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MiniReview

Molecular biology of mycotoxin biosynthesis

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Abstract

Mycotoxins are secondary metabolites produced by many important phytopathogenic and food spoilage fungi including *Aspergillus*, *Fusarium* and *Penicillium* species. The toxicity of four of the most agriculturally important mycotoxins (the trichothecenes, and the polyketide-derived mycotoxins; aflatoxins, fumonisins and sterigmatocystin) are discussed and their chemical structure described. The steps involved in the biosynthesis of aflatoxin and sterigmatocystin and the experimental techniques used in the cloning and molecular characterisation of the genes involved in the pathway are described in detail. The biosynthetic genes involved in the fumonisin and trichothecene biosynthetic pathways are also outlined. The potential benefits gained from an increased knowledge of the molecular organisation of these pathways together with the mechanisms involved in their regulation are also discussed. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mycotoxin; Biosynthetic pathway; Cloning

1. Introduction

Mycotoxins are a group of secondary metabolites which are produced by various filamentous fungi, and which can cause a toxic response, termed a mycotoxicosis, if ingested by higher vertebrates and other animals. The mycotoxigenic fungi involved in the human food chain belong mainly to three main genera: *Aspergillus*, *Penicillium* and *Fusarium*. The former two genera are commonly found as contaminants of foods during drying and storage while the latter are plant pathogens which produce mycotoxins before, or immediately after harvesting.

Aflatoxins are the group of mycotoxins which are of greatest significance in foods and feeds, and are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. The four main aflatoxins produced are B_1, B_2, G_1 and G_2. They are difuranocoumarin derivatives (Fig. 1), with the B and G nomenclature deriving from the blue and green fluorescent colours produced under UV light on thin layer chromatography plates; with the subscript numbers indicating major and minor compounds respectively. Aflatoxin B_1 (AFB_1) is widely regarded as the most potent liver carcinogen known for a wide variety of animal species, including humans [1]. Aflatoxin M_1 and M_2 are hydroxylated derivatives of AFB_1 and AFB_2, which are formed and excreted in the milk of lactating animals including humans, that have consumed aflatoxin-contaminated foods. A precursor of the aflatoxins, sterigmatocystin (ST), is a mycotoxin

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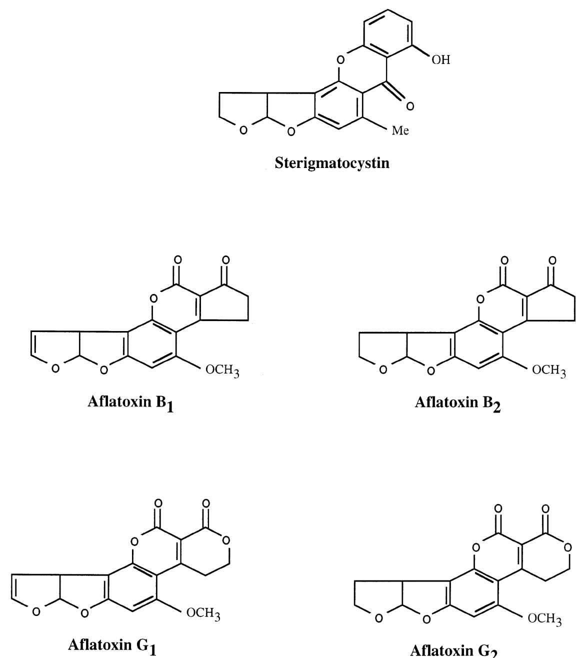
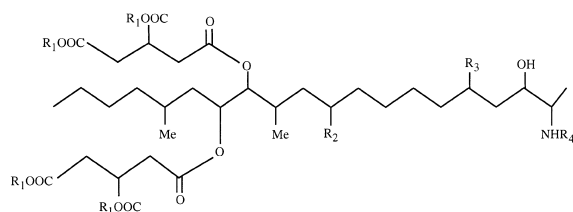


Fig. 1. Common toxins produced by *Aspergillus* and *Penicillium* species.

which is characterised by a xanthone moiety fused to a dihydrodifuran or tetrahydrofuran moiety; it is also acutely toxic and carcinogenic [2].

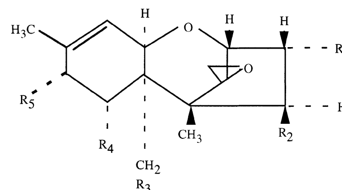
The trichothecenes are a group of mycotoxins which are produced by several fungal genera including *Fusarium, Trichoderma, Trichothecium, Stachybotrys, Cephalosporium* and *Myrothecium*. The trichothecenes are chemically very diverse (Fig. 2), but are all tricyclic sesquiterpenes with a 12,13-epoxy-trichothec-9-ene ring. They can be designated into four subclasses: type A having a functional group other than a ketone at position C-8; type B having a ketone at position C-8; type C having a second epoxy group at C-7, C-8, C-9 or C-10; and type D containing a macrocyclic ring between C-4 and C-5 with two ester linkages [2]. The non-macrocyclic trichothecenes are frequently less cytotoxic, e.g. T-2 toxin, diacetoxyscirpenol and deoxynivalenol, and are primarily produced by *Fusarium equiseti*, *F. graminearum* and *F. sporotrichioides*, whilst the more complex macrocyclic trichothecenes are commonly associated with members of the genus *Myrothecium* [3,4]. Interestingly, two members of the Brazilian plant genus, *Baccharis*, produce macrocyclic trichothecenes, although evidence suggests that the mycotoxin may also be synthesised by an endophytic fungus [4]. Trichothecenes are known to cause alimentary toxic aleukia, fusariotoxicoses and to be cytotoxic to mammalian cells. In addition they are immunotoxic and potent inhibitors of protein synthesis, which can result in a predisposition to other diseases and mask the underlying toxicoses [5]. In addition to their role in animal and human health, many trichothecene-producing fungi are phytopathogenic and its has been suggested that trichothecenes may function as virulence factors in plant disease [4,6,49].

