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Trichothecene Biosynthesis in *Fusarium* Species: Chemistry, Genetics, and Significance

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INTRODUCTION

Trichothecenes are sesquiterpene epoxides that inhibit eukaryotic protein synthesis and thereby impair human and animal health. Several fungal species of the genus *Fusarium* and related genera can produce trichothecenes in agricultural crops and commodities. Interest in these toxins is due primarily to the discovery that trichothecene contamination of human foods and animal feeds is a continuing worldwide problem. The most effective control strategy for trichothecene toxins is prevention of fungal infection and toxin production in the field and in storage. In the long term, understanding the molecular biology of trichothecene production should help the development of practical and specific controls. In recent years, rapid advances in the molecular genetics of filamentous fungi have opened the way for detailed genetic analysis of trichothecene biosynthesis in *Fusarium* species. This review will describe recent progress in understanding the biochemistry and genetics of the trichothecene biosynthetic pathway and in evaluating the significance of trichothecenes in plant diseases caused by *Fusarium* species.

The chemistry and toxicology of trichothecenes were established by early studies, which have been thoroughly reviewed (2, 55, 74). All trichothecenes share a tricyclic nucleus named trichothecene (Fig. 1) and usually contain an epoxide at C-12 and C-13, which is essential for toxicity. The total number of naturally occurring trichothecenes known today exceeds 60. Their chemical structures vary in both the position and the number of hydroxylations, as well as in the position, number and complexity of esterifications. The *Fusarium* trichothecenes, which will be the major focus of this review, are relatively simple alcohols and short-chain esters, whereas trichothecenes of *Myrothecium*, *Verrucaria*, and other genera can be complex macrocyclic esters. Trichothecenes are named after the fungus *Trichothecium roseum*, from which the first trichothecene was isolated in 1948. The discovery of the carcinogenic aflatoxins in the 1960s greatly increased interest in mycotoxins (i.e., fungal toxins that affect animals) and stimulated the development of sensitive analytical methods for mycotoxin detection in various plant products. Subsequent investigations of moldy-grain toxicoses led to the isolation and identification of many new trichothecenes, including T-2 toxin, diacetoxyscirpenol, and deoxynivalenol (Fig. 1) (74). These three closely related compounds are the trichothecenes most commonly found in agricultural commodities infected with *Fusarium* species.

The complex taxonomy of the genus *Fusarium* has led to considerable confusion and misidentification of trichothecene-producing species. The publications of Nelson and coworkers (55, 64) present the most comprehensive and widely used system for the identification of toxigenic *Fusarium* species. According to the taxonomic system of these authors and other authorities, six *Fusarium* species have been well documented worldwide to produce trichothecenes (51, 55). Two species, *F. sporotrichioides* and *F. poae*, of the section Sporotrichiella produce mainly T-2 toxin and diacetoxyscirpenol. Four species, *F. crookwellense*, *F. culmorum*, *F. graminearum*, and *F. sambucinum*, of section Discolor produce mainly diacetoxyscirpenol and deoxynivalenol. A wide variety of other trichothecenes and structurally related compounds can be produced by individual strains of these species under specific growth conditions.

All trichothecene-producing *Fusarium* species are destructive pathogens that can attack a wide range of plant species. The main sources of trichothecenes in the food supply are contaminated cereal grains, namely maize, wheat, rye, barley, and rice. Multiyear surveys in the United States and Canada indicate that maize and wheat are often contaminated with trichothecenes but that the levels are generally below 2 ppm, a recommended tolerance level (66). The severity of *Fusarium* infection and of trichothecene contamination increases with wet weather at harvest and with storage under conditions of relatively high moisture. There are few effective, economical methods for decontamination of trichothecenes in grains. Contaminated grains can be diverted to nonfood uses such as fuel ethanol production, or their toxicity can be reduced by dilution with clean grain.

All animal species that have been tested appear to be sensitive to trichothecene toxins. Disease symptoms vary widely with the species of animal, the particular trichothecenes present, their levels and routes of exposure, and other factors. Experiments with chemically pure trichothecenes at low dosage levels have reproduced many of the features observed in moldy-grain toxicoses in animals, in-

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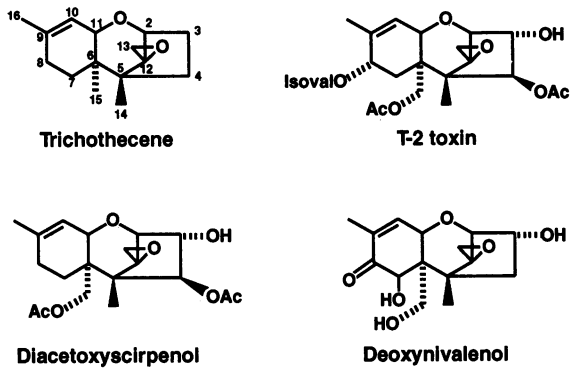

FIG. 1. Structure of trichothecene, T-2 toxin, diacetoxyscirpenol, and deoxynivalenol.

