

Minireview

Molecular biology and biotechnology for reduction of *Fusarium* mycotoxin contamination [☆]

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Abstract

Fusarium head blight (FHB) is a devastating disease of important cereal crops resulting in significant yield loss and mycotoxin contamination. Persistent outbreaks of FHB in Europe and North America have led to various efforts to understand the mechanisms of resistance to this disease and mycotoxin biosynthesis. In this minireview, we summarize basic and applied studies conducted in our laboratories into reducing mycotoxin contamination in FHB.

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1. Introduction

The necrotrophic plant pathogens *Fusarium graminearum* and *Fusarium culmorum* are the causal agents of *Fusar-*

ium head blight (FHB), a devastating disease of wheat, barley, and maize [1]. These pathogens invade early stage spikelets of host plants and cause their kernels to develop improperly. In addition to the loss of yield caused by the disease, the contamination of infected grains with mycotoxins poses a serious health threat. The mycotoxins produced by the FHB pathogens are trichothecenes (e.g., deoxynivalenol; DON and 3-acetyldeoxynivalenol; 3-ADON) and zearalenone (ZEN). DON frequently occurs in cereals and causes toxicoses such as vomiting, dermatitis, immunosuppression, and hemorrhagic septicemia [2]. ZEN is an estrogenic resorcylic acid lactone that causes severe morphological and functional disorders of reproductive organs in livestock [3].

Although several fungicides reduce the disease severity of FHB, mycotoxin contamination is not necessarily correlated with the visible disease symptoms [4].

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For example, a number of field and greenhouse studies demonstrated that application of the strobilurin fungicide azoxystrobin increases the amount of DON in wheat grains [5]. In addition, sublethal concentrations of certain fungicides were reported to increase trichothecene production by *Fusarium* species [6]. Little is known about the complex factors (e.g., herbicides, fungal competition, moisture, and nutritional factors) that influence the regulation of mycotoxin biosynthesis in infected grains. Therefore, care must be taken to secure the safety of cereal crops treated with fungicides.

To develop an efficient method of reducing *Fusarium* mycotoxin contamination in cereals, it is important to understand how mycotoxins are produced at a molecular level. Obviously, it is more important not to accumulate the mycotoxin in crops by preventing the fungal infection and disease development. The contribution of mycotoxin to the pathogenesis of FHB also needs to be molecularly clarified [7] because defense-related genes, whose expression is affected by mycotoxin, have some role in blocking or delaying infections of mycotoxigenic pathogens [8]. A more practical alternative aimed at preventing mycotoxin from accumulating in planta is detoxification. Enzymatic degradation in particular offers a specific and efficient means of decontamination [9]. In this mini review, we summarize basic and applied studies conducted in our laboratories into reducing mycotoxin contamination in FHB.

2. Inactivation of trichothecenes by an acetyltransferase gene

Trichothecenes are a large group of sesquiterpenoid antibiotics that inhibit protein synthesis in eukaryotes. Trichothecene biosynthesis begins with the formation of trichodiene, which undergoes multiple oxygenation, cyclization, and esterification reactions (see Fig. 1). In the case of *Fusarium* trichothecenes, an acetyl group is attached to C-3 of isotrichodermol, the first tricyclic intermediate, to give isotrichodermin [10]. This C-3 acetyl is mostly lost (i.e., deacetylated) but subsequently restored (i.e., re-acetylated) during the metabolism to 3-ADON in the 3-ADON producer *F. culmorum* [11].

We previously found that 3-*O*-acetylation of the trichothecene ring is related to self-protection for the producers and cloned the *Tri101* gene encoding trichothecene 3-*O*-acetyltransferase [12]. Although 3-ADON is still highly toxic to plants [13–16] at low concentrations, its toxicity appears to be attributed to C-3 deacetylation inside the cell. Thus, transgenic expression of *Tri101* (i.e., constantly eliminating C-3 deacetylated trichothecenes within plant cells) is expected to protect cereals from the phytotoxic effect of trichothecenes and to reduce the disease severity.