The fumonisins are another important group of mycotoxins produced primarily by the cereal pathogen, *Fusarium moniliforme*. A number of other fungal species also produce fumonisins including *F. proliferatum, F. anthophilum, F. dlamini, F. napiforme*



	R_1	R_2	R_3	R_4
Fumonisin B_1	H	OH	OH	H
Fumonisin B_2	H	H	OH	H
Fumonisin B_3	H	OH	H	H
Fumonisin A_1	H	OH	OH	$COCH_3$

Structure of some Fumonisins



	R_1	R_2	R_3	R_4	R_5
Deoxynivalenol	OH	H	OH	OH	O
Nivalenol	OH	OH	OH	OH	O
4,15-Diacetoxyscirpenol	OH	OAc	OAc	H	H
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Neosolaniol	OH	OAc	OAc	H	OH

Structure of some Trichothecenes

Fig. 2. Important toxins produced by *Fusarium* species.

and *Alternaria alternata* f. sp. *lycopersici* [7]. Their chemical structure, which is a C-20, diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino group (Fig. 2), resembles sphingosine (sphinganine), which forms the backbone of sphingolipids. The fumonisins are competitive inhibitors of sphingosine *N*-acetyltransferase which results in the blocking of complex sphingolipid biosynthesis and the accumulation of sphingosine [7]. The most abundant fumonisin produced in nature is fumonisin B_1 (FB_1) which can cause leukoencephalomalacia in horses and pulmonary oedema syndrome in pigs and is hepatotoxic and hepatocarcinogenic in rats [7,8]. Fumonisins may also be implicated in the epidemiology of oesophageal cancer in humans. Although no experimental evidence exists the occurrence of *F. moniliforme*-infected maize and production of fumonisins has been correlated with a higher incidence of oesophageal cancer in specific geographical regions of China and South Africa [7,9].

2. Biosynthetic pathways

2.1. Aflatoxins and sterigmatocystin

The aflatoxin biosynthetic pathway is well understood and has recently been reviewed [10,11,50]. Initially, acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase, which is then extended by a polyketide synthase to norsolorinic acid, the first stable precursor in the pathway. The polyketide then undergoes approximately 12–17 enzymatic conversions, through a series of pathway intermediates, which are summarised in Fig. 3. Following the formation of versicolorin B, the pathway branches to form AFB_1 and AFG_1 which contain dihydrobisfuran rings and are produced from demethylsterigmatocystin (DMST); and the other branch forms AFB_2 and AFG_2, which contain tetrabisfuran rings and are produced from dihydrodemethylsterigmatocystin (DHDMST).

While aflatoxins are produced only by certain strains of *A. parasiticus*, *A. flavus* and *A. nominus*, numerous ascomycetes and deuteromycetes including *A. nidulans* produce the mycotoxin sterigmatocystin (ST), the penultimate intermediate in the AF biosynthetic pathway. The ST pathway is believed to include at least 15 enzymatic activities involving each enzyme activity from the AF pathway bar the penultimate steps involving the conversion of ST to AF.

Several of the enzymes involved in the AF pathway have been purified to homogeneity (for review see [11]). A 78-kDa versicolorin B synthase enzyme has been isolated which is involved in the cyclisation of versiconal to versicolorin B. This enzyme is believed to be the pivotal enzyme in determining the stereochemistry of the bisfuran ring in the aflatoxins. In addition, a tetrahydrobisfuran cyclising enzyme vericonyl cyclase has been purified which is responsible for the conversion of versiconal to versicolorin B. Three different enzymes have been characterised which appear to be involved in the conversion of norsolorinic acid to averantin: a 38-kDa norsolorinic reductase (NAR), a 43-kDa isozyme of the reductase and a 140-kDa NAR. Two versiconal hemiacetal (VHA) reductases (VHA I and II), which convert VHA to versiconal acetate, have been purified from *A. parasiticus*. Also three esterases which catalyse the conversion of versiconal acetate to versiconal acetate have recently been isolated.

A number of methyltransferases involved in the pathway have also been characterised. A 168-kDa *O*-methyltransferase and a 40-kDa methyltransferase corresponding to MT-II, which are involved in the conversion of ST to *O*-methylsterigmatocystin and dihydrosterigmatocystin to dihydro-*O*-methylsterigmatocystin, respectively, have been purified. More recently another *O*-methyltransferase (MT-1), with a molecular mass of 150 kDa, which is involved in the conversion of DMST to ST and of DHDMST to dihydrosterigmatocystin (DHST), has been purified [12].

2.2. Trichothecenes

The trichothecene pathway begins with the cyclisation of farnesyl pyrophosphate (FPP) to trichodiene by the enzyme trichodiene synthase (Fig. 4). This is the only enzyme in the biosynthetic pathway that has been purified and characterised to date, and the dimer, with a subunit molecular mass of 45 kDa, has been isolated from four fusaria including *Fusarium sporotrichioides* [13]. The subsequent pathway involves a number of oxygenations, isomerisations,