cluding anemia and immunosuppression, hemorrhage, emesis, and feed refusal in cattle, pigs, and poultry (55). Animal-feeding experiments have also demonstrated that trichothecenes are teratogenic but have provided no evidence that they are carcinogenic (1). Historical and epidemiological data obtained with humans indicate an association between certain disease epidemics and consumption of grain infected with *Fusarium* species that produce trichothecenes. In particular, outbreaks of alimentary toxic aleukia that occurred in the former Soviet Union in the 1940s have been associated with consumption of overwintered grains infected with *F. sporotrichioides*, which is a T-2 toxin-producing species (55). In Japan, outbreaks of a similar disease called akakabi-byo or red mold disease have been associated with grains infected with *F. graminearum*, which is a deoxynivalenol-producing species (55). There is, however, no direct evidence that either T-2 toxin or deoxynivalenol was responsible for these human disease epidemics. On the other hand, symptoms similar to those of alimentary toxic aleukia and akakabi-byo were produced by pure diacetoxyscirpenol in clinical trials conducted with terminally ill cancer patients (1). The most controversial aspect of human exposure to trichothecene toxins has been the charge that they were used as chemical-warfare agents in Southeast Asia in the early 1980s. Most recent assessments of this controversy have concluded that the evidence is not sufficient to warrant such claims (53).

TRICHOTHECENE BIOSYNTHESIS IN *FUSARIUM* SPECIES

Pathway Intermediates

The biosynthesis of trichothecenes proceeds from trichodiene (Fig. 2), a natural product first isolated from *T.*

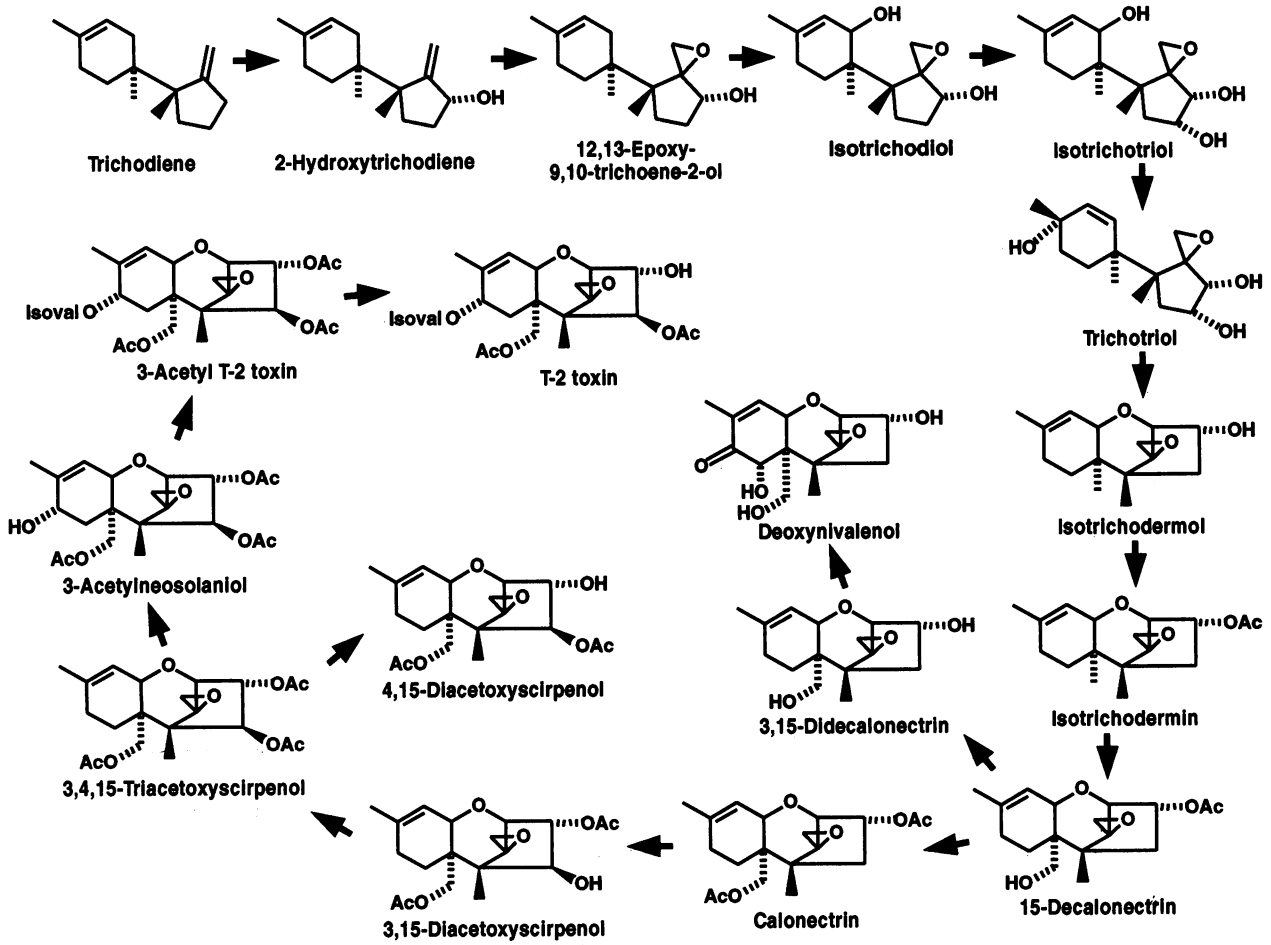

FIG. 2. Trichothecene biosynthetic pathway in *Fusarium* species.

roseum (52). Feeding tritiated trichodiene to *T. roseum* resulted in low-level incorporation of tritium into trichothecolone, which suggested that trichodiene is a precursor of *T. roseum* trichothecenes (52). Treatment of trichothecene-producing strains of *F. sporotrichioides*, *Gibberella pulicaris* (anamorph, *F. sambucinum*), and *F. culmorum* with oxygenase inhibitors such as ancymidol and xanthotoxin resulted in the inhibition of trichothecene production and the accumulation of trichodiene, which suggested that trichodiene is a precursor of *Fusarium* trichothecenes (25, 26, 33, 79). Further evidence was obtained by UV irradiation of *F. sporotrichioides* and the recovery of a mutant that was blocked in T-2 toxin production and accumulated trichodiene (5, 65). Experiments in which synthetic, labeled trichodiene was fed to *F. culmorum* cultures confirmed that trichodiene is a precursor of trichothecenes in *Fusarium* species (68, 80).

