Recent Advances in Phytochemistry 42

David R. Gang *Editor*

Phytochemicals, Plant Growth, and the Environment

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Introduction to the 42nd Volume of the Recent Advances in Phytochemistry Series

This is the second volume since the reintroduction of the *Recent Advances in Phytochemistry* (*RAP*) series, an annual journal supported by the Phytochemical Society of North America. Topics appropriate for *RAP* include the biosynthesis of natural products and regulation of metabolism, the ecology of specialized metabolites and the evolution of their pathways, and the effects of natural products or plants on human health. Research appropriate for *RAP* involves genomics, proteomics, metabolomics, natural product structural determination and new technology development, medicinal chemistry and metabolic engineering, or any of the myriad of fields that are now closely associated with what may be called "traditional phytochemistry" and plant biochemistry. The advent of post-genomics-based ways of thinking, of systems biology, of synthetic biology, of comparative genomics/proteomics/transcriptomics/metabolomics, and especially of the introduction and establishment of a mentality that leads to support of large collaborative projects has opened up many new doors to scientists interested and versed in the (bio)chemistry of plants. The goal of *RAP* is to highlight these developments.

Two main types of articles are printed in *RAP*: Perspectives and Communications. Perspectives in *RAP* are expected to synthesize results from the primary literature and perhaps from new/novel results and place these in perspective relative to the broader field. These articles not only may be similar to review articles, but also are intended to present important ideas and hypotheses, and may present proposals for interesting directions in the field. It is the hope of the Editorial Board that these articles will be of great value to a large audience. Communications are intended to represent new advances in the field that will be of interest to a large audience. Articles of both types are typically solicited from the Society membership based on the content of the annual meeting talks, but in keeping with the title "Recent Advances in Phytochemistry," the editorial board reserves the right to solicit additional perspectives and/or communications from non-attendees as well (e.g., where an editorial board member has knowledge of

an interesting recent advancement that would be of general interest to the society membership).

All submissions to *RAP* go through a rigorous peer review process, overseen by the Editorial Board, which includes external review. *RAP* is indexed with Springer-published journals. All *RAP* papers are available not only in the published volume form but also electronically through Springer's online literature services. This marks a significant change from past volumes of *RAP*, and it is the hope of the Editorial Board that this will lead to broader dissemination of the contents of and greater interest in *RAP*.

This 42nd volume of RAP includes a total of seven articles, many, but not all, based on talks presented at the 50th annual meeting of the PSNA. As was seen in *RAP* Volume 41, these seven perspectives give a very good picture of the breadth of plant (bio)chemistry research in North America, which is also indicative of the state of the field worldwide. Each of these articles describes the integration of several different approaches to ask and then answer interesting questions regarding the function of interesting plant metabolites, either in the plant itself or in interactions with the environment (natural setting or human health application).

Many of these perspectives have a strong ecological focus. McCormick et al. review the discovery of the biosynthetic pathway leading to production of trichothecene mycotoxins such as the T-2 toxin in plant pathogenic and other fungi. These compounds play very important roles in plant–pathogen interaction and are very significant from a human health perspective. In a complementary paper, Duringer et al. describe recent technological advances in monitoring mycotoxins such as ergovaline and lysergic acid in forage crops, using state-of-the-art and highly sensitive mass spectrometric means. Gross reviews the current understanding of how infochemicals mediate interactions between plants and insects and highlights how such knowledge can be used to mitigate crop losses by pests.

Two perspectives discuss how recent technological advances are making an impact on our understanding of the role of plant hormones in plant growth and development. Gouthu et al. outline highly sensitive methods for measurement of plant hormones in tissues such as developing grape berry. In contrast, McDowell and Gang outline how new transcriptional profiling techniques are shedding light on old questions, such as how rhizome development is regulated by different plant growth regulators.

The last two perspectives outline the role of biotechnology in modern plant biochemistry research. Makhzoum et al. review the long history of use of hairy roots and provide perspective on future utility of this tissue type in continuing to uncover mechanisms of plant natural product biosynthesis, among other applications. Dalton et al. outline, on the other hand, recent efforts to produce nonnative polymers of human interest in plants and outline many of the challenges associated with such investigations.