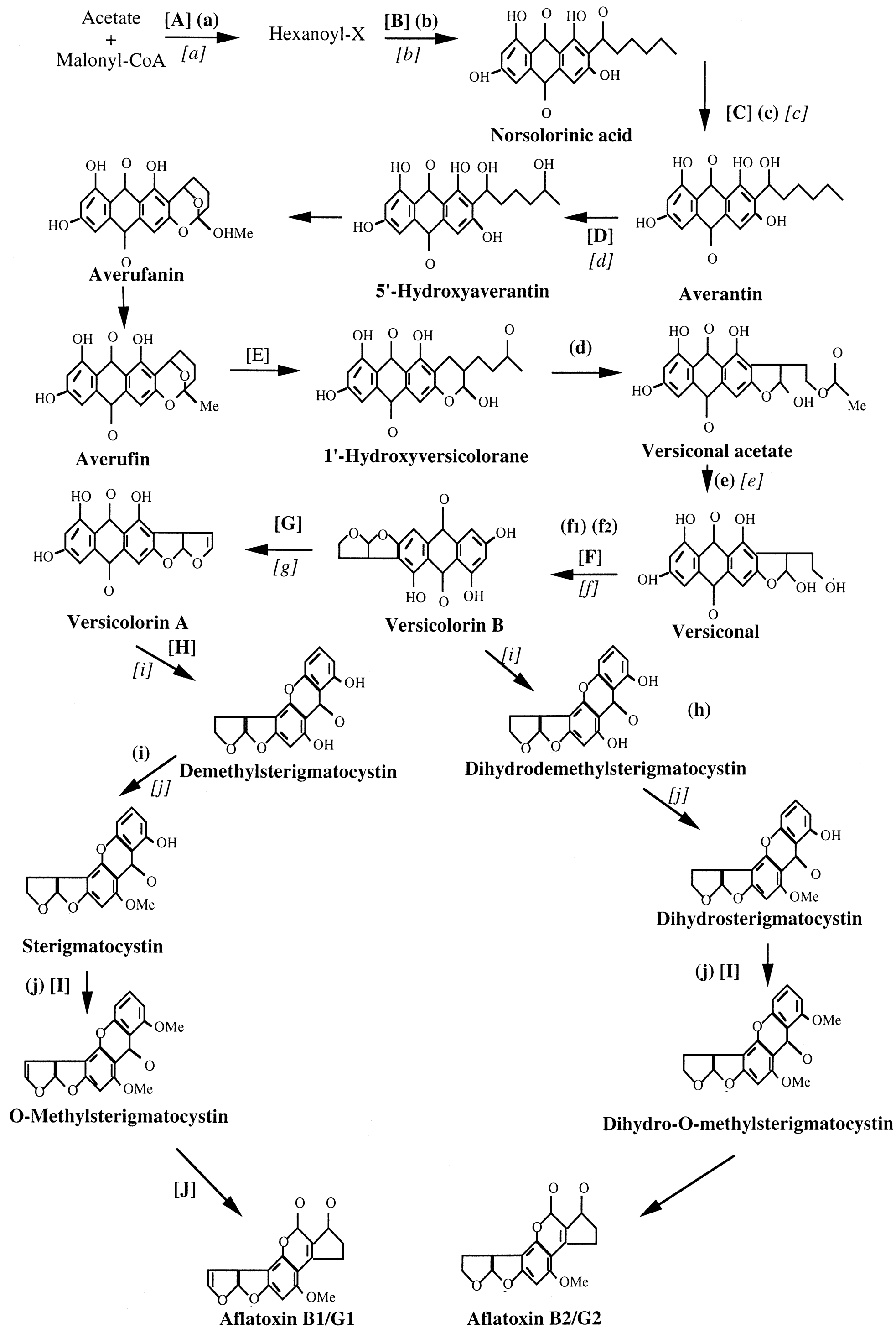

Acetate
+
Malonyl-CoA
[A] (a)
[a]
Hexanoyl-X
[B] (b)
[b]
Norsolorinic acid
[C] (c) [c]
Averantin
[D]
[d]
5'-Hydroxyaverantin
Averufanin
Averufin
[E]
1'-Hydroxyversicolorane
(d)
Versiconal acetate
(e) [e]
Versiconal
(f1) (f2)
[F]
[f]
Versicolorin B
[G]
[g]
Versicolorin A
[H]
[i]
Demethylsterigmatocystin
[i]
Dihydrodemethylsterigmatocystin
(h)
(i)
[j]
Sterigmatocystin
[j]
Dihydrosterigmatocystin
(j) [I]
O-Methylsterigmatocystin
(j) [I]
Dihydro-O-methylsterigmatocystin
[J]
Aflatoxin B1/G1
Aflatoxin B2/G2

Fig. 3. Aflatoxin and sterigmatocystin biosynthetic pathway. Enzymes involved: (a) fatty acid synthase, (b) polyketide synthase, (c) norsolorinic acid reductase, (d) versiconal hemiacetal acetate reductase, (e) esterase, (f_1) versicolorin B synthase, (f_2) versiconyl cyclase, (g) desaturase, (h) *O*-methyltransferase (MT-II), (i) *O*-methyltransferase, (j) *O*-methyltransferase (MT-I). Genes involved in aflatoxin biosynthesis: [A] *fas1A* and *fas2A*, [B] *pksA*, [C] *nor1*, *norA*, [D] *avnA*, [E] *avf1* (*aflB* and *aflW*), [F] *vbs*, [G] *verB*, [H] *ver1A*, *aflS*, [I] *omtA* and [J] *ord1*. Genes involved in sterigmatocystin biosynthesis: [*a*] *stcJ* and *stcK*, [*b*] *stcA*, [*c*] *stcE*, [*d*] *stcF*, [*e*] *stcI*, [*f*] *stcN*, [*g*] *stcL*, [*h*] *stcS*, [*i*] *stcU* and [*j*] *stcP*. (Compiled from [11,18,21,50].)
←

cyclisations and esterifications leading from trichodiene to diacetoxyscirpenol, T-2 toxin and 3-acetyldeoxynivalenol. All of the intermediates except those involved in the earlier steps of the non-macrocyclic biosynthetic pathway have been confirmed by feeding studies [14]. In contrast, the macrocyclic biosynthetic pathway is much less understood; only the end products and late intermediates of the pathway have been isolated and characterised [15].

2.3. Fumonisins

Fumonisins are thought to be synthesised through the condensation of the amino acid alanine to an acetate-derived precursor. Branched-chain methyl groups are added at C-12 and C-16 by an *S*-adenosyl methionine transferase. The subsequent biosynthetic steps involving oxygenation and esterification of the acetate-derived backbone are as yet unknown. It is not clear whether oxygenation and methylation occur before or after condensation with alanine. However, it appears likely that less oxygenated trichothecenes such as FB_2, FB_3 and FB_4 are precursors of the more highly oxygenated FB_1 (Fig. 5) [16]. To date, no enzymes involved in the fumonisin biosynthetic pathway have been isolated.