The sequence of oxygenations, isomerizations, cyclizations, and esterifications leading from trichodiene to the more complex trichothecene toxins such as diacetoxyscirpenol, T-2 toxin, and 3-acetyldeoxynivalenol has been established through experiments with *F. sporotrichioides*, *G. pulicaris*, and *F. culmorum* in several laboratories in the United States, Canada, and England. The characterization of the trichothecenes accumulated by *F. sporotrichioides* mutants led to a scheme in which oxygenation at C-3 or C-15 is followed by hydroxylation at C-4 and then at C-8. (65). Clues to earlier oxygenated precursors began to emerge with the isolation of an array of minor constituents, including several new bicyclic, oxygenated, trichodiene derivatives, from large-scale solid fermentations of *F. sporotrichioides* and *G. pulicaris* (15–18, 67). One of these compounds, trichotriol, was found to cyclize into isotrichodermol in weakly acidic environments, suggesting that C-3 hydroxylation might precede cyclization (16). Solid fermentations of *F. sporotrichioides* UV-induced mutant strains yielded trichotriol and several other bicyclic compounds, including isotrichotriol, the 11α-hydroxy isomer of trichotriol (58).

Subsequent feeding experiments in which cultures of *F. sporotrichioides* mutants were amended with a number of possible precursors of diacetoxyscirpenol and T-2 toxin (i.e., trichothecenes with hydroxylation at one or more of positions C-2, C-3, C-4, C-8, C-9, C-11, and C-15) clearly demonstrated that both isotrichotriol and trichotriol are T-2 toxin precursors, that isotrichotriol isomerizes to trichotriol, and that C-3 hydroxylation precedes cyclization (59). In *F. sporotrichioides* and *G. pulicaris*, the sequence of oxygenation steps in trichothecene biosynthesis is therefore C-11 (isotrichotriol) → C-9 (trichotriol → isotrichodermol) → C-15 (didecalonectrin) → C-4 (diacetoxyscirpenol) → C-8 (neosolaniol → T-2 toxin) (Fig. 2). These studies also determined that the parent compound, trichothecene (Fig. 1), is not a precursor in *Fusarium* trichothecene biosynthesis (59).

In contrast, initial reports of pulse-labeling experiments with *F. culmorum* suggested that this species had an alternate pathway in which trichothecene was an intermediate (77). Subsequent results, however, indicated that isotrichodermol was the first cyclized precursor of 3-acetyldeoxynivalenol (79), suggesting that *F. culmorum*, *F. sporotrichioides*, and *G. pulicaris* share a common pathway.

Another trichodiene derivative, isotrichodiol (Fig. 2), was isolated from cultures of *F. culmorum* that had been amended with large amounts of trichodiene (33, 34). Feeding experiments with labeled isotrichodiol demonstrated that it is a precursor of 3-acetyldexoynivalenol (33, 34, 78). Conversion of trichodiene to isotrichodiol would require three oxygenations, at C-2, C-11, and C-12/13. One possible precursor, 11α-hydroxytrichodiene, has been shown in feeding experiments with UV-induced mutants and *Tox5*$^-$ transformants to be a precursor of apotrichothecenes rather than trichothecenes (57a, 59). Pulse-labeling experiments have identified a dioxygenated compound, 12,13-epoxy-9,10-trichoene-2-ol (Fig. 2) as an efficient precursor of 3-acetyldeoxynivalenol in *F. culmorum*.

In summary, *F. culmorum*, *F. sporotrichioides*, and *G. pulicaris* share most of the initial scheme of oxygenations and cyclizations in trichothecene biosynthesis. The branch point between the *F. culmorum* and *F. sporotrichioides*/*G. pulicaris* pathways appears to occur after didecalonectrin. Although the large number and variety of trichothecenes and pretrichothecene structures identified has led to speculation that a metabolic grid may be in operation (34), feeding experiments indicate that there is an ordered sequence of steps in trichothecene biosynthesis. Some of the additional metabolites have been demonstrated to be involved in shunt pathways to modified trichothecenes such as apotrichothecenes, sambucoins, and sambucinol, whereas others may be dead-end metabolites.

Trichodiene Synthase

The enzyme trichodiene synthase catalyzes the cyclization of *trans*,*trans*-farnesyl PP_i to trichodiene. Because it is the first unique enzyme in the trichothecene pathway, trichodiene synthase has been the primary focus of efforts to understand pathway regulation and function. Trichodiene synthase activity was first found in cell extracts of *T. roseum* (31). The enzyme from *T. roseum* has also been used for mechanistic and stereochemical studies of the trichodiene synthase reaction by Cane et al. (see reference 10 for a recent review). These studies indicate that trichodiene synthase is typical of the terpene cyclase-type enzymes that are involved in the biosynthesis of cyclic terpenoids in both fungi and plants.

