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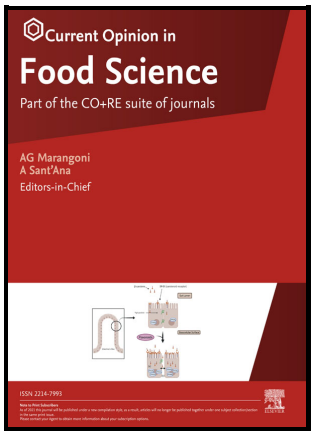

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RECENT ADVANCES IN BIOSYNTHESIS AND REGULATORY MECHANISMS OF PRINCIPAL MYCOTOXINS

Short title: ADVANCES IN BIOSYNTHESIS MECHANISMS OF MYCOTOXINS

Massimo Ferrara[a], Giancarlo Perrone[a]*, Antonia Gallo[b]

[a]Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Amdendola 122/O 70126 Bari, Italy (massimo.ferrara@ispa.cnr.it; giancarlo.perrone@ispa.cnr.it)

[b]Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Provinciale Lecce-Monteroni, 73100 Lecce, Italy (antonia.gallo@ispa.cnr.it)

*corresponding author

Abstract

Filamentous fungi possess a wide diversity of metabolic pathways, among which the production of mycotoxins and the resultant contamination of agricultural commodities cause severe health impacts on humans and animals. Understanding the biology, ecology and genetics of mycotoxins biosynthesis is fundamental to counteract their spread in food and feed products and reduce the human and animal health risk. The gene clusters responsible for the biosynthesis of mycotoxins of agricultural importance, including aflatoxins, fumonisins, ochratoxins, patulin, citrinin and trichothecenes, have been mostly identified and characterized. However, due to the complex organization of fungal secondary metabolisms and interaction with climatic, environmental and biotic factors, numerous new researches have been recently published on structural, regulatory and epigenetics mechanisms underlying mycotoxin biosynthesis. This review provides an overview of the recent new insight into understanding genes, molecular mechanisms and factors involved in biosynthesis regulation of the principal mycotoxins.

Keywords:
aflatoxins, ochratoxins, patulin, citrinin, fumonisins, trichothecenes, epigenetic, RNAseq

Introduction

Filamentous fungi are complex microorganisms characterized by the ability to produce a broad spectrum of secondary metabolites (SMs), among which are the well-known mycotoxins. They can induce toxic effects in humans and animals, resulting in great concern for public health and economic loss [1].. In recent years, progress in the fungal genome and transcriptome sequencing, computational tools, gene disruption techniques and analytical chemistry have allowed the comprehension of several molecular aspects of the biosynthesis of mycotoxins and their regulation [2, 3, 4]. Generally, the genes encoding enzymes acting in the multi-step pathway of mycotoxins biosynthesis are located in a biosynthetic gene cluster (BGC), enabling the regulation of their expression in a coordinated manner. Synthases or synthetases key genes (polyketide synthases, terpene synthases and/or cyclases, nonribosomal synthetases, and isocyanide synthases) are present in the cluster together with additional enzymes modifying and forming the final complex molecular structure of mycotoxins, and possibly permitting transportation or reduction of their toxicity. For some mycotoxins (i.e. ochratoxin A and patulin), genes in BGCs need further characterization to clarify their function and determine the complete biosynthesis pathway [5, 6]. As for the molecular regulatory mechanisms, one or more pathway-specific transcription factors coordinating the expression of biosynthesis genes are placed in the same cluster. In some cases, a transcription factor may modulate the expression of genes in different BGCs [5, 7, 8]. In addition, the production of mycotoxins is controlled by a more complex regulatory system, comprising broad domain transcription factors and multiprotein complexes that positively or negatively regulate the expression of different BGCs in fungal species [9]. Recent research has evidenced the crucial role of signalling molecules and pathways in controlling the

fungal response to a multitude of nutritional, chemical and environmental cues that affect the primary metabolism and the biosynthesis of SMs, including mycotoxins [10]. Also, evidence of the importance of epigenetic mechanisms and post-translational modifications in the regulatory system of fungal secondary metabolism has recently emerged [11, 12].

In this review, we describe the latest findings (Table 1) on molecular mechanisms and factors involved in regulating the main important mycotoxins for food safety and human health: aflatoxins, ochratoxins, patulin, citrinin, fumonisins and trichothecenes (Figure 1).