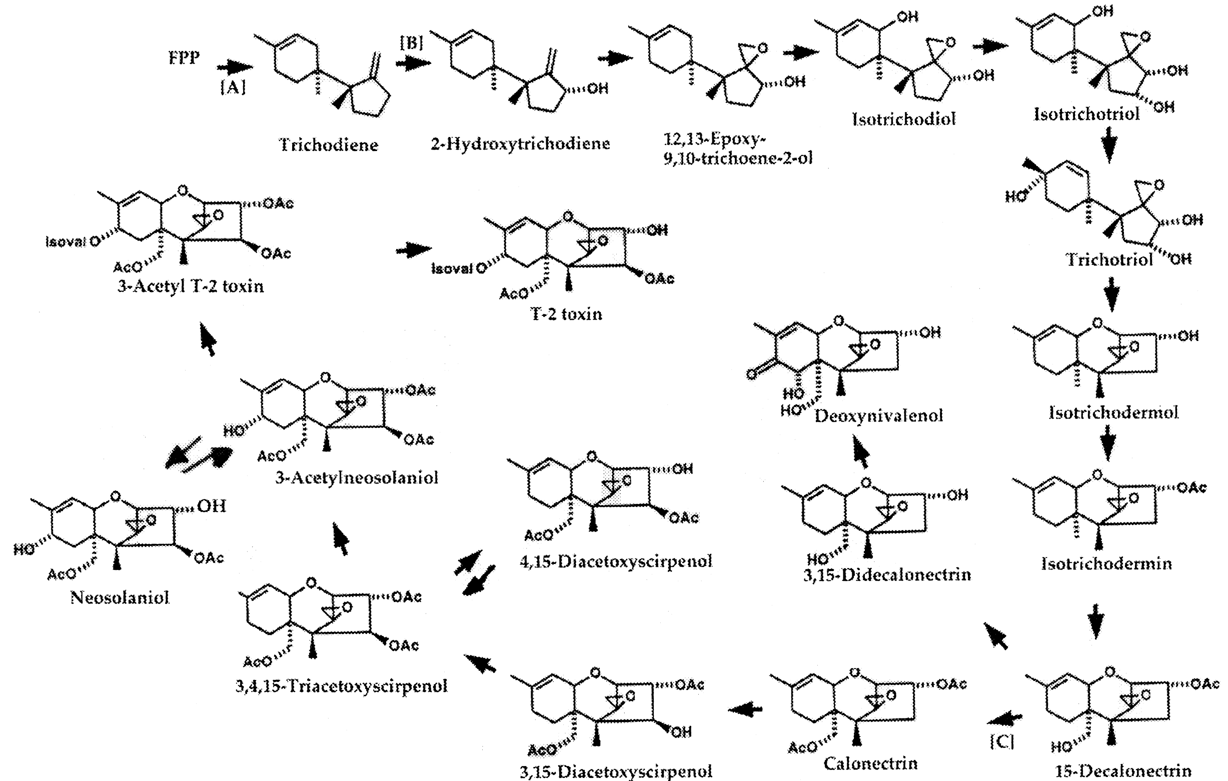


Fig. 4. Trichothecene biosynthetic pathway in *Fusarium* species. Genes involved: [A] *Tri* 5, [B] *Tri* 4 and [C] *Tri* 3. (Taken from [44].)

Table 1
Identity of AF pathway genes (A) and ST pathway genes (B) with other protein sequences[a]

Gene	Putative activity	Accession number	Gene product (aa) (kDa)	Similar polypeptides (aa)	% identity	Number of aa compared	Organism	Accession number
A: AF pathway genes								
fas1A	fatty acid synthase α	L48183	1980	β-subunit of FAS1[1]	47	159	*Saccharomyces cerevisiae*	M31034
				β-subunit of FAS1[2]	40	345	*S. cerevisiae*	M31034
pksA/pksL1	polyketide synthase	Z47198	2109	StcA/pksST (2181)	64	overall	*Aspergillus nidulans*	L39121
				wA PKS[3]	64	100	*A. nidulans*	X65866
				wA PKS[4]	32	130	*A. nidulans*	X65866
				type 1 PKS[5] (3519)	29	100	*Streptomyces antibioticus*	L09654
				type 1 PKS[6] (10288)	25	100	*Saccharopolyspora erythraea*	M63677
nor1	dehydrogenase	L27801	271 (29)	StcE (260)	56	overall	*A. nidulans*	U34740
				PKS[7] (272)	32	103	*Streptomyces violaceoruber*	X16300
				VER1A (262)	26	120	*Aspergillus parasiticus*	M91369
				NAM dehydrogenase[8] (272)	23.2	211	*Flavobacterium* sp. 141-8	D90316
norA/adh2	dehydrogenase	U24698	388 (43.7)	NORA (388)	99	overall	*Aspergillus flavus*	U32377
				AAD[9] (385)	49	overall	*Phanaerochaete chrysosporium*	L08964
				ADH1 (349)	23	overall	*A. flavus*	L27434
				NOR1 (271)	22	overall	*A. parasiticus*	L27801
avnA	CYP-450 monooxygenase	U62774	495 (56.3)	StcF (506)	66	overall	*A. nidulans*	U34740
				StcL (500)	37	overall	*A. nidulans*	U34740
				StcB (435)	15.6	overall	*A. nidulans*	U34740
				StcS (505)	9.3	overall	*A. nidulans*	U34740
vbs	oxidase/dehydrogenase	U51327	643 (70.3)	GOX[10] (605)	38	overall	*A. niger*	X16061
				CDH[11] (556)	34	overall	*Escherichia coli*	X52905
ver1A	ketoreductase	M91369	262	VER1B[12] (86)	95	overall	*A. parasiticus*	U63994
				StcU/VERA (397)	85	overall	*A. nidulans*	L27825
				T_4HN[13] (283)	56	overall	*Magnaporthe grisea*	L22309
				ketoreductase[14] (261)	52	overall	*Streptomyces coelicolor*	M1953141
omtA/omt1	*O*-methyltransferase	L25834	428 (46)	OMT1 (418)	97	overall	*A. flavus*	L25836
ord1	CYP-450 monooxygenase	U81806	528 (60.2)	StcF (506)	69	overall	*A. nidulans*	U34740
ord2	unknown	L40840	286 (30.6)	StcO (297)	52	overall	*A. nidulans*	U34740
				StcQ (274)	30	overall	*A. nidulans*	U34740
aflR/apa2	transcription factor	L26220	437 (46.7)	AFLR/AFL2 (437)	>95	overall	*A. flavus*	L32577
				AFLR (433)	71	42	*A. nidulans*	U34740
				AFLR (433)	31	overall	*A. nidulans*	U34740

Table 1 (*continued*)
Identity of AF pathway genes (A) and ST pathway genes (B) with other protein sequences[a]

Gene	Putative activity	Accession number	Gene product (aa) (kDa)	Similar polypeptides (aa)	% identity	Number of aa compared	Organism	Accession number
adh1	alcohol dehydrogenase	L27434	349	ADH1[15] (349)	82	overall	*A. nidulans*	M16196
				ADH3[16] (352)	82	overall	*A. nidulans*	X02764
				ADH1[17] (348)	57	overall	*A. nidulans*	J01313
aflJ	unknown	AF002660	438	AFLJ (435)	> 95	overall	*A. flavus*	AF0077975
B: ST pathway genes								
stcJ	fatty acid synthase α	U34370	1559	FAS2[18]	44	overall	*Penicillium patulum*	M37461
stcK	fatty acid synthase β	U34370	1914	FAS1[19] (2076)	37	overall	*Yarrowia lipolytica*	X53868
stcA/pksST	polyketide synthase	L39121	2181	wA PKS[3,4]	42	overall	*A. nidulans*	X65866
stcE	ketoreductase	U34740	260	NOR1	56	overall	*A. parasiticus*	L27801
stcF	CYP-450 mono-oxygenase	U34740	506	StcL	41	overall	*A. nidulans*	U34740
				ORD1	69	overall	*A. parasiticus*	U81806
stcI	lipase/esterase	U34740	276	lipase[20] (433)	sig. ident.	overall	*Moraxella* sp. TA144	X53868
				lipase[21] (308)	sig. ident.	overall	*Pseudomonas* sp. B11-1	AF034088
stcN	GMC oxido-reductase[32]	U34740	unp seq.	GDH[22] (612)	sim. ident.	overall	*Drosophila melanogaster*	M29298
				CDH[11] (556)	sim. ident.	overall	*E. coli*	X52905
				GOX[10] (605)	sim. ident.	overall	*A. niger*	X16061
				MOX[23] (664)	sim. ident.	overall	*Hansenula polymorpha*	X02425
stcL	CYP-450 mono-oxygenase	U34740	500	StcF (506)	41	overall	*A. nidulans*	U34740
				ORD1 (528)	40	overall	*A. parasiticus*	U81806
				CYP-450 mono-oxygenase[24] (506)	29	overall	*Nectria haematococca*	X73145
stcS/verB	CYP-450 mono-oxygenase	U34740	505	CYP 4A11[25] (519)	23	overall	*Homo sapiens*	S67581
stcU/verA	ketoreductase	U34740	264	VER1	85			
stcP	*O*-methyl-transferase	U34740	unpub. seq.	OMT1 (428)	sig. ident.	overall	*A. parasiticus*	L22091
				O-methyltransferase[26] (306)	sig. ident.	overall	*S. erythraea*	X60379
				N-methyltransferase[27] (381)	sig. ident.	overall	*S. erythraea*	X51891
stcB	CYP-450 mono-oxygenase	U34370	435	CYP-450 mono-oxygenase[24] (506)	24	overall	*N. haematococca*	X73145
stcC	oxidase	U34370	311	Chloroperoxidase (321)	29	overall	*Caldariomyces fumago*	M19025