We hope that you will find these perspectives to be interesting, informative, and timely. It is our goal that *RAP* will act not only as the voice of the PSNA, but also

that it will serve as an authoritative, up-to-date resource that helps to set the gold standard for thought and research in fields related to plant biochemistry.

We welcome suggestions for future articles and comments on the new format.

The RAP Editorial Board

Contents

Chapter 1
Trichothecene Triangle: Toxins, Genes, and Plant Disease

Susan P. McCormick, Nancy J. Alexander, and Robert H. Proctor

Abstract Trichothecenes are a family of sesquiterpene epoxides that inhibit eukaryotic protein synthesis. These mycotoxins are produced in *Fusarium*-infested grains such as corn, wheat, and barley, and ingestion of contaminated grain can result in a variety of symptoms including diarrhea, hemorrhaging, and feed refusal. Biochemical and genetic investigations have characterized the genes controlling trichothecene biosynthesis. In *Fusarium*, trichothecene genes have been mapped to three loci including a 26-kb cluster of 12 genes. Production of trichothecenes by *Fusarium graminearum* has been shown to be an important virulence factor in wheat head blight. Strains of *F. graminearum* have been categorized into three different chemotypes, nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), and 15-acetyldeoxynivalenol (15ADON), based on polymorphisms observed in PCR assays. Although 15ADON-producing strains predominate in North America, there has been a recent emergence of 3ADON- and NIV-producing strains. The genetic basis for these chemotypes has been elucidated with sequence analysis, genetic engineering, and heterologous expression of trichothecene biosynthetic genes.

1.1 Introduction

Trichothecenes are a diverse family of sesquiterpenoid toxins produced by *Fusarium* and some other genera of filamentous fungi. These mycotoxins are characterized by a tricyclic 12,13-epoxytrichothec-9-ene (trichothecene) ring structure (Fig. 1.1). *Fusarium* trichothecenes have been classified as Type A or Type B based on the

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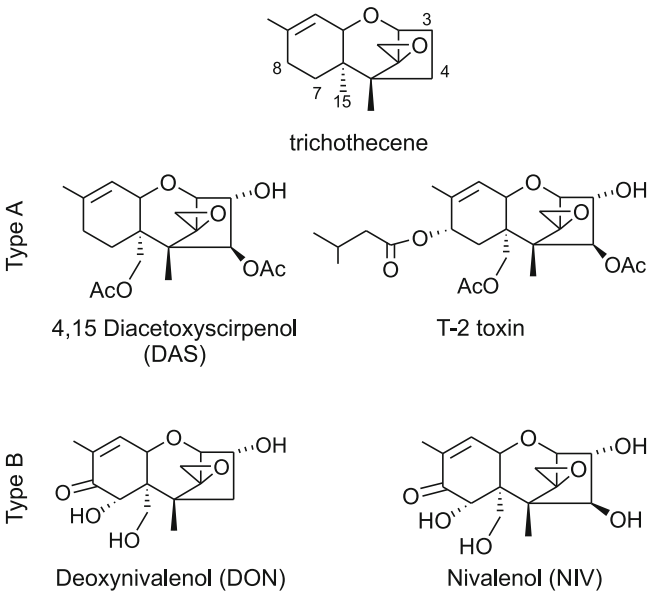

Fig. 1.1 Structures of the trichothecene skeleton with carbons numbered and representative examples of *Fusarium* Type A and Type B trichothecenes

functional group at carbon atom 8 (C-8) of the trichothecene molecule (Fig. 1.1). Type B trichothecenes have a keto function at C-8, while Type A trichothecenes have a hydrogen, hydroxyl, or ester function at C-8. Both Type A and Type B trichothecenes can occur in *Fusarium*-contaminated cereal grains. Type A trichothecenes are generally more toxic to animals, and two Type A compounds, 4,15-diacetoxyscirpenol (DAS) and T-2 toxin, are on the National Select Agent list (http://www.selectagents.gov) [1].

Trichothecenes block protein synthesis in most eukaryotes [2–4], but other cellular effects, including inhibition of mitochondrial enzymes and electrolyte loss, have also been reported [5, 6]. In animals, trichothecene exposure can cause feed refusal, immunological problems, vomiting, skin dermatitis, and hemorrhagic lesions [3, 7]. Trichothecenes are also phytotoxic and can cause chlorosis, inhibition of root elongation, and dwarfism [8, 9].