Aflatoxins

The aflatoxins (AFs) are probably the most widely studied mycotoxins regarding molecular biosynthesis and regulation. More than 19 identified AFs analogues are produced by over 16 species in Aspergillus genus [13]. A biosynthetic gene cluster with about 30 genes has been characterized in both major aflatoxigenic species A. flavus and A. parasiticus, although molecular studies have been conducted mainly on the production of AFB1 by A. flavus [14]. The roles of most AF genes have been established; however, some steps still need to be fully determined. Only recently, the disruption of aflN (verA) gene has led to the identification of a novel intermediate between versicolorin A and demethylsterigmatocystin in the AFs biosynthesis [14, 15]. In addition, site-specific mutagenesis and biochemical studies have shown modifications of two key biosynthesis enzymes, such as the lysine succinylation of aflE [16].
Most recent RNA-seq research focused on various environmental stresses related to climate change scenarios affecting aflatoxin production. The results suggested that co-regulation of different secondary metabolic pathways likely help maintain cellular homeostasis and promote cell survival under stress conditions and, in addition, provided a valuable gene set for further investigation [17, 18]. The protein HexA is the main component of Woronin body, repairing hyphal wounding in filamentous fungi, and its loss in A. flavus was reported to reduce the production of AFB1, conidia, and conidiophores [19]. In the study of Yang et al. [20], the vacuole-related protein Fab1, proposed to maintain the vacuolar/cellular homeostasis, has been shown to impact aflatoxin production, suggesting the regulatory role of fab1 gene.
Transcriptome analyses have been carried out on mutant strains of A. flavus to examine the role of AF biosynthesis regulators previously identified: the homeobox gene hbx1 showed a broad effect on secondary metabolism genes [21]; while the rmtA gene appeared to govern over 2,000 genes, including 200 transcription factors [22].
The RNA-seq analysis of A. flavus treated with a non-aflatoxigenic A. oryzae culture filtrate showed reduced expression of AF positive regulators genes aflS, farB, and mtfA correlated with aflatoxin inhibition [23].
Regarding nutritional regulation, the creA gene was identified in A. flavus, acting in the carbon catabolic repression (CCR) mechanism to use the most favourable carbon source [24]. Also, the antioxidant gallic acid was observed to correlate with the down-regulation of the transcription factor genes creA and farB, the latter participating in peroxisomal fatty acid –oxidation [25]. In addition, the regulators SsnF and RocA were found to control the expression of CCR factors, interact with CreA and positively regulate AFB1 biosynthesis [26].
As for the regulation of nitrogen metabolism, the areA gene was confirmed to play an important role in the aflatoxin biosynthesis in A. flavus [27].
Fungal cellular development and AFs biosynthesis are complex and interconnected processes involving many different types of transcription factors. For example, the plant homeodomain (PHD) transcription factor Rum1 has been reported as a regulator of the growth and the formation of conidia and sclerotia, as well as aflatoxin biosynthesis in A. flavus [28]. The latest findings have indicated that the basic leucine zipper (bZIP) transcription factors Afap1 and AflRsmA are associated with oxidative stress response and aflatoxin synthesis in A. flavus [29, 30]. Furthermore, the antioxidant catalase enzyme CTA1 in A. flavus has been shown to play a crucial role in fungal development, virulence, and aflatoxins biosynthesis [31], confirming that the antioxidant system, involved in the defence response to increased reactive oxygen species (ROS), could regulate several fungal metabolisms. Also, ethanol inhibited AFB1 biosynthesis by enhancing fungal oxidative stress response [32] as well as the antioxidant epigallocatechin gallate was associated with the down-regulation of the bZIP transcription factor AtfA mediating oxidative stress [33].

In A. flavus, the G protein α-subunit GpaB was found to play a role in AF biosynthesis by regulating the adenylate cyclase/cAMP signalling transduction pathway, and the cyclase-associated protein Cap has been demonstrated to contribute to mycotoxin biosynthesis and fungal virulence [34, 35].
In recent years, in A. flavus, three central mitogen-activated protein kinases (MAPK) pathways, representing other essential mechanisms for environmental signal transduction, have been described, which include the kinases SakA (HogA/Hog1), Slt2 (MPKA) and Fus3 (MPKB) and are involved in the development, stress response, pathogenicity and aflatoxin biosynthesis [36, 37, 38]. Furthermore, the Fus3 kinase protein has been recently suggested to affect mycotoxin production by the AF substrate regulation rather than the modulation of AF cluster genes [39]. In the MAPK cascade participating in the high osmolarity glycerol (HOG) pathway in response to hyperosmotic stress, the kinase kinase PbsB, likely involved in the activation of the terminal kinase SakA kinase, was found to upregulate the expression levels of regulatory and structural genes in the AF gene cluster increasing AFB1 biosynthesis [40] and the Msb2 mucin protein has been hypothesized as an osmosensor initiating the signalling response [41]. Lately, the Ras subfamily GTPase proteins have been demonstrated to act as molecular switches in A. flavus signal transduction pathways controlling various cellular processes, including aflatoxin biosynthesis [42].
Epigenetic and post-transcriptional enzymatic modifications (PTM) have been correlated to the modulation of aflatoxin biosynthesis in A. flavus [43].
The transcriptome analysis of A. flavus grown in conducive conditions contributed to identifying the gene lael1 as coding a novel LaeA-like methyltransferase, whose deletion caused a significant increase in AF production [44]. Also, the volatile ether compound benzenamine was proposed to affect the aflatoxin biosynthesis by downregulating the global regulatory factor laeA [45].
New findings have provided additional evidence that the phosphatases CDC14 and Ssu72 [46, 47], the histone deacetylase HosA [48], the histone methyltransferases Set3 and AflSet1 [49, 50], the histone acetyltransferases MystA and MystB [51], and the lysine acetylation of aflO [52], play a critical role in the regulation of AF biosynthesis cluster genes.
Furthermore, a very recent proteomic study has revealed a wide range of PTMs potentially implicated in regulating the AF pathway in A. flavus [53].