Table 1 (*continued*)
Identity of AF pathway genes (A) and ST pathway genes (B) with other protein sequences[a]

Gene	Putative activity	Accession number	Gene product (aa) (kDa)	Similar polypeptides (aa)	% identity	Number of aa compared	Organism	Accession number
stcG	dehydrogenase	U34740	unpub. seq.	*budABC* operon encoded polypeptides[28]	sig. ident.	overall	*Klebsiella terrigena*	L04507
				α-acetolactase decarboxylase[29] (259)	sig. ident.	overall	*Enterobacter aerogenes*	L04506
stcO	unknown	U34740	297	ORD2 (286)	52	overall	*A. parasiticus*	L40840
				StcO (297)	30	overall	*A. nidulans*	U34740
stcQ	unknown	U34370	274	ORD2 (286)	30	overall	*A. parasiticus*	L40840
				StcO (297)	30	overall	*A. nidulans*	U34740
stcT	elongation factor 1γ	U34370	215	elongation factor 1γ[30] (430)	37	overall	*Artemia* sp.	M28020
stcV	dehydrogenase	U34370	387	AAD[31] (385)	46	overall	*P. chrysosporium*	L08964
stcW	FAD mono-oxygenase	U34370	488	cyclohexanone mono-oxygenase (542)	27	overall	*Acinetobacter* sp. NCIB 9871	M19029

[a]Protein sequences included in this analysis are as follows: 1, FAS1, β-subunit enoyl reductase domain of β-subunit of fatty acid synthetase; 2, malonyl/palmityl transferase domain of β-subunit of fatty acid synthetase; 3, β-ketoacyl-acyl-carrier protein synthase functional domain of wA polyketide synthase; 4, acyltransferase domain of wA polyketide synthase; 5, polyketide synthase putatively involved in oleandomycin biosynthesis; 6, erythromycin-producing polyketide synthase (*eryA* gene); 7, granaticin-producing polyketide synthase; 8, *N*-acyl-D-mannosamine dehydrogenase (*nam* gene); 9, ligninolytic aryl-alcohol dehydrogenase; 10, glucose oxidase; 11, choline dehydrogenase (*betA* gene); 12, non-functional truncated polypeptide; 13, tetrahydroxynaphthalene reductase (scytalone reductase); 14, ketoreductase (*actIII* gene); 15, alcohol dehydrogenase I (*alcA* gene); 16, alcohol dehydrogenase III; 17, alcohol dehydrogenase I; 18, α-subunit of fatty acid synthase; 19, β-subunit of fatty acid synthase; 20, triacylglycerol lipase (*lip2* gene); 21, cold-adapted lipase (*lipP* gene); 22, glucose dehydrogenase; 23, methanol oxidase; 24, phytoalexin pisatin demethylase (PDA6-1 gene); 25, fatty acid ω-hydrolyase; 26, erythromycin *O*-methyltransferase (*eryG* gene); 27, *N*-6-aminoadenine-*N*-methyltransferase (*ermE* gene); 28, α-acetolactase decarboxylase (*budA* gene), α-acetolactate synthase (*budB* gene), acetoin (diaceyl) reductase (*budC* gene); 29; α-acetolactase decarboxylase (*budA* gene); 30, translation elongation factor; 31, aryl alcohol dehydrogenase; 32, glucose/methanol/choline oxidoreductase.

Abbreviations: unpub. seq., unpublished sequences; sig. ident., significant identity; sim. ident., similar identity.

3. Cloning and molecular characterisation of the aflatoxin and sterigmatocystin biosynthetic genes

Early studies on the genetics of AF/ST biosynthesis employed *A. flavus* and *A. parasiticus* mutants, either partially or fully blocked in AF production, indicating the possibility that some of the AF genes were clustered [17]. Molecular genetics confirmed these initial reports, as the development of efficient DNA transformation systems for both *A. flavus* and *A. parasiticus* allowed the elucidation of genes corresponding to the enzyme activities of the AF/ST pathway [10,11,18]. Two gene transfer techniques, gene complementation which involves the restoration of gene function in AF blocked mutants and gene disruption where 'knock-out' strains are formed, have been invaluable in assigning the function of the isolated AF/ST genes. The AF biosynthetic genes are clustered in *A. flavus* and *A. parasiticus* and the gene cluster has been located on a 4.9-Mb chromosome in *A. flavus* [19]. Mapping studies and complementation of an AF gene cluster-deleted mutant of *A. flavus* indicated that all of the cloned aflatoxin biosynthetic genes are located within a 75–90-kb region [20,21]. The physical order of the genes in the cluster appears to largely coincide with the sequential enzymatic steps of the pathway and both gene organisation and structure are very conserved within *A. flavus* and *A. parasiticus*. The significance of this gene clustering is not known although the involvement of chromosome structure in gene regulation may be possible. In addition the conserved nature of the AF gene cluster suggests that the function or regulation of AF biosynthesis may rely on an intact structural organisation [10]. Many of the genes encoding enzymes involved in the AF biosynthetic pathway in both *A. flavus* and *A. parasiticus* have been cloned (Table 1A and Fig. 3). The genetics of the ST biosynthetic pathway of *A. nidulans* has been recently elucidated, which has furthered our understanding of the AF pathway (Table 1B and Fig. 3). A 60-kb region of chromosome IV of *A. nidulans* has been identified, which encodes 25 co-regulated transcripts which are thought to encompass most or all of the genes involved in ST biosynthesis [22]. The functions of many of these *stc* genes have been assigned experimentally, whilst the putative functions of the others have been assigned from the identity of the deduced amino acid sequences to enzymes predicted to the involved in the ST/AF biosynthetic pathway. The ST biosynthetic genes in *A. nidulans* are functionally and physically conserved with the AF genes of *A. flavus* and *A. parasiticus*, although differences in gene order and the direction of transcription are evident. The characterisation of the genes of the AF/ST biosynthetic pathway, together with the techniques employed for their isolation and confirmation of function, will be outlined.

