The production of fumonisins by *Fusarium verticillioides*

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Abstract

Plants and fungi produce an abundance of secondary metabolites, of which some are beneficial and others are toxic. The fungus *Fusarium verticillioides* destroys corn by causing rotting diseases and also produces toxic secondary metabolites: fumonisins. These have been found to cause cancer and neural tube defects in humans, and several other diseases in animals. Therefore, much research is done to elucidate the factors involved in the biosynthesis of these toxins. The genes involved, organized in the FUM gene cluster, and the biosynthetic pathway have already been characterized. Several abiotic conditions, such as pH, light and carbon or nitrogen source have been found to influence the production of FB_1, the most abundant fumonisin. By comparing the *F. verticillioides* genome with gene sequences from other fungi, new genes that are involved in regulation of secondary metabolism have been discovered. Amongst these are several transcription factors. G-proteins play a role in the regulation as well. Several epigenetic factors have been identified by using homology studies. The pathways between incoming signals and transcription still need to be elucidated. In the future, insight in the regulation of toxin synthesis may be used to grow and store crops under different conditions, minimizing toxin concentration.

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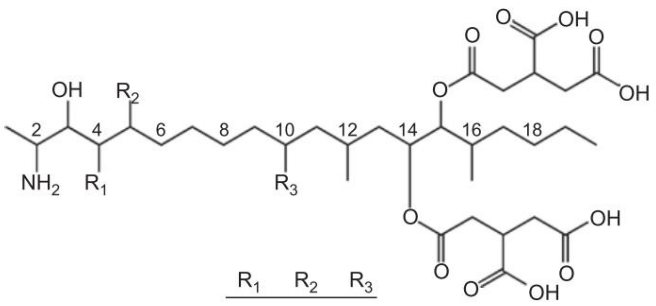
Introduction

Secondary metabolites are a heterogeneous group of organic molecules of low molecular mass that are synthesized by bacteria, fungi and plants. These metabolites are not directly required for processes such as growth or reproduction[1]. The exact function of many secondary metabolites is unknown. But since especially fungi and soil bacteria produce a wealth of different metabolites within their complex ecological niches, it appears that these metabolites developed in the course of evolution to enable the organism to survive in a certain environment. Many metabolites seem to play a role in communication or in inhibition of growth and development of competing organisms to defend their producers niche[1].

Many secondary metabolites are derived from nonribosomal peptides, built from amino acids, or polyketides, which are derived from acetate. Other classes are the terpenes, consisting of isoprene units fused together, the alkaloids, a heterogeneous group of molecules containing a basic nitrogen atom, and fatty acid products[1,2].

Today, secondary metabolites are an important source of pharmacological compounds, with the antibiotic penicillin, derived from the fungus *Penicillium chrysogenum*, as a well known example. Unfortunately, other metabolites can be toxic. This is especially problematic when these are produced by organisms growing on food. A well known example is aflatoxin, which is produced by *Aspergillus nidulans*, growing on peanuts and corn. This compound is known to be carcinogenic in humans[3].



	R_1	R_2	R_3
Fumonisin B_1	H	OH	OH
Isofumonisin B_1	OH	H	OH
Fumonisin B_2	H	OH	H
Fumonisin B_3	H	H	OH
Fumonisin B_4	H	H	H

Figure 1. The different structures of the B-fumonisins[4].

Another important group of toxins are the fumonisins, which are polyketides (figure 1), produced especially by filamentous fungus *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*), although other members of the Fusarium genus can produce the toxin in smaller amounts as well. *F. verticillioides* causes ear rot and stalk rot in maize. These plant diseases cause a decrease in yield as well as a decreased quality of the corn. Besides, the fumonisins produced by the fungus are believed to cause several diseases in both humans and animals. Concentrations of up to 300 mg/kg of maize have been found, although a wide range of concentrations was found in different studies. Fumonisins have also been detected in rice, wheat and soybean meal, and in derived products such as bread and biscuits[5].

In humans, the intake of fumonisins is associated with esophageal cancer. FB_1 is listed by the International Agency for Research on Cancer as a 2B carcinogen, indicating that it is a possible carcinogen in humans. Several epidemiological studies conducted in several African countries, South America, China and Iran show a higher risk of this type of cancer in regions where homegrown, contaminated maize is consumed. Another study done in China, however, did not indicate an

increased risk[5,6]. Another unresolved question is whether the carcinogenicity is influenced by other compounds in the food, or only by FB_1 itself[7].

Other studies indicate that fumonisins can cause neural tube defects (NTD) when ingested during the first trimester of pregnancy. In the United States, an increase in the occurrence of NTDs was found among Mexican-American women, who consume a lot of corn-based food. In other countries a similar trend was observed[5,8].

In animals, several diseases are associated with fumonisin ingestion. In horses and other equine species, the toxin can cause leukoencephalomalacia, a disease affecting the brain. In swine, fumonisin is believed to cause pulmonary edema. Kidney and liver tumors occur in rodents as well as in other species due to fumonisin intake[3,7].

Four main groups of fumonisins have been identified: fumonisins A, B, C and P, based on differences in their structures. *F. verticillioides* produces mainly fumonisin B_1 (FB_1), although FB_2 and FB_3 are produced in significant amounts as well[5].

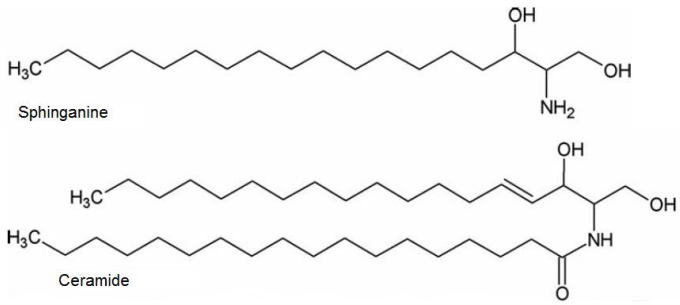


Figure 2. The structures of sphinganine and ceramide, a sphingolipid. *Note the similarities to the fumonisins[5].*

It is believed that fumonisins cause disease by disrupting sphingolipid synthesis. Sphingolipids exist in all eukaryotic cells. Not only are they structural molecules in membranes, but they also regulate several cell functions. Sphingolipids are synthesized from sphinganine by the enzyme ceramide synthase (figure 2). Since the structures of fumonisins and sphingolipids are quite similar, as both possess long carbon chains, it is believed that fumonisins exert their toxicity by inhibiting this enzyme, and thereby the whole pathway. How this exactly leads to the induction of cancer is not clear yet, as others factors may be involved. NTDs might be induced by fumonisins because the related folate biosynthesis is inhibited as well[5].