By applying this strategy, Okubara et al. transformed wheat plants with *Tri101* from *Fusarium sporotrichioides* and obtained a transgenic line with partial resistance to *F. graminearum* in greenhouse trials although this line did not show resistance to FHB in the field [17]. Little is known

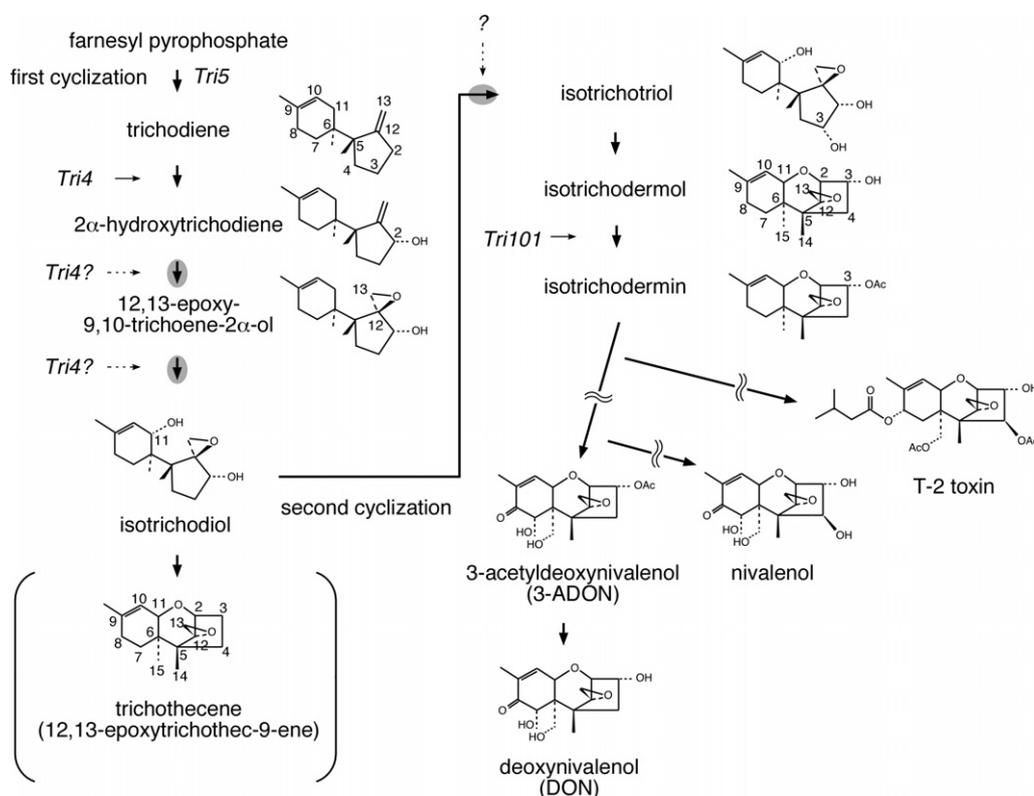


Fig. 1. An outline of trichothecene biosynthesis. In the pathway to the first tricyclic intermediate, the genes for the oxygenation of C-11 and C-3, and epoxidation of C-12,13, have not been identified (see shaded ellipses).

about the molecular mechanism by which DON helps *Fusarium* cause disease in cereal plants. Without detailed information about the temporal, spatial, and quantitative distribution of DON in infected floral tissues, it might not be justified to attribute its toxicity to a simple shut-down of translation (see below).

In animal systems, DON and other trichothecenes, via a mechanism known as the ribotoxic stress response, activate mitogen-activated protein (MAP) kinase and affect downstream gene expression [18,19]. In contrast, there has been no report on the signal transduction cascade and the downstream gene expression in plants treated with trichothecenes. In this regard, it is worth assessing the possibility that trichothecenes function as small molecule effectors involved in the regulation of plant–microbe interactions. In support of this idea, we have recently demonstrated that certain trichothecenes activate MAP kinases and show an elicitor-like activity (e.g., cell death, callose deposition, hydrogen peroxide generation, salicylic acid generation, and pathogenesis-related (*PR*) gene expression) by using a model plant *Arabidopsis thaliana* [20]. The elicitor-like activity of trichothecene-treated plants differed significantly among their molecular species, which is in agreement with the previous theory of molecular evolutionists; i.e., chemotypic differences might affect the pathogenicity of individual *Fusarium* strains in host cereals [21].

