyscirpenol by acetylation at C-3 and deacetylation at C-4. However, the latter reaction is not known to occur in the pathway for the biosynthesis of trichothecenes. Similarly, C-15 deacetylation of CAL (9) (competing against the step catalyzed by the TRI3 enzyme) was not reported in the biosynthesis of 15-hydroxytrichothecene 3-ADON, but F. oxysporum and F. fujikuroi efficiently convert CAL (9) to 15-deCAL (8) (our unpublished results). Although such activities do not contribute to the biosynthesis of trichothecenes, these findings suggest the possibility that evolutionarily different genetic factors act in concert with cluster Tri genes to modify the original structure of the secondary metabolites defined by the gene cluster.

If several trichothecene non-producing Fusarium species carry out one of the steps of biosynthesis, the corresponding pathway gene of the producer might have a different origin from that of the core cluster Tri genes. In this context, metabolism studies of trichothecene intermediates by trichothecene non-producing Fusarium species also gives some clues to the evolutionary history of the pathway Tri genes. There are two such genes, the trichothecene 3-O-acetyltransferase gene (including Tri101) and an as-yet-unidentified DHC C-8 oxidoreductase gene, which have a different evolutionary history from the cluster Tri genes.

Tri201, TAT, and AYT1 (encoding trichothecene 3-O-acetyltransferase). Trichothecene non-producer Gibberella species carrying a dysfunctional copy of Tri101 have been found to be capable of acetylation of the C-3 of trichothecenes in feeding experiments with 3-hydroxytrichothecenes (e.g., DON, T-2 toxin). We cloned a new trichothecene 3-O-acetyltransferase gene, designated Tri201, (about 70% nucleotide sequence identity to Tri101 of trichothecene-producing fusaria) from F. oxysporum and F. fujikuroi. The genes flanking either side of Tri201 (a putative β-galactosidase gene and a homologue of a Drosophila hypothetical protein gene) are adjacent to each other in the genome of F. graminearum.

Further feeding experiments revealed that non-producing Fusarium species belonging to the teleomorph genus other than Gibberella also have trichothecene 3-O-acetyltransferase activity. In addition, BLASTX-based similarity searches of publicly available fungal genome databases revealed the presence of Tri101 homologues in other ascomycetous fungi, including S. cerevisiae. Subsequent analysis resulted in the functional identification of trichothecene 3-O-acetyltransferase genes in Fusarium decemcellulare (teleomorph genus, Albonectria), Fusarium solani (teleomorph genus, Neocosmospora), Magnaporthe grisea, and S. cerevisiae, designated TAT (Fusarium and Magnaporthe) or AYT1 (S. cerevisiae).

In contrast to pathway Tri genes in the Tri5 gene cluster, the phylogeny of the 3-O-acetyltransferase (TRI101, TRI201, TAT) was mostly concordant with the rDNA phylogeny of these ascomycetous fungi. Together with the identification of pseudo-Tri101 in the pho5-to-ura7 syntenic region in the non-producer Gibberella, this result strongly supports a different evolutionary origin for Tri101 from other Tri genes in the Tri5 gene cluster. Therefore, Tri101 might have been just an antibiotic resistance gene before the ancestors of extant trichothecene-producing Gibberella species acquired the ability to produce highly substituted Fusarium trichothecenes (with functional groups at C-3, C-4, C-7, C-8, and C-15 of the trichothecene skeleton) depending on the function of this essential C-3 acetylase gene (see section II-2 above). There are only two other examples of fungal antibiotic-inactivating genes, MPRI of S. cerevisiae and BSD of Aspergillus terreus, but neither of these is known to be involved in the biosynthesis of antibiotics.