Why the Fumaria produce fumonisins remains unclear. Some studies indicate that mycotoxins are produced in response to oxidative stress, having a protective role for the fungus itself. Other studies pointed out that secondary metabolites are produced as a part of cell differentiation. Mycotoxins might be specifically produced to protect the ecological niche of the fungus by inhibiting or even destroying competing organisms. Fumonisins, therefore, probably are involved in ensuring fungal survival in its niche[9].

Fumonisins make the fungus, that already causes significant agricultural damage, even more dangerous. Therefore, much effort is put in elucidating how *F. verticillioides* produces this toxin and how this production is affected by different conditions. This essay will focus on the way *F. verticillioides* synthesizes this compound and what is known about its regulation on a molecular level.

The production of fumonisins

The organization of the gene cluster

The genes necessary for the biosynthesis of secondary metabolites are in general clustered together on one chromosome. Usually, these clusters contain at least one multidomain enzyme that synthesizes the scaffold of the metabolite, such as polyketide synthases (PKS) or non-ribosomal peptide synthases (NRPS). Other genes in the cluster encode regulating proteins, transporters or enzymes , such as oxidases, that catalyze different modifications[1].

The biosynthesis of fumonisins is no exception. The different biosynthesis steps in the production of fumonisins are catalyzed by enzymes clustered together in the so called FUM cluster (figure 3). The FUM cluster encodes for seventeen different proteins, (located on chromosome 1). In other related species, such as *F. oxysporum* or *F. proliferatum*, the FUM cluster is found as well[4].

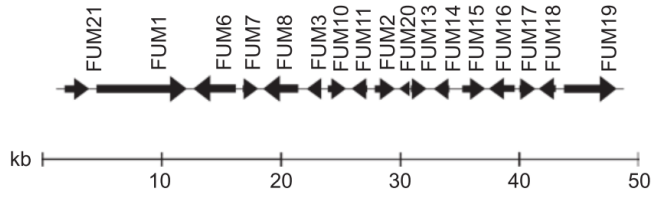


Figure 3. The organization of the FUM gene cluster.
Transcriptional orientation is indicated by the arrows[4].

The biosynthesis of fumonisins

The functions of the proteins that are encoded by the FUM genes were elucidated by comparing the amino acid sequences with those of known enzymes. Furthermore, all the FUM genes, except for FUM20, were disrupted or deleted to assess which metabolites were synthesized by the fungus instead of fumonisin, if any. Also, in vitro experiments were performed with isolated enzymes to elucidate which step in the biosynthesis pathway was catalyzed[4].

Taking all these studies together, a biosynthesis pathway has been proposed (figure 4). In the first step, Fum1p (the protein of FUM1) catalyzes the fusion of nine acetate molecules and two methyl units by a condensation reaction. The product is a linear polyketide consisting of an 18-carbon atom chain. Fum1p is believed to be a PKS. In the second step, the enzyme Fum8p, adds an alanine to the carbon chain by catalyzing another condensation reaction, yielding a 20-carbon chain. It is therefore believed that this protein is an α-oxoamine synthase, an enzyme that catalyzes the condensation of amino acids with another compound. The product, however, has never been isolated**.** Adding glycine, which does not have the methyl group, yields fumonisin C as the product, which differs from the B fumonisins by lacking an extra methyl group, indicating that alanine is indeed incorporated[4].

In the third step, the carbons at position 14 and 15 are hydroxylated by Fum6p. It is believed that this reaction is catalyzed by a protein consisting of a cytochrome P450 monooxygenase and a NADPH-dependent reductase, since the two oxygens that are added are derived from molecular oxygen [9]. Recently it was discovered that these steps do not take place in a fixed order, because the enzymes can accept compounds similar to their natural substrate as well[10].

When any of the genes mentioned above is deleted, the fungus is no longer able to produce fumonisins or any of their precursors. The next three steps are also not believed to take place in a specific order. At one point, the carbonyl group at C-3 is reduced to an alcohol group by Fum13p. Since the gene sequence is very similar to hydrogenases or reductases, it is thought that this enzyme

belongs to the same family. The purified enzyme also catalyzes this reaction. The addition of a hydroxyl group to the C-10 carbon of fumonisin is most likely catalyzed by Fum2p, believed to be a cytochrome P450 monooxygenase because of similarities in gene sequence. When the gene is deleted, fumonisins lacking a hydroxyl group at this position are produced[4].

Fum14p catalyzes the addition of two tricarballylic functions to the C-14 and C-15 hydroxyl groups by esterification. The enzyme appears to consist of two domains, one condensation domain and a peptidyl carrier protein domain[4]. For the addition of these tricarballylic moieties, more FUM genes in the cluster are needed. Fum10p is thought to be an acyl-CoA or an acyl-protein synthase, that plays a role in the activation of the tricarballylic moiety and its addition to the peptidyl carrier protein domain of Fum14p. The other domain of Fum14p can then catalyzes the addition of the moiety to the fumonisin. Fum7p appears to play a role in the formation of the tricarballylic moiety. Its gene sequence is very similar to iron-containing dehydrogenases, which are able to catalyze the reduction of double bonds to single bonds. When Fum7p is deleted, the mutant fumonisins indeed contain tricarballylic moieties with double bonds. Fum11p is thought to transport the double bonded precursor of the tricarballylic moiety out of the mitochondria into the cytosol, although deletion does not completely turn off biosynthesis of fumonisins, indicating that other transporters can perform the same task[4].