Rice is a good model for studies of cereal diseases because genome and full-length cDNA sequencing projects provide essential tools for the molecular characterization of plant defense reactions. We therefore generated transgenic rice plants stably expressing and inheriting *Tri101*. The result of disease assay indicated that they were less susceptible to floral infections of *F. graminearum* than the wild type (Fig. 2), suggesting that trichothecenes are also a virulence factor for the FHB pathogen in the interaction with rice plants. These transgenic rice plants may be used as a model for comparative transcriptome-based analyses of trichothecene-treated flowers. Identification of the host plant genes affected by trichothecenes may contribute to our understanding of the disease at a molecular level.

3. Key enzymes involved in the biosynthesis of trichothecenes

In toxin-producing *Fusarium* species, some, but not all, trichothecene genes were found within a 25 kb core gene cluster (*Tri5*-cluster) [22]. In addition, two trichothecene biosynthesis pathway genes were isolated from a two-gene cluster (*Tri1*-mini-cluster) [23–26], and a gene encoding trichothecene 3-*O*-acetyltransferase (*Tri101*) was identified at a single locus separated from these cluster genes [27,28]. However, other biosynthesis pathway genes have not yet been identified.

Fungal secondary metabolite biosynthesis genes acquired by horizontal gene transfer (HGT) are often clustered, having different evolutionary origins from the non-cluster genes, and each biosynthesis gene in the cluster has the same evolutionary origin. However, trichothecene bio-

synthesis genes are different. In particular, *Tri101* is unique in that it is a key biosynthesis pathway gene physically separated from others in the cluster and its functional homologs are also found in trichothecene non-producer *Fusarium* species and other ascomycetous fungi [29,30]. These acetyltransferase genes are thought to have been vertically transmitted because they did not differ significantly in their positions from the phylogeny of the species from which they are derived [30]. This implies that the trichothecene structure arose as a result of the combined functions of genes with different pathways of evolution (viz., HGT and vertical gene transfer).

In the early biosynthetic pathway before the formation of isotrichodermol, three oxygenation steps (the C-12, 13 epoxidation, and the C-11 and C-3 hydroxylations) remain to have genes functionally assigned to them (see Fig. 1). These oxygenation steps are attractive targets for the specific inhibition of mycotoxin biosynthesis because no trichothecene skeletons are formed without the oxygenations (i.e., isotrichodermol is the first tricyclic intermediate of various trichothecenes). Since all the oxygens in the trichothecene skeleton (viz., the oxygens of position 1 and the 12,13-epoxide) and in the hydroxyl groups at C-3, C-4, and C-15 proved to be derived from molecular oxygen [31], cytochrome P450 monooxygenases (CYPs) are likely to be involved in the above oxygenation steps. Therefore, based on the hypothesis that *CYP* genes involved in trichothecene biosynthesis are specifically induced to express under toxin-producing

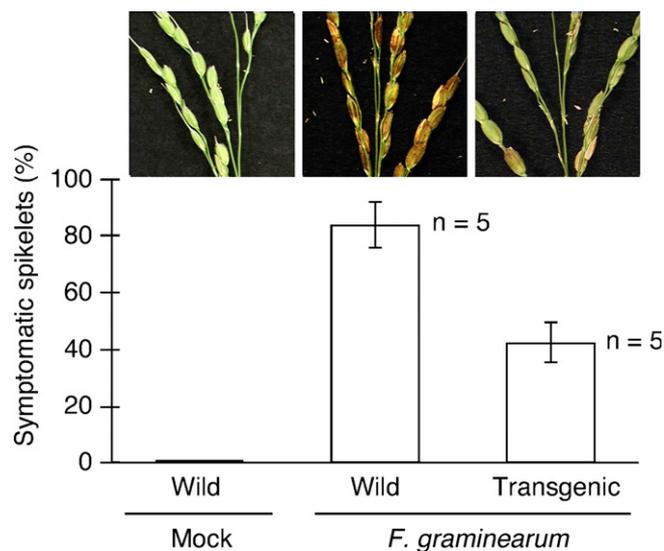


Fig. 2. Resistance to FHB of transgenic rice plants expressing *Tri101*. The flowering spikelets of the wild type and transgenic homozygous T_2 progenies of rice plants (each $n = 5$) were inoculated with *F. graminearum* IFO 5269, a strain that produces 3-ADON. Disease response was determined by visually examining the heads 7 days after inoculation and compared to the mock inoculation with sterile water. The total number of spikelets examined per wild type and T_2 plant was 53.4 ± 17.4 and 62.2 ± 13.8 , respectively. Severity (vertical axis) is represented by the percentage of symptomatic spikelets among the *Fusarium*-treated spikes. Differences between the wild type and the transgenic T_2 lines inoculated with *F. graminearum* were analyzed with Student's *t* test: $*P = 0.000291$. Examples of uninoculated and inoculated spikelets are shown above.