DHC C-8 oxidoreductase gene (as yet unidentified). In feeding studies with F. verticillioides, McCormick and co-workers found that DHC (II) is converted to 3,15-diADON, although such oxidation did not occur spontaneously in the control medium (pH 2.6). This C-8 oxidation of DHC (II) was also observed in feeding studies carried out with FgTri1 disruption mutants of F. graminearum. The DHC C-8 oxidoreductase gene has yet to be cloned from either of these fusaria, but the availability of the F. graminearum genome sequence may aid in the identification of a candidate oxidoreductase gene. In contrast to the presence of C-8 oxidation activity in the non-producing Fusarium species, the T-2 toxin-producer F. sporotrichioides appears not to have a functional C-8 oxidoreductase gene, as suggested by the absence of 8-ketotrichothecenes in the wild-type and a FsTri16− targeted gene disruption mutant.

IV. Perspectives

Since the relative toxicity of trichothecenes is determined by the pattern of oxygenation, acetylation, and/or esterification, it is important to understand the genetic and molecular mechanisms that generate the different substitution patterns of trichothecene side chains at the late stages of biosynthesis. Among various type A and type B trichothecenes, T-2 toxin and 4-ANIV appeared to be suitable for studies of biosynthesis as representatives of the types because other trichothecene chemotype strains appeared to have arisen in evolution (e.g., 4,15-DAS, 3-ADON, NIV) via the inactivation of appropriate pathway Tri genes. With regard to T-2 toxin, Beremand’s group proposed late biosynthetic grids
based on the structure of the metabolites of the FsTri13−, FsTri7− and FsTri1− mutants (Fig. 7),101 but the late biosynthetic grids of 4-ANIV are largely unknown.

If Tri101 and all of the seven pathway genes in the Tri5 gene cluster are functional and other non-cluster pathway genes are inactivated, 4,15-DAS is the expected product (Fig. 8). This is true in the case of T-2 toxin-producing F. sporotrichioides,101) but does not apply to F. graminearum, which produces 4-ANIV (Fig. 3).
unpublished results). Indeed, *F. sporotrichioides* and its *FsTri1* mutant are known to produce 4,15-DAS, but this compound was never isolated as a metabolite of the type B trichothecene-producing strain or its mutant; that is, the *FgTri1* targeted disruption mutant accumulates CAL (9) and 3-deacetylcalonectrin, implying that *FgTri13* cannot contribute to the biosynthesis of NIV-type trichothecenes without a functional copy of *FgTri1*. Therefore, the apparently conserved TR13 enzyme accepts different precursors as a substrate in the biosynthesis of type A and type B trichothecenes (Fig. 8). Presumably due to the structural requirements of the substrate for *FgTR1* to function, perhaps *FgTri13* experienced a different evolution so that the encoded CYP does not add a hydroxyl at C-4 of CAL (9).

Details of the late biosynthetic grids of type A and type B trichothecenes can be determined by analyzing the metabolites of *F. graminearum* and *F. sporotrichioides* transformants in which either *Tri13* or *Tri1* is swapped with the same gene from another species (*F. sporotrichioides* (*FsTri13*/*FgTri13*), *F. sporotrichioides* (*FsTri1*/*FgTri1*), *F. graminearum* (*FgTri13*/*FsTri13*), and *F. graminearum* (*FgTri1*/*FsTri1*)).

It is also important to identify and characterize the C-15 deacetylase gene from various chemotype strains of *F. graminearum*. Indeed, the functionality and substrate specificity of the C-15 deacetylase influences the metabolic flow at the late stage of the biosynthesis. Hence, the distribution and characterization of this enzyme in various chemotype strains should provide clues as to the underlying mechanisms that generate the structural diversity of type B trichothecenes. In addition to study of the C-15 deacetylation step, feeding experiments with various trichothecene precursors using toxin non-producing double gene disruption mutants (e.g., *FgTri15*/*FgTri101*, *FgTri5*/*FgTri3*) should unambiguously define the complex metabolic grids of type B trichothecenes. Using an *F. graminearum* strain that shows a 4-ANIV/4,15-diANIV coproduction phenotype, further details of the late biosynthetic grids are now under investigation in our laboratory in order to identify the molecular and genetic mechanisms generating these biosynthetically diverse and interesting secondary metabolites.

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