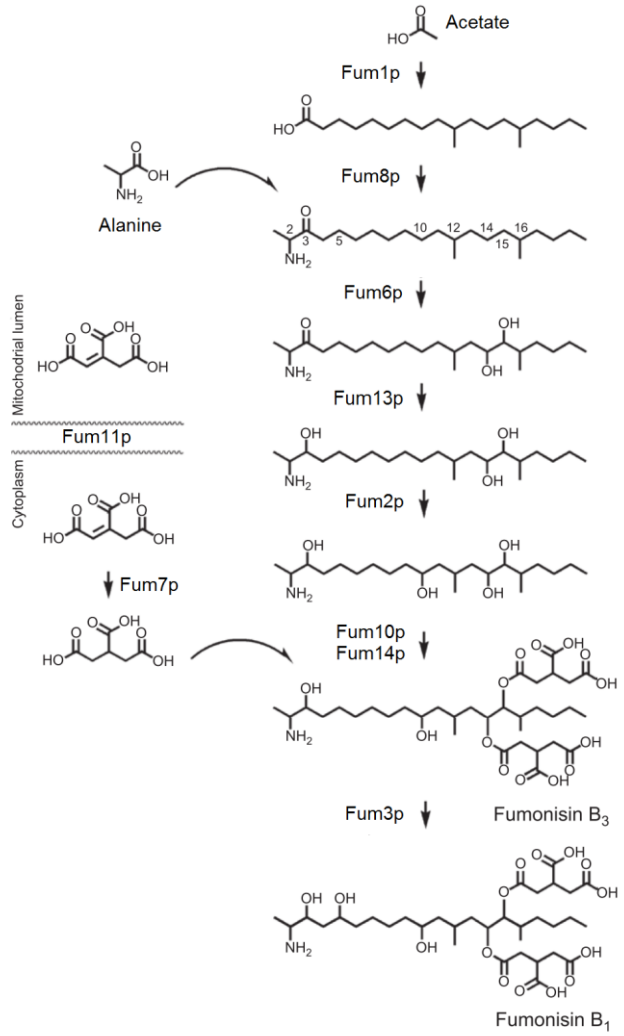


Figure 4. The different steps in fumonisin biosynthesis[4].

Fum3p catalyzes the final step in the biosynthesis pathway. The enzyme adds a hydroxyl group to the C-5 carbon in the chain. Knocking out this gene leads to the production to fumonisins without this group[4].

Other FUM genes are not directly involved in the biosynthesis itself, but have different roles. Fum21p is thought to be a transcription factor belonging to the Zn(II)2Cys6 family. These bind to promoter regions, thereby regulating the expression of the genes downstream. Knocking FUM21 out renders the cell almost unable to produce fumonisins[11]. Fum19p is believed to be an ABC transporter, but its function can be taken over by other transporters after disrupting FUM19. FUM17 and FUM18 were found to influence the synthesis of fumonisins indirectly. Nowadays they are believed to synthesize fumonisin resistant ceramide synthases, protecting the fungus' vital sphingolipid synthesis. The exact functions of FUM15 and FUM16 remain a mystery[4].

Regulation of fumonisin production

Environmental factors

In their natural habitat, fungi are exposed to different (abiotic) environmental stimuli, such as temperature, light, humidity, pH and different carbon or nitrogen sources. These factors are known to influence biosynthesis, including that of fumonisins, although the molecular mechanisms are not clear in many cases[12].

Nitrogen and carbon sources

Fungi can utilize several nitrogen sources. Since many, if not all, secondary metabolites contain nitrogen atoms, e.g. the amine group in fumonisin, different sources of nitrogen could influence biosynthesis pathways. They probably also interconnect with other pathways in the cell. In several species, there are different genes for nitrogen utilization. In *A. nidulans*, the protein *AREA* is involved in nitrogen utilization. AREA binds to GATA sequences in gene promoters to regulate the genes involved in nitrogen utilization under nitrogen restricted conditions. In the case of *F. verticillioides*, this has not been studied extensively, although it was found that its genome does contain these GATA sequences. Strains lacking the putative *AREA* homologue cannot grow on medium containing nitrate, nor can they produce FB_1. It was also found that addition of ammonium phosphate to *F. verticillioides* inhibits fumonisin production, although the mechanism is unclear[12].

Different carbon sources were found to influence biosynthesis in several fungi as well. In *F. verticillioides*, however, only a few studies have been done to assess this. In one study it was found that different concentrations of sucrose in the medium did not influence FB_1 production in Fusarium. In another study, conducted by Bluhm and Woloshuk, it was found that FB_1 production increases almost 50 times when the fungus was grown on medium containing amylopectin, a component of starch, when compared to amylose, dextrose, glucose or maltose containing media. This result is interesting, since in the course of the development of a maize kernel, the exact chemical composition of the kernel also changes, as more and more starch is produced. Fumonisin production thus is connected to the stage in maize development[13].

The influence of light

Another important environmental factor is light. Light is known to affect growth, reproduction and pigment formation in fungi. It also influences secondary metabolism. Fungi have wavelength dependent receptors, containing for example flavin groups or retinal, for light perception.

In a study done by Matic et al. on *F. verticillioides*, a strain grown on rice, it was found that the fungus, grown in culture, produced less FB_1 in darkness than in light. FB_1 synthesis was especially stimulated under yellow and green light, although other wavelengths also improved FB_1 synthesis. The expression of FUM1 was also tested. Highest mRNA levels for FUM1 were found with yellow and green light, exactly as expected[14].

An earlier study, conducted by Fanelli et al., also pointed out that the biosynthesis of FB_1 was increased in light (figure 5). White light slightly decreased FB_1 synthesis. Different wavelengths stimulated FB_1 synthesis more than others. FB_1 was in all cases produced in larger amounts than FB_2 and FB_3. The trends were similar. When testing the expression of FUM1, it was found that yellow, red and blue stimulated FB_1 biosynthesis most, whereas green light led to a decrease of FUM1 expression. FUM21 expression also varied with the different wavelengths of light[15].