Due to the potential health hazards associated with trichothecene ingestion, the US Food and Drug Administration has set advisory levels for deoxynivalenol (a Type B trichothecene) in grain. The advisory level for finished grain products destined for human consumption is 1 ppm, and the levels for animal feed are set up to 10 ppm, depending on the animal; pigs are particularly sensitive to deoxynivalenol. At harvest, infected grain with characteristically bleached, shriveled tombstone kernels can have over 200 ppm, but only grain at 2 ppm or lower is accepted without monetary discount.

Outbreaks of *Fusarium* head blight (FHB), caused primarily by *Fusarium graminearum*, of wheat and barley have been a persistent problem in the midwestern and eastern United States and Canada. Six particularly devastating years in the Red River Valley of the Upper Midwest caused billions of dollars of losses in the 1990s [10]. Not only have grain growers suffered reduced yield and quality of grain due to FHB, they have also received lower financial reimbursement due to the presence of trichothecenes in the grain.

Research during the 1990s demonstrated that trichothecene production is a virulence factor in the *Fusarium*–wheat interaction [11–13]. Deoxynivalenol blocks the development of a heavy cell-wall protection barrier in wheat and thereby facilitates spread of *Fusarium* [14]. When wheat was infected with *Fusarium* strains that had been engineered to produce no trichothecenes, spread of the disease within the head was limited compared to plants infected with wild-type trichothecene-producing strains [13].

Here we review the biochemistry and genetics of the trichothecene biosynthetic pathway, focusing on the T-2 toxin-producing species *F. sporotrichioides*. We also review correlations between trichothecene structures produced by *Fusarium* species and variation in trichothecene biosynthetic (TRI) genes. *Fusarium* carries the genes that are necessary for trichothecene production, and the fungus must invade plant tissue for the mycotoxins contribute to plant disease.

1.2 Trichothecene Biosynthesis in *Fusarium sporotrichioides*

The proposed trichothecene biosynthetic pathway (Fig. 1.2) is based on a number of different analyses, including (1) feeding experiments in which labeled and unlabeled precursors were fed to fungal cultures [15–17], (2) feeding experiments with transgenic yeast carrying an intact trichothecene biosynthetic gene [18–20], (3) experiments with mutant strains of *Fusarium* generated by targeted, molecular genetic-induced changes to trichothecene biosynthetic genes [21–23], and (4) experiments with mutant strains of *F. sporotrichioides* with altered trichothecene production phenotypes that were induced by UV mutagenesis [24–27].

The biosynthesis of trichothecenes begins with the cyclization of farnesyl pyrophosphate to form trichodiene [28]. Trichodiene undergoes an allylic oxygenation at C-2 to form 2α-hydroxytrichodiene. Another oxygenation at C-12 adds an epoxide to form 12,13-epoxy-9,10-trichoene-2-ol, which is converted to isotrichodiol by a second allylic oxygenation at C-11. Oxygenation at C-3 leads to isotrichotriol which undergoes a nonenzymatic isomerization followed by cyclization to form isotrichodermol.

In *F. sporotrichioides*, biosynthesis of T-2 toxin proceeds from isotrichodermol with a series of acetylations and oxygenations. Isotrichodermol is acetylated at C-3 to form isotrichodermin [18], which then undergoes C-15 oxygenation to form 15-decalonectrin followed by C-15 acetylation to form calonectrin [29, 30].