Ochratoxin A

Ochratoxin A (OTA) is a toxic secondary metabolite produced by Aspergillus and Penicillium species that widely contaminates food and feed. Presently, fungal species known to be producers of OTA (more than 20) belong to the genera Aspergillus and Penicillium [54, 55].
An OTA-biosynthetic cluster comparative analysis highlighted a high synteny in OTA cluster organization in five structural genes, namely otaA, otaB, otaC, otaR1, and otaD [56, 57]. Recently a sixth gene coding for a cyclase synthase, otaY, located between the otaA and otaB genes, was identified in all available genomes of Aspergillus and Penicillium OTA producers [54]. The key role of OTA core genes has been demonstrated not only by gene deletion in producing species [58, 59, 60] but also by genome sequencing and transcriptomic analysis of some natural A. carbonarius OTA non-producing strains [61].
One of the most important nutrients for the growth and production of secondary metabolites is the carbon source. The growth on different carbon source components leads to differences in mycotoxin production. CreA is the main transcriptional factor mediating carbon catabolite repression, which is employed in using carbon sources. Wang and co-authors [62] investigated the growth and OTA production of A. ochraceus on different carbon sources (glucose, D-xylose, maltose, fructose, D-galactose, D-mannitol, lactose, D-mannose, and acetate), revealing that glucose and maltose were the most OTA inducing carbon sources for A. ochraceus. The generation of a AoCreA deletion mutant of A. ochraceus demonstrated an effect on fungal morphology, conidiation and OTA production on all media except for PDA.
An extensive understanding of OTA biosynthesis and mycelium development in relation to NaCl-riched medium was recently gained in A. ochraceus and P. nordicum, demonstrating that increasing NaCl concentration induced OTA production. Moreover, in A. carbonarius mycelium growth, sporulation and OTA production were significantly promoted at increasing glucose content [63]. In addition, in P. verrucosum OTA production and the expression of related biosynthetic key genes were significantly decreased when solute or matric stress was imposed [64].
External pH plays an important for the growth, development and metabolism of microorganisms. Fungi have evolved complex signalling pathways responding to external pH in order to adapt to the different

ambient conditions. The transduction factor PacC is one of the most important factors in the fungal regulatory system. A reduction of mycelium growth and an increase in sporulation were observed in the A. ochraceus ΔAopacC mutant. Compared to the neutral condition, OTA content was severely reduced under both acidic and alkaline conditions regardless of ΔAopacC that instead influenced OTA production in the neutral condition [65]. A comparable behavior with some differences was observed for the ΔAcpacC mutant strain of A. carbonarius showing altered fungal growth both at neutral and acid pH, linked to reduced sporulation and conidial germination and inhibition of OTA production at pH 7.0 and 4.0 [66].
The fluctuation of internal peculiar factors that change during fruit ripening, such as sugars, organic acids and pH, also significantly impact A. carbonarius ability to produce OTA. A strong correlation was observed when the fungus was exposed to high malic acid content and limited sugar and low pH levels, resulting in activation of OTA biosynthesis pathway through modulation of laeA [67].
Many studies have shown the involvement of global transcription factors in the regulation of secondary metabolite biosynthesis in filamentous fungi. Gene expression analysis of Velvet complex (laeA/veA/velB) suggested that the exposure to 15-28 °C and 1000 ppm CO_2 directly influences the global regulatory complex and thus OTA production in A. carbonarius [68]. The involvement of laeA in OTA regulation was confirmed recently by its deletion in A. carbonarius, resulting in a significantly reduced OTA production [69].
In A. ochraceus mutant strains for laeA, veA, and velB genes, differences in vegetative growth, conidial and OTA production were observed[70]. Especially, ΔlaeA strain almost lost the ability to generate conidiophores under dark conditions. Moreover, even the disruption of veA gene in A. niger demonstrated its role in positive regulation of conidia production, OTA biosynthesis, and oxidative stress tolerance, regardless of light conditions [71].
More recently, the effect of environmental stimuli has been explored from the perspective of a mutated climate scenario that could occur in the next future. Cervini and co-authors [68] investigated the influence of two different temperature cycles (15-28 vs 18-34 °C, 11.5 h/12.5 h dark/light) and interaction with existing and future CO_2 exposure concentrations (400 vs 1000 ppm) on the growth, OTA production and cluster regulation on a synthetic grape medium. They found that the increase of more than 2.5 fold CO_2 concentration even at a lower temperature cycle (15-28 °C) resulted in an increase in colony growth and OTA production.
Moreover, a similar study has been conducted on P. verrucosum by analyzing the effect of temperature (25 vs 30 °C), CO_2 (400 vs 1000 ppm) and matric/solute stress (-2.8 vs -7.0 MPa) on growth, OTA biosynthesis and cluster regulation [72]. Overall, the growth rate under solute stress was slower in elevated CO_2 than under matric stress when compared with existing conditions, and under elevated CO_2 levels in matric stress treatments otaA (otapksPV) gene expression was increased..