Trichodiene synthase has been purified from *F. sporotrichioides* and shown to be a homodimer with a subunit of 45 kDa (43). Some properties of the purified enzyme, such as its requirement for Mg^{2+} as a cofactor and inhibition by PP_i, are similar to the properties reported for other terpene cyclases (10). However, trichodiene synthase differs from other terpene cyclases in that its 60 nM K_m for farnesyl PP_i is 10- to 50-fold lower. The regulation of trichodiene synthase expression in liquid cultures of both *F. sporotrichioides* and *G. pulicaris* has been investigated (36). Although specific nutritional factors involved in the induction of trichothecene biosynthesis have not been identified, media containing a high ratio of carbon to nitrogen have been found to give the highest yields of trichothecenes. Analysis of cultures grown in such a medium revealed that enzyme activity and trichodiene synthase polypeptide were first detected early in the stationary growth phase and preceded the initial detection of trichothecenes by about 3 h. The kinetics of increasing trichodiene synthase activity were different in *F. sporotrichioides* and *G. pulicaris*. Trichodiene synthase activity increased from undetectable to maximum levels over a 3-h period in *F. sporotrichioides*, whereas activity levels increased gradually over a 144-h period in *G. pulicaris*. The increases in enzyme activity were closely paralleled by increases in trichodiene synthase polypeptide levels as determined by immunoblot analysis. These results suggested that the regulation of trichodiene synthase activity occurs primarily through changes in its cellular concentration (36).

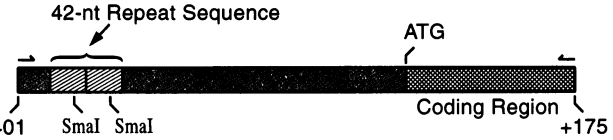

FIG. 3. Diagram of the *Tox5* promoter region of *G. pulicaris* R-6380.

The trichodiene synthase gene (*Tox5*) has been cloned from both *F. sporotrichioides* (37) and *G. pulicaris* (38) and shown to be present in a single copy in each organism. Comparisons between the genes from the two species revealed that the deduced amino acid sequences are 95% homologous and that they differ primarily by the addition of a nine-amino-acid sequence near the C terminus of the *G. pulicaris* enzyme. The levels of *Tox5* mRNA in *G. pulicaris* were observed to increase 47-fold between 18 and 42 h postinoculation (39). During the same period the levels of trichodiene synthase activity increased approximately 10-fold, suggesting that *Tox5* gene expression in *G. pulicaris* is regulated in part by transcriptional controls.

Efforts to characterize the *Tox5* promoter in *G. pulicaris* have identified a 401-nucleotide (nt) sequence that is sufficient to regulate the expression of a β-galactosidase reporter gene in a manner similar to *Tox5* (39). This sequence contains a 42-nt tandem repeat located 280 bp upstream from the ATG (Fig. 3) (38). Analysis of the same 401-nt promoter sequence in geographically distinct strains of *G. pulicaris* has revealed the existence of two alleles (*Tox5-1* and *Tox5-2*) that differ with respect to the presence (*Tox5-1*) or absence (*Tox5-2*) of the 42-nt duplication. Most strains carrying the *Tox5-1* allele were found to produce high levels of trichothecenes in liquid culture, whereas all strains carrying the *Tox5-2* allele produced low or undetectable levels of trichothecenes. Because the distribution of these alleles appeared to be correlated with the ability of *G. pulicaris* strains to produce trichothecenes, efforts were made to determine whether different trichothecene production phenotypes were due to the presence of a specific *Tox5* allele. Genetic crosses between strains differing at the *Tox5* locus resulted in the cosegregation of higher-level trichothecene production with the *Tox5-1* allele. The importance of the 42-nt repeat sequence was further investigated through transformation of the high-level-trichothecene-producing strain R-6380 (*Tox5-1*) with reporter gene constructs differing only by the presence or absence of the duplication. Reporter gene expression in transformants was found to be independent of the 42-nt sequence copy number. These results suggest that the duplication of this sequence is not responsible for the higher levels of trichothecenes produced by some strains of *G. pulicaris* and that a genetic factor(s) controlling the trichothecene production phenotype may be linked to the *Tox5* locus.

The heterologous expression of trichodiene synthase in both *E. coli* and tobacco plants has been investigated. The *Tox5* coding region from *F. sporotrichioides* was expressed in *E. coli* by using the expression vector pDR540 (42). Induced cultures made low levels of trichodiene synthase and trichodiene. The production of trichodiene following the induction of trichodiene synthase expression indicates that the heterologous expression of fungal sesquiterpene cyclase genes can result in biosynthesis of novel sesquiterpenoids by bacteria. Recently, soluble trichodiene synthase has been successfully overproduced to a level of 20 to 40% of total soluble protein in *E. coli* by using a T7 promoter-based expression vector (11). The availability of large quantities of trichodiene synthase will facilitate further studies of its chemical and physical properties. Expression of trichodiene synthase has also been found in transgenic tobacco plants (41). Plants carrying a chimeric trichodiene synthase gene, consisting of the complete trichodiene synthase open reading frame fused to the CaMV 35S promoter, were regenerated from transformed callus. The leaves from transgenic plants were found to contain active trichodiene synthase and low levels of trichodiene. These results demonstrate the feasibility of using fungal sesquiterpene cyclase genes to alter plant sesquiterpenoid metabolism.