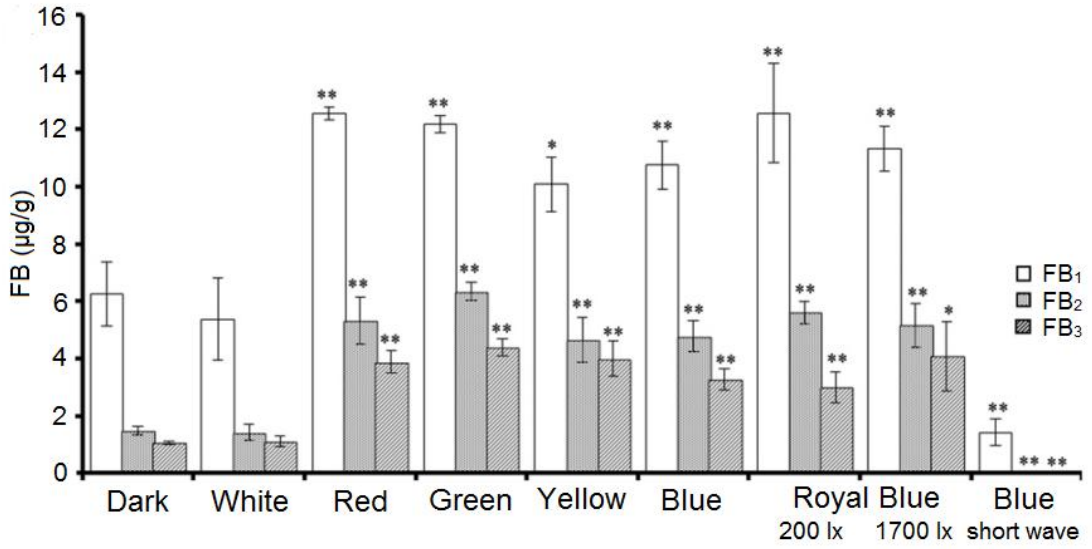


Figure 5. The production of FB_1, FB_2 and FB_3 for different colors of light. *Concentration of FB is in µg/g agar[15]. * P<0.05 **P<0.01.*

The response to light is most likely related to the ecology of the fungus. Since *F. verticillioides* usually grows in a field, it is exposed to sunlight for many hours a day. Therefore it is not surprising that light not only influences growth, but also its secondary metabolism[14,15].

Changes in pH

Another important environmental factor is the pH, which influences many processes. For several toxins it has been found that acidic conditions stimulate their synthesis, whereas alkaline conditions decrease the production of these secondary metabolites. In *A. nidulans*, the protein PacC regulates the response to a change in pH. PacC, a Cys2-His2 zinc finger, activates alkaline-expressed genes and represses acid-expressed genes. A homologue of this protein was found to exist in *F. verticillioides* and was named Pac1. A knock-out mutant was made and its fumonisin production was compared to the wild type and the mutant complemented with PAC1. The mutant produced more FB1 than the other strains. When the fungus was resuspended in an acidic medium buffered at pH 4.5, all three strains produced FB_1, whereas in a medium of pH 8.4 only the mutant produced FB_1 (table 1). At pH 4.5 and 7, all strains grew equally well. At pH 8.4 the mutant did not grow anymore, whereas the wild type and the complemented mutant did grow. Pac1 therefore helps the fungus to survive alkaline pH[16].

Table 1. The influence of pH on FB_1 production and FUM expression[16].

	pH 4.5		pH 8.4	
	FB_1	**FUM1**	**FB_1**	**FUM1**
Wild type	20	10 (8.0-13)	-	1.0 (0.9-1.1)
PAC1	25	140 (120-150)	10	48 (43-52)
PAC1 (comp.)	20	39 (37-43)	-	4.3 (3.9-4.7)

When the expression of FUM1 was investigated, it was found that under acidic conditions, FUM1 was expressed 10 times more than under alkaline conditions. For the mutant, the FUM1 transcription under acidic conditions was 135 times higher than for the wild type. For alkaline conditions, this was 47 times. PAC1 is therefore most likely a negative regulator of the FB_1 biosynthesis[16].

Transcription factors

Several transcription factors are known to regulate the FUM cluster. Within the cluster itself, Fum21p is produced as a transcription factor, as mentioned above. Other transcription factors have been discovered in recent years that also influence fumonisin biosynthesis. In many cases, these have been discovered by searching the genome for homologues of known transcription factors. Here, these transcription factors will be discussed.

MADS-box transcription factors

MADS-box transcription factors belong to a family of proteins involved in the regulation of many important cellular processes. They are also believed to be involved in the regulation of gene expression in response to different stimuli. All share a conserved dimerization domain and a DNA binding domain, which contains a highly conserved 56-60 amino acid region. This so called MADS-box motif can bind to the consensus sequence CC(A/T)6CG[17].

Screening of the *F. verticillioides* genome yielded two putative MADS-box TFs, named MADS1 and MADS2. To characterize their function, three deletion mutants were generated, one for each separate gene and one for both genes. It was found that they did not play a large role in fungal virulence, but they did have influence on the production of FB_1. The MADS1 knockout produced 50% less FB_1, whereas the MADS2 knockout and the MADS1/MADS2 knockout produced 80% less when compared to the wild type[17].

The influence of the deletion on the expression of 15 different PKS (such as Fum1p) was determined too. In the MADS1 mutant, after 10 days the expression levels of the PKS were reduced with almost 50% (see table), with exception for PKS10. For the MADS2 mutant, the expression levels were still comparable with those of the wild type[17].

The MADS-box TFs are therefore believed to be positive regulators of FB_1 synthesis. Since the double deletion yielded similar FB_1 levels as only the MADS2 deletion, MADS2 might be the primary MADS-box regulator of this biosynthesis pathway. Since the deletion of MADS1 influenced the expression of PKS more than MADS2, MADS1 might be a broad regulator of secondary metabolism. The authors of the study argued that MADS1 has a direct impact on FUM1, whereas MADS2 regulates genes more downstream the FUM cluster[17].

Transcription factor Sda1

The availability of different carbon sources, mentioned above, can influence the production of fumonisins. To investigate carbon utilization in relation to secondary metabolism, researchers looked for a transcription factor of the Cys2-His2 family. These TFs are believed to be involved in carbon catabolism and cell growth and development. The influence of these proteins on FB_1 production was assessed as well[18].

Sda1 (Sorbitol Dehydrogenase Activator) was identified in their study as a transcription factor of this Cys2-His2 (C2H2) family. The gene is believed to encode a polypeptide consisting of three zinc finger domains. To study the gene a deletion mutant was generated. The deletion strain was found to produce five times more FB_1 (figure 6) when grown on corn than the wild type. The complemented strain yielded somewhat more FB_1 than the wild type. Sda1 is thus most likely a negative regulator of fumonisin biosynthesis[18].