Patulin

Patulin is a tetraketide mycotoxin frequently found in fruit juices and apple products. Various fungal species of Penicillium, Aspergillus and Paecilomyces genera produce patulin [73].
In the last decade, the patulin gene cluster, containing 15 genes (PatA–PatO) in a genomic region of 41-kb, was identified in A. clavatus, P. griseofulvum and P. expansum. The cluster includes genes encoding a C6 transcription factor (PatL), three transporter proteins (PatM, PatC, PatA), and 11 biosynthetic enzymes [73, 74]. The deletion of patL in P. expansum was found to prevent the expression of all other biosynthesis genes [74]. Successively, Li and co-authors clarified the functions of the genes involved in eight steps of the patulin biosynthetic pathway by using substrate feeding and heterologous expression analyses [75]. In particular, it was established that: i) PatK encoding a 6-methyl salicylic acid synthase is responsible for the initial step of patulin biosynthesis; ii) PatG encoding a putative decarboxylase acts most likely in the decarboxylation of 6-methyl salicylic acid to m-cresol; iii) PatH and PatI encode the enzymes responsible for the hydroxylation of m-cresol to m-hydroxy benzyl alcohol and of m-hydroxy benzyl alcohol to gentisyl alcohol, respectively; iv) PatJ and PatO catalyze the conversion of gentisaldehyde to isoepoxydon that is transformed to phyllostine by PatN. In addition, the authors also demonstrate for the first time that PatF (neopatulin synthase), PatD (alcohol dehydrogenase) and PatE (glucose-methanol-choline oxidoreductase) are involved in the last three steps of patulin biosynthesis by catalyzing the conversion from phyllostine to neopatulin, neopatulin to ascladiol, and finally ascladiol to patulin, respectively [75, 76]. In particular, the

secretion of PatE protein outside the cell is probably due to reducing the risk of cell toxicity. In addition, the authors demonstrated for the first time that the inactivation of patulin genes resulted in changes in other phenotypic characteristics such as the production of a dark-red pigment, slower colony expansion (ΔPePatI, J, K, N, L), and reduced sporulation (ΔPePatF). Finally, no substantial differences in the pathogenicity were observed with respect to apple fruit in deletion mutants compared with wild-type strain [76].
However, the enzyme responsible for converting gentisyl alcohol to genitsaldehyde, remains unknown, and the PatB gene, encoding a carboxylesterase, is the only gene not yet assigned to any step of the patulin biosynthesis [75, 76]. The specific regulation mechanism of patulin biosynthesis depends on a C6 transcription factor PatL located in the cluster.
Interestingly, it was evidenced that sporulation in P. expansum is not related to the regulation of patulin biosynthesis. The deletion of brlA gene, usually expressed in the early conidiation process, altered the fungal morphology, inhibited conidiation and enhanced the in vivo aggressiveness. As for the production of mycotoxins, a significant increase in patulin production was observed in vivo in relation to the higher aggressiveness [77].
More recently, Chen et al. [78] have demonstrated that the bZIP transcription factor PeMetR, involved in sulfur assimilation, participates in controlling virulence and patulin biosynthesis in P. expansum. Furthermore, the effect of sulfur assimilation on the patulin production was confirmed by the deletion of other sulfur assimilation pathway genes, PesA, PesB, PesC, PesF, which generated defects in growth, virulence and patulin production similar to ΔPeMetR [78].
Patulin biosynthesis is also regulated at the epigenetic level. Among the velvet family proteins in P. expansum, VeA and VelB were found to positively regulate the expression of patulin genes, as well as VelC [75, 76, 79]. Patulin biosynthesis also responds to carbon sources and pH through the general transcription regulators CreA and PacC [80, 81, 82]. Also, the epigenetic reader SntB governs patulin biosynthesis by positively modulating the regulators PacC, LaeA, and CreA, and its expression results significantly reduced under postharvest storage conditions such as low temperature and high CO_2 [73, 82]. However, regulatory mechanisms behind the patulin production in response to most environmental factors remain to be clarified [76]. Additionally, the in silico prediction of Paecilomyces niveus draft genome for secondary metabolite genes revealed a cluster of 15 putative genes responsible for patulin biosynthesis [83]. However, a deeper investigation on cluster regulation is still needed for this species.

Citrinin

Citrinin (CIT), discovered in 1930, was isolated firstly from Penicillium citrinum, but it is also produced by P. expansum, P. verrucosum, A. fumigatus, A. parasiticus, and by M. purpureus, M. aurantiacus, and M. ruber. Citrinin exhibits a polyketide structure related to the polyketide component of OTA and is a potent mycotoxin with nephrotoxic activities [84, 85]. The citrinin biosynthesis involves seven structural genes, encoding the nrPKS (known as CitS) synthetizing a ketoaldehyde, the hydrolase mrl1/CitA, the iron oxidase mrl2/CitB, the oxidase mrl7/CitC and the aldehyde dehydrogenase mrl4/CitD. In the last step, the dehydrogenase mrl6/CitE leads to the formation of citrinin [84].
Citrinin could occur with OTA or patulin, but its biosynthesis results genetically linked to OTA and not to PAT, with a shift between the two toxins versus OTA at high NaCl level or versus CIT under oxidative stress in P. verrucosum [86]. Moreover, in P. expansum, toxins production was shifted to citrinin at higher pH values, suppressing the patulin biosynthesis, occurring at lower pH (4–6) [87].
The regulation of citrinin in Penicillium and Monascus is not yet well understood; a major transcriptional activator (CtnA) is present in the cluster, but additional transcription factors, may be involved in the biosynthesis as identified in a transcriptional analysis in M. purpureus [88]. In general, it is well known that pigments and citrinin share the biosynthetic pathway up to a certain branch point. An increased pigments production in Monascus leads to a decrease in citrinin biosynthesis [89]. Hong et al. [90] demonstrated by comparative transcriptomic analysis that NH_4Cl or NH_4NO_3 as a nitrogen source can significantly enhance the synthesis of pigment precursors in M. purpureus, but downregulates the expression of citrinin genes. Recently, Wang et al. [91] revealed that overexpression of PexanC, encoding a bZIP transcription factor of the xanthocillin gene cluster, leads to high citrinin production in P. expansum, indicating the evolutionary relevance of functional divergence of BGC regulatory elements. [91].