By using molecular disruption, *Tox5*$^-$ mutants of both *F. sporotrichioides* (57a) and *G. pulicaris* have been generated (38). Transformants carrying the disrupted *Tox5* allele do not produce trichothecenes and appear to be indistinguishable from the progenitor strain with respect to mycelial growth rate, asexual spore development, and sexual fertility. The absence of a functional trichodiene synthase in *Tox5*$^-$ mutants will permit investigations into the role of this enzyme in trichothecene and isoprenoid pathway regulation.

Other Pathway Enzymes

In *Fusarium* species, the biosynthesis of trichothecenes proceeds via an ordered sequence of oxygenations and esterifications (Fig. 2). Although the succession of oxygenated intermediates is largely established, the oxygenation enzymes appear to be unstable, and none have yet been purified. Oxygen isotope incorporation studies involving whole-cell cultures of *F. sporotrichioides* showed that the pyran, epoxide, and hydroxide oxygenations of T-2 toxin are all catalyzed by molecular oxygen-dependent monooxygenases or dioxygenases (27). Further evidence of the involvement of cytochrome P-450 monooxygenases in trichothecene biosynthesis comes from the demonstration that cytochrome P-450 inhibitors effectively block trichodiene oxygenation in a number of *Fusarium* species (25, 26, 33). In addition, Gledhill et al. (32) demonstrated epoxidation of a trichodiene derivative by a cell-free homogenate of *F. culmorum*. This epoxidase activity required NADPH and molecular oxygen and was inhibited by carbon monoxide, all of which are characteristics of a cytochrome P-450 monooxygenase. Because of its low activity and its instability, the epoxidase of *F. culmorum* was not purified further.

The failure to isolate enzymes that catalyze trichothecene oxygenation has stimulated the development and application of genetic techniques to trichothecene biosynthesis. Such genetic studies have involved both meiotic recombinational analysis of *G. pulicaris* and molecular genetic analysis of UV-induced mutants of *F. sporotrichioides*. Sexual genetic analyses of trichothecene biosynthesis have focused on strains of *G. pulicaris* isolated from soils and from a variety of diseased plants (6, 20). These field strains differ widely in the amount of trichothecenes they produce in liquid culture. Genetic crosses between such strains indicate that multiple genes affect trichothecene yields, although the biochemical bases of these differences in yield have not been identified. The majority of field strains of *G. pulicaris* produce only diacetoxyscirpenol in liquid culture, but a small number (3 of more than 150 strains tested to date) also produce trichothecene derivatives with an additional oxygen at the C-8 position of the trichothecene nucleus (7, 21). The heritability of C-8 hydroxylation was studied by crosses between field strains that differed in this trait. Random ascospore and

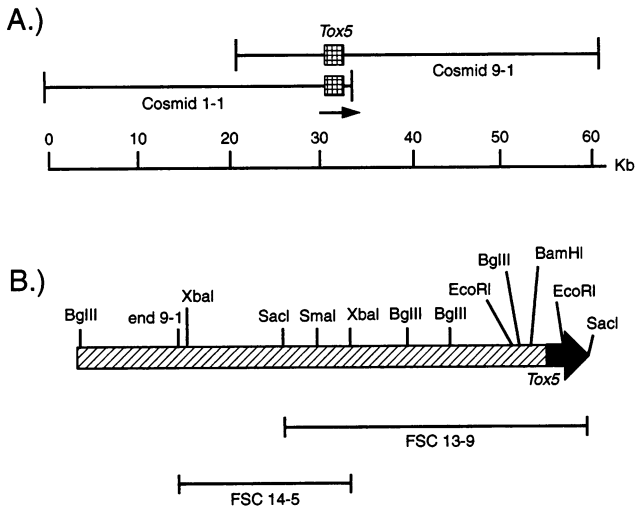

FIG. 4. Analysis of cosmids Cos1-1 and Cos9-1 and DNA flanking the *Tox5* gene. (A) Physical map of cosmids Cos1-1 and Cos9-1. (B) Restriction map of DNA flanking the 5′ end of the *Tox5* gene showing the locations of fragments FSC14-5 and FSC13-9. Data and figure from reference 40 with permission.

tetrad analyses indicated that the ability to hydroxylate at C-8 always segregated as a single gene or group of closely linked genes (6). The identification of two *Tox5* alleles in *G. pulicaris* (see the section on trichodiene synthase, above) has permitted meiotic segregation analysis of the C-8 hydroxylation phenotype and the *Tox5* locus. The C-8 hydroxylation phenotype was found to segregate independently of the *Tox5* locus in all eight tetrads analyzed in one genetic cross (23).

From a T-2 toxin-producing strain of *F. sporotrichioides*, Beremand and coworkers (5, 58, 65) isolated and characterized three mutants that accumulate different pathway intermediates (trichodiene, calonectrins, or diacetoxyscirpenol) and thus appear to be blocked at different steps of the trichothecene pathway. Although the chemical phenotypes of these three mutants were well defined, it was not known which, if any, represented defective monooxygenase genes or other structural genes. Although complementation (cross-feeding) tests indicated that these mutations were nonallelic, the absence of a sexual stage in *F. sporotrichioides* has prevented further characterization of these mutations with respect to their genomic location. Parasexual genetic analysis has not yet been applied to this problem, although such methods should be possible in *F. sporotrichioides*, which is normally haploid.