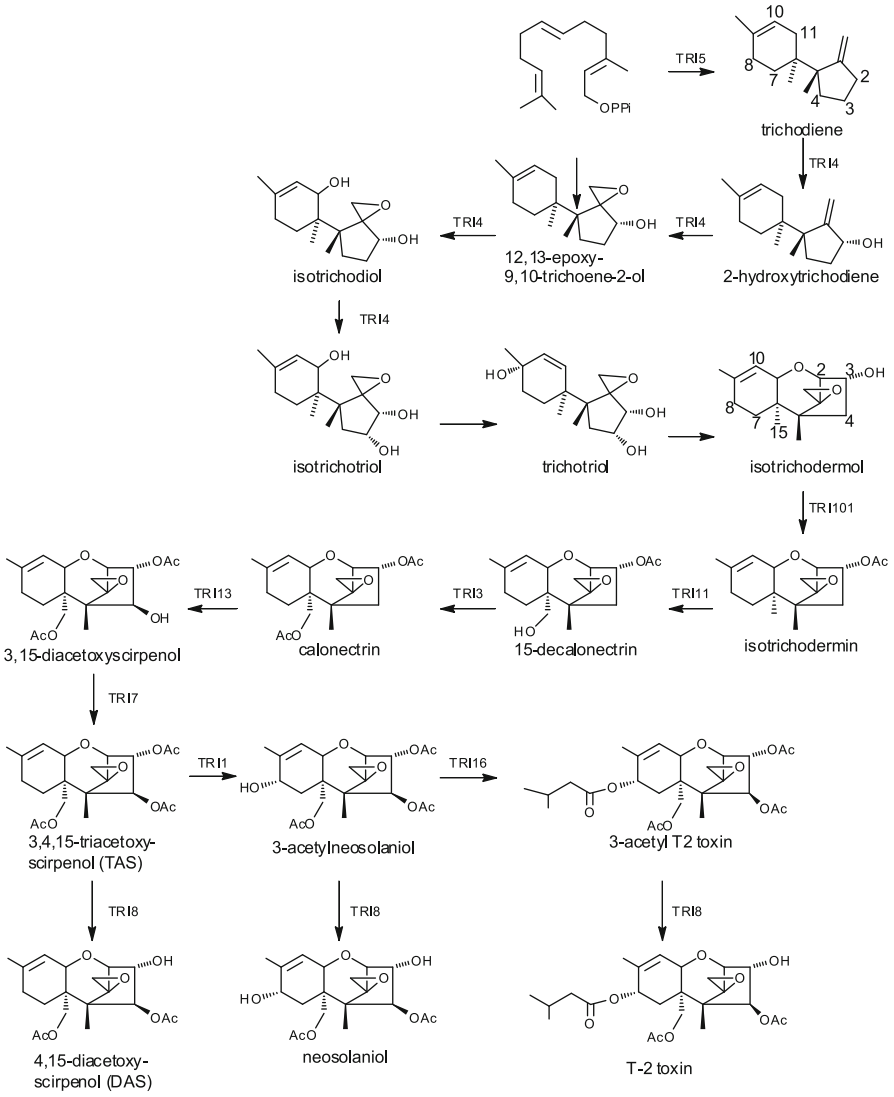

Fig. 1.2 *Fusarium sporotrichioides* trichothecene pathway leading to T-2 toxin, 4,15-diacetoxyscirpenol (DAS), and neosolaniol (8-hydroxy-4,15-diacetoxyscirpenol)

Calonectrin undergoes C-4 oxygenation to form 3,15-diacetoxyscirpenol followed by C-4 acetylation to form 3,4,15-triacetoxyscirpenol (TAS). 3-Acetyl T-2 toxin results from oxygenation at C-8 followed by addition of an isovaleryl group derived from leucine [26]. The final step in T-2 toxin biosynthesis is removal of the C-3 acetyl group [19].

1.3 *Fusarium sporotrichioides* Trichothecene Biosynthetic Genes

Most of the known trichothecene biosynthetic (*TRI*) genes were first characterized in *Fusarium sporotrichioides* (Table 1.1). In this species, the genes occur at three genetic loci: the 12-gene core *TRI* cluster, the two-gene *TRI1/TRI16* locus, and the single-gene *TRI101* locus (Fig. 1.3). The core TRI cluster includes genes responsible for formation of the trichothecene molecule, as well as most modifications to it, while the *TRI1/TRI16* genes are responsible for modification at C-8 [21, 22], and the *TRI101* gene is responsible for the C-3 acetylation [31].