conditions and are unique to the toxin-producing strains, we carried out comprehensive screening of such genes using the complete genome database of *F. graminearum* (<http://www.broad.mit.edu/annotation/fungi/fusarium/>). However, targeted gene disruption of all such candidate *CYP* genes demonstrated that they were unrelated to trichothecene biosynthesis [32]. This result raised a possibility that a cluster gene has multiple functions and is responsible for multiple oxygenation steps as do other fungal *CYP*s involved in the biosynthesis of secondary metabolites.

Currently, we are investigating the function of *Tri4*, a known cluster gene, with an *in vivo* assay using *Saccharomyces cerevisiae*. *Tri4* is a *CYP* gene encoding trichodiene C-2 oxygenase in the biosynthesis of trichothecenes [33], but previous study did not rule out the possibility that the gene encodes a multifunctional oxygenase used in the successive oxygenation of bicyclic intermediates (see Fig. 1). TLC of the hexane-extractable fraction of transgenic yeast strains co-expressing *Tri5* and *Tri4* revealed several metabolites that are not found in the control yeast strains carrying *Tri5* alone. Preliminary structural analyses suggested that a small amount of trichothecene (12,13-epoxytrichothec-9-ene; see Fig. 1) is formed in the culture of the transgenic yeasts, implying that *Tri4* encodes a key multifunctional *CYP* responsible for oxygenation of C-2, C-12,13, and C-11 of the bicyclic intermediates (our unpublished results). *Tri4* is thus a good target gene with which to develop a high-throughput system for the screening of specific inhibitors of trichothecene biosynthesis.

4. Detoxification of ZEN by a lactonohydrolase gene

ZEN (**1** in Fig. 3) is found in contaminated maize and perturbs the hormonal equilibrium of fed animals. This mycotoxin consists of a resorcinol moiety fused to a 14-membered macrocyclic lactone. Previously, El-Sharkawy and Abul-Haji found microbial cleavage of ZEN and determined the structure of the transformed product to be 1-(3,5-dihydroxyphenyl)-10'-hydroxy-1'-undecen-6'-one (**2** in Fig. 3) [34]. While screening for microorganisms capable of degrading various *Fusarium* mycotoxins, we found that the lactone ring of ZEN is sensitive to hydrolysis by *Clonostachys rosea*. When incubated with the fungus, ZEN is converted to the same cleavage product **2**, which proved to be far less estrogenic than ZEN [35].

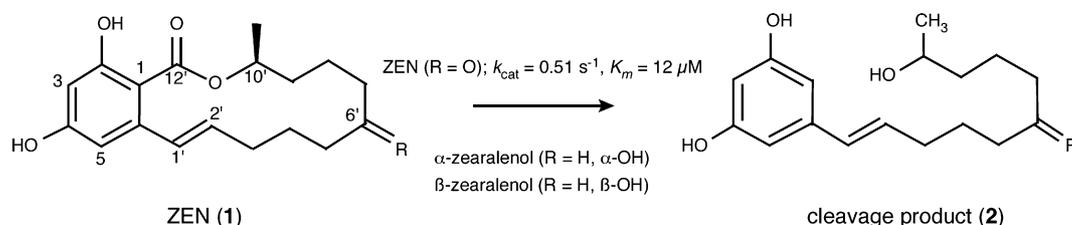


Fig. 3. Detoxification of ZEN and its derivatives (α -zearalenol, and β -zearalenol) by the enzyme ZHD101. ZHD101 detoxifies ZEN via a two-step reaction: (1) enzymatic hydrolysis of the lactone ring, and (2) subsequent decarboxylation of the resulting carboxylic intermediate. The cleaved product shows no estrogenicity as assessed by a proliferation assay with human breast cancer MCF-7 cells [35].