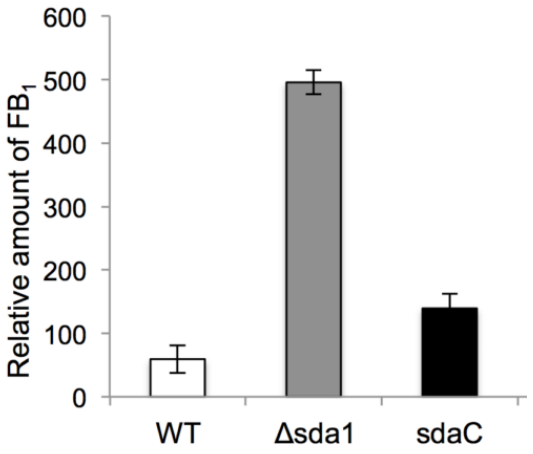


Figure 6. Fumonisin production in wild type, Sda1 mutant and complemented mutant. *Amount of FB_1 is normalized to growth[18].*

ZFR1

FUM21 is not the only gene that encodes for a Zn(II)2Cys6 transcription factor. When screening for more members of this family, another one, dubbed ZFR1, was discovered. The gene is believed to belong to this family because it contains the Zn(II)2Cys6 cluster DNA binding motif. To test its effect on the fungus, including its ability to produce fumonisins, a knock out strain was generated. When grown on degermed cracked maize kernels, it was found to produce approximately 13 times less FB_1 than the wild type strain (figure 7). When grown on the germs, there was no difference. With Northern blot transcripts of FUM1 and FUM8 were not found, accounting for the decreased production of FB_1. Zfr1 is probably not a specific regulator of the FUM cluster, but a broader transcription factor. It is also believed to regulate the expression of some putative sugar transporters[12,19].

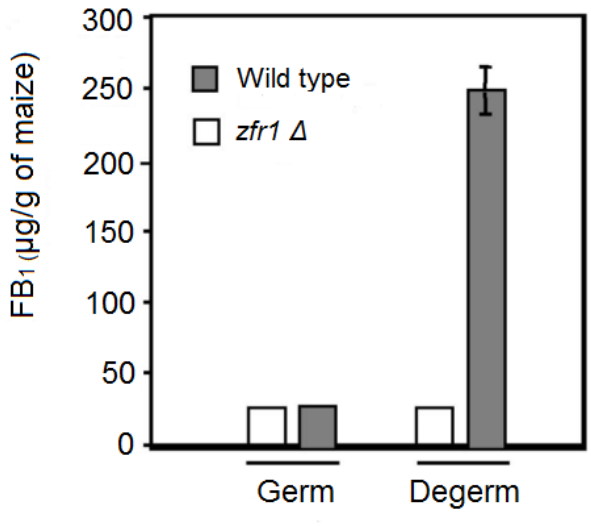


Figure 7. The production of FB1 of the wild type strain and the zfr1 mutant on corn germ or degermed corn[19].

How these transcription factors are activated themselves remains unclear and needs to be investigated in the future in order to get more insight in its role in regulating biosynthesis. Other factors involved in this regulation have been discovered already and will be discussed in the following sections.

Epigenetic factors

Not only transcription factors have transcriptional control. In recent years, epigenetic factors such as the modification of chromatin or nucleosome structure have been shown to influence transcription. For several secondary metabolites, including penicillin, epigenetic factors are known to influence their production[20].

In *A. nidulans*, the Velvet protein complex, which consists of LaeA, which is a protein methyltransferase, VeA and VelB, is believed to influence the production of secondary metabolites through histone methylation. In *F. verticillioides*, a homologue for the Velvet VeA gene has been found. When this gene, called FVVE1, is knocked out in *F. verticillioides*, FB_1 production decreased[21].

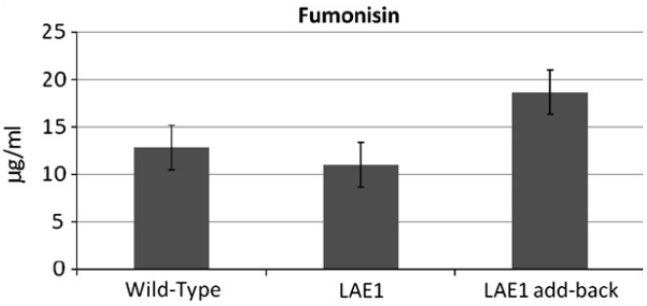


Figure 8. The influence of Lae1 on fumonisin production. *Concentration in mg fumonisin per mL culture[22]. P<0.05.*

A homologue of LaeA has also been discovered in *F. verticillioides*. When the gene was knocked out, the relative transcription of several gene clusters, among which also the FUM cluster, was measured. The transcription of all FUM genes was reduced. There was, however, no significant reduction in fumonisin production (figure 8), implying that other factors are involved downstream[22].

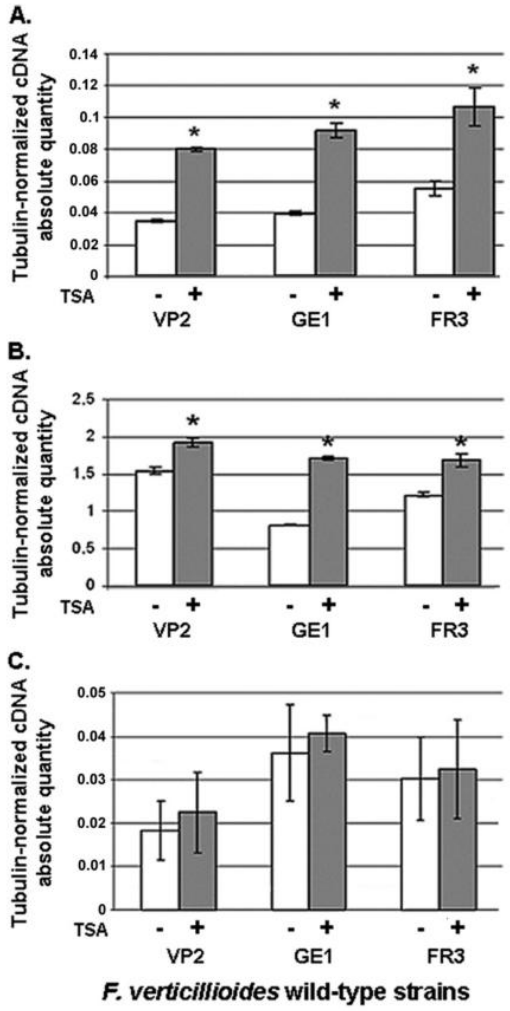


Figure 9. The influence of TSA (+) on expression of A. FUM1 B. FUM21 C.FUM9 in three wild type strains of F. verticillioides[23]. *P<0.05.