Two of the genes of the ST gene cluster in *A. nidulans*, *stcJ* and *stcK*, encode the α- and β-subunit of a fatty acid synthase (FAS) which is specific for the formation of the hexanoate starter of ST. Disrupted *stcJ*/*stcK* mutants do not synthesise ST, but retain the ability to do so when provided with hexanoic acid [23]. Two functional homologues of *stcJ* and *stcK* were isolated in *A. parasiticus*, *fas1A* and *fas2A*. *fas1A* was cloned by complementation of an *A. parasiticus* double mutant (blocked at *nor1*, responsible for the conversion of norsolorinic acid, and at a preceding step) resulting in the restoration of norsolorinic acid (NA) production [24]. The subsequent disruption of *fas1A* prevented NA accumulation in a *nor1*-blocked *A. parasiticus* mutant which normally accumulated NA. Homology of the predicted product of *nor1* with functional domains of the β-subunit of a yeast FAS suggests that the *fas1A* gene encodes a FAS which synthesises part of the hexanoate starter of AF. A second FAS gene, *fas2A*, has been located next to *fas1A* and is suspected to function as the β-subunit of this specific FAS [25].

The subsequent extension of the hexanoate starter by a specialised polyketide synthase (PKS) was supported by the isolation of *stcA* (formerly *pksST*). *stcA* was revealed by transcriptional mapping of genomic DNA cosmids of *A. nidulans* which hybridised to a *nor1* cDNA fragment and a 55-kb deleted region of a non-sterigmatocystin-producing mutant, and shows significant amino acid identity to two other PKSs of *A. nidulans* [26,27]. The functional homologue of *stcA* in *A. parasiticus*, *pksA* (formerly *pksL1*), was independently cloned by polymerase chain reaction (PCR) amplification with degenerate primers and by gene disruption of an *O*-methylsterigmatocystin-accumulating strain of *A. parasiticus*, which resulted in a mutant unable to produce NA

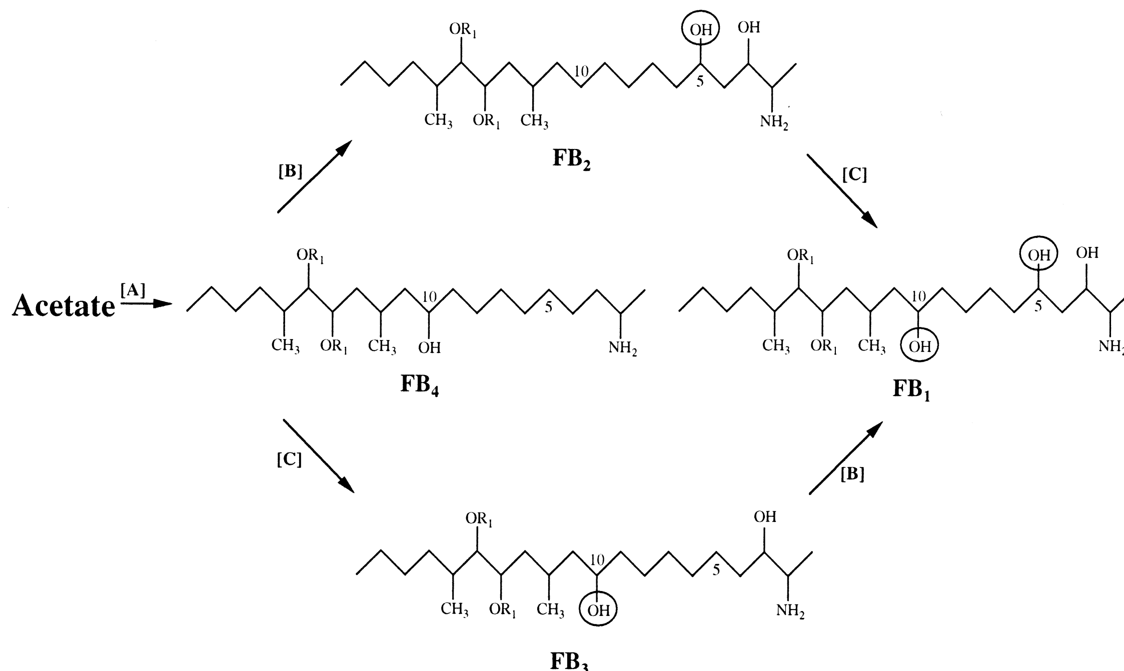


Fig. 5. Proposed pathway for fumonisin biosynthesis. R_1 designates tricarballylic acid esters. Genes involved: [A] *fum* 1, [B] *fum* 3 and [C] *fum* 2. (Taken from [16].)

[28,29]. The next step in the AF/ST pathway, where NA is converted to averantin (AVN), may involve the putative ketoreductase encoding gene in *A. nidulans*, *stcE*, whilst subsequent conversion of AVN to averufin (AVF) may involve *stcF* which encodes a putative P-450 monooxygenase, although this has not been confirmed [22]. The apparent homologue of *stcE*, *nor1*, was isolated by the complementation of an *A. parasiticus* mutant which accumulated NA. The role of *nor1* was confirmed by the disruption of *nor1* in aflatoxigenic isolates of *A. parasiticus* which resulted in the accumulation of NA [30]. The identity of *nor1* as an NADPH-dependent reductase was confirmed by a *nor1*/maltose-binding protein fusion assay where transformed *Escherichia coli* converted NA to AVN in the presence of NADPH. A second reductase capable of converting NA, which shows little homology to *nor1*, *norA*, was located in the AF gene cluster of *A. flavus* and *A. parasiticus* and was cloned using monoclonal antibodies to NAR [31]. *stcV* in *A. nidulans* encodes a similar deduced product to that of *norA*; however, the function of *stcV* has not been confirmed [22]. The putative homologue of *stcF, avnA* (formerly *ord1*), was initially identified from the region between *ver1* and *omtA* and associated with an oxidoreductive step in the AF pathway. Disruption of *avnA* resulted in a non-aflatoxigenic averantin-accumulating mutant and precursor feeding studies with AF intermediates with this mutant indicated that *avnA*, which encodes a cytochrome P-450 type enzyme, is involved in the conversion of AVN to AVF [32].