Fumonisins

Fumonisins are a group of long-chain amino polyalcohols, of which fumonisin B1 is the most toxic and most occurring in cereals. Fusarium verticillioides is considered worldwide the main fumonisin contamination source in maize, although many species of the F. fujikuroi species complex and F. oxysporum have been reported as fumonisin producers.

The FUM cluster consists of 16 genes encoding biosynthetic enzymes and regulatory proteins [92]. The function of several genes involved in fumonisin biosynthesis has been widely studied in different Fusarium species. Recently, Sun and co-authors [93] found that deletion of fum1, fum6, fum8, or fum21 results in a strong reduction in fumonisin production in F. proliferatum, while loss of fum19 does not. In addition, fumonisin-deficient strains display significantly decreased pathogenicity. The role of fum1 in F. proliferatum fumonisin biosynthesis was also confirmed by CRISPR/Cas9 genome editing [94]. Moreover, comparative analyses of FUM cluster genes between F. fujikuroi fumonisin producing and non-producing strains revealed that natural mutations in the FUM cluster, especially in fum21 and fum7 genes, determined the fumonisin nonproduction [95, 96].

Regulation of fumonisin biosynthesis is strictly related to carbon sources. For example, the absence of sucrose in the medium could greatly induce the production of fumonisin in F. proliferatum, as confirmed by fum1 and fum8 up-regulation. In contrast, additional supplementation of sucrose to the culture medium significantly reduced fumonisin production [97].

The complex process of fumonisin production is regulated not only by specific genes of the biosynthetic cluster but also by global regulators able to control mycotoxin production at different levels.

The bZIP transcription factors play an essential role in regulating vegetative growth, secondary metabolite production and environmental stress tolerance. In F. verticillioides the bZIP-type transcription factor, FvAtfA, is involved in fumonisins regulation. Expression levels of fum1 and fum8 genes in the ΔFvatfA mutant were down-regulated, resulting in defected fumonisin production [98].

Also, the APSES-class transcription factors are well-known fungal regulatory elements. The deletion of stuA gene in F. verticillioides led to reduced vegetative growth, stunted aerial hyphae, and significant reductions in microconidiation, as well as reduced fumonisin production and virulence. Additionally, the transcriptomic analysis revealed the downregulation of the expression of several genes in the fumonisin and fusarin C biosynthetic clusters [99].

The transduction of environmental stimuli at the membrane level plays a crucial role in the regulation of fumonisins biosynthesis. The G protein-coupled receptors (GPCRs) are the largest group of membrane receptors that transduce signals from the external environment into the cell [100]. Earlier studies demonstrated that Gα and Gβ subunits are positive regulators of FB1 biosynthesis and that the expression of two regulators of G-protein signalling genes, FvFlbA1 and FvFlbA2, were induced in Gβ deletion mutant ΔFvgbb1. Notably, FvFlbA2 has a negative role in FB1 regulation. While many fungi contain a single copy of FlbA, F. verticillioides harbors two putative FvFlbA paralogs, FvFlbA1 and FvFlbA2. Yan et al. [101] characterized the functional role of FvFlbA1 and FvFlbA2. While the ΔFvflbA1 mutant exhibited no significant defects, ΔFvflbA2 and ΔFvflbA1/A2 mutants showed thinner aerial hyphal growth while promoting FB1 production. Thus, FvFlbA2 is required for the proper expression of key conidia regulation genes and fumonisin cluster genes.

Membrane trafficking and vesicular transportation are other processes strictly involved in the secretion of secondary metabolites in fungi. The Rab GTPases, particularly those homologous to Saccharomyces cerevisiae Sec4, are known to be associated with protein secretion, vesicular trafficking, secondary metabolism and pathogenicity. However, the role of Rab GTPases in many toxigenic fungi remains elusive. Recently in F. verticilliodes the role of a Rab GTPase, namely FvSec4, was investigated [102]. The ΔFvsec4 mutant produces dramatically lower levels of FB1 than the wild-type progenitor, as confirmed by the down-regulation of fum1 and fum8 genes. Moreover, the mutant demonstrated that FvSec4 plays essential roles also in hyphal development, virulence, and stress responses.