Besides methylation, acetylation is also involved in epigenetic control. Acetylation changes the chromatin structure, thereby influencing the transcription of the DNA. Histone acyltransferases (HATs) and histone deacetylases (HDACs) play an essential role in this process. Hypoacetylation is associated with gene silencing through the formation of heterochromatin, whereas hyperacetylation is associated with gene activation through the formation of euchromatin. In a study done by Visentin et al., the influence of acetylation on the expression of FUM1, FUM21 and FUM8 was investigated under FB_1-inducing conditions[23].

After inhibiting HDACs with the compound trichostatin A (TSA), the level of transcription of FUM1, FUM21 and FUM8 was determined in three wild type strains of F. verticillioides (figure 9). There was significantly more FUM1 and FUM21 transcribed, but the level of FUM8 did not increase significantly. Strangely, the FB production did not show significant differences. With help of antibodies targeting the hyperacetylated form of histone H4, the position of the histones relative to the promoter sites of the FUM genes was determined. It was found that the promoters for FUM1 and FUM21 were hyperacetylated under FB inducing conditions, but not significantly for FUM8[23].

Since the production of FB_1 was not significantly affected, but the FUM proteins themselves were, it is likely that there are more factors involved in repressing fumonisin synthesis than epigenetic factors[23].

G-proteins: other regulators of the FUM gene cluster

Environmental stimuli activate cellular cascades, which eventually lead to changes in the transcription of genes by activating or deactivating transcription factors or epigenetic modulators. A few proteins are known to influence FUM transcription upstream of the cascade. The ones mentioned below are all related to G-proteins, which are heterotrimers consisting of an α-, β- and γ-monomer, that relay signals received by G-protein coupled receptors (GPCRs) to other proteins. G-proteins are highly conserved among eukaryotes. In other fungal species, they were already known to be related to secondary metabolism, among other important cellular functions[24].

GBB1

In the search of G-proteins involved in fungal development and biogenesis, GBB1 was identified after searching the *F. verticillioides* genome by comparing the sequence with that of Gβ subunits of known heterotrimeric G-proteins in related species. When a deletion mutant was made and the production of FB_1 was investigated, it was found that the levels of FB_1 were significantly lower in the mutant, although the growth of the fungus was not different (figure 10). FUM1 and FUM8 transcription were also found to be much lower, so Gbb1 plays a positive role in the transcription of these genes. But to understand how exactly Gbb1 regulates this, one should find out which proteins are acting downstream the cascade, characterize the other subunits and find out whether there are other associated factors[24].

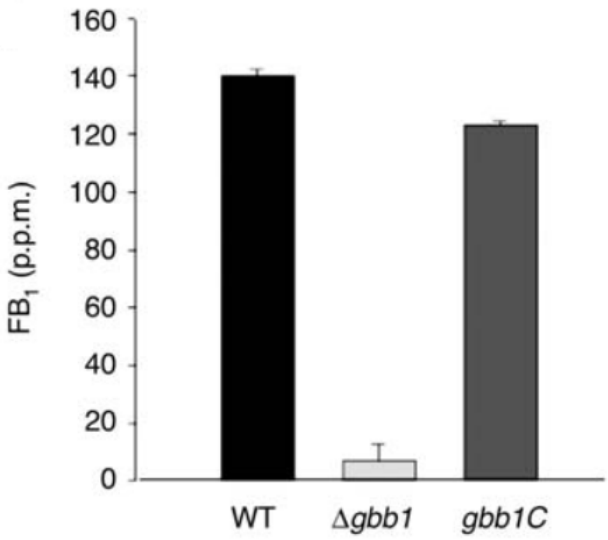


Figure 10. FB_1 production in the wild type, gbb1 mutant and complemented mutant[24].

G-proteins themselves can be regulated as well. In a study done by Mukherjee et al. several proteins belonging to the group of regulators of G-protein signaling (RGS) were identified and investigated. In general, RGS proteins are negative regulators of G-protein cascades. In *A. nidulans*, this group of proteins is known to influence toxin biosynthesis. In *F. verticillioides*, all RGS deletion mutants showed an increase in FB_1 production. RGS was also found to be upregulated when GBB1 was knocked out, but it remains unclear how GBB1 and RGS interact exactly to influence FB_1 synthesis[25].

GBP1

Since G-proteins are ubiquitous and important for many functions, the search for more G-proteins in *F. verticillioides* continues. Another G-protein that was discovered to influence fumonisin production is Gbp1, a GTP-binding protein. When the gene was deleted, the fungus produced 58% more FB_1 than the wild type (figure 11). Deletion of the gene was found to upregulate FUM1 transcription 3-fold and FUM8 transcription 2.5 fold. So GBP1 is probably a negative regulator of fumonisin biosynthesis. Overexpression of the gene, however, did not lead to more repression of FB_1 production. Probably, there is a threshold that cannot be crossed[26].

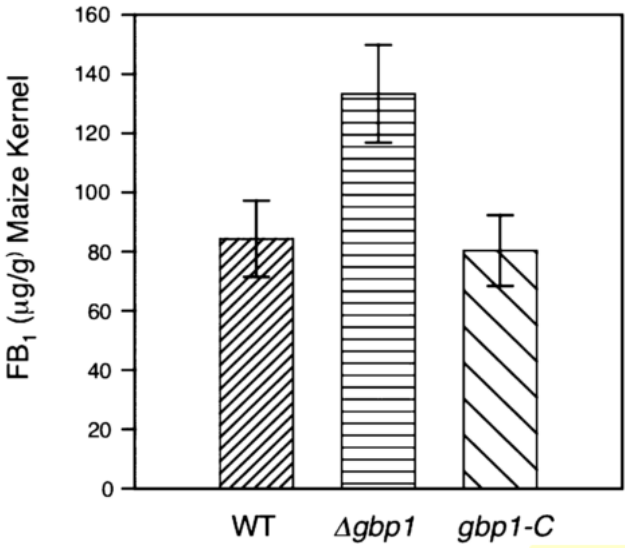


Figure 11. FB_1 production in wild type, gbp1 mutant and complemented strain[26]. *P<0.05*

FvMK1

G-proteins transduce their signals in many cases through cAMP signaling pathways. However, in the case of *F. verticillioides*, these are probably not involved in the regulation of fumonisin biosynthesis. Other important targets of G-proteins are the mitogen-activated protein kinases (MAPK). MAP kinases are threonine or serine kinases that regulate many important cellular mechanisms. In *F. graminearium* MAPK are known to influence secondary metabolism. In a study done by Zhang ea. an orthologue of another fungal MAPK was identified. It was determined that FvMK1 works downstream of GBB1 in fumonisin biosynthesis regulation. The orthologue, named FvMK1, was deleted and the effects were investigated. The growth rate was lower and the mutant produced significantly less FB_1 than the wild type (figure 12). The expression of FUM1 and FUM8 was found to be respectively 12 and three times reduced. FvMK1 thus is a positive regulator of fumonisin synthesis. It probably regulates some transcription factors via other signaling proteins[27].