The *avf1* locus is believed to be involved in the conversion of AVF to VHA [21]. An AF gene cluster-deleted mutant of *A. flavus* transformed with a series of overlapping cosmids containing the AF cluster accumulated averufanin (AVNN) and AVF and was relieved by complementation with the *avf1* locus. Sequence analysis of the *avf1* locus revealed two genes, *aflB* and *aflW*, which encode products with similar amino acid identity to *stcB* and *stcW* respectively in *A. nidulans* but their functions are as yet unknown [18].

Conversion of versiconal acetate to versiconal and subsequently to versicolorin B (VER B) may involve the putative esterase gene *stcI*, which has no known

AF gene homologue, and *stcN*, a putative oxidoreductase [22]. The homologue of *stcN*, *vbs*, was cloned using degenerate primers designed from the amino acid sequences of peptide fragments of the VBS protein which catalyses the conversion of versiconal to VER B [20].

The *stcL* gene encodes a P-450 monooxygenase putatively involved in the conversion of VER B to versicolorin A (VER A) as gene inactivation of *stcL* resulted in the accumulation of dihydrosterigmatocystin (DHST) [27]. *verB*, the apparent homologue of *stcL* in *A. parasiticus* and *A. flavus*, has been cloned [18]. The conversion of VER A and VER B to demethylsterigmatocystin (DMST) and dihydrodemethylsterigmatocystin (DHDMST) involves *stcS* and *stcU* as gene disruption of the two genes resulted in the accumulation of VER A [33–35]. In addition, a disrupted double mutant (*stcL* and *stcU*) of *A. nidulans* accumulated VER B demonstrating the specific requirement of *stcU* for the conversion of VER B to DHDMST [27]. *aflS*, a gene similar to *stcS*, has been located between *ver1A* and *avnA* in the AF gene cluster [18]. *ver1A* (homologue of *stcU*), which encodes a NADPH-dependent ketoreductase involved in the conversion of VER A to ST, was isolated by the complementation of an *A. parasiticus* mutant that accumulated VER [36]. The subsequent conversion of DHDMST to DHST and DMST to ST may involve the methyltransferase *stcP*, which has no known AF gene homologue, as disruption of *stcP* results in the accumulation of DMST [35].

Homologues of *omtA* and *ord1*, genes involved in the final conversion steps of the AF biosynthetic pathway, are notably absent in the non-aflatoxigenic ST-producing *A. nidulans*. *omtA* (formerly *omt1*) was isolated by antibodies raised against the purified enzyme, OMT-A, and subsequently cloned into an *E. coli* expression system which overexpressed an OMT-A-β-galactosidase fusion protein capable of converting ST to *O*-methylsterigmatocystin (OMST) [37]. The *ord1* gene was identified by transforming an *A. flavus* AF gene cluster- deleted mutant with a 3.3-kb genomic fragment and the regulatory gene *aflR* of the AF gene cluster which allowed the transformant to convert OMST to AFB_1. Sequence analysis of the inserted AF fragment revealed *ord1* which encodes a cytochrome P-450-type monooxygenase. Transformation of *Saccharomyces cerevisiae* with *ord1* resulted in the ability to convert OMST to AFB_1, indicating that the *ord1* gene product is sufficient to complete the last step in the AF pathway [21].

There is growing evidence suggesting that gene expression is involved in the regulation of multiple parts of the AF/ST biosynthetic pathway. The observation of co-ordinate transcription of *nor1*, *ver1* and *omtA* suggests that AF genes may be regulated, at least in part, at the transcriptional level by a common regulatory factor [37,38]. *A. flavus* mutants blocked at *aflR* (previously *afl2*) could not convert various intermediates to AF and complementation of these mutants with *afl2* restored the expression of several AF pathway enzyme activities, which is characteristic of a gene encoding a *trans*-acting regulatory factor [39]. *aflR* (previously *apa2*) was also isolated from *A. parasiticus* by complementation of a non-aflatoxigenic *aflR* mutant [39]. Preliminary studies demonstrated that the transcription of *nor1*, *ver1* and *omtA* is activated by the *aflR* gene product AFLR [40]. Inactivation of *aflR* in *A. nidulans* results in the absence of expression of *stcW*, *stcV*, *stcU* and *stcT* transcripts and transformation of *A. nidulans* with the *aflR* homologue from *A. parasiticus* regulates ST production, demonstrating that the AFLR of *A. nidulans* is a functional homologue of the AFLR of *A. parasiticus* even though overall amino acid identity is low [41]. The predicted amino acid sequence of *aflR* contains a cysteine-rich zinc finger DNA-binding domain which is characteristic of some fungal transcriptional activators. The expression of *aflR* may be autoregulated as AFLR has been shown to specifically bind upstream of the AFLR translation start site [40]. *aflJ*, which is located adjacent to *aflR*, is required for the conversion of AF pathway intermediates to AF as disrupted strains of *A. flavus* at the *aflJ* locus do not accumulate any AF pathway intermediates and do not convert NA, ST or OMST to AF. Although speculative, the deduced amino acid sequence suggests that the *aflJ* product may be involved in transmembrane transport of AF intermediates or the localisation of AF pathway enzymes to an organelle [42].