Trichothecenes

Trichothecenes belong to the sesquiterpene type of toxins and are produced by fungi belonging to Sordariomycetes, Eurotiomycetes and Dothideomycetes classes [103]. Over the last three decades, the genetic and biochemical bases of trichothecene biosynthesis in several species of Fusarium and Trichoderma have been elucidated. Trichothecenes may differ in structure, and these changes significantly

impact toxicity and the biological activity of these compounds. Phylogenetic and functional analyses of trichothecene biosynthetic genes in multiple genera have provided evidence that the loss, acquisition, and changes in functions of TRI genes have determined the diversity of trichothecene structures. In F. graminearum, the biosynthetic TRI cluster is composed of 15 genes located on different chromosomes at three different loci [104]. The core TRI cluster is represented by 10 co-regulated genes, including the tri5, encoding a trichodiene synthase. Moreover, in F. sporotrichioides and F. graminearum two additional genomic regions are involved in trichothecene biosynthesis: the Tri1-Tri16 locus and the Tri101 locus [104]. Regulation of trichothecenes biosynthesis is strictly correlated to environmental conditions.

Climate change factors may have a significant impact on growth and trichothecenes production. Abiotic factors such as water activity, temperature and CO_2 exposure could affect growth, gene expression of TRI cluster, and associated metabolites. In F. langsethiae, Verheecke-Vaessen and co-authors demonstrated that tri5 gene expression was significantly increased (5.3-fold) at 30 °C, 0.98 aw, elevated CO_2 (1000ppm) and the tri6 and tri16 genes were overexpressed, especially under high CO_2 conditions. Moreover, at 0.98 aw, in stored oats, they observed that elevated CO_2 led to a significant (73-fold) increase in T2/HT-2 toxin, especially at 30 °C [105].

Nakajima and co-authors showed that continuous acidification of the culture medium plays an important factor in the stimulation of trichothecene biosynthesis, especially during the early growth stage [106]. Also, the nitrogen regulatory GATA transcription factor AreA is indispensable for transcriptional activation of TRI cluster genes. In F. graminearum AreA (FgAreAp) is unessential for the functioning of the Tri6 promoter, but it contributes to some extent to the increased production of mycotoxin under mildly acidic conditions [106]. Recently, the suppressive effects of amino acids, used as the sole nitrogen source, on trichothecene biosynthesis were also demonstrated in F. graminearum. When the medium pH was maintained at 4.0, Gly, L-Ser, and L-Thr suppressed trichothecene production, while when the medium pH was 3.5 the trichothecene production was induced, with only the exception of L-Thr, which suppressed their biosynthesis [107].

Histone acetyltransferase (HATs) could play a central role in the regulation of gene expression and other processes by inducing post-translational modifications of chromatin structure in eukaryotes. Even though these genes were investigated in various fungal species, few of them have been functionally characterized. In F. graminearum the role of four putative HATs (FgGCN5, FgRTT109, FgSAS2, FgSAS3) in sensitivity to osmotic and oxidative stress, conidiation and DON production was demonstrated. Interestingly, both ΔFgSAS3 and ΔFgGCN5 mutants inhibit the production of DON, inducing a significant downregulation of TRI genes expression. Moreover, for the first time, it was demonstrated that FgSAS3 and FgGCN5 are indispensable for the acetylation of histone at different sites [108, 109]. This evidence was further confirmed by a comparative acetylome analysis revealing as the deletion mutant of Fggcn5 failed to produce DON, supporting the hypothesis of the central role of lysine acetylation for DON biosynthesis in F. graminearum [110].

Conclusions

In the last few years, new findings have expanded the understanding of the complex regulatory network underlying the biosynthesis of some main mycotoxins.

Several biotic and abiotic factors trigger the production of mycotoxins through different steps, including the detection of environmental cues at the membrane level, the signal transmission into the fungal cell, the response to the resulting oxidative stress and the activation of broad domain transcription factors and multiprotein complexes, that finally regulate the expression of the mycotoxin biosynthesis genes. In addition, epigenetic and post-transcriptional enzymatic modifications have been demonstrated to be crucial in modulating mycotoxins production.

The most advanced results have been achieved for the main studied mycotoxins, such as aflatoxins and fumonisins, while for other mycotoxins, such as OTA and patulin, the comprehension of the molecular biosynthesis mechanisms is more recent and requires further investigation.

In the future, the latest progress in omic techniques and genome-editing approaches will help clarify the still unknown aspects of biosynthesis and regulation even in deeply studied mycotoxins. Furthermore, elucidating the complex interconnection between the specific and global biological processes behind the biosynthesis of mycotoxins is fundamental for developing new prevention and control solutions.