Recently, molecular genetic methods have been used to determine whether other trichothecene biosynthetic pathway genes are closely linked to the *Tox5* gene (40). Two cosmids, Cos1-1 and Cos9-1, carrying *Tox5* were isolated from a library of *F. sporotrichioides* NRRL 3299 genomic DNA (Fig. 4A) and used to transform three different trichothecene-deficient mutants of a T-2 toxin-producing strain of NRRL 3299 (Table 1). Transformation with either Cos1-1 or Cos9-1 resulted in restoration of T-2 toxin production in strains carrying mutations at the *Tox3* and *Tox4* loci but not at the *Tox1* locus. Additional transformation experiments with subcloned cosmid DNA fragments have localized the *Tox3* and *Tox4* complementing DNAs to two different fragments, plasmid 13-9 and plasmid 14-5, respectively (Fig. 4B), that are located within a 10-kb region adjacent to the *Tox5* gene. The production of T-2 toxin by the complemented *Tox3-1*$^-$ and *Tox4-1*$^-$ mutants, as well as the production of diacetoxyscirpenol by the cosmid-transformed *Tox1-2*$^-$ mutant, was 2- to 10-fold higher than was toxin production by strain NRRL 3299. In addition, transformants carrying Cos9-1 produced significantly higher levels of trichothecenes than did transformants carrying Cos1-1. The overall higher levels of trichothecenes produced by Cos9-1 transformants suggest that this cosmid may carry pathway genes that are not present on Cos1-1. If so, these genes are most probably located downstream from *Tox5*, since Cos1-1 extends only 1 kb beyond *Tox5*. These results have provided the first evidence that three or more trichothecene biosynthetic genes are clustered in *F. sporotrichioides*.

The clustering of trichothecene biosynthetic genes contrasts markedly with the organization of other fungal biosynthetic pathway genes, which are typically not closely linked. Trichothecenes represent the third fungal antibiotic pathway for which there is evidence of gene clustering. The genes for two enzymes in the biosynthetic pathway of the polyketide aflatoxin were recently reported to be closely linked in *Aspergillus parasiticus*. A single cosmid isolated from an *A. parasiticus* genomic library (70) contains both the *nor-1* and *ver-1* genes. The genes for enzymes in the β-lactam pathways of *Penicillium chrysogenum* (71), *Cephalosporium acremonium* (56), and *A. nidulans* (57) have also been shown to occur as gene clusters.

The fact that gene clustering has now been observed for pathways involved in the biosynthesis of three biogenetically distinct antibiotics suggests that this type of gene organization is a general feature of antibiotic pathways in fungi. One possible explanation for the clustering of antibiotic pathway genes is that they have a different evolutionary origin from that of other fungal biosynthetic pathways. Comparisons between the β-lactam pathway gene clusters in actinomycetes and fungi have led to the suggestion that fungi acquired this pathway via horizontal transfer from a prokaryote (12, 44). More recent studies focusing on the isopen-

TABLE 1. Genetic complementation of mutant strains blocked in trichothecene biosynthesis

Strain no.	Genotype	Trichothecene phenotype of complemented mutants[a]				
		Untransformed	Cosmid 1-1	Cosmid 9-1	FSC13-9	FSC14-5
T-926[b], NRRL3299	Wild type	T-2	—	—	—	—
T-927[b], MB5493	*Tox4*$^-$	Trichodiene	T-2	T-2	Trichodiene	T-2
T-932[b], MB2972	*Tox3*$^-$	DECAL	T-2	T-2	T-2	DECAL
T-937[b], MB1716	*Tox1*$^-$	DAS	DAS	DAS	—	—

[a] *F. sporotrichioides* UV-induced mutants were transformed with cosmids and plasmids carrying a selectable marker for resistance to hygromycin B. Abbreviations: DECAL, deacetylated calonectrins; DAS, diacetoxyscirpenol; —, not tested (39).

[b] Strain deposit numbers in the Fusarium Research Center collection, The Pennsylvania State University, University Park, Pa.

icillin N synthetase and the deacetoxycephalosporin C synthetase genes indicate that the presence of the β-lactam pathway in fungi could also be the result of conventional evolutionary processes (14, 72). Comparisons of this type can not be performed with trichothecene pathway genes, since no prokaryote is known to produce trichothecenes. An alternative explanation for the clustering of fungal antibiotic pathway genes is that the close physical linkage of pathway genes may play a role in the regulation of their expression. For example, it is possible that cluster-related gene regulation occurs through the formation of chromosomal environments that affect interactions between pathway genes and transcription factors. The importance of gene clustering for the regulation of fungal antibiotic pathway gene expression remains to be determined.

Besides the several genera of fungi that are known to produce trichothecenes, two members of the plant genus *Baccharis* also accumulate trichothecenes (47). Some of the trichothecenes found in the tissues of these plants are identical to the complex macrocyclic trichothecenes produced by *Myrothecium* species (45). On the basis of these observations it has been proposed that *Baccharis* species acquired the genes for trichothecene biosynthesis through horizontal transfer from a trichothecene-producing fungus (46). The demonstration that several trichothecene biosynthetic genes are clustered in *F. sporotrichioides* indicates that the horizontal transfer of this pathway from fungi to plants is feasible. Characterization of trichothecene pathway genes will facilitate efforts to determine whether related genes are present in *Baccharis* or *Myrothecium* species.