4. Cloning and molecular characterisation of other mycotoxin biosynthetic genes

Several genes of the trichothecene biosynthetic pathway appear to be clustered in *F. sporotrichioides* [43]. Analysis of the gene cluster revealed nine genes within a 25-kb region and the function of eight of these genes has been assigned [6]. Two of these genes, *Tri* 3 and *Tri* 4, were identified following complementation of UV-induced mutants, blocked in trichothecene T-2 toxin production. *Tri* 3 encodes a 15-*O*-acetyltransferase, which converts 15-decalonectrin to calonectrin [43] (Fig. 4). *Tri* 4 encodes a cytochrome P-450 monooxygenase involved in the first step in the pathway converting trichodiene to an as yet unidentified oxygenated product. *Tri* 11, a second cytochrome P-450 monooxygenase, has also been identified which appears to oxygenate the trichothecene ring at the C-15 position [44]. Two additional specific acetyltransferases may also be present in the gene cluster for the hydroxylation at the C-3 and C-4 positions [43]. The other biosynthetic gene, *Tri* 5, encodes trichodiene synthase involved in the cyclisation of farnesyl pyrophosphate to trichodiene (Fig. 4). The biosynthetic pathway appears to be regulated by the product of the *Tri* 6 gene, a Cys_2, His_2 zinc finger protein [45]. More recently a *Tri* 101 gene has been isolated from *F. graminearum*, encoding a protein which catalyses the acetyl CoA-dependent *O*-acetylation of the trichothecene ring at the C-3 position. This *O*-acetyl group introduction acts as a resistance mechanism for the type B trichothecene producer, *F. graminearum* [46]. Interestingly, the *Tri* 101 gene is located between a putative UTP-ammonia ligase gene and the phosphate permease gene and mapping analysis with two of the least overlapping cosmid clones containing *Tri* 101 revealed that this gene is not clustered with *Tri* 4, *Tri* 5 and *Tri* 6 [46]. In addition to the structural genes and transcription factor, a *Tri* 12 gene which encodes a putative transport protein has also been identified [6]. The genes of the biosynthetic pathway of macrocyclic trichothecenes have been investigated in *Myrothecium roridum* [4]. Homologues of the non-macrocyclic trichothecenes pathway genes *Tri* 4, *Tri* 5 and *Tri* 6 have been reported within a 40-kb region of *M. roridum*. The deduced amino acid sequences of the products of *MrTri* 6 and *MrTri* 4 are 75% and 63% identical and similar in molecular mass to the apparent counterpart proteins in *F. sporotrichioides*. However, *MrTri* 6 encodes a protein which is almost twice the size of the product of *Tri* 6 and only the C-terminal region containing the Cys_2, His_2 zinc finger motif shows significant homology (65% identity) to *Tri* 6 in *F. sporotrichioides* The putative cytochrome P-450 monooxygenase product of *MrTri* 4 appears to be a functional homologue of *Tri* 4 as complementation of a *F. sporotrichioides* mutant lacking *Tri* 4 resulted in the accumulation of T-2 toxin, sambucinol, deoxysambucinol and the intermediates, trichothecene and isotrichodiol. Although mapping data indicate that the macrocyclic genes of *M. roridum* are clustered, the organisation and orientation of these genes differ from those of the trichothecene gene cluster in *F. sporotrichioides*. In *F. sporotrichioides* the *Tri* 4, *Tri* 6 and *Tri* 5 genes are located in that order within an 8-kb region whilst their putative homologues are located within a 40-kb region in *M. roridum*. In addition, the relative orientation of *Tri* 6 and *Tri* 4 differs from that of *MrTri* 6 and *MrTri* 4 in *M. roridum.* While differences in gene organisation have been observed between the AF and ST pathways of *A. parasiticus* and *A. nidulans*, the differences in the trichothecene pathways of *F. sporotrichioides* and *M. roridum* are more pronounced [4,10,22,38]. The less conserved gene organisation of the trichothecene pathways, in contrast to the AF/ST pathways, may be indicative of the presence of genes required for the production of unique structural features of the trichothecenes of *F. sporotrichioides* and *M. roridum* or may reflect the comparison of two more distantly related fungal species, i.e. *F. sporotrichioides* and *M. roridum* are taxonomically more distant than *A. parasiticus* and *A. nidulans.* Overall, it is evident that the clustering of genes for trichothecene biosynthesis is maintained in distantly related fungi and that the evolution of these gene clusters can involve substantial genetic rearrangements [4].

To date, no fumonisin biosynthetic genes have been cloned although several genes have been identified in *F. moniliforme* by classical genetics. Variants of *Gibberella fujikuroi* (*F. moniliforme*), blocked in the production of FB_1 and whose phenotypes segregate as single genetic loci were identified by crossing with high-producing FB_1 strains. Four classes of pu-

tative fumonisin biosynthetic genes, *fum* 1, *fum* 2, *fum* 3 and *fum* 4, were identified by this meiotic genetic analysis. *Fum* 1 represents strains that do not produce FB_1, FB_2, FB_3 or FB_4, whilst *fum* 4 represents a single strain that shows reduced fumonisin production and appears to be closely linked to *fum* 1. *Fum* 3 and *fum* 4 affect the hydroxylation of FB1 at C-10 and C-5 respectively and do not affect the overall level of fumonisin production. Both *fum* 3 and *fum* 4 are closely linked to *fum* 1. Tentative estimations suggest that *fum* 4 and *fum* 2 are situated 250 kb and 360 kb from *fum* 1; however, gene order has not been elucidated. Close linkage of the four genes indicates that the biosynthetic genes are arranged in a gene cluster on chromosome 1 of *G. fujikuroi.* These genetic data are consistent with the scheme in Fig. 5 where *fum* 2 may encode a C-10 hydroxylase that converts FB_4 to FB_3 and FB_2 to FB_1 while *fum* 3 may encode a C-5 hydroxylase that can convert FB_4 to FB_2 and FB_3 to FB_1 [16]. However, the *fum* loci could alternatively encode regulatory genes and not the structural genes of the biosynthetic pathway.

5. Conclusions

The cloning and molecular characterisation of mycotoxin biosynthetic genes is vital in order to gain a fuller understanding of the organisation, regulation and expression of these genes. Firstly this will be valuable in our overall understanding of the number, type and order of the enzymatic steps involved in the various biosynthetic pathways and of the physiological factors controlling these processes. Secondly it will aid in the development of improved molecular-based detection systems for mycotoxins and mycotoxigenic fungi in food systems. For example, sequence variability in the *aflR* gene generates distinct DNA fingerprints which allow the non-aflatoxigenic species of *A. sojae* and *A. oryzae* in the *Aspergillus flavi* group to be distinguished from the aflatoxigenic species *A. parasiticus* and *A. flavus* [47]. In addition PCR has been successfully used to detect aflatoxigenic fungi in grains, using primers based on the coding regions of *ver1*, *omt1* and *aflR* [48]. Finally this knowledge may allow (through the use of techniques such as gene disruption and AF gene/reporter constructs), the development of strategies for the biological control of mycotoxigenic fungi and the development of genetically engineered resistant crop plants.

The recent field application of atoxigenic strains of the trichothecene-producing wheat pathogen, *Gibberella zeae* (*Fusarium graminearum*), obtained by disruption of the *Tri* 5 gene, resulted in disease reduction and indicates the potential of genetically engineered atoxigenic fungi for the suppression of mycotoxigenic fungi in the field [49].

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