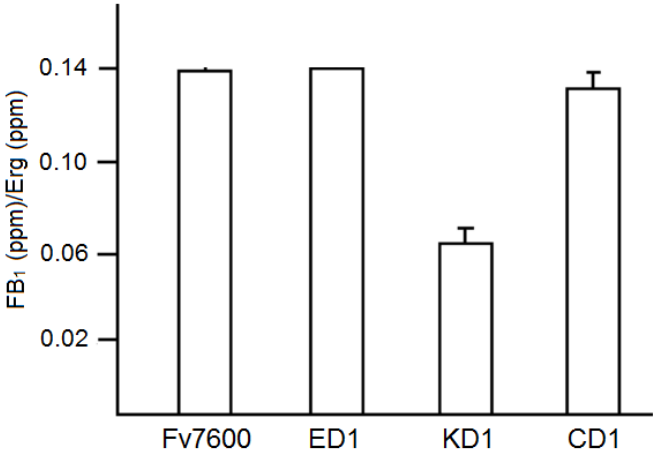


Figure 12. FB_1 production in wild type strain Fv7600, ectopic transformant* (ED1), FvMK1 mutant KD1 and complemented mutant CD1. *Amount of FB1 was normalized for ergosterol as biomarker for growth. *contains the gene in another position than in wild type[27].*

Discussion

The production of toxins by fungi living on food crops has been the topic of much research in the past years and still is. More and more the impact of toxins in food becomes clear now it is known that they can cause higher incidence of disease in regions were the crop is consumed, in both humans and animals. Since fumonisins were discovered to increase the incidence of esophageal cancer in humans and also to be the causing agents of several epidemics in swine and horses, many studies were started to find out how the fungus produces this toxin and how its production is regulated.

The studies described above mostly show that the understanding of the regulation of fumonisin biosynthesis is very incomplete. Basically, it is known that certain factors have a certain effect, but the exact cellular pathways are not clear. Therefore, it is not possible to draw clear conclusions from these data. The regulation of the biosynthesis of the carcinogenic aflatoxins has already been characterized in more detail in *A. nidulans*. Although the compounds are chemically quite different, there are similarities in the regulation of their production. Since many genes that were found to play a role in fumonisin biosynthesis are homologues of known genes in *A. nidulans* and other Fumaria, comparison can not only explain some phenomena, but also predict which genes and pathways could be involved.

Certain nitrogen sources cause an increase in FB_1 biosynthesis, whereas other ones decrease it. This might depend on the way the nitrogen containing compound is processed by the cell. Whereas one compound may be useful for eventual insertion in the toxin, another compound could disturb an essential pathway that normally stimulates the biosynthesis. This also applies for carbon sources. Biosynthesis can be limited when there is a shortage of one of the components that is necessary for the final product. This was found to be the case in *A. nidulans*[28]. In this fungus it is believed that carbon source does not have specific regulatory effects. Carbon source was also found to influence fumonisin biosynthesis. The toxin was produced in great amounts when grown on amylopectin, one of the components of starch. This indicates that fumonisins might play a role in maintaining *F. verticillioides*' ecological niche. Sda1, a Cys2-His2 transcription factor, possibly could relate carbon utilization and FB_1 synthesis. On several carbon sources, the knock out gene led to reduced fungus growth, but to an increase in FB_1 production. It could be that the protein is involved in balancing these two processes[13].

Light was also found to influence the production of fumonisins. FB_1 production is stimulated by light, especially yellow and green, because light of these wavelengths stimulates FUM1 transcription. In another study, yellow light was again found to increase FB_1 biosynthesis, whereas green light was found to decrease FUM1 transcription. This seems contradictory. It might be that the wavelengths used are not exactly the same, but it is also likely that it depends on the intensity of the light. In the study done by Fanelli et al. it was found that light intensity also influences the biosynthesis. Since these two parameters both have influence on FB_1 biosynthesis, they should be standardized in other studies looking into other parameters as well. Otherwise it can be problematic to account for some of the observed phenomena. Why the growth is optimal under yellow light is unclear, since sunlight contains all colors. It could be that this wavelength optimally stimulates a light receptor[14,15].

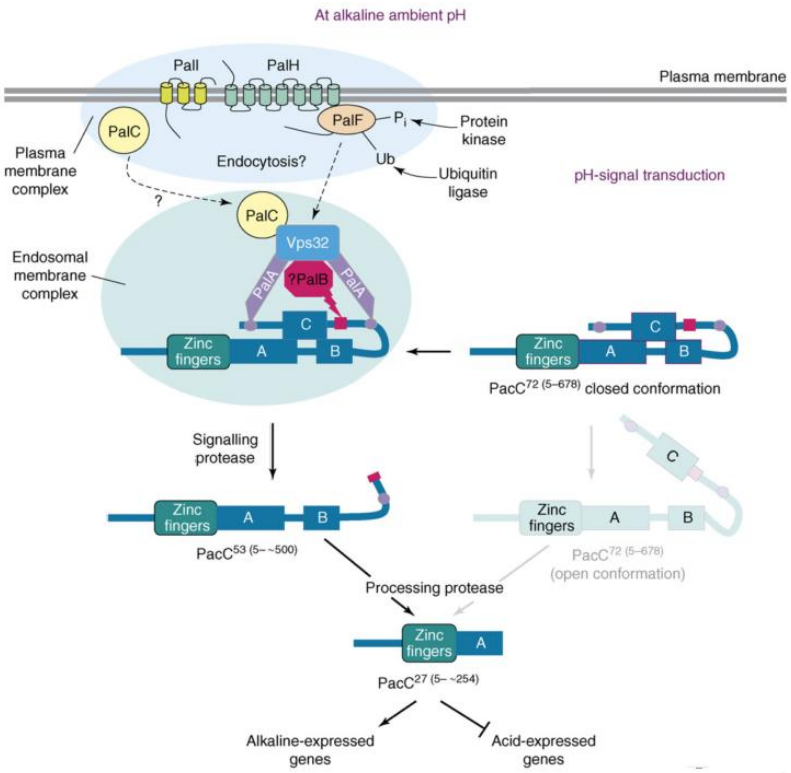


Figure 13. The regulation of the response to an increase in pH in A. nidulans[29].