SIGNIFICANCE OF TRICHOTHECENES IN PLANT PATHOGENESIS

Although the major biological activity of the trichothecene toxins is known to be the inhibition of protein synthesis (60), their specific function in the fungi that produce them is not obvious. In common with many other fungal secondary metabolites (4), trichothecenes apparently are not essential for fungal growth or reproduction in vitro. Field strains, mutants, and transformants that do not produce trichothecenes appear to grow as vigorously as trichothecene-producing strains do and to retain their sexual fertility (7, 38). Trichothecenes have been well documented to be host nonspecific in their toxicity and to inhibit protein synthesis in a wide range of eukaryotic organisms, including animals, fungi, and higher plants. There are several lines of evidence that trichothecenes are virulence factors in some *Fusarium* diseases; i.e., they can affect "the amount or extent of disease caused" (76). Trichothecenes are potent phytotoxins and at very low concentrations (10^{-5} to 10^{-6} M) can produce wilting, chlorosis, necrosis, and other symptoms in a wide variety of plants (19). For example, simple trichothecenes such as T-2 toxin and deoxynivalenol inhibited protein synthesis in maize leaf disks and kernel sections (13) and inhibited the growth of wheat coleoptiles (75), and the more complex macrocyclic trichothecenes of *Myrothecium roridum* produced chlorotic and necrotic lesions on muskmelon leaves (50). In addition, trichothecenes have been found in a variety of plant tissues infected with *Fusarium* species, including wheat and maize kernels, and in dry-rotted potato tubers and parsnip roots (24, 28, 55, 73). On the other hand, attempts to detect trichothecenes in diseased plants have not always been successful; trichothecenes were not found in muskmelon seedlings infected with trichothecene-producing strains of *M. roridum* (3). As discussed by Yoder (76), Mitchell (63), and others, inability to detect fungal toxins in a complex plant matrix does not necessarily mean that toxins are not involved in pathogenesis. Toxins may be unstable or inactivated by plant enzymes or may not be detectable because of interference by plant constituents. Correlations of virulence with the ability of field strains to produce trichothecenes in vitro provide additional circumstantial evidence that trichothecenes play a role in plant pathogenesis (7, 54). However, such data are not convincing because quantitative correlations among field strains of different genetic backgrounds might be entirely fortuitous.

More critical tests of the role of trichothecenes in plant pathogenesis have used *Fusarium* and *Gibberella* strains in which trichothecene production was blocked by UV-induced mutation or specifically altered by gene disruption. Three UV-induced *F. sporotrichioides* mutants were shown by complementation tests and by chemical analysis to each be blocked at a different step in the T-2 biosynthetic pathway (Table 1) (5, 8, 65). These three mutants and the wild-type parent were used to test the causal relationship between trichothecene production and virulence on parsnip root. Only the T-2 toxin-producing wild-type strain and a diacetoxyscirpenol-producing mutant were of high virulence. A calonectrin analog-producing mutant and a trichodiene-producing mutant were low in virulence, but they complemented each other to restore T-2 toxin production in vitro and to partially restore virulence on parsnip root (29). These results strongly suggest that production of certain highly oxygenated trichothecenes is required for high virulence of *F. sporotrichioides* on parsnip root.

To determine whether trichothecene production is a general virulence factor for *Fusarium* species, diacetoxyscirpenol biosynthesis in *G. pulicaris* was blocked by disruption of the *Tox5* gene encoding trichodiene synthase (22, 38). Of the 82 hygromycin-resistant transformants that were tested, 5 produced no detectable trichothecenes in liquid culture or in diseased parsnip or potato tissues. The virulence of these five toxin-nonproducing transformants on parsnip roots was significantly reduced when compared with the virulence of the progenitor strain. Furthermore, reduced virulence on parsnip cosegregated with the trichothecene-nonproducing phenotype among tetrad progeny of a cross between a nonproducing transformant and a producing parent (Fig. 5). These observations in *G. pulicaris* are consistent with the previous findings that the virulence of a UV-induced *F. sporotrichioides* mutant that produced no trichothecenes was highly reduced on parsnip root. On potato tubers, in contrast, trichothecene-nonproducing transformants and tetrad progeny were equal in virulence to the trichothecene-producing progenitor strain. These results indicate that production of trichothecenes is important for the virulence of *G. pulicaris* and *F. sporotrichioides* on parsnip root but has no role in the virulence of *G. pulicaris* on potato tubers. These results also suggest that one should be cautious in generalizing results from one plant species to another when assessing the role of trichothecenes in plant disease.

An increasing body of evidence indicates that the chemical interactions between plant-pathogenic fungi and higher plants are both complex and highly integrated. For instance, as fungi have developed toxins that increase their virulence on plant tissues, plants have developed a variety of ways to limit the effectiveness of these fungal toxins (48). Current information is not sufficient to identify all of the plant factors that affect trichothecene biosynthesis and phytotoxicity, but some possibilities are under investigation. There is no evidence to date that any plant constituent specifically induces