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Figure and table caption

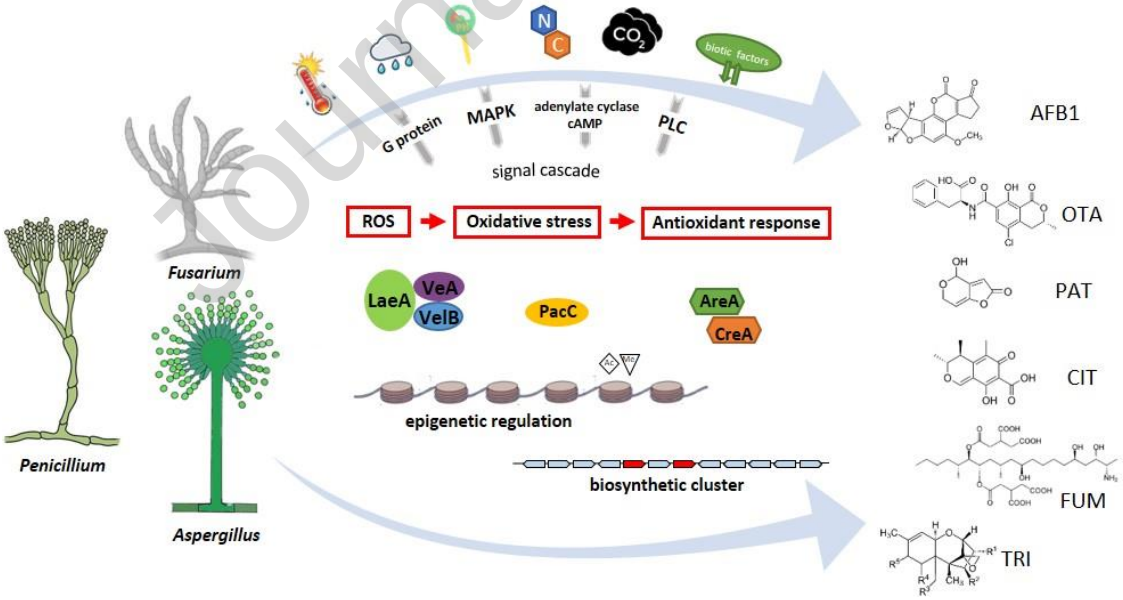

Fig.1 Representation of principal mechanisms governing mycotoxins biosynthesis. The main producing fungal genera (Aspergillus, Fusarium and Penicillium), some of the most studied regulatory elements and mycotoxins (AFB1, aflatoxin B1; OTA, ochratoxin A; PAT, patulin; CIT, citrinin; FUM, fumonisin; TRI, trichothecenes) are depicted.

Table 1. List of the latest findings on biosynthesis and main regulatory mechanisms of mycotoxins.

mycotoxin	Biosynthesis genes	reference
ochratoxin	**otaY** gene is involved in the cyclization of OTA molecule in A. carbonarius	[59]
patulin	functions of several enzymes in the PAT biosynthetic pathway are confirmed in P. expansum	[75,76]
fumonisin	role of **fum1**, **fum6**, **fum7**, **fum8**, **fum21** genes is confirmed in producing species in F. proliferatum and F. fujikuroi	[93,94,95,96]
	Pathway-specific transcription factors	
ochratoxin	bZIP transcription factor **otaR1** regulates biosynthesis OTA cluster genes in A. ochraceus/westerdijkiae, A. carbonarius, A. niger	[57,58,60]
	Regulators of mycotoxin biosynthesis	
aflatoxin	**HexA** protein, the main and essential component of Woronin body, is required for AF biosynthesis in A. flavus	[19]
aflatoxin	vacuole-related protein **Fab1** maintaining cellular homeostasis regulates AF biosynthesis in A. flavus	[20]
aflatoxin	AF regulator **Hbx1** shows a broad effect on SM genes in A. flavus	[21]
aflatoxin	AF regulator **RmtA** controls the expression of around 2,000 genes in A. flavus	[22]
aflatoxin	**non-aflatoxigenic A. oryzae** downregulates AF regulators **aflS, farB** and **mtfA** genes in A. flavus	[23]
aflatoxin	CCR gene **creA** regulates AF biosynthesis and virulence in A. flavus	[24]
aflatoxin	**gallic acid** downregulates AF biosynthesis regulators **farB** and **creA** genes in A. flavus	[25]
aflatoxin	**SsnF** and **RocA** positively regulate AF biosynthesis in A. flavus as corepressors of CreA	[26]
aflatoxin	global nitrogen regulatory gene **areA** is important for AF production in A. flavus	[27]
aflatoxin	PHD factor **Rum1** involved in conidia e sclerotia production plays a role in AF biosynthesis in A. flavus	[28]
aflatoxin	bZIP transcription factors **Afap1** and **AflRsmA** mediate oxidative stress response and AF production in A. flavus	[29,30]
aflatoxin	antioxidant related catalase **CTA1** regulates AF biosynthesis in A. flavus	[31]
aflatoxin	**ethanol i**nhibits AF biosynthesis upregulating oxidative stress response genes in A. flavus	[32]
aflatoxin	**epigallocatechin gallate** downregulates bZIP **AtfA** mediating oxidative stress and inhibiting AF biosynthesis in A. flavus	[33]
aflatoxin	novel LaeA-like protein **Lael1** plays a specific role in the regulation of AF biosynthesis in A. flavus	[44]
aflatoxin	**benzenamine** affects AF biosynthesis by downregulating the global regulatory factor **leaA** in A. flavus	[45]
ochratoxin	**AoCreA** gene regulates OTA biosynthesis in response to carbon sources in A. ochraceus.	[62]
ochratoxin	**glucose** and **salt** content in substrate regulates OTA production in A. carbonarius, A. ochraceus, P. nordicum	[63]
ochratoxin	**solute** and **matric potential stress** affect OTA production in P. verrucosum	[64]
ochratoxin	**pacC** gene regulates OTA biosynthesis in response to ambient pH in A. ochraceus and A. carbonarius	[65,66]
ochratoxin	Velvet complex **laeA**, **veA**, **velB** genes regulate OTA production in A.	[67,68,69,70,71]