The functions of most of the trichothecene genes were determined using gene disruption, heterologous expression, as well as precursor feeding experiments with whole cells and cell-free extracts. However, two of the genes were identified by UV mutagenesis [25, 30], and one (*TRI5)* was identified by purifying the

Table 1.1 Summary of *Fusarium sporotrichioides* trichothecene biosynthetic genes and their functions

Gene	Function	Enzyme substrate
TRI1	C-8 oxygenase	3,4,15-Triacetoxyscirpenol
TRI3	C-15 acetyltransferase	15-Decalonectrin
TRI4	Trichodiene oxygenase	Trichodiene
TRI5	Trichodiene synthase	Farnesyl pyrophosphate
TRI6	Transcriptional regulator	
TRI7	C-4 acetyltransferase	3,15-Diacetoxyscirpenol
TRI8	C-3 esterase	3-Acetyl T-2 toxin
TRI9	Unknown	
TRI10	Global regulator	
TRI11	C-15 oxygenase	Isotrichodermin
TRI12	Trichothecene pump	
TRI13	C-4 oxygenase	Calonectrin
TRI14	Unknown	
TRI15	Regulatory	
TRI16	C-8 acyltransferase	3-Acetylneosolaniol
TRI101	C-3 acetyltransferase	Isotrichodermol

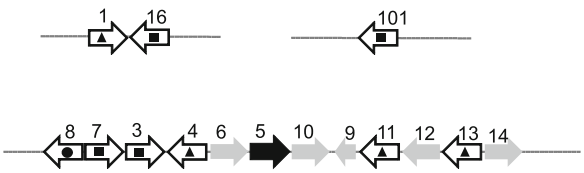

Fig. 1.3 *Fusarium sporotrichioides* loci containing genes involved in trichothecene biosynthesis: *black*, terpene cyclase; *square*, acetyl/acyltransferase; *circle*, esterase; *triangle*, P450 oxygenase; *gray*, genes for regulation, trichothecene pump, and other uncharacterized genes

corresponding enzyme, raising antibodies to the purified enzyme, and screening a library of *F. sporotrichioides* genomic DNA expressed in *E. coli* with the antibody [28, 32]. Once *TRI5* was identified, sequence analysis of adjacent regions of DNA revealed the presence of additional genes, and the role of two of these genes in trichothecene biosynthesis was determined by complementation of UV-induced mutants of *F. sporotrichioides* that were blocked in T-2 toxin production; that is, introduction of large pieces of DNA (cosmid clones) carrying *TRI5* and multiple adjacent genes into mutants restored T-2 toxin production [33].

TRI4 was identified with a UV-induced mutant [24, 33, 34] and characterized more fully by gene deletion analysis [35]. These analyses indicated that *TRI4* encodes a cytochrome P450 enzyme involved in the initial oxygenation of trichodiene at C-2. However, heterologous expression in yeast and *F. verticillioides,* organisms that do not produce trichothecenes or have trichothecene biosynthetic genes, revealed that the *TRI4* monooxygenase is multifunctional and can catalyze four oxygenation reactions that result in conversion of trichodiene to isotrichotriol [36, 37].

The next proposed step in the trichothecene biosynthetic pathway in *Fusarium sporotrichioides*, the conversion of isotrichodermol to isotrichodermin, is controlled by *TRI101* [8, 18]. This gene was initially identified from a cDNA library, prepared from *Fusarium*. When a *TRI101* cDNA was expressed in yeast, it conferred resistance to high concentrations of the trichothecenes 4,15-DAS, T-2 toxin, and deoxynivalenol by acetylating these toxins at the C-3 position [8]. Because C-3 acetylated trichothecenes are less toxic to microorganisms [8, 38], *TRI101* is considered to be a resistance gene that protects trichothecene-producing organisms from their own toxins.

The next proposed step in the pathway, the hydroxylation of isotrichodermin to form 15-decalonectrin (Fig. 1.2), is catalyzed by another cytochrome P450 encoded by *TRI11* [29, 30]. 15-Decalonectrin is then converted to calonectrin by an acetyltransferase encoded by *TRI3*. Calonectrin is then hydroxylated at the C-4 position by the *TRI13*-encoded P450 monooxygenase to form 3,15-diacetoxyscirpenol [39], which then undergoes a C-4 acetylation, catalyzed by the *TRI7*-encoded acetyltransferase [40] to form 3,4,15-triacetoxyscirpenol.