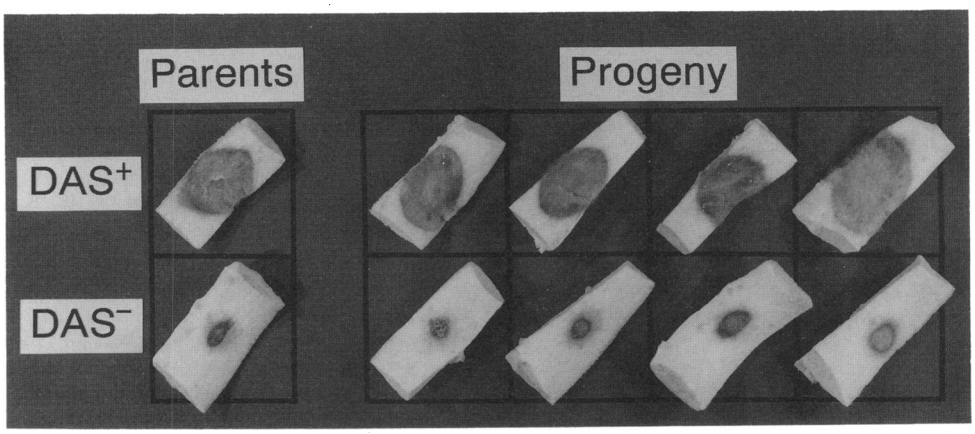

FIG. 5. Segregation of diacetoxyscirpenol production and virulence among an eight-spored tetrad from a cross between a *Tox5*$^+$ parent and a *Tox5*$^-$ transformant. For each strain, parsnip root strips were inoculated and incubated for 5 days at 25°C in the dark. Data and figure from reference 22 with permission.

or enhances trichothecene biosynthesis in *Fusarium* species. Biosynthesis can, however, be blocked in vitro by the addition of certain naturally occurring plant metabolites at concentrations that are not inhibitory to fungal growth. Xanthotoxin and other furanocoumarins, which are produced by parsnips and a wide variety of other plants, were especially effective inhibitors and appeared to block the pathway after trichodiene but before its oxygenated derivatives, suggesting an effect on the enzyme(s) catalyzing trichodiene oxygenation (26). Furthermore, accumulation of trichodiene in *Fusarium*-infected parsnip roots suggests that furanocoumarins may inhibit trichothecene biosynthesis in planta. On the other hand, plant production of furanocoumarins that block trichothecene biosynthesis is countered by fungal production of enzymes that detoxify furanocoumarins and apparently override the plant response (28). The parsnip-*Fusarium* system thus illustrates the potential complexity of chemical interactions between a fungus and its plant host.

That trichothecenes themselves could be metabolized by plant enzymes was suggested by the experiments of Miller and Young (62) and Scott et al. (69), in which levels of deoxynivalenol increased and then declined in wheat heads naturally and experimentally infected with *F. graminearum*. When deoxynivalenol was added to wheat cell suspension cultures, it was metabolized to a variety of products (61). Similarly, diacetoxyscirpenol was rapidly deacetylated by potato tuber slices to 15-monoacetoxyscirpenol and scirpentriol, which were metabolized to undetected products (22). Preliminary experiments with a variety of plants indicate a correlation between plant resistance to the effects of trichothecenes and higher rates of trichothecene metabolism by plant tissues (22, 61).

APPLICATIONS

Knowledge of the biosynthesis, regulation, and function of the trichothecene toxins suggests new strategies for controlling trichothecene contamination of agricultural commodities. For example, if a trichothecene is a virulence factor on particular host plants, inhibition of trichothecene biosynthesis could decrease virulence and protect such plants from infection. Strategies for inhibiting trichothecene biosynthesis include the application of synthetic or naturally occurring trichothecene inhibitors directly to plants and the incorporation of genes for such inhibitors into plants by classical plant breeding or genetic engineering. It might also be possible to decrease the virulence of trichothecene-producing fungi by altering genes encoding plant proteins that are target sites for trichothecenes. One can assume that this approach might be successful because it appears to be the mechanism used by fungi that are resistant to trichothecenes. The 60S ribosomal subunits of the trichothecene-producing fungus *M. verrucaria* were shown to be insensitive to high concentrations of macrocylic trichothecenes (35). Although most plants are very sensitive to trichothecenes, certain Brazilian shrubs, such as *Baccharis coridifolia*, are resistant to their effects and actually accumulate them in seed coats (46); however, the mechanism of *Baccharis* resistance to trichothecenes is not known. If the trichothecene biosynthetic gene cluster is analogous to antibiotic gene clusters of streptomycetes, genes for trichothecene resistance may be closely linked to genes for their biosynthesis. It should be noted that successful introduction of trichothecene tolerance or resistance into crop plants could have undesirable results, such as increased trichothecene levels in *Fusarium*-infected agricultural commodities.

If trichothecene toxins do not enhance virulence on a particular host plant, an alternative strategy for reducing trichothecene production levels is to use nonproducing strains to displace trichothecene-producing pathogens through competition. In fact, preliminary studies of mixed populations of *G. pulicaris* from potato dry rot lesions suggest that trichothecene-nonproducing strains might be more competitive than producer strains (21). Also, the presence of the *Tox1* gene in *Cochliobolus heterostrophus* reduced its competitiveness or pathogenic fitness on maize in the greenhouse and in the field (49). A possible control strategy for trichothecene contamination could thus be introduction of trichothecene-nonproducing strains or species in the field or in storage. Little work of this type has yet been published, and most of that has been concerned with aflatoxin in cotton, peanuts, and maize. These studies indicate that aflatoxin contamination can be decreased by inoculation with competitive strains or species that do not produce aflatoxin (9, 30). Competitive exclusion of mycotoxins by field release of pathogenic fungi such as *Fusarium* and *Aspergillus* spp. is a highly controversial strategy.

Understanding the biosynthesis and regulation of trichothecenes should also provide insights into the biosynthesis of

other biologically active sesquiterpenes of fungi and of higher plants. Continued success in understanding these complex systems and in applying new information to improving food quality and safety will require multidisciplinary team research that can integrate the diverse fields of chemistry, molecular biology, and plant pathology.

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