A lactonohydrolase responsible for the detoxification was purified to homogeneity and its gene, designated *zhd101*, was subsequently isolated from the fungus [36]. Maximal activity of recombinant ZHD101 protein toward ZEN was observed at pH 10.5 with an extremely low molar activity ($k_{\text{cat}} = 0.51 \text{ s}^{-1}$ at 30°C) [37]. In addition, ZHD101 showed even less activity toward a frequently co-occurring natural ZEN metabolite, β -zearalenol ($k_{\text{cat}} = 0.075 \text{ s}^{-1}$). Regardless of these features of ZHD101, biological decontamination of ZEN using genetically modified (GM) organisms has been shown to be practical, as follows.

The first example is based on an experiment with *S. cerevisiae*, which can be used as a live vehicle to transiently confer a new genetic trait in an animal's digestive environment [38]. While wild-type yeasts could not detoxify ZEN in the culture, a high concentration of ZEN ($2 \mu\text{g/ml}$) was completely removed by GM yeasts expressing a synthetic *zhd101* gene whose codons were optimized for *S. cerevisiae* [39]. Given that increasing attention is being paid to GM yeasts as live vehicles for the detoxification of various xenobiotics, the use of the ZEN-detoxifying yeasts as feed additives may offer a more efficient and cost-effective solution than other physicochemical strategies of decontamination [40,41].

The second example is based on model cereal rice plants transformed by *zhd101*. Wild-type and transgenic T_2 seeds were treated with ZEN by absorption of a mycotoxin solution ($5 \mu\text{g/ml}$). While wild-type grains contained approximately $30 \mu\text{g}$ of ZEN per gram of seed, the transgenic grains contained just $16 \mu\text{g}$ [42]. Considering the previously reported levels of mycotoxin in grains, namely $0.04\text{--}12 \mu\text{g}$ per gram of seed [43–45], it will be possible to reduce the level of ZEN in the transgenic seeds.

In this way, GM microbes and crops were shown to be useful for the development of an efficient and cost-effective system to secure the safety of food and feed. Although public and scientific concerns have been raised about the environmental and food safety of GM crops, appropriate risk assessment can be used to evaluate the probability of such negative and undesired impacts. The consumer acceptance of commercial GM products, especially in Europe, seems unlikely at present [46]. However, since the world is facing such serious problems with global food and nutrition security, we might not afford to turn away from GM crops. Apart from the debate on GM crops, we are currently

evaluating the usefulness of *zhd101* for the detoxification of ZEN in maize, which is actually susceptible to contamination from mycotoxins in the field.

5. Perspective

As we mentioned in Section 1, a fundamental solution to the problems of mycotoxin contamination is to establish an efficient disease control strategy for FHB. When contamination cannot be prevented, detoxification could offer an alternative solution. In this context, transgenic inactivation of ZEN may be a good example of this, provided that the public being more generous to GM organisms in the future. With regard to detoxification of trichothecenes, a rumen bacterial strain capable of irreversible de-epoxidation of the 12,13-epoxy ring was isolated and characterized [47]. This bacterial strain was already commercialized as feed additives from Biomin® GmbH although the responsible detoxification system (viz., genes involved in the process) remains to be identified.

Classical breeding efforts using natural resistance sources are thought to be the most promising and environmentally friendly approach to develop wheat lines with FHB resistance. Compared to GM technologies, this approach is an integral and accepted tradition in agriculture. Unfortunately, FHB resistance so far identified is only partial and quantitative in nature, although some progresses have been made [48,49]. The slow progress is due to expense in disease screening, complex nature of resistance, and coincidence of quantitative trait loci of FHB with other phenotypes such as plant development and spike morphology [50]. Therefore, different approaches for controlling FHB need to be considered simultaneously.

Although several *PR* genes involved in defense against *F. graminearum* have been identified in wheat [51–55], the molecular breeding of FHB-resistant plants is not currently possible because of the ineffectiveness and side effects of these transgenes when expressed in GM crops [56,57]. This is quite different from the case of insect-resistant maize or herbicide-resistant soybean, which have already been commercialized and are commonly used for processed foods in some industrialized countries [58]. To develop an efficient transgenic strategy to control FHB, isolation of fungal molecules essential for the pathogenesis and affected target proteins of host plants are indispensable [59,60]. With such knowledge in molecular plant pathology, GM crops may offer a possible contribution to the problems of FHB in the future. For the moment, however, the problems associated with FHB are most likely to be dealt with natural genetic resistance, management practice, and/or fungicide application.

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