	ochraceus, A. carbonarius, A. niger	
ochratoxin	**temperature and CO_2** impact OTA production in A. carbonarius and P. verrucosum	[68,72]
patulin	**LaeA, VeA**, **VelB** and **VelC**, are involved in the regulation of PAT biosynthesis in P. expansum	[75,76,79]
patulin	deletion of the conidiation regulator gene **brlA** does not impair PAT biosynthesis in P. expansum	[77]
patulin	bZIP transcription factor **PeMetR** mediating sulfur assimilation is essential for PAT biosynthesis in P. expansum	[78]
patulin	pH-responsive **PePacC** transcription factor regulates PAT biosynthesis in P. expansum	[80]
patulin	**apple intrinsic factors** modulate the global regulator **LaeA** and impact PAT biosynthesis in P. expansum	[81]
citrinin	CIT is produced at high pH while PAT is produced at low pH in P. expansum	[87]
citrinin	transcriptomic analysis indicates possible transcription factors regulating CIT biosynthesis in M. purpureus	[88]
citrinin	increased pigments production leads to a decrease in CIT biosynthesis in Monascus	[89]
citrinin	nitrogen source **NH4Cl** or **NH4NO3** increases pigments production and decreases CIT formation in Monascus spp.	[90]
citrinin	transcription factor **PexanC** regulates pathway-specific **ctnA** gene and promotes CIT biosynthesis in P. expansum	[91]
fumonisin	**sucrose** content in substrate regulates FUM production in F. proliferatum	[97]
fumonisin	bZIP transcription factor **FvAtfA** regulates the expression of FUM biosynthesis genes in F. verticillioides	[98]
fumonisin	APSES-class transcription factor **FvstuA** regulates the expression of FUM biosynthesis genes in F. verticillioides	[99]
trichothecene	**climate change factors** (T, a_w, CO_2) impact expression of Tri genes and T-2/HT-2 production in F. langsethiae	[105]
trichothecene	transcription factor **AreA** contributes to TRI production under mildly acidic conditions in F. graminearum	[106]
trichothecene	certain **amino acids** (Gly, Ser, Thr) negatively affect TRI production in F. graminearum	[107]
	Signalling pathways	
aflatoxin	G protein α-subunit **GpaB** regulates cAMP signalling and is required for AF biosynthesis in A. flavus	[34]
aflatoxin	cyclase-associated protein **Cap** regulates cAMP signalling and contributes to AF biosynthesis in A. flavus	[35]
aflatoxin	kinases **Slt2** (MPKA) and **Fus3** (MPKB) are involved in MAPK signalling pathways modulating AF biosynthesis in A. flavus	[37,38,39]
aflatoxin	kinase **SakA** (HogA/Hog1) and kinase kinase **PbsB** in the MAPK/HOG pathway regulate AF biosynthesis during osmotic stress in A. flavus	[36,40]
aflatoxin	**Msb2** acts as an osmosensor in the MAPK/HOG pathway regulating AF biosynthesis in A. flavus	[41]
aflatoxin	**Ras subfamily** GTPase proteins act as molecular switches in signalling pathways regulating AF biosynthesis in A. flavus	[42]
fumonisin	RGS protein **FvFlbA2** is negatively associated with FUM production in F. verticillioides	[101]
fumonisin	Rab GTPase protein **FvSec4** is critical for FUM production in F. verticillioides	[102]
	Epigenetic and Post Transcriptional Modifications	
aflatoxin	**lysine succinylation** of aflE and **lysine acetylation** of aflO contribute to	[16,52]

	AF biosynthesis in A. flavus	
aflatoxin	phosphatases **CDC14** and **Ssu72** regulate AF biosynthesis in A. flavus	[46,47]
aflatoxin	histone deacetylase **HosA** regulate AF biosynthesis by modulating AF cluster genes in A. flavus	[48]
aflatoxin	histone methyltransferase **Set3** and **AflSet1** are involved in AF biosynthesis in A. flavus	[49,50]
aflatoxin	histone acetyltransferases **MystA** and **MystB** impact AF biosynthesis in A. flavus	[51]
patulin	epigenetic reader **SntB** positively regulates the expression of **LaeA**, **CreA**, **PacC** in P. expansum	[82]
trichothecene	histone acetyltransferases **FgGCN5**, **FgRTT109**, **FgSAS2**, **FgSAS3** are involved in DON biosynthesis in F. graminearum	[108,109,110]

Highlights

- An update on molecular mechanisms behind the biosynthesis of main mycotoxins
- Global and cluster specific regulatory elements govern mycotoxin biosynthesis
- Effect of nutritional, chemical and environmental signals on mycotoxin biosynthesis
- New advances in epigenetic and post transcriptional modifications for AFs and TRI