In *F. sporotrichioides*, the *TRI1*-encoded P450 monooxygenase then catalyzes C-8 hydroxylation to form 3-acetylneosolaniol [21, 41]. The *TRI16*-encoded acyltransferase catalyzes esterification of an isovaleryl moiety to the C-8 oxygen to yield 3-acetyl T-2 toxin [22, 41]. The final step in the T-2 toxin biosynthetic pathway is C-3 deacetylation of 3-acetyl T-2 toxin, a reaction catalyzed by the *TRI8*-encoded esterase [19]. This last reaction reverses the self-protecting C-3 acetylation catalyzed by the *TRI101*-encoded acetyltransferase and produces a trichothecene with increased toxicity.

Thus, T-2 toxin biosynthesis requires a sesquiterpene cyclase gene (*TRI5*), four P450 monooxygenase genes (*TRI4*, *TRI11*, *TRI13*, and *TRI1*), four acetyltransferase/acyltransferase genes (*TRI101*, *TRI3*, *TRI7*, and *TRI16*), and an esterase gene (*TRI8*). However, the core TRI cluster also includes a transport protein-encoding gene, *TRI12*, which is most likely responsible for pumping T-2 toxin out

of *Fusarium* cells into the surrounding environment [20]. The cluster also includes two genes, *TRI6* and *TRI10*, that regulate expression of other *TRI* genes inside and outside the core cluster [42, 43]. The mechanism by which *TRI10* affects *TRI* gene expression is not yet known. In contrast, *TRI6* is predicted to encode a transcription factor, a class of proteins that bind specific sequence motifs in the promoter regions of genes and induce transcription. The sequence motifs to which the Tri6 protein binds were originally demonstrated for the promoter regions of *TRI4* and *TRI5*, but the motifs were subsequently identified in the promoter regions of all known *TRI* genes within and outside the cluster [18, 19, 40, 43]. Disruption of *TRI6*, *TRI10*, or *TRI12* reduced T-2 toxin production. Transcriptional regulators are often associated with the expression of co-regulated proteins, such as those found to be in a metabolic pathway.

1.4 Variations on a Theme

The diverse number of trichothecenes produced by species in the order Hypocreales suggests that there are variations in the biosynthetic pathways and the genes that control their production. Although most of the trichothecene biosynthetic genes were first identified in *F. sporotrichioides*, homologues of some of the genes have also been identified in other trichothecene-producing organisms.

Fusarium trichothecenes have an oxygen function at the C-3 position. But trichothecenes produced by other genera, such as *Myrothecium*, *Trichothecium*, *Trichoderma*, and *Stachybotrys*, lack this C-3 oxygen. In *M. roridum*, which produces the trichothecene roridan A (Fig. 1.4), the predicted Tri4 amino acid sequence is 64% identical and 80% similar to that of *F. sporotrichioides* [44]. When the *M. roridum TRI4* was expressed in a mutant of *F. sporotrichioides* in which *TRI4* was inactivated, T-2 toxin production was not restored, but trichothecene, deoxysambucinol, and sambucinol were produced [16, 44]. This experiment, and later experiments in which *M. roridum TRI4* was expressed in *F. verticillioides* [45] and *Trichothecium roseum TRI4* [37] was expressed in yeast, indicate that in *Myrothecium* and *Trichothecium*, the *TRI4* monooxygenase catalyzes three oxygenation reactions to covert trichodiene to isotrichodiol. Therefore, the function of *TRI4* in *Myrothecium* and *Trichothecium* differs from the function in *Fusarium*, and the difference gives rise to structural variation in the trichothecenes produced by *Myrothecium*/*Trichothecium* versus *Fusarium*.

Variation in function of *TRI* gene homologues in different species of *Fusarium* has also been observed. In *F. sporotrichioides,* the *TRI1*-encoded P450 monooxygenase gene catalyzes hydroxylation of the C-8 position only [21]. In contrast, the *F. graminearum TRI1* enzyme, FgTri1, catalyzes hydroxylation of both C-7 and C-8 positions (Fig. 1.5) [23]. The predicted amino acid sequence of FgTri1 is 59% identical to the *F. sporotrichioides* Tri1 [23]. In addition, the function of *TRI16* differs in *F. sporotrichioides* and *F. graminearum*. Tri16 catalyzes esterification of