For the response to pH changes, the Pac1 protein is involved, which is known to be a Cys2-His2 zinc finger. In *A. nidulans* and other fungi, parts of the pathway, which is believed to be conserved among fungal species, have been elucidated (figure 13). In *A. nidulans*, PacC is in a closed conformation under acidic conditions. When the fungus is exposed to alkaline conditions, two membrane proteins, PalH and PalF, are believed to be endocytosed by the cell. Interaction with PalC helps to transmit the pH signal through the cell to the endosomal membrane complex. PacC, bound to PalA, then undergoes its first proteolysis step, probably catalyzed by PalB, a cysteine protease. PacC opens up and becomes accessible for a non-pH dependant protease, which cleaves of another part of PacC, which is then fully activated. It would be interesting to study whether this pathway is the same in *F. verticillioides*. More insight in the pathway of pH activation and which genes are impacted by Pac1 could give more information on the exact regulation of FB1 synthesis[29].

Other transcription factors than Fum21p and Pac1 are also found to regulate fumonisin biosynthesis. In *F. verticillioides*, the MADS-box genes were found when looking into the genome comparing it with homologous genes known to encode these transcription factors. For Mads1, the homologous gene in several fungal species, Mcm1, was found to be involved in mating and virulence. Mads2 homologues were shown to be the target of a MAPK cascade in the other fungi, involved in cell wall reinforcement and remodeling. This is interesting, because in *F. verticillioides* MAPK cascades are probably involved in the regulation of fumonisin biosynthesis. It would be interesting to find out whether these pathways are linked. It was also shown in the study that MADS1 disruption had a negative impact on all PKS genes. This probably also includes also FUM1, but it was nowhere mentioned which of the PKS genes was FUM1[17].

Another transcription factor, Zfr1, was found when searching for a member of the Zn(II)2Cys6 binuclear cluster family in *F. verticillioides*. These proteins are known to regulate many processes in the cell. AflR from *A. nidulans*, which is involved in specific regulation of aflatoxin biosynthesis, also belongs to this family. This gene is found to be essential for aflatoxin synthesis. Zfr1, however, is not likely to be a specific regulator of the FUM cluster. In *F. graminareum*, a very similar gene sequence is found, but this fungus does not have a FUM cluster. Finding out to which DNA sequence this transcription factor binds could give more information on the genes it influences. Also, it is important to investigate how Zfr1 itself is activated, so eventually the regulatory pathway can be unraveled[19].

Epigenetic factors impose another layer of regulation. The epigenetic factors found in *F. verticillioides* were all found by looking for homologues of known genes from *A. nidulans*. The functions of the homologous genes are similar. It is not clear yet which pathways influence these epigenetic factors. A surprising result of the performed studies was that in knock out strains FUM1 and FUM8 transcription was much higher, but the increase in FB_1 synthesis was not significant. This indicates that there are other factors involved in FB_1 synthesis, probably later in the biosynthesis pathway. It could also be that there was a response to the loss of normal epigenetic regulation to compensate for the increased FUM transcription. More research is needed to elucidate how this can be[21-23].

G-protein pathways are important in all eukaryotes, and fungi are no exception. Only a few genes are currently known to play a role in FB_1 biosynthesis. Gbb1, part of a heterotrimeric G-protein, was found to have a positive influence on FB_1 production, whereas GBP1, a GTPase, was found to have a negative influence. It is not possible to tell why this is as long as the downstream cascades remain unknown. Comparison with other fungi might be difficult, since there is a lot of crosstalk and pathways are not necessarily conserved. Therefore, the discovery that G-proteins impact the MAPK pathway in *F. verticillioides* is interesting. FvMK1 is a positive regulator of fumonisin biosynthesis in *F. verticillioides*. It is believed that this protein acts downstream of GBB1[24-27].

The influence of G-protein cascades in *A. nidulans* is better characterized than in *F. verticillioides*. Several G-proteins are known to influence aflatoxin biosynthesis, but since G-protein pathways are not conserved very well between species, comparing is difficult. A difference is that in *A. nidulans*, the cAMP pathway does play a role in biosynthesis, whereas this is thought not to be the case in *F. verticillioides*. The family of Ras-GTPases, in humans well known for their role in cancer, are in *A. nidulans* found to be involved in secondary metabolism, although these pathways are not as well characterized in the fungus as other pathways. Ras-GTPases might be interesting topic for future study of the regulation of fumonisin production in *F. verticillioides*, since currently nothing is known about its role in biosynthesis[28].

The pathways, genes and other factors that are known in *A. nidulans* could be used in the future as further cues to find new regulators in *F. verticillioides*. Aflatoxin biosynthesis is for example known to be influenced by oxidative stress and metals or other trace elements. It might be interesting to study whether these factors are involved in fumonisin biosynthesis[28].

Conclusion

The steps in the biosynthesis of fumonisins in *F. verticillioides* are understood rather well, but much of the regulation of the FUM cluster remains a mystery. Several environmental factors have been found to impact the production of fumonisins, such as light, pH, nitrogen and carbon sources. How they function on a molecular level is in many cases not clear yet. New transcription factors, such as the Zfr1 protein and Sda1 have been found to influence FB_1 biosynthesis. G-proteins, such as GBB1 and GBP1 are found to be involved as well, but the pathways in between remain unknown.

Hopefully, a better understanding of the regulation of toxin synthesis will help to decrease the amounts of fumonisin on food crops. If it is not possible to eradicate this ubiquitous fungus, it would be an improvement to find out under which circumstances *F. verticillioides* produces the least fumonisins. Increasing knowledge could be used to cultivate crops differently, or, which might be easier to achieve, to store food under different conditions.

To achieve this, more research needs to be done to elucidate the regulation of fumonisin biosynthesis. Conditions should be standardized as much as possible so the results will be comparable. Furthermore, comparison with better characterized fungi can be a helpful means to find interesting homologues. Eventually, more knowledge of fumonisin production will make crops even safer for consumption.

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